THE INTESTINAL MICROBIOTA IN EARLY LIFE UNDERNUTRITION

by

Kelsey Elizabeth Huus

B.Sc., University of Ottawa, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

November 2020

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

The Intestinal Microbiota in Early Life Undernutrition

submitted by Kelsey Elizabeth Huus in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

in Microbiology and Immunology

Examinining Committee:

B. Brett Finlay, Professor, Microbiology & Immunology
Supervisor

Amee R. Manges, Professor, School of Population and Public Health
Supervisory Committee Member

Harry Brumer, Professor, Chemistry
University Examiner

Erin Gaynor, Professor, Microbiology & Immunology
University Examiner
Abstract

Child undernutrition is a global health issue that is exacerbated by poor sanitation and infectious disease; however, the microbial and immune contributions to child growth remain poorly understood. To understand how nutrition impacts immune-microbe interactions, I assessed interactions between intestinal bacteria and immunoglobulin A (IgA), the antibody responsible for mucosal homeostasis, in mouse and human undernutrition. In contrast to healthy control mice, undernourished mice failed to develop IgA recognition of commensal Lactobacillus. Glycan-mediated interactions between Lactobacillus and IgA were lost in undernourished mice; this was driven by bacterial adaptation to the nutritional environment, independently of host antibody, and was associated with bacterial mutations in carbohydrate processing genes. Together these data indicate that diet-driven bacterial adaptations shape IgA recognition in the gut, which may have implications for the use of probiotics and oral vaccines in undernutrition. To extend these findings to human populations, I measured IgA-microbiota interactions in the fecal microbiota of 200 children with or without linear growth stunting from Madagascar and the Central African Republic. Stunted children had increased abundance of several pathobionts; two of these, Haemophilus and Campylobacter, were strongly recognized by IgA regardless of nutritional status, while Lactobacillus was broadly IgA-negative. Stunted children also had a greater number of IgA-positive fecal bacteria overall, a phenotype previously seen in inflammatory bowel disease patients. Together, IgA-binding patterns in mice and humans suggest that undernutrition alters intestinal homeostasis. To understand how pathobiotic communities respond to nutrient limitation, I further examined metabolic interactions between human Bacteroidales and E. coli, strains which exacerbate inflammation and growth stunting in undernourished mice. These bacteria experienced a mutual growth advantage which was
enhanced by protein-limited, carbohydrate-rich conditions, and which led to outgrowth of \textit{B. fragilis} and \textit{E. coli} at the expense of other strains. Thus, cross-feeding between pathobionts might contribute to community dysbiosis in the undernourished gut. Taken together, I show that undernutrition drives not only the composition of the intestinal microbiota but also its metabolic and immune functionality. A better understanding of intestinal microbial function in undernutrition may lead to improved intervention strategies.
Lay Summary

Child malnutrition is a global health issue that is driven by poor diet, but also by poor sanitation and infectious disease. Malnourished children have imbalances in the community of microbes that inhabits the intestinal tract. To better understand how intestinal bacteria are involved in child malnutrition, I studied (1) how bacteria are recognized by the immune system in the malnourished gut, and (2) how intestinal bacteria grow and interact with one another during nutrient limitation. I found that pro-inflammatory *E. coli* grew better in a microbial community during protein restriction, while *Lactobacillus*, a popular probiotic, evolved to interact less closely with the immune system. I also found that undernourished children had more immune-recognized bacteria in the gut, which is consistent with a high abundance of inflammatory microbes. Together, this work contributes to our understanding of the microbial dysbiosis in child malnutrition, which may lead to better treatments.
Preface

Portions of Chapter 1 and 5 are being prepared for publication as a review paper.

**Huus KE, Petersen C, Finlay BB. IgA-microbiota interactions and host nutrition.**

All portions included in the thesis were written by me.

A version of chapter 2 has been published:

**Huus KE, Bauer KC, Brown EM, Bozorghmehr T, Woodward S, Serapio A, Boutin RCT, Petersen C, Finlay BB (2020).** Commensal bacteria modulate immunoglobulin A binding in response to host nutrition. Cell Host & Microbe. doi: 10.1016/j.chom.2020.03.012. I was responsible for designing the experiments, analyzing the results and writing the manuscript. I conducted all IgA-SEQ experiments and 16S analysis, all *Lactobacillus* isolation, quantification and growth experiments, all IgA ELISA assays, and all whole-genome analysis. EMB, KCB, TB, SW and RCTB helped conduct mouse experiments, particularly oral gavage and tissue collection. AS generated microscopy images of *Lactobacillus*. CP and BBF provided critical feedback and editing of the manuscript. Ethical approval for this project was obtained by the University of British Columbia Animal Care Committee (Protocols A18-0279, A17-0264, and A15-0236).

A version of chapter 3 has been published:

This research was conducted as a collaborative effort with Afribiota, an international research project co-coordinated by Prof. Philippe J. Sansonetti and Dr. Pascale Vonaesch at the Institut Pasteur, Paris, France. I was responsible for performing IgA-SEQ on human fecal samples, processing and analyzing the data, and writing the paper. I conducted most of the preliminary data analysis in the laboratory of PJS during a three-month visit in the spring of 2019. ARP and PV performed immunoglobulin quantification experiments. NK and AN measured the fecal inflammatory biomarkers. AH provided the helminth data. JMC provided supervision for AH, assisted with fecal biobanking and provided critical editing of the manuscript. AM provided critical statistical and epidemiological review of the manuscript. PJS, PV and BBF obtained funding, assisted with data analysis and interpretation, and provided critical editing of the manuscript. The study protocol for Afribiota was approved by the Institutional Review Board of the Institut Pasteur (2016-06/IRB), the National Ethical Review Boards of Madagascar (55/MSANP/CE) and the Central African Republic (173/UB/FACSS/CSCVPER/16), and the Human Ethics Board of the University of British Columbia (H18-01108).

In Chapter 3 I also discuss findings from a paper on which I was a co-author:

I assisted with some of the microbiota analysis and critical editing of this manuscript. PV was responsible for designing the experiments, analyzing the results and writing the manuscript. Other author contributions can be found in the acknowledgement section of the paper. All discussion of this paper in the thesis was written by me.

A version of Chapter 4 is being prepared for publication:

**Huus KE*, Hoang TT*, Knuff K, Vogt S, Finlay BB. Mutual growth promotion of Bacteroidales and E. coli in protein-restricted nutritional environments. In preparation.**

(*authors contributed equally).

Chapter 4 is based on work conducted by myself and by Thomas Hoang, an undergraduate student under my supervision, in the lab of B. Brett Finlay. I was responsible for conceiving the project, designing the experiments, analyzing the results and writing the manuscript. I generated one of the *E. coli* mutant and complement sets (ΔnanA and nanA:: in strains 3_2_53 and 4_1_47), conducted approximately half of the cross-feeding growth assays, and supervised experiments performed by TH. TH generated two of the *E. coli* mutants used in this work (ΔnagE and ΔfucI) and conducted the remainder of the cross-feeding growth assays under my guidance. KK and SV provided cloning reagents and guidance for the mutant and complement construction.

A version of Appendix B has been submitted for publication:

These data are listed in the appendix because they do not relate to the central theme of malnutrition, but are of interest for general understanding of IgA-microbiota interactions. I was responsible for generating the IgA-SEQ dataset, analyzing the data and writing the manuscript. MF, MBP, FV, GL, BM and TMM generated the metabolite datasets used in the correlation analyses. DK led the cohort study and provided the patient samples. MMT, DK and BBF supervised the work, helped write the manuscript and provided critical editing and feedback.

Publications arising from my PhD work that are directly related to this thesis:

6. **Huus KE***, Hoang TT*, Knuff K, Vogt S, Finlay BB. Mutual growth promotion of *Bacteroidales* and *E. coli* in protein-restricted nutritional environments. *In preparation*. (*authors contributed equally*).
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAT</td>
<td>Alpha-1-antitrypsin</td>
</tr>
<tr>
<td>ASV</td>
<td>Amplicon sequence variant</td>
</tr>
<tr>
<td>CAR</td>
<td>Central African Republic</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>EED</td>
<td>Environmental enteric dysfunction</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>HAZ</td>
<td>Height-for-age z-score</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>MAL</td>
<td>Malnourished</td>
</tr>
<tr>
<td>MAL-b</td>
<td>Malnourished plus bacterial gavage</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>WASH</td>
<td>Water, sanitation, and hygiene</td>
</tr>
<tr>
<td>WHZ</td>
<td>Weight-for-height z-score</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
Acknowledgements

This work would not have been possible without such an amazing network of people in my life. I want to acknowledge my supervisor Brett for his enthusiasm, flexibility and unwavering support of his students’ well-being. I will be forever grateful to Brett for allowing me free reign with my research, for providing me with unparalleled opportunities to travel and collaborate, and for insisting to everyone we met that I was an ‘expert’. I also owe a huge debt of gratitude to the incredibly supportive Finlay Lab. Thanks in particular to Eric, my first lab mentor, for introducing me to EED, teaching me all about his mouse model and suggesting that “IgA-SEQ might be a cool technique to try”; and to Kylynda, my EED co-conspirator, for lots of combined experiments, generous sample collection and staunch moral support. To my lab mentorship team, Charisse, Roz, Kylynda, and Mihai: meetings and conversations with you have kept me motivated, directed, and enthusiastic about science through both highs and lows. To Thomas Hoang, my first student mentee: thank you for joining me on team cross-feeding with such capability and enthusiasm, and for making me so proud. And of course, to literally everyone in the Finlay Lab, all of whom have offered advice, help and comradery: Sarah, Tahereh, Jorge, Kylynda, Rozyln, Avril, Mihai, Haggai, Zack, Paula, Nina, Zakhar, James, Jen, Audrey, Katelyn, Andrew S., Deng, Lisa T., Antonio, Anna, Weronika, Stef, Natalie, Lisa R., Eric, Charisse.

I am full of gratitude and admiration for everyone involved in the Afribiota project. I have learned so much from Pascale, not only about science but also about managing projects and motivating people. Thank you to Pascale for tons of feedback and advice, and for being an amazing host and guide on my travels last spring. A heartfelt thank-you as well to Philippe for hosting me in his lab and for much thoughtful insight into this project, and to the entire Sansonetti group in Paris for welcoming me during my stay and helping me practice my French.
I moreover want to thank all of the health care, science, and community workers involved in the Afribiota project, and, most importantly, all the families and children who participated. Thank you especially to the anthropologists Elliot and Anéan and to the four families who graciously shared their time and experiences with us during one unforgettable morning in Antananarivo.

I am lucky and grateful to have received significant financial support for this degree. I want to thank CIHR, Killam, and UBC for their generous funding; Keystone for funding me to attend the Banff 2018 microbiome conference; and the CIHR Michael Smith Foreign Study Supplement for allowing me the incredible experience to work directly with my collaborators in Afribiota and at the Pasteur Institute.

I want to thank my committee, who has consistently given me thorough and helpful feedback and guidance. To Cara, Amee, and Mike: thank you for caring about my research and for asking so many good and tricky questions. Your expertise in genomics, biostatistics, IgA, and grad school were exactly what I needed!

To my Green College community and all the friends I’ve made there – you taught me a lot about the world beyond science. Thank you for the ideas and friendship.

Last but certainly not least, I couldn’t have done any of this without my family. From the bottom of my heart: thank you to my Mom, my Dad and my brother Evan for supporting me throughout the process. And to my partner, Andrew: you are the best thing that happened to me in grad school. I love you all so much.
Dedication

This thesis is dedicated to the young children I met in Antananarivo. They have so much curiosity, energy, and potential; they also have many obstacles in their paths. They deserve a brighter future.
Chapter 1: Introduction

1.1 Early life growth faltering

1.1.1 Undernutrition

Malnutrition is a global health issue, responsible for an estimated 45% of all deaths in children under five years old\(^1\). Malnutrition encompasses both under- and over-nutrition and there is increasingly a ‘double burden’ of both types in regions experiencing rapid industrialization\(^2,3\). Reducing the global prevalence of malnutrition is a central development goal of the World Health Organization, but progress has been inconsistent\(^4,5\). Most recently, the pandemic spread of the respiratory virus SARS-CoV-2 has disrupted development projects and global supply chains, leading to warnings from the United Nations that an additional 100 million people worldwide are at imminent risk of food shortages and undernutrition\(^4\).

Undernutrition increases susceptibility to infectious disease\(^6\), delays cognitive development, and affects long-term education, mental health, and economic output\(^7,8\). Acute undernutrition, measured as low weight-for-height or ‘wasting’, is associated with health complications and high mortality rates\(^1,9\). However, chronic undernutrition is also incredibly serious due to its long-lasting consequences on physical and cognitive health\(^1,7\). Chronic undernutrition manifests as linear growth faltering in children, resulting in ‘stunting’ or a height-for-age z-score of more than two standard deviations below the mean of a reference population, typically the WHO reference population\(^10\). Multiple dietary insufficiencies contribute to early-life undernutrition, including suboptimal breastfeeding, inadequate caloric intake, and the lack of specific macronutrients (e.g. protein undernutrition) and micronutrients (e.g. deficiencies in iron,
zinc and vitamin A). Notably, the prenatal period and first two years of a child’s life represent a particularly critical developmental window, after which the impacts of undernutrition become even more difficult to reverse.

Effective methods for treating and preventing early life undernutrition are surprisingly elusive. Even when breastfeeding practices are optimized or young children are provided with nutrient-rich therapeutic foods, children in low and middle income countries often fail to achieve normal height and weight. Indeed, it has been estimated that the universal implementation of all existing nutritional interventions would only reduce stunting by one-third. This suggests that factors beyond diet contribute to undernutrition. It has long been recognized that infectious disease, and particularly diarrheal diseases, exacerbate linear growth stunting; subclinical infections and microbial dysbiosis may be additional contributors. Currently, the contributions of non-dietary factors to stunting represents an area of intensive research and interest for the prevention and treatment of childhood malnutrition.

1.1.2 Environmental enteric dysfunction (EED)

Environmental enteric dysfunction (EED) is an acquired, subclinical inflammatory disease of the small intestine that is thought to contribute to child growth faltering in low-income countries. EED occurs in the absence of diarrhea or other clinical signs of infection, and may be nearly ubiquitous in resource-poor areas that lack adequate sanitation. It was first recognized as an environmental disease due to its reversibility in adults who migrated between low-income regions and the United States. EED is characterized by blunting of the small intestinal villi, increased intestinal permeability and inflammation, and reduced absorptive capacity of the gut (Fig 1.1). EED has been linked to growth faltering.
cognitive development, oral vaccine failure, and increased risk of metabolic diseases later in life. Intestinal inflammation in particular has been correlated with subsequent growth faltering across multiple independent studies, pointing to the importance of intestinal homeostasis in child growth.

EED features are strongly correlated with inadequate sanitation, and are also associated with small intestinal bacterial overgrowth, microbial dysbiosis in the gut, and subclinical carriage of enteropathogens. These associations have led researchers to propose a microbial etiology for EED. It is thought that persistent fecal-oral contamination allows the overgrowth of pathobionts in the intestine, leading to chronic inflammation and intestinal damage; in turn, this intestinal dysfunction inhibits nutrient absorption, thus exacerbating undernutrition (Fig 1.2).
Figure 1.1 Schematic depicting the main features of environmental enteric dysfunction (EED). The small intestine of EED patients is characterized by bacterial overgrowth and overabundance of pathobionts; blunting of the small intestinal villi; local intestinal inflammation; and epithelial damage combined with leaky gut barrier.
Figure 1.2 The vicious cycle of EED. In low-income regions, poor sanitation and poor diet both contribute to growth faltering and EED. Fecal-oral contamination causes infection and chronic pathogen carriage, leading the intestinal damage and inflammation characteristic of EED. This leads to nutrient malabsorption and thus exacerbates dietary undernutrition. Undernutrition increases susceptibility to infection and dampens vaccine responses, propagating the cycle.
1.1.3 Microbial ecology in the undernourished gut

1.1.3.1 Microbiota composition

Multiple studies have demonstrated that undernutrition is associated with dysbiosis of the intestinal microbiota, supporting the proposed association between fecal-oral contamination and growth faltering. One of the most consistent findings is an increased abundance of *Proteobacteria* species in undernourished children, especially pathogens such as *Campylobacter*, *Klebsiella*, and entero-adherent *E. coli*\(^{58,66,71-73}\). Other bacterial pathobionts, such as enterotoxin-producing *Bacteroides fragilis*, are also common in the fecal microbiome of stunted children; so are helminths, parasites, and enteroviruses\(^{63,66}\). This overabundance of pathogens is accompanied by a lack of strictly anaerobic bacteria, particularly those of the *Firmicutes* phylum\(^{17,58,74}\). This lack of *Firmicutes* and overabundance of *Proteobacteria* has been conceptualized as an “immaturity” of the gut microbiota, since it reflects the natural composition of the microbiome in infancy and early life\(^{17,58}\); this bacterial signature is also a hallmark of intestinal inflammatory dysbiosis in children and adults across multiple diseases\(^{75}\).

Malnourished mice that are infected or co-infected with pathogens such as enteroaggregative *E. coli*, *Giardia*, and enterotoxin-producing *B. fragilis* show exacerbated inflammation and stunting\(^{63,76,77}\). Our lab has reported that a combination of dietary malnutrition and fecal-oral exposure to human *Bacteroidales* and *E. coli* replicates the features of EED in mice, even though these bacteria lack known virulence factors\(^{78}\). Moreover, a combination of *Bacteroidetes*, *Enterobacteriaceae* and *Enterococcus* from children with severe acute malnutrition was independently shown to induce enteropathy in malnourished gnotobiotic mice\(^{59}\). These studies provide proof-of-principle that specific microorganisms, including those not traditionally considered pathogens, can induce features of EED during malnutrition.
Together, they point to a microbial etiology for EED in which multiple pathobionts act within the context of a complex community to induce inflammation and damage in the intestine.

1.1.3.2 Metabolic function

The assembly, maintenance, and functional output of microbial communities are dependent on metabolic exchange between microbes\textsuperscript{79,80}. In the mammalian gut, bacteria compete for nutritional niches\textsuperscript{81}; engage in symbiotic cross-feeding\textsuperscript{80}; and alter their gene expression and metabolism in response to microbial, host and dietary cues\textsuperscript{82,83}. Together, microbial metabolism and the resultant metabolites contribute to the synthesis, digestion and absorption of nutrients\textsuperscript{84}, impact host inflammation and immunity\textsuperscript{85}, and influence the virulence of enteric pathogens\textsuperscript{83}. Metabolic interactions between bacteria also help to determine ecosystem stability, including the susceptibility of the microbial community to pathobiotic ‘blooms’ or to invasion by novel pathogens\textsuperscript{81}. Alterations in host diet and disease can further perturb this ecosystem and affect its functionality\textsuperscript{86}.

Under nutrient-restricted conditions, competition arises between the host and its intestinal microbiome, and amongst the members of this microbiome, for limited energetic resources. When lacking dietary alternatives, bacteria turn to host nutrient sources to fuel their metabolism. For example, bacterial foraging of the mucous layer is enhanced in mice lacking dietary fiber, and this degradation of the host mucous layer increases susceptibility to intestinal pathogens\textsuperscript{87}. Bacterial N-linked glycan metabolism is also altered in undernourished neonatal mice, pointing to changes in the microbial metabolism of mucus and other polysaccharides\textsuperscript{88,89}. One study found that undernourished children showed signatures of increased microbial proteolysis\textsuperscript{90}, another possible sign of bacterial starvation response. Further supporting the presence of altered microbial metabolism in undernutrition and EED, multiple studies have detected differences in
bile acids (host cholesterol derivatives that are extensively metabolized by members of the microbiota) in these populations.\textsuperscript{78,91–93}

Mucosal inflammation can also impact microbiota metabolism. The intestinal mucosa responds to infection and inflammation by increasing the production of reactive oxygen species, nitric oxide species, and oxidized sugars such as galactarate and glucarate\textsuperscript{94,95}, as well as by enhancing mucus shedding\textsuperscript{96}. These responses can, paradoxically, make growth substrates more available to pathogens that are adapted to take advantage of them\textsuperscript{94,95}. Pathogens may also be better adapted to take advantage of growth substrates released by the commensal microbiota during inflammatory dysbiosis\textsuperscript{97,98}. The inflamed gut is typically a more oxygenated environment, associated with blooms of facultative anaerobic \textit{Proteobacteria} at the expense of strict anaerobic bacteria. These facultative anaerobes carry a distinct metabolic repertoire that is often less beneficial to the host compared to anaerobic microbiota members\textsuperscript{75}. As noted previously, \textit{Proteobacteria} are more abundant in the stunted microbiota, a potential indication of this inflammatory oxidation of the gut environment, and suggestive of its reduced metabolic functionality. In children with severe acute malnutrition in Malawi, mortality was associated with lower circulating butyrate\textsuperscript{99}, a short chain fatty acid produced by strict anaerobes in the gut.

Thus, not only the composition of the microbiota, but also its metabolic output, are changed during undernutrition and EED. This may have important consequences for host health.

\subsection*{1.1.4 Microbial interventions for EED}

Antibiotics improve clinical outcomes in children recovering from severe acute malnutrition\textsuperscript{100}, and prophylactic antibiotic use has been shown to increase both weight gain and linear growth in children from low-income regions\textsuperscript{101}. However, administering prophylactic
antibiotics to human populations is highly undesirable, given that it may favour the emergence of antibiotic resistance\textsuperscript{101}. In the last five years, two large clinical trials were implemented that aimed to improve child growth by directly reducing fecal-oral contamination in early life\textsuperscript{102,103}. These randomized controlled trials introduced a number of household-level water, sanitation and hygiene (WASH) interventions such as handwashing stations, drinking water chlorination, and latrines, in combination with standard nutritional interventions. WASH interventions had high adherence, significantly reduced fecal parasite loads in two of the study sites\textsuperscript{104,105}, and reduced the prevalence of diarrhea and of respiratory illnesses at one study site\textsuperscript{105–108}. Disappointingly, however, WASH interventions did not reduce bacterial pathogen load in the household environment or in the children\textsuperscript{105,109} and had no measurable impact on linear growth at any study site\textsuperscript{106,110}. Baseline sanitation was nevertheless a highly significant risk factor for stunting in the same study populations\textsuperscript{110}. Given that enteropathogen levels remained high even in the WASH intervention arms, it remains impossible to conclude on the causal link between sanitation and child health\textsuperscript{105,110,111}. This has led to calls for radically more effective or “transformative” WASH interventions, including infrastructure interventions at the community rather than the household level\textsuperscript{110,111}.

In addition to preventative WASH measures, there is intense interest surrounding the potential of probiotics - live microorganisms with beneficial health effects - to ameliorate child stunting\textsuperscript{112}. A body of work from François Leulier and colleagues demonstrates that \textit{L. plantarum}, an intestinal commensal of \textit{Drosophila}, successfully mitigates the impact of host undernutrition in both flies and mice\textsuperscript{113–117}. An early trial with \textit{Lactobacillus} and \textit{Bifidobacterium} probiotics in undernourished children failed to show any benefit; however, this study did not test or measure the colonization and engraftment of their probiotic strains\textsuperscript{118}. A more recent clinical
trial for severe acute malnutrition found that *Lactobacillus* and *Bifidobacterium* probiotics decreased the incidence of diarrhea in undernourished children, but only in children where the strains stably colonized. Jeffrey Gordon and colleagues have also shown the benefit of several ‘non-traditional’ probiotics, such as *Akkermansia* and *Clostridiales* species, in reversing the effects of undernutrition and EED in mice. Although challenges remain in viably administering such bacteria to humans, these results suggest that diverse bacteria may have beneficial effects on host health.

Prebiotics are defined as food compounds that enhance the growth of probiotic microbes, and may be an effective and straightforward route for modulating the microbiota in humans. The presence of specific oligosaccharides in breastmilk, considered ‘natural’ early-life prebiotics for the microbiota, have been associated with improved growth outcomes in children via their impact on microbial growth. For older infants, “microbiota-directed” foods also induced promising signatures in acutely malnourished children, including reduced inflammatory markers, although these foods did not impact weight gain. A prebiotic food also improved oral vaccine responses in mice colonized with undernourished human microbiota. Synbiotics, which are combinations of pre- and probiotics, also show promise: a large, double-blind placebo-controlled trial in India found that synbiotic treatment with *L. plantarum* and fructo-oligosaccharides significantly reduced mortality from infectious diseases in the first 60 days of life. However, not all human prebiotic trials have been successful: an intervention trial that administered resistant-starch prebiotics to children in Malawi noted that, in contrast to the expected beneficial effects of the prebiotics, the intervention caused an increase in the fecal inflammatory marker calprotectin.
Taken together, the mixed evidence for the efficacy of microbial interventions in early life undernutrition is both exciting and cautionary. There is an urgent need to better understand the mechanisms by which intestinal microbes contribute to undernutrition, EED, and intestinal health, in order to design targeted interventions and successfully prevent the vicious cycle of EED and growth faltering in early life.

1.2 Host immunity during undernutrition and EED

Undernourished children are more susceptible to infectious diseases and mount dampened immune responses to oral vaccination. They nevertheless show heightened inflammatory responses at the intestinal mucosa, which may exacerbate intestinal damage and nutrient malabsorption. The early-life microbiota is essential for the development of productive immune responses. Reciprocally, the host immune system plays a critical role in shaping the microbiota and responding to microbial insults. An understanding of immune-microbial interactions in early life is therefore central to the understanding of intestinal homeostasis in undernutrition and EED.

1.2.1 Intestinal mucosa and EED

Physical barriers are one of the fundamental innate immune defenses of the intestine. Epithelial cells of the intestine are connected by tight junctions and form a mechanical barrier containing the microbiota within the lumen. Mature epithelial cells also consume oxygen at high rates, contributing to anaerobic conditions in the intestine that help to support microbial homeostasis. The epithelial layer folds into finger-like projections (villi) and invaginations (crypts), which serves to increase the overall surface area for nutrient absorption and provides microenvironments for stem cell growth and epithelial renewal. In addition, specialized goblet
cells secrete mucins, large glycoproteins that form the intestinal mucous layer and contribute to mechanical defense\textsuperscript{96}.

In the small intestine, the primary site of intestinal nutrient absorption, the mucous layer is loose and penetrable; this allows both nutrients and microbes to come into closer contact with the epithelium\textsuperscript{127}. In the colon, where microbial load is typically much higher, the mucous layer is divided in two: a loose outer layer that allows bacterial colonization, and a tight inner layer which excludes bacterial-sized particles and protects the epithelial surface from direct contact with the microbiota\textsuperscript{96}. Breakdown of the mucosal and epithelial barriers leaves the host susceptible to systemic microbial invasion.

Damage to the intestinal epithelium was one of the first features used to define EED. Early biopsy-based studies noted blunting of the small intestinal villi in asymptomatic people from low-income regions; this was coupled with functional malabsorption of xylose\textsuperscript{23,24,27}. Sugar absorption tests, such as the lactulose:mannitol test, are now often used to non-invasively test intestinal barrier function as a biomarker of EED\textsuperscript{26}. Intestinal permeability reported from these tests tends to be higher in children from low-income countries compared to healthy western populations, and are correlated with growth in several studies\textsuperscript{27,31,36,38}. Decreased barrier function in EED is also seen through the increased leak of proteins from serum to feces, as reflected in the fecal biomarker alpha-1-antitrypsin, and through reduction of total enterocyte mass, as reflected in the loss of enterocyte-secreted citrulline\textsuperscript{20,41}. Subjects with EED also show disruption in the epithelial expression of tight junction proteins and mucins\textsuperscript{20,41}. Thus, the intestinal mucosa is an important mechanical defense against the microbiota that is disrupted in EED.
1.2.2 Inflammatory responses and EED

In addition to forming a physical barrier between the epithelium and the microbiota, the intestinal mucosa is filled with immune factors, such as antimicrobial peptides, cytokines, and defensins, which control microbial growth and colonization\textsuperscript{128}. These factors are produced by epithelial cells and innate immune cells (such as mononuclear phagocytes, natural killer cells and innate lymphoid cells) upon recognition of microbial patterns via specific cell-surface receptors\textsuperscript{128,129}. Cytokines and chemokines, intercellular signaling proteins, are also released by stimulated host cells and help to recruit additional immune cells and contribute to the inflammatory milieu. In addition to stimulating adaptive immunity, local inflammatory responses can be directly microbiocidal, contributing to the clearance of intestinal infections\textsuperscript{128,130}.

Heightened inflammatory responses are a hallmark feature of EED. Fecal calprotectin and myeloperoxidase, both of which are produced by neutrophils and reflect innate immune activation, are inflammatory biomarkers of IBD which have also become central biomarkers in the study of EED\textsuperscript{19,20,49}. Intestinal cytokines are also elevated in EED, and activation of the adaptive immune system has been observed via T cell infiltration of the epithelium\textsuperscript{27,43}. Of all the features of EED, intestinal inflammation is one of the most consistent correlates with growth stunting\textsuperscript{49}.

It is not clear if inflammatory responses are beneficial or detrimental in the context of EED\textsuperscript{131,132}. Although inflammation is essential for defense against pathogens, it is also destructive to the host since chronic responses contribute to intestinal damage\textsuperscript{132}. In addition, immune cell activation is metabolically costly, and could contribute to further energy deficits in an undernourished host\textsuperscript{131,132}. Indeed, inflammatory mediators can directly suppress the insulin-like growth factor (IGF)-1 signaling axis, suggesting a direct link between inflammation and
child stunting\textsuperscript{131,133}. Nevertheless, the inflammation observed in EED may be a necessary immune response in the face of excessive and repeated microbial insults.

\textbf{1.2.3 Immunoglobulin A and EED}

Immunoglobulin A (IgA) is the main mucosal antibody and is the most abundant antibody isotype in humans overall; as such, it is one of the key adaptive immune effectors in the intestinal tract\textsuperscript{134}. Secreted as a protease-resistant dimer from specialized M cells in the epithelium, IgA acts at mucosal surfaces both to prevent infections, and to maintain homeostasis of the commensal microbiota. Its dual and context-dependent role makes it a fascinating and relevant player in the paradigm of the intestinal microbiota in human health\textsuperscript{134,135}.

IgA acts at mucosal surfaces from the first days of life, delivered through maternal breastmilk as well as produced endogenously in the infant gut\textsuperscript{136–138}. Its presence during this early window is critical to prevent overgrowth of \textit{Proteobacteria} and pathogens and to shape the establishment of a beneficial microbiota\textsuperscript{136,139–143}. As the microbiota matures, endogenously produced IgA targets the successive colonizers of the gut and the increasingly abundant \textit{Firmicutes} species\textsuperscript{137,138}, with a preferential binding of small intestinal and mucosal bacteria\textsuperscript{144–147}. At maturity, members of the \textit{Firmicutes} are among the most frequently IgA-recognized bacteria in healthy humans, while adults with secretory IgA deficiency show a persistent dysbiosis of the gut microbiota that includes overabundance of \textit{Proteobacteria} species and loss of specific \textit{Firmicutes}\textsuperscript{148–151}.

It is now a popular concept that IgA can both prevent and promote bacterial colonization, depending on the type and context of the interaction\textsuperscript{146,152}. The mechanisms by which IgA can provide both positive and negative selection of the microbiota are not fully understood, but modelling suggests that adhesion, intestinal fluid dynamics and bacterial growth rate help to
explain the context dependency of this interaction\textsuperscript{147,153}. In addition, beyond colonization success, IgA-binding affects bacterial gene expression and epitope production\textsuperscript{154–158}. These IgA-mediated bacterial responses affect broad functional properties of the microbiota, including motility and metabolism, thus allowing IgA to shape a beneficial microbiota at a molecular level\textsuperscript{156,159}. Overall, IgA has an important functional impact on bacterial colonization and phenotype (Fig 1.3).

Altered antibody-microbiota interactions occur during infections, as well as during chronic inflammatory conditions, and this has been extensively studied in the context of inflammatory bowel disease (IBD). Mucosal antibodies directed against intestinal bacteria are consistently increased in IBD patients\textsuperscript{160–165} and correlate with clinical disease severity\textsuperscript{164}. Ig-recognized bacteria in IBD patients include \textit{Proteobacteria} as well as a number of bacteria associated with oral cavities, including \textit{Gemella}, \textit{Streptococcus} and \textit{Haemophilus}\textsuperscript{162–164}. Further, Ig-recognized bacteria from IBD patients have been shown to penetrate the mucous layer of their hosts\textsuperscript{166} and to cause increased inflammation and damage when transferred into gnotobiotic mice\textsuperscript{162,163}.

The relevance of IgA to undernutrition remains poorly understood. Undernourished children mount weaker IgA responses to oral vaccines; indeed, it is a matter of serious public health concern that oral vaccinations are less effective in low- and middle-income countries compared to industrialized settings\textsuperscript{30,167}. However, acutely undernourished children appear to have normal or increased levels of fecal IgA and B cell populations at baseline\textsuperscript{168–170}. Anti-flagellin and anti-LPS IgA and IgG titres in serum correlate positively with child stunting and inflammation\textsuperscript{27,37}, and with the total burden of enteropathogens in feces\textsuperscript{171}. Furthermore, Kau \textit{et al.} found that acutely undernourished children had higher IgA-targeting of fecal
Enterobacteriaceae, but reduced IgA-targeting of other commensals such as Akkermansia\textsuperscript{59}. Isolates of IgA+ Enterobacteriaceae induced intestinal damage and weight loss when transferred into gnotobiotic mice, while IgA+ Akkermansia were protective\textsuperscript{59}. Together, these data suggest a broad dysbiosis in IgA-microbial interactions in the undernourished gut.

Excessive stimulation by intestinal pathogens may cause an exhausted mucosal immune response, explaining both the heightened baseline levels of IgA and the weakened vaccine responses\textsuperscript{30,45,46}. In mice, microbiota composition does impact IgA responses to oral vaccination\textsuperscript{123}. However, data for this axis in humans is conflicting\textsuperscript{167,172}, and the determinants of IgA-bacterial interactions in undernutrition remain poorly understood.
Figure 1.3 Functions of immunoglobulin A (IgA) in the gastrointestinal tract. IgA acts in a context-dependent manner to (1) prevent bacterial colonization, through the aggregation and exclusion of fast-growing bacteria; (2) promote bacterial colonization, through the facilitation of adhesion and biofilm formation in slow-growing bacteria and (3) directly alter bacterial gene expression and functionality, for example the down-regulation of bacterial flagellar genes.
1.2.4 Immune interventions for undernutrition and EED

It is not known whether immune responses in undernutrition and EED are appropriate or inappropriate for the intestinal environment. Hypothesizing that the intestinal inflammation is excessive and pathological, as is the case for IBD, one trial gave children with severe acute malnutrition mesalazine, a mild anti-inflammatory drug used to treat IBD\textsuperscript{173}. This drug was safe but did not cause a significant reduction in inflammatory markers, although the study was not powered to detect small differences. A “hypoallergenic” therapeutic food which has been successful in treating Crohn’s disease patients also did not perform any better than standard therapeutic foods in children recovering from SAM, and did not reduce inflammatory biomarkers\textsuperscript{174}. An ongoing trial will test additional immunomodulatory interventions, including an anti-inflammatory drug budesonide which acts in the small intestine rather than the colon and is more potent than mesalazine\textsuperscript{175}.

Trials that have attempted to stimulate the early life immune system have also achieved mixed success. Educational interventions to promote breastfeeding reduce infectious disease in infants, but do not affect child stunting\textsuperscript{13}. A trial that supplemented infants with lysozyme and lactoferrin, two protective immune factors found in breast milk, found promising signs, with improvements in a marker of intestinal permeability and reduced rates of hospitalization and acute undernutrition\textsuperscript{176}. In addition to testing budesonide, Kelly \textit{et al.} (2019) will trial bovine colostrum, which as an early proteinaceous form of maternal milk is rich in immune factors\textsuperscript{175}.

No microbial or immunomodulatory intervention has had any measurable impact on the effectiveness of oral vaccination in low-income settings\textsuperscript{167}. Methods to improve oral vaccination success rates in undernourished populations are urgently needed and would have significant impact on global health. Taken together, the mixed efficacy of immune-intervention trials
highlight our need to better understand the mechanisms of immune-microbial interaction during undernutrition.

1.3 Research Objectives

As outlined above, there is substantial evidence for the involvement of the intestinal microbiota in child undernutrition. However, intervention trials have yielded conflicting and often negative results, and the mechanisms by which bacteria contribute to child stunting remain unclear. A better understanding of microbiota activity and immune interactions during child undernutrition is critical for the effective treatment of this global health challenge.

My doctoral work has focused on the mechanisms by which intestinal bacteria interact with one another and with the host immune system during early life undernutrition and EED. I hypothesized that dietary undernutrition and fecal-oral contamination alter (a) immune-microbiota interactions and (b) metabolic interactions between bacteria, with consequences for intestinal health.

To better understand host-microbial dynamics in undernutrition, I have studied the interactions between mucosal antibodies and gut bacteria in a mouse model of undernutrition and fecal-oral exposure (Chapter 2) and in two populations of stunted children through the Afribiota project (Chapter 3). To better understand the growth of pathobiotic communities in undernutrition, I have further explored inter-bacterial interactions among key pathobionts under different nutritional environments (Chapter 4). Together this research aims to clarify the mechanisms by which host-bacterial and bacterial-bacterial interactions develop during dietary undernutrition. Improved understanding of microbial contributions to undernutrition could help inform better treatments for this persistent global health challenge.
Chapter 2: Commensal bacteria modulate immunoglobulin A binding in response to host nutrition

2.1 Synopsis

IgA controls host-microbial homeostasis in the gut. IgA recognition of beneficial bacteria is decreased in acutely undernourished children, but the factors driving these changes in IgA-targeting are unknown. To understand how nutrition impacts immune-microbe interactions, we used a mouse model of undernutrition with or without fecal-oral exposure and assessed IgA-bacterial targeting from weaning to adulthood. In contrast to healthy control mice, undernourished mice failed to develop IgA recognition of intestinal *Lactobacillus*. Glycan-mediated interactions between *Lactobacillus* and host antibody were lost in undernourished mice due to rapid bacterial adaptation. This adaptation occurred in direct response to nutritional pressure, independently of host IgA, and was associated with reduced mucosal colonization and with bacterial mutations in carbohydrate processing genes. Together these data indicate that diet-driven bacterial adaptations shape IgA recognition in the gut.

2.2 Introduction

The mucosal antibody IgA is crucial for the maintenance of host-bacterial homeostasis in the mammalian gut. IgA preferentially binds to small intestinal bacteria and to microbes that interact closely with the host mucosa, and appears to play a dual and context-dependent role of excluding pathogens while promoting the colonization of commensals. IgA-bacterial targeting is altered in patients with inflammatory bowel disease and in children.
with severe protein undernutrition\textsuperscript{59}, and these IgA-targeted bacteria have an outsized influence on intestinal health\textsuperscript{59,162,163}. However, our understanding of IgA-microbe interactions contains critical gaps, including the specific immune or microbial factors which drive altered IgA-binding during dysbiosis, and the impact of IgA on bacterial physiology\textsuperscript{177}. Intestinal IgA may impact motility and other virulence factors in Proteobacteria\textsuperscript{156,158}, while studies in \textit{Bacteroides} sp. suggest that IgA enhances adhesion and colonization of commensals in the intestinal mucosa, in addition to altering bacterial gene expression and metabolism\textsuperscript{154,159,178,179}. However, it is not clear how these results extend to other intestinal commensals, particularly the highly IgA-recognized Firmicutes\textsuperscript{148,180}. Given the crucial role of IgA in host-bacterial homeostasis, there is a need to better understand these factors during both health and intestinal disturbance.

Measuring the IgA-targeting of bacteria provides an attractive technique for uncovering dysbiotic host-microbe interactions in undernutrition, particularly given the pivotal role of the small intestine in both IgA production and bacterial targeting, and in nutrient absorption and EED\textsuperscript{19,49,127,181,182}. In a small cohort of severely undernourished children, IgA responses were skewed towards decreased recognition of beneficial bacteria and increased recognition of pathobionts \textsuperscript{59}. However, it is not clear whether this was due to differential colonization of bacterial strains, altered host barrier function, or altered host immune responses.

To better understand the development of host-bacterial interactions in undernutrition and intestinal dysbiosis, we measured IgA-targeting of bacteria in undernourished mice from weaning to adulthood, with or without exposure to a pathobiotic bacterial cocktail which has been shown to induce features of EED\textsuperscript{78} and which contains several species identified as IgA-targeted in undernourished children\textsuperscript{59}. We demonstrate that dietary undernutrition alone impacts immune recognition of the microbiota, independently of fecal-oral bacterial exposure. The IgA
recognition of several commensals known to benefit the host, including *Lactobacillus*, is dramatically reduced in undernourished mice. Unexpectedly, this loss of interaction is driven by rapid and reproducible bacterial adaption to limited nutrient availability which alters glycan-mediated antibody interactions. These data provide important mechanistic insights into the dynamics of IgA-bacterial interactions during disease. Moreover, they reinforce the importance of nutritional pressure in host-*Lactobacillus* interactions, a salient point given the current interest in *Lactobacillus* probiotics for the treatment of child undernutrition\textsuperscript{116,119}.

### 2.3 Materials and Methods

#### 2.3.1 Mouse strains and model of undernutrition

All mice were maintained at the University of British Columbia Modified Barrier Facility on a 12-hour light-dark cycle according to Animal Care Protocols A18-0279, A17-0264, and A15-0236. Mouse models of undernutrition and environmental enteric dysfunction were performed as described previously\textsuperscript{78}. Three-week-old female C57BL/6J mice from Jackson were placed on a control (CON) diet (20% protein, 15% fat, 65% carbohydrate; D09051102 from Research Diets) or low-protein, low-fat isocaloric (MAL) diet (7% protein, 5% fat, 88% carbohydrate; D14071001 from Research Diets). Mice were randomly assigned to dietary groups at the beginning of each experiment, while attempting to maintain similar average starting weight of mice between CON and MAL groups. Mice were housed in groups of 4 or 5 per cage and at least two cages of mice included per group in each experiment to account for cage effects. At 5 weeks of age, a subset of undernourished mice (MAL-b group) were gavaged three times, once every two days, with a defined mixture of *Bacteroidetes* and *E. coli* strains (*Bacteroides fragilis* 3/1/12, *B. vulgatus* 3/1/40, *B. ovatus* 3/8/47, *B. dorei* 9/1/42, *Parabacteroides distasonis*
2/1/33B, *E. coli* 3/2/53 and *E. coli* 4/1/47) at 10⁹ CFU/ml in reduced PBS (PBS containing 0.05% w/v L-cysteine). Mice were sacrificed at 7 weeks of age. During the dietary reversal experiment, mice were switched at 7 weeks of age onto the opposite model (CON or MAL-b) for an additional four weeks and were sacrificed at 12 weeks of age.

For Rag2-/- experiments, both wild-type and Rag2-/- female mice were obtained at 4 weeks of age (due to reduced availability of this background compared to wild-type mice). Upon arrival, mice were cohoused for four to five days on regular chow to normalize the starting microbiota between genotypes (2 wild-type and 2 Rag2-/- mice per cage). Mice were then separated into their experimental groups (4 mice of the same genotype per cage) and fed either a CON or MAL diet for three weeks. Mice were sacrificed at 7.5 weeks of age.

For the germ-free experiment, adult (5-6 months old) male C57BL/6J mice were obtained from an in-house colony at the University of British Columbia Centre For Disease Modeling. The choice of adult male mice for this experiment was limited by sample availability but we do not feel these variables are likely to have an important influence, given that adult mice in the reversal experiment showed comparable phenotypes to young mice and that we also observe similar undernutrition phenotypes in male conventional mice as in female (unpublished observation). Three cages of mice were placed on an autoclaved CON diet (n=2 mice per cage; total n=6) within a single germ-free isolator and two cages of mice were placed on an autoclaved MAL diet (n=4 mice per cage; total n=8) within a second isolator. All germ-free mice were monocolonized with a strain of high-IgA-binding *L. johnsonni* isolated from a young control mouse (strain Wk3C3AF; see Table A.2.3). Oral gavage of this strain was prepared from an overnight culture that was resuspended in reduced PBS at 10⁸ CFU/mL. Monocolonized mice
were sacrificed after three weeks on a CON or MAL diet and we confirmed by plating that they were not colonized with bacteria other than *Lactobacillus* at endpoint.

Small intestinal samples from mice monocolonized with murine norovirus were a gift from Dr. Lisa Osborne at the University of British Columbia.

### 2.3.2 Bacterial strains and culture

Strains used in the MAL-b gavage were described previously (see section 2.3.1)\(^78\) and were grown anaerobically on Fastidious Anaerobe Agar (LAB090) at 37\(^\circ\)C. *Lactobacillus* strains were isolated and grown in De Man, Rogosa and Sharpe (MRS) selective media (Difco 288130), under anaerobic conditions for solid media and 5\% CO\(_2\) for liquid media at 37\(^\circ\)C. A modified nutrient-poor MRS broth was additionally used for growth of *Lactobacillus* where indicated, in which case both the rich (CON) and modified (MAL) MRS broths were prepared as described in Table A.2.1. MRS media was stored at 4\(^\circ\)C in the dark for up to one month.

*Lactobacillus* isolates used in this study are described in detail in Table A.2.3.

### 2.3.3 HT-29 MTX cell culture

HT29-MTX cells (human colonic, mucus-producing) were obtained directly from the European Collection of Authenticated Cell Cultures (Cat#12040401). Cells were grown routinely in DMEM supplemented with 10\% v/v heat-inactivated fetal bovine serum (FBS), 1\% v/v non-essential amino acids (Gibco 11140-050), 1\% v/v Glutamax (Gibco 35050061) and 100 U/mL PenStrep (Gibco 15140122) in T-25 or T-75 flasks at 37\(^\circ\)C and 5\% CO\(_2\). Cells were split at approximately a 1:3 dilution every 2-3 days depending on their health and confluency, using 0.25\% trypsin. After at least 4 routine passages, healthy HT29-MTX cells were seeded into a 96-well plate at 25 000 cells/well (1.25 \(\times\) 10\(^5\) cells/mL) in the modified DMEM described above.
Cells were allowed to differentiate for 1 week as described previously\textsuperscript{178} to induce mucus production, and media was changed 2-3 times throughout the week.

2.3.4 IgA-Sequencing

IgA-sequencing (IgA-SEQ), also known as “Bug FACS”, was performed as described previously\textsuperscript{59,162}. Intestinal samples were homogenized in PBS (HyClone DPBS-/-, SH30028.02) and spun gently to settle debris; intestinal bacteria were then filtered through a 0.7 µm filter, washed in FACS buffer (PBS containing 1% bovine serum albumin), blocked for 20 minutes in FACS buffer containing 10% fetal bovine serum, and stained with anti-mouse IgA-PE (eBioscience 12-4204-81, 1:25 dilution) for 30 minutes in the dark. Samples were washed twice, stained with SYTO-BC (Invitrogen S-34855, 1:4000 dilution) for bacterial DNA, washed again and sorted by flow cytometry. Isotype controls (Rat IgG1 kappa Pe, eBioscience 12-4301-82) and feces from Rag\textsuperscript{-/-} (IgA-deficient) mice were used as negative controls to set gates. A minimum of 50 000 events were collected in the IgA+ and IgA- fractions, and the sorted and pre-sorted fractions were frozen at -20ºC.

Pre-sorted and sorted bacterial suspensions were boiled for 15 min at 100ºC and 2 µL of lysate was used as template for 16S PCR, using Illumina-tagged and barcoded primers specific for the 16S V4 region\textsuperscript{183}. Reactions were run on a gel to ensure successful amplification, and were purified and normalized using the 96well Sequal-Prep kit (ThermoFisher A1051001). All reactions were subsequently pooled and gel extracted (GeneJet K0692) to remover primer-dimers. Sequencing was performed on an Illumina MiSeq using a v2 kit for 2x250 bp reads with 30% PhiX.
2.3.5 Bioinformatics analysis of 16S rRNA data

Demultiplexed forward reads were analyzed in QIIME2\textsuperscript{184}, using the Deblur option\textsuperscript{185} for sequence quality control and trimming to 250 bp. Taxonomic assignment was performed using the Greengenes database\textsuperscript{186} version 13-8-99-515-806. Taxa were binned at the species level and further filtering was then performed in R to remove known contaminants (Archaea, mitochondria and chloroplast, as well as the bacterial families Halomonadaceae and Shewanellaceae, as these last two are not associated with mammalian hosts and were abundant in sequenced PCR blanks) and to remove taxa with a low fractional abundance (<0.005% of all sequenced reads), resulting in 61 identified species. Downstream analysis and visualization was performed in R using phyloseq\textsuperscript{187} and ggplot2\textsuperscript{188}. For sorted samples, a log-adjusted IgA index was calculated as described previously\textsuperscript{59,137}:

\[ I_{\text{Aindex}} = -\frac{\log(I_gA^+\text{taxon}) - \log(I_gA^-\text{taxon})}{\log(I_gA^+\text{taxon}) + \log(I_gA^-\text{taxon})} \]

2.3.6 MACS-Sorting

To confirm IgA-sequencing results, samples were sorted by magnetic beads using magnetic activated cell sorting (MACS) as described previously\textsuperscript{162} with anti-PE microbeads and LS columns (Miltenyi 130-105-639 and 130-042-401), into an IgA-enriched and IgA-depleted fraction. DNA was extracted from these fractions using traditional phenol-chloroform extraction, and purified using a PCR purification kit (GeneJet K0702).

2.3.7 Bacterial-specific qPCR

\textit{Lactobacillus}-specific qPCR primers (Table A.2.2) were designed based on the \textit{Lactobacillus} amplicon sequence variant (ASV) of interest in the IgA-SEQ dataset using NCBI Primer-BLAST. Primer specificity to taxa of interest was confirmed using SILVA TestPrime (https://www.arb-silva.de/search/testprime/). MACS-sorted samples were amplified by qPCR
using primers for *L. johnsonii/gasseri* 16S and for total eubacterial 16S (Table A.2.2), and quantified using a standard curve of purified *L. gasseri* ATCC 33323 DNA. Quantification of Enterobacterial DNA was performed on small intestinal tissue using primers for *Enterobacteriaceae* (Table A.2.2) and a standard curve of purified *E. coli* DH10B DNA.

**2.3.8 Sanger sequencing**

For traditional Sanger sequencing, colony lysate or purified DNA was PCR-amplified (see Table A.2.2 for a list of primers), and the PCR product was purified (GeneJet K0702). Sequencing was performed commercially by Genewiz.

**2.3.9 Total IgA**

To process samples for detection of free IgA, a fecal pellet from each mouse was weighed and homogenized in 1 mL PBS. Content of the jejunum was carefully squeezed out with forceps, and the remaining tissue was sliced longitudinally and washed in a petri dish of sterile PBS; small intestinal content and tissue were then weighed and suspended in 1 mL PBS. Feces and small intestinal content were homogenized by vigorous vortexing and with the aid of a pipette tip to mechanically disperse sample chunks in feces. Small intestinal tissue was homogenized with the aid of a tungsten bead in a Mixer Mill tissue homogenizer (1/25s for 2 min). The supernatant was separated from bacteria and other debris by centrifugation (20 min at 16,000xg) and frozen at -70°C prior to analysis. Total IgA was measured using a Ready-Set-Go! IgA ELISA kit (eBioscence 88-50450-22) as per the manufacturer’s instructions using a sample dilution of 1:500-1:1000.

**2.3.10 Lactobacillus-specific IgA ELISA**

A *Lactobacillus*-specific IgA ELISA was developed. Culture conditions, coating conditions, and antibody dilutions were optimized to obtain the best signal without saturation for
Lactobacillus isolates. To obtain active, epitope-expressing bacteria, overnight cultures of *Lactobacillus* were subcultured in MRS broth (1/100 dilution) for 5 hours in a 5% CO₂ incubator at 37°C, until an O.D. of ~0.5. *Lactobacillus* subcultures were washed and normalized to O.D. 0.45 in a sodium acetate buffer (pH 5.5), and 50 µL of bacterial suspension was used to coat each well (4°C overnight). The ELISA plate was then blocked (PBS with 1% BSA, 2h at room temperature), and incubated with IgA-containing intestinal supernatants (obtained from small intestinal content as per total IgA measurements, above) normalized to 10 µg/mL of total content or approximately 1 µg/mL total protein. Alternatively, to detect anti-*Lactobacillus* antibody in serum, mouse serum was used in place of intestinal content at a series of 1:2 dilutions; the 1:100 dilution was ultimately chosen for detection of IgA, and 1:200 dilution for detection of IgG. Negative control wells using *Lactobacillus* isolates but no host antibody were used on every plate to assess background. Bound IgA was detected with a goat anti-mouse IgA-HRP (Abcam ab97235, 1/10 000 dilution), or bound IgG with a goat anti-mouse IgG-HRP (Jackson Immunoresearch, 1/4000 dilution) for 1 hour at room temperature. Plates were developed with TMB solution (BD 555214), stopped with 2N H₂SO₄, and read at 450 nm minus a background of 570 nm. Unless otherwise indicated, in graphs of ELISA results, each data point represents the average O.D. of an individual *Lactobacillus* isolate exposed to pooled IgA from 4-8 different mice, minus the background O.D. of *Lactobacillus* in the absence of IgA.

2.3.11 Light microscopy

Overnight cultures of *Lactobacillus* were subcultured as described for ELISA, above. 100 µL of subculture seeded into 8-well microscopy chamber slides (Lab-Tek™ II) and incubated with 1:50 dilution of IgA-containing intestinal supernatants in 100 µL of MRS for 30 min at
37°C and 5% CO2. After the incubation time, slides were analyzed using a Phase Contrast Inverted Microscope with a 40x objective.

2.3.12 Quantification of adherent bacteria in vivo

To quantify tissue-adherent bacteria, content of the jejunum was carefully squeezed out with forceps, and the remaining tissue was sliced longitudinally and washed in a petri dish of sterile PBS. Samples were processed on ice and tools were flamed in ethanol between each sample. Content was weighed and homogenized in 1 mL of PBS by vigorous vortexing; tissue was weighed and homogenized in 1 mL of PBS with a sterile tungsten bead using a FastPrep machine (2x 1 min at setting 5.5). Bacterial colonization of homogenized samples was then quantified by CFU plating onto selective media (MRS), or by bacterial-specific qPCR as described above, and normalized to sample weight.

2.3.13 Adhesion to mucus-producing cells in vitro

For mucosal adhesion assays, Lactobacillus was coated with intestinal IgA by incubating log-phase bacteria (5 hour sub-culture to an O.D. of ~0.5) for 1h at room temperature with small intestinal supernatants diluted 1/100 in PBS-BSA. Then as modified from Donaldson et al.178, mixes were added to differentiated, PBS-washed HT29-MTX cells in DMEM for 2 hours at 37°C and 5% CO2. After incubation, cells were washed twice with PBS, trypsinized with 50 µL of 0.25% trypsin for 15-20 minutes to lift cells and adherent bacteria, and vigorously resuspended in FBS. Lifted cells were plated onto MRS agar for colony enumeration. Data from four separate wells (technical replicates) were averaged into a single data point for each biological replicate.

2.3.14 In vitro evolution of Lactobacillus

For each in vitro evolution experiment, one Lactobacillus isolate was grown overnight in rich MRS broth and used to inoculate multiple tubes of CON-MRS or MAL-MRS (see media
recipie in Table A.2.1). All inoculations and passages were done at 1/1000 (2 µL per 2 mL media) and bacteria were grown at 37°C and 5% CO₂. Bacteria were passaged every 24h into a fresh tube for a total of 1-3 weeks, and glycerol stocks were saved every 2-4 days for subsequent phenotypic and genotypic analysis of the strains. This experiment was repeated three times with three different high-IgA-binding isolates, and twice with two different low-IgA-binding isolates, using 3-4 independently passaged tubes per media per experiment. Ancestral strains are indicated in Table A.2.3.

2.3.15 Whole Genome Sequencing

Library preparation and sequencing for whole genome analysis was performed commercially by Genewiz according to a standardized pipeline. Briefly, genomic DNA extracted from Lactobacillus isolates (using a genomic DNA extraction kit, GeneJet K0722) was fragmented, ligated to Illumina adaptors, and sequenced on a MiSeq platform (2x 150bp paired-end reads).

2.3.16 Bioinformatics analysis of whole genome sequencing data

Analysis was performed in-house: resultant sequences were filtered and trimmed for quality and adaptors using Trimmomatic189, and run through Spades error correction190. The optimal reference genome was determined using PyParanoid191 and FastTree192 to determine which of the available sequenced Lactobacillus johnsonii genomes was most closely related to our isolates. Cleaned reads were then aligned to the chosen reference (Lactobacillus johnsonii Byun-jo GCA_003316915.1)193 using Bowtie2 (Langmead and Salzberg, 2012), and single nucleotide variants (SNVs) were called with the Samtools pipeline195. Low-confidence SNVs were filtered out if the phred-adjusted quality score was <100, the sequencing depth <10, or it was ≤ 3 base pairs from an INDEL. The impact of SNVs on coding regions with respect to the
reference genome was determined using snpEff\textsuperscript{196}. In order to identify genetic variants which distinguished isolates, pairwise comparisons were made between CON and MAL-b isolates within each biological experiment (cross-sectional approach). A longitudinal analysis was further performed by comparing each strain back to an ancestral isolate prior to dietary intervention. To validate the filtration approaches used, several mutations of interest were additionally confirmed by Sanger sequencing (see Table A.2.2 for a list of primers and genes).

2.3.17 N-glycosidase assays and sugar adsorption

To remove glycans, intestinal IgA was incubated with N-glycosidase F (Sigma #11365169001) at 0, 0.1, 1 or 10 U/mL for 4 h at 37°C with gentle agitation. Glycan-stripped IgA was then added to the ELISA plate (which had been blocked, as normal, with 1% PBS-BSA) and the \textit{Lactobacillus}-specific IgA ELISA assay was continued as described above. Alternatively, \textit{Lactobacillus}-coated ELISA plates were incubated with N-glycosidase at 0, 0.1, 1 or 10 U/mL, or with carbohydrate solutions at 5% w/v, for 4 h at 37°C without agitation, prior to the regular blocking step with 1% PBS-BSA. ELISA assays were then finished normally as described above.

2.3.18 Statistical Analysis

Statistical analysis was performed in GraphPad Prism (\url{www.graphpad.com}) or in RStudio (\url{https://www.rstudio.com/}). Unless otherwise stated, analysis was performed using a Mann-Whitney test to compare two groups and ANOVA for more than one group, with post-hoc Sidak’s or Tukey’s test for multiple comparisons as appropriate. Aggregate results represent the mean +/- SEM, and statistical significance is represented by \( \ast p \) value <0.05, \( \ast \ast p \) value <0.01, \( \ast \ast \ast p \) value <0.001 and \( \ast \ast \ast \ast p \) value <0.0001. Sample size, center and dispersion metrics, and statistical tests are also reported in the figure legends. R code is attached in File S1.
2.4 Results

2.4.1 IgA-targeting of Lactobacillus is lost in undernutrition

Mice were placed on a control diet (CON), isocaloric low-protein, low-fat undernourished diet (MAL), or undernourished diet with bacterial gavage to mimic EED etiology (MAL-b), and sampled every two weeks for total and IgA-targeted microbiota and for total intestinal IgA (Fig 2.1A). As expected, diet-dependent differences in the total microbiota were observed, and MAL-b mice had substantial blooms of Bacteroides and Parabacteroides in the colon as a result of their bacterial exposure (Supplemental Fig A.2.1 A-D).

Newly weaned three-week-old mice had low levels of total fecal IgA, which increased with age regardless of diet or bacterial gavage (Fig 2.1B). Flow cytometry sorting of IgA-positive bacteria (Supplemental Fig A.2.1E) indicated that as mice aged, increases in total IgA levels were accompanied by increased proportions of IgA-positive fecal bacteria by flow cytometry (Fig 2.1C), increased alpha-diversity of the IgA-positive sorted fraction (Supplemental Fig A.2.2A-B), and by the development of IgA responses to specific bacterial taxa (Fig 2.1D). Overall, mice on a CON or MAL diet shared similar patterns of IgA-bacterial targeting, including the development of IgA responses to Akkermansia and Ruminococcus and a persistent depletion in IgA responses to Lactococcus (Fig 2.1D, Supplemental Fig A.2.2C-D). Importantly, and as described previously, the IgA-targeting of a taxon had no correlation with its relative abundance within the microbiota (Supplemental Fig A.2.2E), and therefore provided new insights not offered by traditional microbiota screens. Instead, IgA-targeting of taxa in the small intestine correlated with targeting of the same taxa in the feces (Supplemental Fig A.2.2F), and PCoA separation of fecal and small intestinal microbiota was observed in unsorted, but not in
IgA-sorted, fractions (Supplemental Fig A.2.2G), consistent with the existing hypothesis that IgA-targeting originates mainly in the small intestine and is merely reflected in the feces\textsuperscript{144}.

Despite overall similarities in the IgA-targeting patterns of CON, MAL and MAL-b mice, multiple interactions were significantly altered by host nutritional status (Supplemental Fig A.2.3A-B). Contrary to our expectations, MAL-b mice did not display increased IgA-targeting of the bacterial gavage members (\textit{Bacteroides, Parabacteroides} and \textit{Enterobacteriaceae}), although blooms of these bacteria were detected in both the large and small intestine of MAL-b mice two weeks after gavage (Supplemental Fig A.2.3C-D). Instead, MAL and MAL-b mice appeared to have decreased IgA-targeting of a number of beneficial commensals, including \textit{Lactobacillus}, \textit{Bifidobacterium} and \textit{Eubacterium}.

Notably, loss of \textit{Lactobacillus} IgA-targeting was both statistically and biologically consistent. \textit{Lactobacillus} was IgA-targeted in healthy adult BL/6 mice (Fig 2.1D), in agreement with previous reports\textsuperscript{162}, and this response increased over time concordantly with the increase in total IgA (Fig 2.1D-E). However, IgA recognition of \textit{Lactobacillus} failed to develop in mice fed a MAL diet, and IgA-targeting of \textit{Lactobacillus} was significantly decreased in both the jejunum and colon of adult MAL and MAL-b mice (Fig 2.1E-F, Supplemental Fig A.2.3A-B). IgA-targeting was specific to a single, dominant amplicon sequence variant (ASV) of \textit{Lactobacillus} sp. which was most closely related to the \textit{L. johnsonii/gasseri} cluster by 16S identity. Differential IgA-targeting of \textit{Lactobacillus} was still observed when samples were re-processed using magnetic activated cell sorting (MACS) and probed by qPCR using taxa-specific primers (Supplemental Fig A.2.3E-F). Together, these data demonstrate that host diet influences IgA-bacterial interactions, and that canonical IgA-\textit{Lactobacillus} interactions fail to develop in undernourished mice.
Figure 2.1 IgA-targeting of Lactobacillus is lost in undernutrition. (A) Schematic of experimental approach, as described in Methods. Wk, Week. (B) Concentration of free fecal IgA in CON, MAL and MAL-b mice at 3 weeks (n=8), 5 weeks (n=8) and 7 weeks of age (n=3-4). Significance is relative to the same group at week 3, by two-way ANOVA with post-hoc Tukey’s test. (C) Percentage of IgA-positive bacteria by flow cytometry (n=4-6). (D) Bubble plot of IgA-targeted taxa of CON, MAL and MAL-b mice, in the feces at weeks 3, 5 and 7, and in the jejunum at week 7 (Week7j, highlighted in grey). Colour of circles indicates the direction of IgA-targeting (red=positively targeted, blue=negatively targeted), and saturation of the colour indicates the degree of significant difference from zero, as determined by a one-sided Wilcoxon test. The size of the circle indicates overall effect size as measured by average IgA Index. The formula for IgA Index is also given; RelAbun, the relative abundance of a taxon in the sorted fraction. (E-F) IgA Index to Lactobacillus sp. as measured by IgA-SEQ in the feces (E) and jejunum (F) (n=8, two-way ANOVA with post-hoc Tukey’s test). Data are represented as mean ± SEM.
2.4.2 Lactobacillus from undernourished mice have reduced antibody-binding ability

IgA-bacterial interactions are influenced by both host and microbial factors\textsuperscript{177}. As noted previously, the IgA-targeting of a taxon had no correlation to its relative abundance in the total microbiota (Supplemental Fig A.2E), and there was no difference in the relative abundance of Lactobacillus by diet in either the fecal or the small intestinal microbiota (Fig 2.2A). When Lactobacillus was isolated from the CON, MAL or MAL-b intestine, genotyping by 16S and additional housekeeping genes indicated that all culturable strains belonged to the L. johnsonii/gasseri cluster identified in the IgA-SEQ dataset and were most closely related to L. johnsonii (data not shown). There was no difference in total fecal IgA by diet over time (Fig 2.1A), or in small intestinal IgA in either the luminal content or tissue of the jejunum (Fig 2.2B). These data suggest that the loss of IgA-Lactobacillus interactions in undernutrition is not due to loss of total IgA or total Lactobacillus.

As there was no difference in total Lactobacillus or IgA levels between dietary groups, we next investigated whether IgA repertoire specificity could explain the observed differences in IgA-targeting. A customized ELISA protocol was developed to assay Lactobacillus-IgA interactions, using various combinations of Lactobacillus isolates and intestinal IgA taken from mice with high (CON) or low (MAL-b) IgA-Lactobacillus interactions (Fig 2.2C). This revealed that both the source of Lactobacillus and the source of IgA had a significant influence on IgA-Lactobacillus interactions. Intestinal IgA from healthy CON mice had slightly higher affinity for Lactobacillus than IgA from MAL-b mice (Fig 2.2D). However, more strikingly, Lactobacillus isolated from MAL-b mice was significantly less able to bind IgA, regardless of the source (Fig 2.2D), suggesting that Lactobacillus itself had adapted for IgA evasion. This low-binding phenotype extended to recognition by other immunoglobulins, as MAL-b Lactobacillus also
displayed reduced binding of serum IgA and IgG from both mouse groups (Fig 2.2E-F). Further, the antibody specificity was irrelevant; intestinal IgA from murine norovirus-monocolonized mice, which had never been exposed to *Lactobacillus* or indeed to any bacterial antigens, showed an identical strain-specific binding profile to *Lactobacillus* (Fig 2.2G). Loss of IgA-*Lactobacillus* interactions in undernourished mice is therefore driven by broad bacterial antibody-binding properties.
**Figure 2.2 Lactobacillus from undernourished mice have reduced antibody-binding ability.** (A) Relative abundance of unsorted Lactobacillus in the feces and jejunum (n=8, NS by Kruskal-Wallis). (B) Concentration of free IgA in the luminal (n=10) and mucosal (n=5) compartments of the jejunum (NS by two-way ANOVA). (C) Schematic of custom ELISA to assay Lactobacillus-IgA interactions. (D) Binding of Lactobacillus and free IgA from the small intestinal content of CON or MAL-b mice (n=17-21 isolates; two-way ANOVA with post-hoc Sidak’s test). (E-F) Recognition of Lactobacillus isolates by (E) serum IgA and (F) serum IgG. Each data point represents the average binding of a single isolate to serum from CON and MAL-b mice. (G) Recognition of Lactobacillus isolates by intestinal IgA from mice monocolonized with murine norovirus. Significance determined by Mann-Whitney (E-G). Data are represented as mean ± SEM. Lactobacillus isolates used in Fig E-G are the core isolates designated in Table S3. Lacto, Lactobacillus.
2.4.3 IgA-binding induces aggregation of Lactobacillus but has minimal impact on mucosal adhesion

IgA-bacterial interactions have been reported to mediate aggregation, adhesion, and colonization of bacteria within the intestinal mucosa.\textsuperscript{178,197} In vitro, IgA-binding of *Lactobacillus* was positively correlated with the clumping of bacterial cells during flow cytometry (Fig 2.3A). Further, exposure to IgA caused a measurable decrease in CFU counts that was rescuable by bead-beating dispersal of cells (Fig 2.3B-C), and this was observed to a greater extent in isolates with high IgA-binding ability. Light microscopy confirmed that extensive clumping occurred in high-binding isolates in the presence of IgA, compared to a more limited clumping in low-binding isolates (Supplemental Fig A.2.4A). IgA recognition therefore impacts aggregation of *Lactobacillus*, consistent with traditional antibody function.

Moreover, we observed that *Lactobacillus* was more adherent to the small intestinal mucosa of CON mice compared to MAL or MAL-b mice \textit{in vivo} (Fig 2.3D, Supplemental Fig A.2.4B). In contrast, the mucosal bacterium \textit{Akkermansia muciniphila} remained strongly IgA-targeted during undernutrition (Fig 2.1D, Supplemental Fig A.2.2D) and did not display any dietary defect in small intestinal adhesion (Supplemental Fig A.2.4C). We therefore assayed the mucosal-binding ability of *Lactobacillus in vitro*. In the absence of IgA, high- and low-binding isolates appeared to adhere equally well to the mucus-producing cell line HT29-MTX (Supplemental Fig A.2.4D). Upon the addition of IgA, however, high-IgA-binding strains showed enhanced adhesion to mucus-producing cells, while low-IgA-binding strains were minimally affected (Fig 2.3E). IgA can thus directly enhance the mucosal binding of *Lactobacillus in vitro*. To determine the \textit{in vivo} impact of IgA binding on *Lactobacillus* adhesion, we next examined Rag2-/- mice, which lack an adaptive immune system and do not
produce IgA. Rag2-/- mice were fed a CON and MAL diet for three weeks in parallel with the wild-type groups, and the abundance of *Lactobacillus* within the lumen and tissue of the jejunum was measured. Contrary to our predictions, and in contrast to published results in *Bacteroides*\textsuperscript{178}, the adhesion profiles of *Lactobacillus* in Rag2-/- mice exactly mirrored those seen in wild-type animals: *Lactobacillus* was significantly less adherent within the malnourished small intestine of Rag2-/- mice compared to the small intestine of Rag2-/- mice fed a control diet (Fig 2.3F). There was no significant difference between *Lactobacillus* adhesion in wild-type and Rag2-/- mice fed the same diet (Fig 2.3F). Thus, although IgA has some ability to enhance *Lactobacillus* mucosal adhesion *in vitro* and may be important in certain contexts, diet appears to be the major factor shaping *Lactobacillus* adhesion within the mouse intestine.
Figure 2.3 IgA-binding induces aggregation of *Lactobacillus* but has minimal impact on mucosal adhesion.
(A) Correlation between the percentage of IgA-bound bacteria and the percentage of clumped bacterial cells by flow cytometry, in *Lactobacillus* isolates exposed to murine small intestinal IgA *in vitro* (n=34, linear regression). (B) CFU counts of *Lactobacillus* incubated with or without IgA (two-way ANOVA with post-hoc Sidak’s test). (C) Fold change in the CFU of IgA-incubated *Lactobacillus*, before and after bead-beating disruption to remove cell aggregates (n=8). (D) Proportion of adherent *Lactobacillus* in the jejunum of CON, MAL and MAL-b mice, determined by CFU counting in tissue and luminal content. (E) Adhesion of *Lactobacillus* to HT29-MTX cells after incubation with IgA (n=5, each replicate an average of 4 wells from an independent assay). (F) Proportion of adherent *Lactobacillus* in the jejunum of wild-type (WT) and Rag2−/− (RagKO) mice fed a CON or MAL diet, determined by 16S qPCR. WT CON vs RagKO CON mice, and WT MAL vs RagKO MAL mice, are both non-significant (padj>0.9999). Statistical significance determined by two-way ANOVA with post-hoc Sidak’s test (B, C, E) or by Kruskal-Wallis with post-hoc Dunn’s test (D, F). Data are represented as mean ± SEM.
2.4.4  IgA-binding ability of *Lactobacillus* is altered dynamically in response to diet and is independent of host IgA

Our results thus far suggested that *Lactobacillus* alters its surface properties in response to the undernourished gut environment. Indeed, all *Lactobacillus* isolates from young mice were able to bind IgA (Fig 2.4A). Isolates taken from the same MAL-b mice at adulthood, however, had lost their affinity for IgA, while isolates from CON mice remained IgA-binding (Fig 2.4A). To test whether *Lactobacillus* phenotypes were reversible by diet, adult 7-week-old mice were maintained on a CON or MAL-b model, or were switched to the opposite dietary model (CON-R, MAL-b-R) for a further 4 weeks until 11 weeks of age, and *Lactobacillus* was isolated every 2 weeks to assay IgA-binding abilities (Fig 2.4B). Strikingly, phenotypic differences in the IgA-binding of *Lactobacillus* isolates from young mice were observable within only two weeks on an undernourished diet (Fig 2.4C) and IgA-binding ability was rapidly lost in isolates when CON mice were switched to a MAL-b model at adulthood (Fig 2.4C). A partial and delayed rescue of IgA-binding was observed in MAL-b mice placed onto a CON diet (Fig 2.4C). Moreover, IgA-binding of MAL-b *Lactobacillus* isolates was low even when the IgA was obtained from the same MAL-b mice at endpoint, indicating that even over a long time period the host did not mount alternative IgA responses to non-binding *Lactobacillus* (Fig 2.4D).

To determine whether intestinal IgA itself was necessary for the antibody-binding phenotypes of *Lactobacillus*, we isolated *Lactobacillus* from Rag2-/- mice maintained on a CON or MAL diet for three weeks. Rag2-/- mice experienced a similar growth deficit on the malnourished diet compared to wild-type mice (Fig 2.4E). Further mimicking results in the wild-type mice, *Lactobacillus* from MAL mice were dramatically less able to bind IgA than those
from CON mice, even on the IgA-deficient Rag2/− background (Fig 2.4F). Surprisingly, therefore, the host adaptive immune system does not influence the IgA-binding adaptations of *Lactobacillus*.

Inter-species interactions in the microbiota contribute to the metabolic environment of the gut and can be influenced by IgA\(^{159}\). To evaluate a role for the microbiota in *Lactobacillus* adaptations, germ-free mice were monocolonized with a high-IgA-binding *Lactobacillus* isolate and placed onto a CON or MAL diet for three weeks. Colonization levels were similar between the two groups but were slightly higher in mice fed a MAL diet (Supplemental Fig A.2.5A-B). *Lactobacillus* isolates did not show a loss of IgA-binding ability in either dietary group after monocolonization; in fact, the IgA-binding ability of *Lactobacillus* was higher in monocolonized MAL mice than in CON mice at experimental endpoint (Supplemental Fig A.2.5C). Total intestinal IgA also trended higher in MAL mice (Supplemental Fig A.2.5D). This outcome is in direct contrast to results in conventional mice, and suggests that the presence of an intestinal microbiota can indeed impact IgA-binding adaptations in *Lactobacillus*. However, it was not clear whether the impact of the microbiota occurred via direct inter-bacterial interactions or via indirect effects of the metabolic or immune environment.

To exclude all host and microbial influence, we directly tested whether nutrient limitation was sufficient to alter IgA-binding ability in *Lactobacillus*. High IgA-binding isolates were repetitively passaged *in vitro* in a rich media (classic “CON” MRS) or nutrient-limited media (“MAL” MRS with reduced protein, lipid and sugar; Table A.2.1). Within only one week of daily passages *in vitro*, isolates grown in the nutrient-limited media showed a significant and consistent decrease in IgA-binding ability (Fig 2.4G). In contrast, low-IgA-binding strains grown in rich media did not regain high IgA-binding properties, even after 3 weeks of daily passages.
(Supplemental Fig A.2.5E-F). Together our data indicate that nutritional pressure is sufficient to drive loss of Lactobacillus IgA-binding, independently of host IgA and indeed of any host factors. Further, the microbiota appear to influence this outcome in vivo, most likely by shaping the metabolic environment and nutritional availability of the gut.
**Figure 2.4 IgA-binding ability of *Lactobacillus* is altered dynamically in response to diet and is independent of host IgA.** (A) IgA recognition of *Lactobacillus* isolated from CON or MAL-b mice at week 3 and from the same mice at week 7 (n=5-6; two-way ANOVA with post-hoc Sidak’s test). (B) Schematic of reversal experiment: mice were placed either on the CON model (CON) or MAL-b model (MAL-b) for eight weeks, or were reversed to the opposite model after four weeks (CON-R and MAL-b-R), and *Lactobacillus* was isolated every two weeks to assess IgA-binding ability. (C) IgA recognition of *Lactobacillus* isolates from the reversal experiment (n=3 at weeks 3 and 9; n=6 at other time points). P-values derive from two-way ANOVA with post-hoc Dunnett’s test, and represent the difference between CON and all other groups: CON vs MAL and CON vs MAL-b are significant at week 7, 9 and 11 as reported, and CON vs CON-R is also significant at week 11 (****p<0.0001). (D) IgA-binding of *Lactobacillus* isolates taken at week 11 endpoint, using small intestinal IgA from the same CON or MAL-b mice (n=5-6). (E) Weights of wild-type (WT) and Rag2/- (RagKO) mice after three weeks on a CON or MAL diet. Kruskal-Wallis with post-hoc Dunn’s test. WT CON vs RagKO CON, and WT MAL vs RagKO MAL, were both non-significant (padj>0.9999). (F) IgA-binding ability of *Lactobacillus* isolates from wild-type (WT) or Rag2/- (RagKO) mice fed a CON or MAL diet (n=14-16 isolates from unique mice, Kruskal-Wallis with post-hoc Dunn’s test). WT CON vs RagKO CON, and WT MAL vs RagKO MAL, are both non-significant (padj>0.9999). (G) Binding of *Lactobacillus* by murine small intestinal IgA, after one week of daily passages in either rich media (CON) or nutrient-deficient media (MAL). Each data point represents an individually passaged strain. Results are pooled from three independent experiments starting with three different *Lactobacillus* isolates and are normalized per experiment to the average O.D. of the CON strains. Data are represented as mean ± SEM. See also Fig S5.
2.4.5 IgA-binding properties of *Lactobacillus* are associated with mutations in carbohydrate transport and metabolism

Loss of IgA-binding was seen in *Lactobacillus* isolates from every cohort of conventional MAL mice examined: this highly reproducible adaptation is suggestive of a strong selective pressure in the undernourished gut. To pinpoint genes or epitopes responsible for IgA-binding in *Lactobacillus*, whole genome sequencing was performed on a selection of high-IgA-binding and low-IgA-binding isolates, using strains taken at experimental endpoint from five independent cages of CON and MAL-b mice over four separate *in vivo* experiments (n=5 strain pairs). For three of the five strain pairs, additional isolates were collected prior to dietary intervention to allow for longitudinal genomic analysis. Phylogenetic alignment indicated that isolate genomes were most closely related to *Lactobacillus johnsonii* Byun-jo, a strain isolated from the murine jejunum\textsuperscript{193}, followed by *L. johnsonii* NCC533, a well-studied probiotic candidate\textsuperscript{198} (Supplemental Fig A.2.6A). Non-synonymous mutations were therefore annotated for each strain with respect to the *L. johnsonii* Byun-jo reference genome. Each pair of CON and MAL-b strains from the endpoint of a single experiment (mouse cohort) were then compared to one another to identify variants uniquely present in one diet in cross-sectional analysis (Fig 2.5A). Where initial isolates were available, each strain was further compared back to its ancestral strain to identify mutations that had appeared during the course of the experiment.

Consistent with the presence of dietary adaptations, broad shifts in genes linked to nutrient transport and metabolism were apparent in both cross-sectional and longitudinal analysis. MAL-b strains accumulated multiple non-synonymous mutations in genes linked to carbohydrate transport and metabolism, most notably in phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) transporters, in ATP-binding cassette (ABC) transporters, and
in Hpr kinase/phosphorylase, a master regulator of PTS and ABC transport systems. MAL-b strains also acquired mutations in enzymes of carbohydrate metabolism (e.g. tagatose/fructose 1,6-bisphosphate aldolase, glucose epimerase) (Fig 2.5A). Conversely, CON strains accumulated mutations in amino acid permeases and in multiple peptidases (e.g. C1, C69, C45, and S8), consistent with an adaptation to a more protein-rich environment. By experimental endpoint, MAL-b strains harboured a greater proportion of mutations in transporters and glycosidases, while CON strains had significantly more mutations in annotated peptidases (Supplemental Fig A2.6B-C). Indeed, supporting the presence of advantageous metabolic adaptations, we observed that low IgA-binding isolates from MAL-b mice grew to higher densities in vitro than high IgA-binding controls, particularly when challenged with nutrient-poor media (Supplemental Fig A2.6D). Overall, mutations in Lactobacillus isolates appear to reflect metabolic adaptation to dietary conditions of the murine intestine.

To identify mutations that were independent of the host, we further performed longitudinal whole genome analysis of Lactobacillus strains that had been evolved in vitro in rich or nutrient-poor media. Strikingly, many mutations found in vivo also occurred in vitro: multiple PTS sugar transporters, ABC transporters, and other carbohydrate metabolic genes (ex. tagatose/fructose 1,6-bisphosphate aldolase) once again uniquely accumulated in low-IgA-binding Lactobacillus compared to the initial inoculum (Fig 2.5B). Loss of IgA-binding in Lactobacillus is therefore accompanied by a consistent genetic signature in vivo and in vitro, characterized by alterations in carbohydrate transport and metabolism. These data indicate that IgA adaptation of Lactobacillus is part of a metabolic adaptation in direct response to altered nutritional availabilities.
Figure 2.5 IgA-binding properties of *Lactobacillus* are associated with mutations in carbohydrate transport and metabolism. (A-B) Distribution of mutations in *Lactobacillus* isolates from (A) the murine intestine of CON or MAL-b mice and (B) in vitro evolution in rich or nutrient-deficient media (designated CON and MAL respectively). Mutations are presented in the heatmap if they are unique by diet in at least one strain pair and have a predicted functional impact in annotated genes from the *L. johnsonii* Byun-jo genome. For size and clarity of the heatmaps, only genes with an annotated function in transport or metabolism are included (excluded categories include hypothetical proteins, transcriptional regulators, and unclassified functions). Starred genes in (B) are mutated in both *in vitro* and *in vivo* isolates. Cross, cross-sectional analysis comparing CON and MAL/MAL-b strains from the same experiment. Long, longitudinal analysis comparing each strain back to an ancestral isolate collected before dietary intervention. INDEL, insertion/deletion; missense_variant *n* indicates there were *n* amino acids changed in the same gene in the same strain.
2.4.6 IgA-Lactobacillus interactions are glycan-mediated

IgA is a heavily glycosylated protein, and gram-positive bacteria have been shown to interact with secretory IgA via glycan side chains\textsuperscript{200}. In light of the consistent genetic association between carbohydrate processing and IgA-binding, we hypothesized that IgA-Lactobacillus interactions in the gut were being mediated by surface glycan interactions. To test whether glycans contributed to binding between IgA and Lactobacillus, murine intestinal IgA was incubated with a promiscuous N-glycosidase to cleave off glycan side chains. Exposure of IgA to N-glycosidase led to a dose-dependent decrease in its ability to bind Lactobacillus, indicating that glycosylation of IgA is important for this interaction (Fig 2.6A). N-glycosidase treatment of Lactobacillus itself led to a similar decrease in IgA binding, implicating both bacterial- and immunoglobulin-derived glycans in this interaction (Fig 2.6B). Notably, N-glycosidase successfully reduced IgA-Lactobacillus interactions whether we used non-specific IgA from murine norovirus-monocolonized mice (MNV) or IgA from Lactobacillus-exposed conventional mice (SPF) (Fig 2.6A-B). This suggests that whether or not intestinal IgA is produced in the presence of Lactobacillus, the binding interactions between them remain significantly glycan-mediated. Further, adsorption of Lactobacillus with carbohydrates significantly reduced IgA-binding, although with slightly different profiles depending on the source of IgA (Fig 2.6C). Collectively, these data demonstrate that Lactobacillus interacts with secretory IgA via surface glycan-glycan interactions, and this IgA-binding ability is influenced by bacterial adaptation to the nutritional environment.
Figure 2.6 IgA-\textit{Lactobacillus} interactions are glycan-mediated. (A-B) Binding of \textit{Lactobacillus} to intestinal IgA after either the IgA (A) or the \textit{Lactobacillus} (B) was treated with N-glycosidase. Percent binding is expressed relative to the untreated control. SPF, IgA derived from conventional specific pathogen free (SPF) mice; MNV, IgA derived from murine norovirus (MNV)-monocolonized mice. (C) Binding of \textit{Lactobacillus} to intestinal IgA after adsorption with carbohydrates. Gluc, glucose; Fruc, fructose; GlcNac, N-acetyl glucosamine. Significance determined by Mann-Whitney. Data are represented as mean ± SEM. \textit{Lactobacillus} isolates used this figure are the core isolates designated in Table S3.
2.5 Discussion

IgA-bacterial interactions in the intestine have been increasingly studied in recent years and are presumed to reflect important immune-bacterial interactions during homeostasis or dysbiosis; however, the actual immune or microbial factors driving altered IgA-recognition have rarely been determined. A recent review discussed this knowledge gap and emphasized the need for mechanistic studies to further our understanding of bacteria-IgA interactions\(^\text{177}\). Here we demonstrate that dietary undernutrition is sufficient to induce rapid metabolic change in a population of intestinal *Lactobacillus*, abolishing glycan-mediated IgA binding. Together these data shed light on the complex nature of IgA-bacterial interactions. Further, they emphasize the importance of host diet in shaping immune recognition of the microbiota.

IgA-targeting of *Lactobacillus* developed consistently in healthy adult mice, concurrent with the increase in total intestinal IgA post-weaning. In contrast, IgA recognition of *Lactobacillus* was almost entirely lost in undernourished mice due to rapid bacterial adaptation. The loss of *Lactobacillus* antibody-binding properties was consistent across a variety of antibody sources, including serum IgG and virally-induced IgA, and was mediated by surface interactions with the glycan side chains that decorate immunoglobulins. This is consistent with findings that both wild-type and T-cell-deficient BL/6 mice display robust IgA-targeting of *Lactobacillus*, implying a T-cell-independent mechanism for *Lactobacillus*-IgA binding\(^\text{162}\). Gram-positive bacteria are known to bind extensively to the glycans present on secretory IgA\(^\text{200}\). Indeed, it has been estimated that the majority of intestinal IgA is polyreactive in both mice\(^\text{201}\) and in humans\(^\text{202}\), and that this is at least partly due to glycan cross-reactivity\(^\text{202}\). Contrary to common assumptions, glycan-glycan interactions can be extremely high-affinity\(^\text{203}\), and it has recently been proposed that they constitute the “elephant in the room” of IgA-microbiota studies\(^\text{152}\). Our
data suggest that *Lactobacillus* from the healthy murine gut is adapted to strongly but non-specifically bind to host immunoglobulin via surface glycans. Moreover, we find that glycan-mediated antibody interactions can be rapidly evaded by commensal bacteria.

Contrary to our initial expectations, *Lactobacillus* properties were directly modulated in response to the nutritional environment and were independent of host IgA. Loss of *Lactobacillus* IgA-binding occurred in malnourished Rag2-/- mice that lack an adaptive immune system, and even occurred in low-nutrient media in the complete absence of host factors. Interestingly, however, loss of *Lactobacillus* IgA-binding was not observed in germ-free malnourished mice, suggesting a role for the microbiota in shaping these adaptations *in vivo*. We speculate that competition with other members of the microbiota increases the nutritional pressure experienced by *Lactobacillus in vivo*.

Mucosal adhesion of *Lactobacillus* also decreased in the malnourished small intestine of conventional mice, independently of host IgA. Thus, in contrast to exciting reports that IgA can enhance the mucosal colonization of *Bacteroides*\(^{178}\), diet appeared to be the most important factor shaping *Lactobacillus* phenotype and host interactions in our study. Like IgA, mucins are heavily glycosylated proteins; our data are therefore most consistent with extensive surface glycan adaptations in *Lactobacillus* in response to host undernutrition, broadly impacting binding to glycosylated host proteins.

Loss of *Lactobacillus* IgA-binding during nutritional deprivation was associated with mutations in carbohydrate import and metabolism, both *in vitro* and *in vivo*. In particular, a large proportion of the mutations in low-IgA-binding strains occurred in PTS and ABC transporters and in the master regulator HPr kinase/phosphorylase, which regulates substrate competition between these two transport systems\(^{199,204}\). Together, these data suggest a broad shift in sugar
transport, regulation and metabolism in the undernutrition-adapted strains. Importantly, these transport systems can also export sugars to the extracellular surface\textsuperscript{204,205}. Given the overlapping functions of carbohydrate transport systems, it is possible that altered IgA-binding is an unintended consequence of metabolic adaptation in \textit{Lactobacillus}; indeed, as noted, adaptation occurred in the absence of host IgA. However, studies in \textit{Bacteroides} indicate that IgA-binding can itself regulate bacterial polysaccharide metabolism\textsuperscript{154,155,159}, raising the intriguing possibility that \textit{Lactobacillus} is evading host recognition in part to avoid host metabolic regulation.

The importance of \textit{Lactobacillus} adaptation to host diet has recently been explored in a body of work by Leulier and colleagues\textsuperscript{113–117}. This group demonstrated that \textit{L. plantarum}, a commensal of \textit{Drosophila}, successfully mitigates the impact of host undernutrition in both flies and mice\textsuperscript{116,117}. Importantly, the protective effects of \textit{L. plantarum} in flies were mediated partly through enterocyte recognition of bacterial cell surface components\textsuperscript{115}. Moreover, the undernourished diet of the flies was sufficient to drive \textit{L. plantarum} evolution for improved bacterial fitness and, subsequently, improved host health during undernutrition\textsuperscript{114}. Our results complement these studies by showing that a natural mouse commensal, \textit{L. johnsonii}, also demonstrates rapid diet-dependent evolution within its host; further, this evolution also alters host recognition of the bacterium by an adaptive immune system only present in vertebrates. Although it is widely acknowledged that host diet has a substantial influence on microbiota composition at the taxonomic or gene abundance level, the impact of diet on strain-level genetics and adaptation is not often considered. Together these studies indicate that diet significantly and rapidly shapes the genotype and phenotype of individual bacterial populations, a nuance often missing from traditional microbiota screens.
Our data also provide a cautionary note, as not all bacterial adaptations to diet may be beneficial to the host. *Lactobacilli* are nutritionally fastidious bacteria, adapted to use the relatively abundant resources of the small intestine; indeed, a close relative to the isolates in the study, *L. johnsonii* NCC533, is a well-studied probiotic which is also auxotrophic for most amino acids\(^{198}\). Clear evolutionary pressure therefore exists for *Lactobacillus* in protein-restricted host environments, with a variety of possible outcomes regarding host-microbe interaction.

Although we were not able to evaluate the probiotic ability of the *L. johnsonii* strains used in our study, it is tempting to speculate that decreased IgA-binding of protective bacteria may contribute to the increased susceptibility of undernourished hosts to intestinal infections, for example by reducing colonization resistance within the mucosa; we believe this to be an important hypothesis to address in future work. Interestingly, in mice with a genetic over-abundance of sIgA, *Lactobacillus* IgA-targeting was strongly increased and correlated with host weight gain; moreover, isolates of *Lactobacillus* altered glucose and fat homeostasis when transferred to healthy mice\(^{206}\). IgA binding or induction is commonly used as an indicator of probiotic ability in lactic acid bacteria, and IgA-bound *Lactobacillus* from a healthy human was recently found to improve barrier function and lipid regulation in mice\(^{207}\). Currently, the use of *Lactobacillus* probiotics for undernutrition shows strong potential in pre-clinical studies\(^{116,117,208}\). Excitingly, a recent clinical trial for severe acute malnutrition found that *Lactobacillus* and *Bifidobacterium* probiotics decreased the incidence of diarrhea in undernourished children, but only in children where the strains stably colonized\(^ {119}\). Dietary compatibility and the ability to bind host glycans are variables that might contribute to the colonization success of the probiotics.
In future clinical trials for probiotics in undernutrition, it will therefore be essential to test for colonization success, and to remain aware of the possibility of ongoing bacterial adaptation.

We did not investigate whether nutrient restriction was able to alter IgA-binding properties in commensals other than *Lactobacillus*; however, it is interesting to note that other gram-positive bacteria with probiotic potential, including *Bifidobacterium* and *Eubacterium*, also become less IgA-targeted in the undernourished mice. Moreover, a recent study in mice fed a high-fat diet also found broad changes in the IgA-targeting of intestinal bacteria\textsuperscript{209}. Future studies will be necessary to determine whether dietary adaptation is a broad driver of IgA recognition amongst diverse commensals or whether alternative mechanisms contribute.

The role of IgA in intestinal homeostasis is well established, and there is increasing interest in identifying microbes recognized by IgA during health and disease. Our data provide important mechanistic insight into the dynamics of bacterial IgA-interactions in the gut, while advancing our understanding of bacterial adaptations to host nutrition.
Chapter 3: Immunoglobulin recognition of fecal bacteria in stunted and non-stunted children: findings from the Afribiota study

3.1 Synopsis

Child undernutrition is a global health issue that is associated with poor sanitation and an altered intestinal microbiota. IgA mediates host-microbial homeostasis in the intestine, and acutely undernourished children have been shown to have altered IgA recognition of the fecal microbiota. We sought to determine whether chronic undernutrition (stunting) or intestinal inflammation were associated with antibody recognition of the microbiota using two geographically distinct populations from the Afribiota project. Fecal bacteria from 200 children between two and five years old in Antananarivo, Madagascar and Bangui, Central African Republic (CAR) were sorted into IgA-positive (IgA+) and IgA-negative (IgA-) populations by flow cytometry and subsequently characterized by 16S rRNA gene sequencing to determine IgA-bacterial targeting. We additionally measured IgG+ fecal bacteria by flow cytometry in a subset of 75 children.

Stunted children (height-for-age z-score ≤-2) had a greater proportion of IgA+ bacteria in the fecal microbiota compared to non-stunted controls. This trend was consistent in both countries, despite higher overall IgA-targeting of the microbiota in Madagascar, but lost significance in each country individually. Two of the most highly IgA-recognized bacteria regardless of nutritional status were Campylobacter (in CAR) and Haemophilus (in both countries), both of which were previously shown to be more abundant in stunted children; however, there was no association between IgA-targeting of these bacteria and either stunting or
inflammatory markers. IgG-bound intestinal bacteria were rare in both stunted and non-stunted children, similar to levels observed in healthy populations. This study furthers our understanding of host-microbiota interactions in undernutrition and identifies immune-recognized microbes for future study.

3.2 Introduction

Undernutrition is responsible for nearly half of all deaths in children less than five years old\(^1\). As discussed in Chapter 1, the contribution of non-dietary factors to stunting represents an area of intensive research and interest for the prevention and treatment of undernutrition, and the intestinal microbiota has been increasingly implicated\(^17,18,49\). In particular, undernutrition is associated with dysbiosis and immaturity of the gut microbiota\(^58,60,71,72\) and with the intestinal inflammatory disease EED\(^49\).

The Afribiota project is an international, collaborative effort that aims to understand the microbial contributions to chronic undernutrition in early life\(^210\). We showed that stunted children in Madagascar and CAR have a dysbiotic fecal microbiota characterized by an overabundance of pathobionts and of bacteria typically associated with the oral cavity, including *Haemophilus, Campylobacter* and *Escherichia-Shigella*\(^211\). Using duodenal aspirates to sample the microbiota of the upper gastrointestinal tract, we further showed that small intestinal bacterial overgrowth (SIBO) was common and that oral taxa were abundant and viable in the upper gastrointestinal tract of stunted children\(^211\). On the basis of this research we proposed that stunted children experienced a ‘decompartmentalization’ of the intestinal tract whereby oral and environmental bacteria more easily persisted throughout the small and large intestine. However,
it remained unclear to what extent these bacteria were functionally interacting with the host and the host immune system.

As discussed in Chapter 2, IgA regulates host-bacterial homeostasis in the mammalian gut. Studies of IgA-targeted bacteria indicate that acute undernutrition and inflammatory bowel disease are both associated with altered interactions between IgA and the intestinal microbiota. Acutely malnourished children had higher IgA-targeting of Bacteroidales and E. coli, while patients with IBD had higher targeting of a variety of bacteria including Haemophilus and E. coli, and these IgA+ bacteria induced intestinal inflammation and disease when transferred into germ-free mice. Moreover, as I showed in Chapter 2, chronic undernutrition in mice directly alters IgA recognition of the microbiota, in part by driving bacterial dietary adaptation. Here, I investigated whether chronic undernutrition and EED are also associated with altered Ig-recognition of the microbiota in children, using two geographically distinct populations from the Afribiota study.

3.3 Materials and Methods

3.3.1 Study participants and sample collection

Study participants were recruited as part of the Afribiota project. The study protocol for Afribiota was approved by the Institutional Review Board of the Institut Pasteur (2016-06/IRB), the National Ethical Review Boards of Madagascar (55/MSANP/CE) and the Central African Republic (173/UB/FACSS/CSCVPER/16), and the Human Ethics Board of the University of British Columbia (H18-01108). All participants received oral and written information about the study and the legal representatives of the children provided written consent to participate in the study. Subject characteristics such as age, sex, and breastfeeding history...
were assessed by standardized questionnaire. The detailed inclusion and exclusion criteria and recruitment procedures of the Afribiota study are described in the published protocol\textsuperscript{210}.

All children were aged 2-5 years living in Antananarivo, Madagascar and in Bangui, Central African Republic. Children were categorized as either stunted cases (height-for-age z-score \(\leq -2\)) or non-stunted controls (height-for-age z-score \(>-2\)), using the World Health Organization (WHO) Anthro Software and growth standards\textsuperscript{213}. For the analysis presented in this study, samples from 100 children in Madagascar and 100 children in CAR were selected for IgA-sorting and sequencing. Participants were selected from the full study based on sample availability; within these constraints, samples were divided equally between stunted and non-stunted children, and matched for country, sex and age (+/- 3 months). Stunted children were initially included if they met the criteria for severe stunting (height-for-age z-score \(\leq -3\)) in order to better differentiate cases and controls; however, 12 out of 96 stunted children were re-categorized after inclusion as moderately stunted (height-for-age z-score \(>-3\) and \(\leq -2\)) and were maintained in the analysis.

Biobanking of fecal samples was performed by the Clinical Investigation and Access to BioResources Platform (ICAReB) at the Pasteur Institute, Paris, and by the Pasteur Institutes of Madagascar and of Bangui. An aliquot of fecal material for each sample was shipped on dry ice to Vancouver, Canada and frozen immediately upon arrival at -70°C.

### 3.3.2 Ig-sorting

IgA-sequencing was performed as described by Kau \textit{et al.}\textsuperscript{59}. Approximately 50 mg of each fecal sample (+/- 10 mg) were homogenized in 1 mL of phosphate buffered saline (PBS; HyClone DPBS/-, SH30028.02) and spun gently to settle debris; intestinal bacteria were then
filtered through a 0.7 µm filter. A volume of suspension equal to 5 mg of sample was washed in FACS buffer (PBS containing 1% bovine serum albumin) and blocked for 20 minutes in FACS buffer containing 10% fetal bovine serum. Samples were then stained with anti-human IgA-PE (Miltenyl 130-093-128) or an isotype control (eBioscience, 12-4714-42) at 1:25 dilution for 30 minutes in the dark. For IgG-sorting, an anti-human IgG Fc APC (BioLegend 409306) and isotype control (BioLegend Mouse IgG2a kappa isotype, 400222) were used at the same 1:25 dilution. Samples were washed twice more and fixed overnight in 2% paraformaldehyde (PFA) at 4°C in the dark without shaking. The next day, the PFA was washed off and samples were stained with SYTO-BC (1:4000 dilution) for bacterial DNA, washed again and sorted by flow cytometry into Ig-positive and Ig-negative populations. A minimum of 50 000 events are collected in the IgA+ and IgA- fraction and frozen at -20°C for further analysis. Each sample was stained with both an anti-human IgA antibody and an isotype control to distinguish between specific and non-specific binding, and the final percentage of IgA-positive bacteria was reported after subtraction of the isotype-positive population. Samples in which the isotype and antibody-specific populations could not be distinguished were excluded from further sequencing analysis.

3.3.3 16S Library preparation

Sorted bacterial suspensions were boiled for 15 min at 100°C and 2 µL of lysate was used as template for 16S PCR, using Illumina-tagged and barcoded primers specific for the 16S V4 region. PCR was performed with Phusion polymerase under the following cycling conditions: 5 minutes initial denaturation at 98°C, 30 cycles of 20 seconds at 98°C, 15 seconds at 55°C, 30 seconds at 72°C, and 10 minutes final extension at 72°C. Reactions were run on a gel to ensure successful amplification, and were purified and normalized using the 96well Sequel-Prep kit.
(ThermoFisher A1051001). All reactions were subsequently pooled and gel extracted (GeneJet K0692) to remove primer-dimers. Sequencing was performed on an Illumina MiSeq using a v2 kit for 2x250 bp reads with 30% PhiX at the Biomedical Research Centre (BRC) Sequencing Core of the University of British Columbia.

3.3.4 Bioinformatics analysis of 16S rRNA data

Demultiplexed forward reads were analyzed in QIIME2 (https://qiime2.org)\textsuperscript{214}, using the Dada2 option\textsuperscript{215} for sequence quality control and trimming to 250 bp. Taxonomic assignment was performed using the SILVA database\textsuperscript{216}. Further filtering was then performed in R using phyloseq\textsuperscript{187}. Filtering included removal of unintended targets (archaea, mitochondria and chloroplast), removal of singleton taxa, and rarefaction to 5000 reads. A log-adjusted IgA index was calculated as described previously\textsuperscript{59,137}: \( IgA_{Index} = -\left( \frac{\log(IgA^{+}taxon) - \log(IgA^{-}taxon)}{\log(IgA^{+}taxon) + \log(IgA^{-}taxon)} \right) \), after adding a pseudocount of 0.0000001 relative abundance to allow for zero values. Taxa were maintained at either the ASV level or the genus level for calculation of the IgA Index. IgA Index data was further filtered for prevalence within each sequencing batch, by excluding taxa in which \( \geq 75\% \) of samples had zero values in either batch individually. Multiple additional statistical methods were explored to further reduce batch effect (ex., ComBat package in R, percentile normalization in qiime2), but were discarded as they did not substantially reduce batch effect in our data. Instead, sequencing batch was taken as a co-variate in all models (see statistical analysis) and analyses were repeated to ensure similar trends in each batch individually. Raw sequencing data has been deposited to the Sequence Read Archive (SRA) under BioProject PRJNA603512.
3.3.5 Quantification of total fecal IgA and IgG

For total Ig quantification, 200 mg of fecal samples were homogenized in 1ml of PBS containing Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany), incubated for 30 min on ice, and centrifuged for 10 min at 10000 g at 4°C. The supernatant was taken for analysis and stored at -80°C. The concentration of antibodies (IgA, IgG1, IgG2, IgG3, IgG4) was measured on supernatants at a dilution of 1/100-1/10000 with the Bio-Plex Pro Human Isotyping Panel (Bio-Rad Laboratories, Hercules, CA, USA) using the DropArray method (Curiox Biosystems Pte Ltd, Singapore) on a Bioplex 200 machine (LUMINEX) according to manufacturer instructions. Values were measured in duplicates and all samples with more than 20% difference in between the two measurements or values below or above the standard curve were repeated. Final values were normalized to the initial fecal weight used for extraction. Analysis was performed with Bio-Plex Manager Software version 6.1.1.

3.3.6 Assessment of inflammatory markers

Fecal calprotectin and alpha-1 antitrypsin (AAT) were measured as described previously. Briefly, stool samples were diluted 1:5 in 0.15M NaCl and vortexed vigorously until complete homogenization; the homogenate was then centrifuged and the supernatant collected for analysis. Calprotectin concentrations were assayed in duplicate by sandwich ELISA using a polyclonal antibody system (Calprest; Eurospital, Italy) according to the manufacturer’s instructions. Fecal alpha-1 antitrypsin (AAT) was measured using an immuno-nephelemetric method adapted on the BN ProSpec system (Siemens, Germany).
To measure serum C-reactive protein (CRP), venous blood was collected through the AFRIBIOTA project using Ethylene Diamine Tetra Acetic Acid (EDTA) microtainer tubes (BD Vacutainer). C-reactive protein (CRP) was measured at the Clinical Biology Center of the Institut Pasteur the Madagascar and the Laboratoire d’Analyse Médicale at the Institut Pasteur de Bangui within 4 hours after blood collection according to accredited methods.

### 3.3.7 Helminth detection

Fecal samples were examined microscopically for the presence of helminths using the Merthiolate-Iodine-Formaldehyde (MIFs) and Kato-Katz (KK) techniques\(^2\). Briefly, MIF solution (200 mL merthiolate tincture, 25 mL formalin 10%, 5 mL glycerin, 250 mL distilled water) was prepared and fecal samples were triturated in the MIF solution in a haemolysis tube at approximately 250 mg stool per 2.5 mL. Stool was allowed to stand in the MIF solution at room temperature for 30 minutes. Using a Pasteur pipette, the stool was then removed and deposited in a microscope slide, and the sample was visually examined for the presence of helminth eggs or larva, and on the surface of the sediment for protozoa trophozoites, cysts, or oocysts. For KK technique, a solution of 3% malachite green-glycerol solution was prepared in advance and cellophane strips the size of a slide were immersed in the solution for 24 hours prior to use. After thorough homogenization of the samples, approximately 1 gram of feces was placed on a tissue paper and covered with a wire mesh. With the aid of a spatula, pressure was applied and the feces that passed through the mesh were deposited onto a standard template holding 41.7 mg of feces located on a glass slide. The template was then removed and the sample was spread onto a strip of cellophane paper embedded in the 3% malachite green-glycerol solution. Finally, the
preparation was left at rest for at least one hour before observation under optical microscope (10x objective) for the visual identification of helminth eggs.

3.3.8 Statistical analysis

All statistical analysis was performed in R studio using the phyloseq and ggplot2 packages. Unless otherwise indicated, all analyses were performed both in the pooled dataset and in each country individually, as well as in each batch individually, and multiple correction of statistical tests was applied using the False Discovery Rate (FDR). Data structure of the IgA Index was explored using a Principle Component Analysis based on Euclidean distance. A PERMANOVA analysis was iteratively applied to each variable of interest to determine its contribution to the distribution of the IgA Index. Non-parametric Wilcoxon rank sum tests were used to compare data with two groups (ex. stunted versus non-stunted or Madagascar versus CAR). A one-sided Wilcoxon rank sum test was used to identify taxa with an IgA Index significantly different from zero. Spearman’s correlation was applied for continuous variables. To look for associations between the IgA Index and metadata of interest, we iteratively fitted a linear model correcting for confounders (batch, relative sequencing depth (the difference in sequencing depth between IgA+ and IgA- fractions for a given taxon), country, age and sex) for each individual taxon and corrected the p using FDR. Where indicated, bootstrapping analysis was further applied to models that contained co-variates, in order to obtain a non-parametric estimate of the statistical significance.
3.4 Results

3.4.1 IgA+ fecal bacteria are higher in stunted children

Two hundred children aged two to five years old who were enrolled in the Afribiota study in Madagascar and the Central African Republic (CAR)\textsuperscript{210} were selected for analysis of the IgA-targeted microbiota (Supplemental Fig A.3.1). IgA-coating of fecal bacteria was measured using flow cytometry (Supplemental Fig A.3.2) and cytometric data was ultimately available for 188 children of whom 98 were stunted and 90 were non-stunted (Supplemental Fig A.3.1). The proportion of fecal bacteria coated by IgA (IgA+) varied widely, from zero to nearly 50%, which is consistent with findings from other human studies\textsuperscript{148,162}. The percentage of IgA+ bacteria in a sample by FACS correlated strongly with total fecal IgA levels (Fig 3.1A, \(p=7.8\times10^{-6}, \rho=0.35\) by Spearman’s correlation), validating our technique and indicating a real biological variation in the availability of secretory IgA within the gut.

The population of IgA+ fecal bacteria was significantly higher in children from Madagascar than in children from CAR (Fig 3.1B, \(p=2.1\times10^{-5}\) by Wilcoxon Rank Sum Test). It did not vary by age or by sex (Fig 3.1C-D). Interestingly, there was a modest association between IgA-coating and height-for-age z-score (HAZ; Supplemental Fig A.3.3A, \(p=0.039, \rho=-0.019\) by Spearman’s correlation), with stunted children having a greater proportion of IgA-coated bacteria in the fecal microbiota compared to non-stunted controls (Supplemental Fig A.3.3B, \(p=0.029\) by Wilcoxon Rank Sum test). The proportion of IgA-coated bacteria also increased stepwise with the severity of stunting (Fig 3.1E, \(p=0.030\) by Jonkheere’s nonparametric trend test). Further, IgA-coating was increased in stunted children compared to non-stunted children in both countries individually, although it did not reach statistical
significance in the country subsets (Fig 3.1F; p=0.13 and p=0.22 by Wilcoxon Rank Sum test in Madagascar and in CAR, respectively). Removing 12 outliers also led to borderline significance (p=0.054 by Wilcoxon Rank Sum test of stunted vs non-stunted in the pooled dataset). This may be partly due to reduced statistical power in smaller sample sizes but could also reflect bias in the data. Due to the large number of samples, IgA-sorting was performed in two major batches over several days each: although there was some variation in the data by batch, trend directions by stunting and by country were consistent in both main sorting batches (Supplemental Fig A.3.3C-F). Further, the association between %IgA+ and HAZ was robust to non-parametric bootstrapping when including country (p=0.045) or batch (p=0.042) as grouping factors to constrain permutations.

Total fecal IgA was higher in Madagascar than in CAR, mirroring the flow cytometry data, but to a much smaller degree (Supplemental Fig A.3.3G, p=0.072 by Wilcoxon Rank Sum test). In contrast, there was no difference in total fecal IgA by stunting (Supplemental Fig A.3.3H; p=0.571 by Wilcoxon Rank Sum Test).

We additionally did not find any association between %IgA-targeting and the inflammatory ‘EED’ biomarkers fecal calprotectin, fecal alpha-1-antitrypsin (AAT) or serum C-reactive protein (CRP), although all trends were positive (Supplemental Fig A.3.4A-C; p=0.862 for CRP by Wilcoxon Rank Sum Test; p=0.139 and p=0.103 for AAT and calprotectin, respectively, by Spearman’s correlation).

Together our data indicate a large difference in IgA recognition of fecal microbiota between geographically distinct populations. IgA recognition of fecal bacteria may moreover be higher in chronically undernourished children; however, larger sample sizes are needed to validate this result.
Figure 3.1 IgA-coating of fecal bacteria. The percentage of IgA-positive (%IgA+) fecal bacteria was measured by flow cytometry relative to an isotype control. (A-D) The relationship between %IgA+ bacteria and (A) the total concentration of fecal IgA in each sample (ng/g of feces, wet weight); (B) the country of origin; (C) child age in months and (D) sex. (E) %IgA+ bacteria by stunting severity. NN, non-stunted; MCM, moderately stunted; MCS, severely stunted. (F) Percent IgA-targeting by stunting status in each country individually. Mada, Madagascar; CAR, Central African Republic. N=188 total children; N=93 in Madagascar and N=95 in CAR. Significance was determined by Spearman’s correlation (A, C), Wilcoxon Rank Sum test (B, D, F) and Jonkheere’s trend test (E). A linear fit is shown in A and C.
3.4.2 Highly IgA-targeted taxa include *Firmicutes, Prevotella and Haemophilus*

To characterize the IgA-targeted microbiota, 16S rRNA gene sequencing of the V4 region was applied to the sorted IgA+ and IgA- fractions in a method known as “IgA-SEQ”\(^59\). After filtering and rarefaction, a total of 138 children had valid sequencing data from both the IgA+ and IgA- fractions (Supplemental Fig A.3.1), allowing us to calculate a log-normalized IgA Index as reported previously\(^59\). Demographics of this IgA-SEQ subpopulation are reported in Tables 3.1 and A.3.1.

IgA-targeting of the microbiota was taxonomically diverse, differing between closely related amplicon sequence variants (ASVs) as has been reported in previous studies (Supplemental Fig A.3.5A)\(^137,162,219\). At the genus level, twenty-one taxa were considered to be significantly IgA-targeted in the total dataset (Fig 3.2A; FDR-adjusted p <0.05 by one-sided Wilcoxon Rank sum test, and mean IgA Index >0). Of these, 12 (57.1%) belonged to the *Firmicutes* phylum, 5 (23.8%) to the *Bacteroidetes* and the remainder to the *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, and *Epsilonbacteraeota*. The distribution was similar at the ASV level (Supplemental Fig A.3.5A), with 24 out of 35 highly targeted ASVs (68.6%) belonging to the *Firmicutes*. These data are consistent with previous findings that *Firmicutes* are the most frequently IgA-recognized taxa in human feces\(^148\). Notably, *Lactobacillus* had a negative IgA Index in both countries (Fig 3.2A).

To identify the most highly IgA-targeted taxa in a given subpopulation, we selected taxa with (a) an IgA Index significantly different from zero (FDR-adjusted p <0.05) and (b) a positive median IgA Index. Because IgA-targeting is highly variable between individuals, the use of median IgA index excluded many taxa identified above where targeting may have been driven by a small number of individuals. By these criteria, therefore, eight genera were highly and
consistently targeted in the total study population: six members of the phylum *Firmicutes* (*Eubacterium*, *Coprococcus*, *Dorea*, *Lachnoclostridium*, unclassified *Ruminococcaceae* UCG-002, and *Subdoligranulum*), one *Bacteroidetes* (*Prevotella* 2), and one *Gammaproteobacterium*, *Haemophilus* (Fig 3.2B). Most of these taxa were targeted in both countries: *Eubacterium*, *Coprococcus*, *Subdoligranulum*, *Prevotella* 2 and *Haemophilus* were significantly targeted in Madagascar and in CAR (Fig 3.2C-D). In addition, *Solobacterium* was highly recognized in Madagascar (Fig 3.2C), and *Campylobacter* was highly recognized in CAR (Fig 3.2D). These patterns were similar at the ASV level and often seemed to be driven by one or two dominant ASVs (Supplemental Fig A.3.5B-D). A larger number of taxa met the criteria for ‘consistently untargeted’ (FDR<0.05 and median negative IgA index) than for significantly targeted, in the pooled dataset and in each country individually, including *Streptococcus*, *Faecalibacterium*, *Pseudomonas* and several *Alphaproteobacteria* of likely environmental origin (Table A.3.2). As expected, for most taxa there was no relationship between IgA-targeting and their relative abundance in the unsorted intestinal microbiota of the same children; the exception was a negative correlation in *Faecalibacterium* (Supplemental Fig A.3.6A; FDR-corrected p=0.003, rho=-0.35 by Spearman’s correlation).

Together, these data suggest a relatively conserved pattern of IgA-targeting in Madagascar and CAR, with high IgA recognition of *Haemophilus*, *Prevotella* and multiple *Firmicutes*. 
Table 3.1. Demographics of stunted and non-stunted children with valid IgA-seq data (N=138). Values are presented as the group median (continuous variable) or as counts (categorical variable) with missing values excluded. Significance was determined by Wilcoxon Rank Sum test (continuous variable) or Fisher’s exact test (categorical variable). Hemoglobin was adjusted by altitude (-0.2 g/100 mL in Madagascar to account for the height above sea level). Anemia was defined as an adjusted hemoglobin level below 11 g/100 mL. Elevated CRP was defined as >10 mg/l serum. Breastfeeding duration represents the total number of months a child was previously breastfed for; all but four children had been weaned by the time of sampling.

<table>
<thead>
<tr>
<th>Description</th>
<th>Nonstunted (N=67)</th>
<th>Stunted (N=71)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Country</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Madagascar</td>
<td>35/67 (52.2%)</td>
<td>43/71 (60.6%)</td>
<td>0.3909</td>
</tr>
<tr>
<td>Central African Republic (CAR)</td>
<td>32/67 (47.8%)</td>
<td>28/71 (39.4%)</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29/67 (43.3%)</td>
<td>35/71 (49.3%)</td>
<td>0.4993</td>
</tr>
<tr>
<td>Female</td>
<td>38/67 (56.7%)</td>
<td>36/71 (50.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (months)</td>
<td>42.7</td>
<td>38.4</td>
<td>0.3553</td>
</tr>
<tr>
<td>2-3 years</td>
<td>24/67 (35.8%)</td>
<td>30/71 (42.2%)</td>
<td></td>
</tr>
<tr>
<td>3-4 years</td>
<td>18/67 (26.9%)</td>
<td>17/71 (23.9%)</td>
<td></td>
</tr>
<tr>
<td>4-5+ years</td>
<td>25/67 (37.3%)</td>
<td>24/71 (33.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Nutritional status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median HAZ score</td>
<td>-1.05</td>
<td>-3.48</td>
<td>2.2e-16</td>
</tr>
<tr>
<td>Median WHZ score</td>
<td>-0.21</td>
<td>-0.54</td>
<td>0.0600</td>
</tr>
<tr>
<td>Median hemoglobin (g/100 mL serum)</td>
<td>11.6</td>
<td>10.9</td>
<td>0.0040</td>
</tr>
<tr>
<td>Presence of anemia</td>
<td>18/64 (28.1%)</td>
<td>34/66 (51.5%)</td>
<td>0.0076</td>
</tr>
<tr>
<td><strong>Breastfeeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median duration (months)</td>
<td>20</td>
<td>20</td>
<td>0.8842</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median AAT (mg/g dry fecal weight)</td>
<td>42.0</td>
<td>43.5</td>
<td>0.4979</td>
</tr>
<tr>
<td>Median calprotectin (µg/g dry fecal weight)</td>
<td>367.5</td>
<td>405.0</td>
<td>0.7026</td>
</tr>
<tr>
<td>Elevated CRP (&gt; 10 mg/l serum)</td>
<td>4/61 (6.5%)</td>
<td>13/66 (19.7%)</td>
<td>0.0374</td>
</tr>
<tr>
<td><strong>Parasites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of helminths</td>
<td>34/59 (57.6%)</td>
<td>36/66 (54.5%)</td>
<td>0.8569</td>
</tr>
<tr>
<td>Presence of Giardia</td>
<td>11/59 (18.6%)</td>
<td>17/66 (25.7%)</td>
<td>0.3942</td>
</tr>
<tr>
<td><strong>Sequencing Batch</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 1</td>
<td>26/67 (38.8%)</td>
<td>27/71 (38.0%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Batch 2</td>
<td>41/67 (61.2%)</td>
<td>44/71 (62.0%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.2 Highly IgA-targeted taxa at genus level. (A) IgA-targeting profiles at genus level in the full dataset (All), Madagascar (Mada) and Central African Republic (CAR). Genera are included if the IgA Index was significantly different from zero by a one-sided Wilcoxon (FDR-adjusted p < 0.05) in at least one subset. Colour of circles indicates the direction of IgA-targeting (red=positive IgA Index, blue=negative IgA Index), and saturation of the colour represents FDR-corrected statistical significance. The size of the circle indicates overall effect size as measured by average IgA Index. (B-D) Most highly IgA-targeted taxa in (B) the full dataset, (C) Madagascar and (D) CAR, as defined by a median IgA Index greater than zero with FDR-adjusted p < 0.05. N=138 total children; N=78 in Madagascar and N=60 in CAR.
3.4.3 IgA-targeting of putative pathobionts does not correlate with stunting or inflammation

Two of the most targeted taxa, *Haemophilus* (in both countries) and *Campylobacter* (in CAR), are putative pathobionts that were previously found to be more abundant in the fecal microbiota of stunted children\(^{211}\). Both *Haemophilus* and *Campylobacter* were positively IgA-targeted in stunted and non-stunted children, regardless of country (Supplemental Fig A.3.6B-C). Notably, *Campylobacter* was significantly IgA-positive in Madagascar (as well as in CAR) when mean IgA Index, rather than median IgA index, was used as a criterion, suggesting a positive but less well distributed IgA-recognition of this taxon (Fig 3.2A). In addition, both taxa had a mean positive IgA Index in each sequencing batch, although there was some batch variability in the magnitude of the index (Supplemental Fig A.3.6D-E). We did not find any association between the IgA Index of either *Haemophilus* or *Campylobacter* and the inflammatory biomarkers fecal calprotectin, fecal AAT or serum CRP (Supplemental Fig A.3.7A-F; \(p>0.1\) for all tests by Spearman’s correlation (calprotectin, AAT) or Wilcoxon Rank Sum Test (binary CRP levels)).

Together, these data indicate that both *Campylobacter* and *Haemophilus* are highly recognized by IgA, with IgA recognition of *Haemophilus* being particularly widespread among children in Madagascar and CAR. However, there is no significant association between IgA recognition of these taxa and chronic undernutrition or inflammatory markers.

3.4.4 Variation in IgA-targeting is influenced by geography, age and breastfeeding

Principle component analysis of the IgA Index shows high inter-individual variability of IgA-bacterial targeting (Fig 3.3A). To identify factors which explain variation in IgA-targeting between individuals, we performed PERMANOVA analysis of the IgA Index against a set of
rationally chosen metadata variables (Fig 3.3B). Country of origin had a large and statistically significant effect on the IgA Index (Fig 3.3B); indeed, several variables which might influence IgA or the microbiota (ex., breastfeeding duration and helminth carriage) differed significantly by country (Table A.3.1). In addition to a strong country effect, sources of technical variation (sequencing batch and the relative sequencing depth of the IgA+ and IgA- fractions before rarefaction) influenced the IgA Index (Fig 3.3B). Notably, sorting and sequencing batches largely corresponded (i.e., the same samples were included in each) and therefore we have only included one batch variable here, although both processes might be contributing to the batch effect. To account for country and batch variation in PERMANOVA, we repeated this analysis with (a) these variables as grouping factors to constrain permutations (using the ‘strata’ parameter) and (b) analysis performed separately in each country and in each batch (fully stratified data). Significance was determined by an FDR-corrected p<0.05.

None of the tested measures of nutrition (HAZ, WHZ, anemia) explained variation in the IgA Index in either full or stratified datasets (Fig 3.3B). Note that WHZ was within normal range for this study and that no children were considered acutely undernourished; however, WHZ tended to be lower in stunted children (Table 3.1) and was therefore included for analysis. The inflammatory markers calprotectin and CRP were also non-significant. Fecal AAT was significant in the pooled dataset and when permutations were constrained by country (Supplemental Fig A.3.8A); however, AAT was not a significant factor in either country individually (Fig 3.3B). There was also some variability in the significance of these nutritional and inflammatory markers between batches (Supplemental Fig A.3.8A), indicating noisiness in the data. Thus, nutritional or inflammatory markers measured in this study do not have a consistent measurable influence on the IgA-targeting of fecal bacteria.
Helminth carriage was also significant in pooled analysis but was almost fully confounded by country (88.5% prevalence in Madagascar versus 2.1% in CAR; Table S1) and was no longer significant when data were stratified or constrained by country (Fig 3.3B, Supplemental Fig A.3.8A).

The prior duration of breastfeeding (i.e., total months the child was breastfed for; all but four children had been weaned by the time of sampling) was also significant in pooled analysis but differed by country (Table A.3.1). Duration of breastfeeding was no longer significant in country-constrained PERMANOVA after FDR correction; however, it was a marginally significant factor in CAR alone (FDR-adjusted p=0.06).

In general, PERMANOVA results for these variables were similar if data were binned taxonomically at the genus level rather than the ASV level (Supplemental Fig A.3.8B). However, in genus-level data, age was a statistically significant factor after FDR correction in the full dataset (Supplemental Fig A.3.8B) and was marginally significant in Madagascar alone (FDR-adjusted p=0.084). Age was also a marginally significant factor at the ASV level but did not pass FDR correction (FDR-adjusted p=0.07, p=0.23, and p=0.35 in the pooled dataset, Madagascar, and CAR respectively).

Taken together, variation in IgA-targeting among children in this population is strongly influenced by geography. Age and prior duration of breastfeeding might also contribute, but the influence of these variables is less significant and differs by country. In contrast, markers of undernutrition and inflammation do not show an important influence.
Figure 3.3 Distribution of IgA-targeting by study metadata. (A) Principal coordinate analysis based on Euclidian distance of the IgA Index, coloured by country of origin. (B) Summary of PERMANOVA analysis of the IgA Index in the full dataset (All) and in each country individually. Analysis is based on taxa maintained at the ASV level. Starred variables are significant with an FDR-corrected p <0.05. Each variable was tested individually in the PERMANOVA without other co-variates. CAR, Central African Republic; Mada, Madagascar; Coef, the coefficient of variance by PERMANOVA. Briefly, the tested variables were as follows: whz_cont, weight-for-height z-score; total IgA, total fecal IgA (ng/g of feces, wet weight); percent IgA+, the percentage of IgA+ bacteria by flow cytometry; helminth, presence or absence of helminths; haz, height-for-age z-score; depth, the relative sequencing depth of the IgA+ fraction compared to the IgA- fraction of a given sample; crp, serum c-reactive protein in mg/l; calprotectin, fecal calprotectin in µg/g dry weight; breastfeeding, total duration of breastfeeding in months; batch, sequencing batch; anemia, presence or absence of anemia; age, child’s age in months; aat, fecal alpha-1 anti-trypsin in mg/g dry weight. N=138 total; N=78 in Madagascar and N=60 in CAR.
3.4.5 IgA-targeting of specific taxa differs by geography but not by stunting

We further explored whether the IgA-targeting of any individual taxa was correlated with chronic undernutrition, or with additional factors identified by PERMANOVA (country of origin, age, breastfeeding duration). Reflecting the contribution of technical factors, a handful of taxa correlated significantly with batch effect by FDR-corrected Wilcoxon Rank Sum test (Table A.3.3); of these, however, most had already been identified as ‘significantly un-targeted’ by IgA (Table A.3.2), underlining the utility of IgA-SEQ in distinguishing host-interacting bacteria.

The IgA-targeting of several taxa differed significantly by country in FDR-corrected linear models adjusted for sequencing depth, batch effect, age and sex (Supplemental Fig A.3.8C-F). Although linear models were used as an exploratory approach to allow for the inclusion of co-variates, we verified that significant hits were also robust to non-parametric methods, including non-parametric bootstrapping of the model and basic uncorrected Wilcoxon Rank Sum test (all taxa in Supplemental Fig A.3.8C-F remained significant by these tests at p<0.05; see R Markdown for further detail). Interestingly, differences in IgA Index by country did not seem to reflect differences in unsorted relative abundance of the same taxa (Supplemental Fig A.3.8G-J), although differences in the processing of these two datasets could have biased detection. Notably, in the case of *Solobacterium* ASV 461 (Supplemental Fig A.3.8C, G) this 16S sequence was barely detected in the unsorted relative abundance data (Supplemental Fig A.3.8G).

No taxa were correlated with age or with breastfeeding in the full dataset at FDR-corrected p<0.05. However, at a more relaxed cutoff of p<0.1, the IgA Index of *Intestinimonas* was negatively correlated with age in a linear model adjusted for sequencing depth, batch effect, country of origin and sex of the child (FDR-adjusted p=0.09). This association was also
significant by uncorrected Spearman’s correlation (Supplemental Fig A.3.9A; rho=-0.29, p=0.0004). An ASV of *Christensenellaceae* was also modestly associated with months of breastfeeding in a linear model adjusted for depth, batch, country, age and sex (FDR-adjusted p=0.09). However, *Christensenellaceae* was not significant by uncorrected Spearman’s (Supplemental Fig A.3.9B; rho=0.14, p=0.10 in the full dataset). Both *Intestinimonas* and *Christensenellaceae* were sparsely detected, and associations with these taxa were also significant after removal of zero values (supplemental R markdown; rho=-0.50 and p=0.0003 for *Intestinimonas* and age; rho=0.24 and p=0.025 for *Christensenellaceae* and breastfeeding, by Spearman’s correlation). No taxa were significant by age or by breastfeeding in either country individually.

We next looked for taxa whose IgA Index correlated with chronic undernutrition (as either a continuous variable (HAZ) or binary factor (stunting)) in linear models corrected for relative sequencing depth, batch effect, country and age. No taxa were associated with HAZ or with stunting in the full dataset or in CAR using an FDR-adjusted p <0.1. In Madagascar, a single ASV of *Lachnospiraceae* NK4A136 was negatively associated with HAZ (FDR-corrected p=0.042) and with stunting status (FDR-corrected p=0.012) and this association remained significant after non-parametric bootstrapping or by uncorrected Spearman’s correlation (Supplemental Fig A.3.9C; p=6.7e-05, rho=-0.43).

Together, these data indicate that the IgA-targeting of specific taxa varies strongly by geography. In contrast, while a small number of taxa correlate with age, breastfeeding status and HAZ, these correlations are inconsistent by geography and should be interpreted with caution.
3.4.6 IgG-targeting of fecal bacteria is rare in stunted and non-stunted children

Given that bacterially-targeted IgG is elevated in the intestines of IBD patients\textsuperscript{164}, and that many biomarkers of intestinal inflammation appear to be shared between EED and IBD\textsuperscript{20}, we investigated whether IgG-targeting of intestinal bacteria was similarly elevated in this population. Fecal samples from 75 children were screened, and most samples demonstrated little to no measurable IgG+ bacteria when compared to an isotype control. However, a small number of samples did show IgG-coated bacterial populations, with 12 out of 75 children having an IgG+ population ≥2% above background and one child displaying a population of nearly 20% IgG+ bacteria (Fig 3.4). IgG-coating did not correlate with levels of total IgG in the feces (Fig 3.4A; \( p=0.6 \) and \( \rho=0.07 \) by Spearman’s correlation). Fecal IgG+ bacteria were significantly higher in children from Madagascar than CAR (Fig 3.4B, \( p=0.0008 \) by Wilcoxon Rank Sum test), similar to the observed increase in IgA+ bacteria in these children (Fig 3.1B). There was no significant relationship between IgG+ bacteria and stunting (Fig 3.4C; \( p=0.39 \) by Wilcoxon Rank Sum test) or markers of inflammation (\( p=0.425 \) and \( p=0.347 \) for calprotectin and AAT, respectively, by Spearman’s correlation and \( p=0.152 \) for CRP by Wilcoxon Rank Sum test). However, all of these observations should be interpreted with caution due to the outlier-skewed distribution of the data.

We selected five highly IgG-targeted samples for a pilot analysis of IgG-targeted microbiota in this population (data points highlighted in red, Fig 3.4B-C). All of these children were from Madagascar; four out of five were severely stunted; two reported a history of diarrheal illness in early life; and three had higher than average AAT in the fecal sample (Table A.3.4). IgG+ and IgG- populations of these five children were sorted, sequenced, and used to calculate
an IgG Index analogous to the IgA Index above. IgG+ bacteria in these children included many members of the normal and IgA+ microbiota, including multiple *Clostridiales* and *Prevotellaceae*, as well as *Bifidobacterium* and *Escherichia-Shigella* (Fig 3.4D). Although the sample size is too small for statistical analysis, these data support previous reports that IgG broadly recognizes many members of the intestinal microbiota. Together, our findings indicate that unlike IBD, chronic undernutrition and EED are not generally associated with elevated IgG-coating of the microbiota; however occasional IgG+ populations exist, and IgG may broadly recognize fecal bacteria.
**Figure 3.4 IgG targeting of the fecal microbiota.** The percentage of IgG-positive (%IgG+) fecal bacteria by flow cytometry relative to an isotype control and (A) total IgG concentration (ng/g wet weight); (B) country of origin and (C) stunting status. N=75. Significance was determined by Spearman’s correlation (A) and Wilcoxon Rank Sum test (B-C). Red datapoints in B-C indicate the five children selected for IgG-SEQ analysis. (D) Top IgG-targeted taxa in five children with high IgG-coating of the fecal microbiota. Colour saturation reflects the numerical IgG Index (red=positively targeted, blue=negatively targeted). The top 10 most and least targeted taxa in each child were selected to display in the heatmap.
3.5 Discussion

Studies on the microbiota of undernourished children have established a signature of dysbiosis that includes increased abundance of a variety of pathogens and pathobionts, and that may drive the inflammatory intestinal condition called EED\textsuperscript{66,68,72,73,211}. IgA is a critical mediator of intestinal homeostasis, and recent studies suggest that IgA recognition of the intestinal microbiota is altered during undernutrition and intestinal inflammation\textsuperscript{59,162,163,212}. We examined IgA-bacterial targeting in almost 200 stunted and non-stunted children across two study sites, constituting one of the largest datasets examined to date and providing valuable insights into IgA-bacterial interactions during chronic undernutrition.

We found here that the overall proportion of fecal microbes bound by IgA was slightly but significantly higher in stunted children. Child stunting has been previously correlated with increased serum antibodies against flagellin and LPS\textsuperscript{27,31,38}. Interestingly, high IgA-recognition of fecal bacteria is also consistently observed in IBD patients, who exhibit an intestinal dysbiosis and inflammation that shares many biomarkers with EED\textsuperscript{20,160,162,163}. High IgA-recognition of intestinal bacteria in stunted children might reflect past or current disruption of intestinal homeostasis, and is consistent with the increased pathogen loads previously observed\textsuperscript{66,68}. However, although this finding was significant in pooled datasets and was consistent in both countries, the trend was non-significant in stratified data and may have been biased by outliers. Reproduction of this finding in other populations of stunted children would therefore be valuable.

We did not find that IgA-recognition of specific bacteria correlated with stunting or inflammatory markers. However, \textit{Lactobacillus} IgA-targeting was low in children from both Madagascar and CAR, regardless of nutritional status. Further, several of the highly IgA-
recognized bacteria in both stunted and non-stunted children are noteworthy for their potential as pathogens or pathobionts. Oral bacteria have been proposed as pathobionts in both undernutrition and IBD\textsuperscript{164,211,220}, and several of the highly targeted genera in this study, including \textit{Haemophilus}, \textit{Prevotella}, \textit{Solobacterium}, and \textit{Campylobacter}, are traditional members of oral microbiomes\textsuperscript{221}. Moreover, multiple species of both \textit{Haemophilus} and \textit{Campylobacter} cause human disease. Both \textit{Haemophilus} and \textit{Campylobacter} are more abundant in stunted children of the larger Afribiota population\textsuperscript{211}, and \textit{Campylobacter} has been strongly and consistently associated with linear growth faltering in children\textsuperscript{65,211,222}, suggesting an important relationship with undernutrition. Both \textit{Haemophilus} and \textit{Campylobacter} are additionally associated with host inflammation, including the species \textit{H. parainfluenzae} to which the ASV observed here was most closely related\textsuperscript{65,223,224}. In fact, in a population of adults from high-income countries, highly IgA-coated \textit{H. parainfluenzae} was unique to IBD patients\textsuperscript{162}. Overall, these data thus highlight immunogenic members of the microbiota, to which children in Madagascar and CAR are widely exposed. Coupled with the lack of IgA-bound \textit{Lactobacillus}, these data may indicate a pervasive dysbiosis in immune-bacterial interactions in children from low-income settings; however, future research is needed to associate these findings with host health.

It is noteworthy that \textit{Haemophilus}, in particular, seems to have an important functional relationship with IgA. Abundance of intestinal \textit{Haemophilus} expands in patients with secretory IgA deficiency\textsuperscript{148} and commensal \textit{Pasteurellaceae}, the family which \textit{Haemophilus} belongs, are persistently expanded in mice born to IgA-deficient dams\textsuperscript{140}. Indeed, pathogenic strains of \textit{H. influenzae}, which are a major cause of bacterial pneumonia in children\textsuperscript{225}, encode IgA proteases as an important virulence factor\textsuperscript{226}. The literature therefore supports the importance of IgA in
controlling *Haemophilus* colonization, suggesting a functional relevance for the IgA-*Haemophilus* interaction observed so pervasively in this population.

Another major finding of this study was the large difference by geography. Total IgA recognition of fecal bacteria was significantly higher in Madagascar than in CAR; in addition, several taxa were differentially targeted. The unsorted fecal microbiota of children in Madagascar and CAR differs significantly\(^\text{211}\), and many environmental and immunological differences exist between these populations (Table A.3.1). This makes it difficult to conclude whether differences in IgA-bacterial targeting are driven by host or microbial factors. However, as has been observed previously, IgA-targeting tended not to correlate with taxonomic abundance\(^\text{137,212}\). One notable difference between the two study populations is the burden of intestinal helminths, which are almost absent in CAR due to successful deworming campaigns but are widespread in Madagascar\(^\text{227}\). Helminths are known to elicit robust IgA responses, and some of this induced IgA has off-target specificity to the bacterial microbiota\(^\text{228}\), offering a possible explanation for the increased IgA-targeting in Madagascar. Although it is not possible to infer causality, these data show clearly for the first time that IgA-targeting patterns are different between geographically distinct populations. In addition, we saw trends in IgA-targeting by both age and the history of breastfeeding, two factors well known to influence the development of the microbiota and of the mucosal immune system\(^\text{137,138,229}\).

Although IgA is the major mucosal antibody, other isotypes may also bind to the intestinal microbiota\(^\text{135}\). In addition to IgA, bacterially-targeted IgG is elevated in the intestines of IBD patients\(^\text{160,165}\), and specific taxa may be differentially recognized by IgG during IBD\(^\text{164,230}\). As noted, many biomarkers of intestinal inflammation are shared between EED and IBD, such as elevated fecal calprotectin and blunting of small intestinal villi\(^\text{20}\); we therefore
hypothesized that children with EED markers might also have increased IgG-recognized fecal bacteria. However, fecal IgG-coating of the microbiota was low in almost all samples measured, similar to values reported in healthy human populations\textsuperscript{219}. Interestingly, a small number of samples in Madagascar did have measurable IgG-coating of the microbiota; two out of five of these samples had reported a previous diarrheal infection and another two had elevated levels of fecal inflammatory markers (AAT or calprotectin). We speculate that transient IgG-coating reflects recent or ongoing intestinal infections. IgG-targeting in this small set of samples also supports findings that IgG, like IgA, recognizes diverse members of the bacterial microbiota\textsuperscript{219,231}.

Our study has several limitations. Ig Index data is extremely heterogeneous and sparse, and popular analysis tools like Deseq2 cannot be applied to normalized data like the log-adjusted IgA Index, making it challenging to find appropriate statistical methods. Compared to other published studies\textsuperscript{59,162}, our FDR-corrected models were relatively stringent and were further limited by the use of non-parametric methods; thus, although our sample size was large for a study of this kind, we were likely underpowered to detect small differences after FDR correction. We may therefore have underestimated differences in IgA-targeting by stunting or inflammation. We additionally had significant technical variation between sorting and sequencing batches, and although we attempted to correct and account for this as rigorously as possible, technical variation further limited our statistical power. Of note, we also found that the IgA Index was influenced by the difference in sequencing depth between the IgA+ and IgA- fractions, even after rarefaction; to our knowledge, this depth bias has not been previously accounted for in IgA-SEQ datasets. Nevertheless, our study benefited from the acknowledgement of these technical biases, as well as from a relatively large sample size and from geographically distinct populations which
allowed us to find reproducible trends. Given the high inter-individual variability in bacterial IgA-targeting, we feel the detection of consistent signatures between countries is particularly valuable.

A correlation between undernutrition and poor sanitation has now been recognized for many years, but it is not certain how these factors are linked or how they relate to the heterogenous disease EED\textsuperscript{49}: a better understanding of host-microbial interactions during human undernutrition is required. Our data suggest that stunted children have a greater proportion of IgA-recognized bacteria in the fecal microbiota; validation of this finding in other populations would be valuable. We moreover identify two putative pathobionts, \textit{Haemophilus} and \textit{Campylobacter}, that are highly targeted by intestinal IgA. These data improve our understanding of immune-microbiota interactions during undernutrition and may inform causative studies on the role of intestinal microbes in child growth.
Chapter 4: Mutual growth promotion of Bacteroidales and E. coli in protein-restricted nutritional environments

4.1 Synopsis

Child undernutrition is associated with dysbiosis of the intestinal microbiota and with EED. Specific communities of human-derived Bacteroidetes and Enterobacteriaceae have been shown to induce EED features in undernourished mice; this phenotype is dependent on the combined presence of both taxonomic groups and on a low-protein diet. Here, we show that EED-inducing communities of Bacteroidetes and Enterobacteriaceae are capable of synergistic growth in protein-limited, carbohydrate-rich nutritional environments. Through the production of secreted factors, B. ovatus supports the growth of E. coli, and E. coli reciprocally supports the growth of B. fragilis, leading to a substantial increase in total community growth. This bi-directional cross-feeding is partly dependent on metabolism of sialic acid by E. coli, and on heme uptake by B. fragilis. Together, these data support the hypothesis that microbiota dysbiosis is exacerbated by pathobiont cross-feeding in the undernourished gut.

4.2 Introduction

As outlined in Chapter 1, there is substantial evidence for a microbial etiology of EED; however, the mechanisms by which microbiota dysbiosis arises and influences host health remain unclear. The model of EED developed by our lab shows that a specific combination of both Bacteroidales (B. fragilis, B. vulgatus, B. ovatus, B. dorei, and Parabacteroides distasonis) and E. coli (E. coli strains 3/2/53 and 4/1/47) is required to induce the features of EED in mice:
providing either *Bacteroidales* or *E. coli* alone is insufficient. Moreover, this EED-inducing interaction must take place in the background of protein and fat- malnutrition, since mice that receive the same bacterial cocktail but are fed an isocaloric control diet do not develop EED. The mechanism driving this context-dependent synergy between *Bacteroidales* and *E. coli* is currently unknown. I showed in Chapter 2 that these *Bacteroidales* and *E. coli* strains successfully colonized both the colon and the small intestine of EED mice; surprisingly, however, none of these strains were strongly recognized by host IgA (Supplemental Fig A.2.3C-D). Indeed, the data presented in Chapter 2 emphasized the importance of the metabolic and microbial environment – and not necessarily the immune environment - in shaping bacterial function during undernutrition. I therefore hypothesized that *Bacteroidales* and *E. coli* were capable of metabolic cross-feeding, and that this would be exacerbated in protein-deficient, carbohydrate-rich environments, leading to synergistic growth of these bacteria and intestinal dysbiosis in EED mice.

Metabolic interactions between individual *Bacteroidales* and *Enterobacteraceae* strains have previously been described, and are known to contribute to bacterial dysbiosis and infection in other disease contexts. *Bacteroides* digest complex polysaccharides in the host diet and host mucosa, liberating simple sugars which can act as growth substrates and signaling cues for *Enterobacteriaceae*. These metabolites may be used by pathogenic *E. coli* and *Salmonella* to control the expression of virulence factors and establish an infection in the intestinal tract. Conversely, *Bacteroides spp.* have been shown to benefit from the presence of *Enterobacteraceae* during both intestinal and extraintestinal infection, thanks to the production of heme and heme-scavenging chelators by *E. coli* or *Salmonella*. Whether both
types of cross-feeding occur simultaneously in mixed communities, and how these interactions might be altered by changes in nutrient availability, have not yet been studied.

Here we show that the undernutrition-associated pathobionts Bacteroidales spp. and Escherichia spp. are capable of mutual, bi-directional cross-feeding, resulting in synergistic growth of a mixed community in vitro. Growth synergy requires the presence of both host mucin and dietary carbohydrates, and involves metabolic pathways for sialic acid consumption in E. coli and heme uptake in B. fragilis. This synergy is dampened in protein- and iron- rich media, indicating a role for host nutrition in shaping pathobiotic co-dependencies within the microbiota.

4.3 Materials and Methods

4.3.1 Bacterial strains and mutant construction.

All bacterial strains and mutants used in this study are listed in Table A.4.1. Bacteroidales and E. coli strains from the BG community were human microbiome-derived strains, provided originally as a gift from Dr. Emma Allen-Vercoe, University of Guelph, and used previously by our lab78. B. fragilis wild-type strain 608R and ΔfeoAB were a generous gift from Dr. Edson Rocha, East Carolina University239. B. thetaiotaomicron was provided by the American Type Culture Collection (ATCC).

ΔnanA, ΔfucI, and ΔnagB mutants were generated using homologous recombination to replace the target gene with a kanamycin resistance cassette (KanR) following a protocol based on lambda red recombination that has been previously described240. Briefly, the KanR locus on pkD13 was PCR-amplified using primers with 5’ overhangs homologous to the target gene. Amplicons were purified by phenol chloroform extraction and electroporated into target E. coli
carrying pkD46 expressing the lambda red recombinase. Transformants were selected on LB agar with 40 μg/ml kanamycin. Mutants were genotypically confirmed by PCR using primers flanking KanR. Mutant phenotypes were confirmed by growth in M9 minimal media with fucose, sialic acid, or N-acetylglucosamine (NAG) as a sole carbon source. Growth was assessed by \( \text{OD}_{600} \) using a plate reader (Tecan). PCR was performed using a T100™ Thermal Cycler (Bio-Rad) with the following cycling conditions: 98°C for 3 min; 35 cycles of 98°C for 10 sec, 68°C for 30 sec, 72°C for 2 min; 72°C for 10 min.

4.3.2 Bacterial media and growth conditions.

*Bacteroidales* and *E. coli* strains were routinely grown on Fastidious Anaerobic Agar (FAA) for 48h at 37°C and in Fastidious Anaerobic Broth (FAB) for 24h at 37°C with shaking at 250 rpm, under anaerobic conditions created by a sealed anaerobic jar and GasPak (BD). *E. coli* mutant strains were routinely grown with the addition of 40 μg/mL kanamycin and *B. fragilis* ΔfeoAB was routinely grown with the addition of 10 μg/mL erythromycin.

Complex media for growth experiments were prepared according to the macronutrient quantities shown in Tables A.4.2 and A.4.3. In addition, all complex media contained, per 1 L: 13.6 g of KH₂PO₄; 0.875g of NaCl; 1.125g of \((\text{NH}_4)_2\text{SO}_4\); 1.0 mL of histidine-hematin solution (1.9 mM hematin in 0.2M L-histidine); 1.0 mL MgCl₂ solution (0.1 M in water); 1.0 mL vitamin K₃ solution (1 mg/mL in ethanol); 1.0 mL CaCl₂ solution (0.8% w/v in water); and 0.5 mL of vitamin B₁₂ solution (0.01 mg/mL in water). Media pH was adjusted to 7.2 with concentrated NaOH. Mucin was Type II Mucin from Porcine Stomach (PGM) (Sigma-Aldrich).
4.3.3 Bacteroidales-E. coli community growth assays.

Liquid cultures of Bacteroidales and E. coli in FAB were resuspended and washed twice in a carbon-free buffer (either PBS-/ or a minimal media containing no carbon source) and normalized to OD_{600} = 0.35 in PBS. Inoculation mixes were prepared from these normalized suspensions at 1:1 ratios: for example, 100 µL of each of the 7 strains for a full-community mix. Missing strains were replaced by PBS: for example, an E. coli-only inoculation contained 100 µL of each E. coli strain and 500 µL of PBS. Of this inoculation mix, 70 µL was added to 2 mL of degassed complex media. Inoculations were performed within an anaerobic chamber into media degassed for at least 24h. Co-cultures were grown anaerobically at 37°C for 24 hours with shaking. To assess growth outcomes, samples were serially diluted in PBS, plated, and grown on LB agar aerobically at 37°C for 24 hours to quantify E. coli growth, on FAA plates anaerobically to estimate total community growth, or on antibiotics-containing plates to enumerate mutant growth as appropriate. In addition, culture samples were frozen at -20°C to quantify bacterial abundance by qPCR.

4.3.4 16S rRNA qPCR to assess bacterial growth.

Total abundance of Bacteroidales and E. coli was assessed using taxa-specific primers (Table A.2.2) and the Quantitect SYBR-Green Mastermix (Qiagen) following manufacturer’s protocols. Culture samples were heat-lysed at 100°C for 15 min to release DNA. Samples taken past 12 hours were diluted 1:10 or 1:20. qPCR was performed on cell lysates using an Applied Biosystems™ 7500 Fast Real-Time PCR Machine. C_{T} values were converted to 16S copy
number using a standard curve generated from qPCR amplification using taxa-specific primers of DNA purified from ATCC bacterial strains. Results were expressed in 16S copies/μl of culture.

4.3.5  E. coli growth assessment in Bacteroidales supernatant.

Bacteroidales and E. coli cultures were grown in FAB, washed in PBS, and normalized to an OD$_{600}$ = 0.35 as previously described. Bacteroidales or E. coli were subcultured 1 in 200 into a final volume of 2 ml of experimental media (MAL+M IMM as defined in table A.4.2). Cultures were grown for 16 or 24 hours at 37°C in an anaerobic chamber with shaking. Endpoint cultures were centrifuged for 20 min at 16000 g and supernatants were filter-sterilized through a 0.22 μm filter. E. coli was normalized to OD$_{600}$ = 0.35 as previously described and were subcultured 1 in 200 into a final volume of 200 μl of culture supernatant in a 96-well plate and grown at 37°C. OD$_{600}$ was recorded every 15 minutes for 24 hours using a plate reader (BioTek).

4.3.6  High throughput 16S sequencing for analysis of community growth kinetics.

Bacteroidales and E. coli cultures were grown, washed in BMM, and normalized to an OD$_{600}$ = 0.35 as previously described. Cells were subcultured 1 in 200 into a final volume of 10 ml MAL+M to form a mock community. Community was grown in an anaerobic chamber at 37°C for 24 hours. Samples collected at 0 and 24 hours were heat-lysed at 100°C for 15 min. The 16S rRNA gene (V4 region) was amplified directly from lysates using Illumina-tagged, bar-coded primers 515F-806R from Table S5 (49). PCR was performed using a T100™ Thermal Cycler (Bio-Rad) with the following cycling conditions: 98°C for 5 min; 30 cycles of 98°C for 20 sec, 55°C for 15 sec, 72°C for 30 min; 72°C for 10 min. Reactions were run on a gel to ensure successful amplification, purified and normalized using the 96well Sequal-Prep kit. Amplicons
were pooled and gel-purified using a GeneJet Gel Extraction Kit (ThermoFisher) to remove primer dimers, and DNA yield was quantified by PicoGreen and qPCR. The final library was pooled and sequenced on an Illumina MiSeq platform with 30% PhiX, using the v2 kit for paired-end 250 bp reads.

4.3.7 Bioinformatic analysis.

Demultiplexed forward reads were analyzed in QIIME2 (https://qiime2.org/), using the DADA2 option for sequence quality control and trimming to 250 bp. Taxonomic assignment was performed using a Naïve Bayes classifier trained on the SILVA database (v132, 99% OTU sequences, region 515F/806R). For Bacteroides which were not identified to the species level by the SILVA database, the 16S sequence was further queried using BLAST to rationally assign all 5 BG community members. Filtering at the amplicon sequence variant (ASV) level was performed in R using the phyloseq package, to remove specific taxa (Archaea, Mitochondria, Chloroplasts, and the common reagent contaminants Halomonas and Shewanella), samples with low read counts (blanks, <200 reads) and rare taxa (appearing less than three times in the whole dataset). Taxonomic barplots were then created using ggplot2. To calculate total abundance of each individual Bacteroidales species over time, relative abundances were multiplied by the total Bacteroidales abundance, as determined by 16S Bacteroidetes-specific qPCR, at each time point.

4.3.8 Statistical analysis

Statistical analysis was performed in GraphPad Prism (www.graphpad.com). Aggregate results represent the mean +/- SEM, and statistical significance is represented by *p value <0.05,
**p value <0.01 , ***p value <0.001 and ****p value <0.0001. Sample size, center and dispersion metrics, and statistical tests are also reported in the figure legends.

4.4 Results

4.4.1 Synergistic growth of Bacteroidales and E. coli in a carbohydrate-rich media

We designed a complex medium that was rich in dietary plant polysaccharides and host mucin, but deficient in protein and fat, to reflect the macronutrients available in the intestine during chronic protein-energy undernutrition (Fig 4.1A, Table A.4.1). This medium was used to co-culture seven strains of human intestinal bacteria used previously to induce inflammation and barrier disruption in a mouse model of EED: five strains of Bacteroidales (Bacteroides fragilis, B. vulgatus, B. ovatus, B. dorei, Parabacteroides distasonis) and two Enterobacteriaceae (Escherichia coli 4_1_47 and Escherichia coli 3_2_53 (Fig 4.1A). Unless otherwise indicated, in this chapter Bacteroidales will refer to all five strains of Bacteroidales grown in combination, while E. coli will refer to both strains of E. coli grown together (“B” and “E”, respectively, in the figures; Fig 4.1A).

Both Bacteroidales and E. coli were capable of growing in this carbohydrate-rich medium, attaining $10^7$ and $10^8$ CFU/mL, respectively, after 24 hours of anaerobic growth (Fig 4.1B). When all seven species were grown in combination, the co-culture grew to $10^9$ total CFU/mL, a 10-fold increase that was substantially more than expected based on simple additive growth (Fig 4.1B).
Unexpectedly, this synergistic increase in bacterial CFU was driven by improved growth of both Bacteroidales and E. coli simultaneously. The growth of E. coli roughly doubled in the presence of Bacteroidales (Fig 4.1C), while the growth of Bacteroidales increased by 10-fold or more in the presence of E. coli (Fig 4.1D). These bacteria thus mutually support one another’s growth through cooperative interactions.

Figure 4.1 Synergistic growth of Bacteroidetes and Enterobacteriaceae in a carbohydrate-rich media. (A) Schematic of the experimental set-up. After a 24h culture, bacterial endpoint growth was determined for (B) the total community, (C) E. coli and (D) Bacteroidetes. Mean +/- SEM are displayed. Significance was determined by Mann-Whitney (C,D). B, Bacteroidetes mix; E, E. coli mix; CFU, colony forming units.
4.4.2 Synergistic growth is dependent on both host mucus and dietary carbohydrates

To better understand the importance of the nutritional environment in promoting *Bacteroidales - E. coli* growth synergy, we compared growth in this original medium, designated “MAL-M” (for malnourished + mucin), with medium in which the ratio of dietary protein to carbohydrate was reversed (“CON-M” for control + mucin) and to CON and MAL media in the absence of host mucin (Supplemental Fig A.4.1A, Table A.4.2). In these complex media, the presence of mucin promoted total growth of the co-culture (Supplemental Fig A.4.1B). However, only the carbohydrate-rich mucin medium (MAL-M) led to enhanced growth of both *Bacteroidales* and *E. coli* simultaneously compared to their respective monocultures (Supplemental Fig A.4.1B-D).

To more clearly distinguish the importance of carbohydrate versus protein in promoting synergistic growth, we cultured bacteria in media containing only dietary carbohydrates (inulin, starch, and cellulose; “Carb”) or only dietary protein (casein; “Prot”) as the carbon source, with or without mucus as a source of host glycoprotein (Fig 4.2A, Table A.4.3; “CarbM” and “ProtM” designate mucin-containing media). Importantly, when grown separately, *Bacteroidales* and *E. coli* showed similar growth in the mucin-containing CarbM and ProtM media, indicating that the inherent availability of nutrients is comparable between these two media (Fig 4.2C-D). However, total bacterial counts in co-culture were significantly higher in the carbohydrate- and mucus-rich CarbM medium than in any other condition, with both *E. coli* and *Bacteroidales* showing a distinct advantage in CarbM co-culture compared to ProtM co-culture (Fig 4.2B-D).

Together, these data indicate that the nutritional environment is important for *Bacteroidales-E. coli* synergism. *Bacteroidales* and *E. coli* show a mutual growth advantage under protein-limited conditions in the presence of mucus and dietary carbohydrates.
Figure 4.2 Synergistic growth of Bacteroidales and Enterobacteriaceae is dependent on the presence of mucus and dietary carbohydrates. (A) Media macronutrient compositions. After a 24h culture, bacterial endpoint growth was determined for (B) the total community, (C) E. coli and (D) Bacteroidetes. Mean +/- SEM are displayed. Significance was determined by two-way ANOVA with Tukey’s post-hoc test. B, Bacteroidales mix; E, E. coli mix; CFU, colony forming units; Carb, carbohydrate media; CarbM, carbohydrate plus mucus media; Prot, protein media; ProtM, protein plus mucus media.
4.4.3  *B. fragilis* benefits from *E. coli* at the expense of other *Bacteroidales* spp., but is not sufficient for reciprocal growth of *E. coli*

To determine whether the expansion of *Bacteroidales* was driven by the abundance of one or more of the individual species present in our cocktail, we performed 16S rRNA sequencing of *Bacteroidales* cultures in MAL+M media in the presence or absence of *E. coli*. Relative abundances were converted to total abundances by means of *Bacteroidetes*-specific 16S rRNA qPCR. All five *Bacteroidales* strains were able to grow in co-culture in the absence of *E. coli* (Fig 4.3A-C, Supplemental Fig A.4.2B-D). In the presence of *E. coli*, however, *B. fragilis* bloomed disproportionately (Fig 4.3A) at the expense of *B. vulgatus* and *B. dorei* (Supplemental Fig A.4.2B-C). Indeed, after 24 hours of growth in co-culture, *B. fragilis* constituted over 50% of the *Bacteroidales* by relative abundance (Supplemental Fig A.4.2A).

The enrichment of *B. fragilis* in co-culture could indicate a keystone role for this taxon in supporting mutualism. However, this species was not sufficient to confer a reciprocal growth advantage to *E. coli* (Fig 4.3D). Instead, *B. ovatus* provide the most substantial individual growth benefit to *E. coli*, recapitulating the benefit seen with all five strains (Fig 4.3D-E). These data suggest that multi-strain interactions are involved in the synergistic growth of this community.

To further delineate strain interactions, we grew co-cultures with various combinations of *B. fragilis*, *B. ovatus*, and *E. coli*. Removing *B. ovatus* from the full *Bacteroidales-E.coli* community caused only a slight and non-significant decrease in *E. coli* growth, suggesting that the cumulative effect of other *Bacteroidales* species might contribute to *E. coli* growth independently of *B. ovatus* (Fig 4.3E). Nevertheless, *B. ovatus* alone was sufficient to fully support the growth of *E. coli* (Fig 4.3D-E). Interestingly, we found that growth of *B. ovatus* was enhanced in the presence of *E. coli* when other *Bacteroidales* were absent (Fig 4.3F). A simple
three-strain community of *B. fragilis*, *B. ovatus*, and *E. coli* also achieved a mutual growth advantage of both *E. coli* and *Bacteroidales*, which was similar to that observed with *B. ovatus* and *E. coli* alone (Fig 4.3E-F). The similar total growth of *Bacteroidales*, whether or not *B. fragilis* was present, suggests that *B. fragilis* and *B. ovatus* do not support one another’s growth but instead compete for finite resources. The ability of *B. ovatus* to benefit from *E. coli* alone, but not in the full community, also supports the conclusion that *B. ovatus* is out-competed by other *Bacteroidales* in mixed co-culture (Fig 4.3C,F).

Together, these data suggest that both cooperative and competitive interactions contribute to the growth of *Bacteroidales* and *Enterobacteriaceae* in co-culture. While *B. ovatus* provides a benefit to *E. coli* and receives a reciprocal advantage, *B. fragilis* appears able to take advantage of this exchange, obtaining an apparently selfish benefit within the mixed community.
Figure 4.3 B. fragilis takes advantage of B. ovatus-E. coli synergy. (A) Total growth of each Bacteroidales species at 24 endpoint, in a mixed community with or without E. coli. (B-C) Total growth of B. fragilis (B) and B. ovatus (C) over time in a mixed community with or without E. coli. (D) Growth of E. coli at 24h endpoint with or without each individual Bacteroidales species or the full mix of all five Bacteroidales. (E) Growth of E. coli at 24h endpoint with iterative combinations of B. fragilis and B. ovatus. (F) Total growth of B. ovatus and B. fragilis at 24h endpoint, with or without E. coli. Mean +/- SEM are displayed. Significance was determined by two-way ANOVA with Sidak’s post-hoc test (A) or by Kruskal-Wallis with post-hoc Dunn’s test (D-F). B, full Bacteroidales mix; E, E. coli mix; BF, B. fragilis; BO, B. ovatus; BV, B. vulgatus; Pdis, P. distasonis; Bdo, B. dorei.
4.4.4 *Bacteroidales* and *E. coli* exchange soluble metabolites, including sialic acid and heme

To determine whether growth synergism in co-culture was mediated by secreted factors, we assessed bacterial growth in sterile supernatants from blank media or from 24-hour cultures of *Bacteroidales* or *E. coli*. As expected, *E. coli* grew significantly better in supernatants from *Bacteroidales* culture compared to blank media or its own spent growth media, while *Bacteroidales* grew significantly better in *E. coli* culture supernatants compared to blank media or its own spent growth media (Fig 4.4 A-B). Thus, both *E. coli* and *Bacteroidales* produce secreted factors in carbohydrate-rich environments that benefit the other community member.

To identify metabolic pathways involved in synergistic growth, we began by testing the role of mucin-derived sugars, since mucus stimulated co-culture growth (Fig 4.2, Supplemental Fig A.4.1) and has been previously shown to fuel microbial cross-feeding. Indeed, co-culture of *E. coli* with *B. thetaiotaomicron*, a species previously shown to cooperatively liberate sialic acid from mucus\(^{97}\), gave a dramatic growth benefit to these *E. coli* strains (Supplemental Fig A.4.3 A). We therefore generated knockout strains of *E. coli* lacking key metabolic enzymes for consumption of sialic acid (\(\Delta nanA\)), N-acetylglucosamine (\(\Delta nagB\)) and fucose (\(\Delta fucI\)), three sugars that decorate mucin.

Mutants of *E. coli* deficient in sialic acid consumption were at a significant competitive disadvantage compared to wild-type strains in the presence of *Bacteroidales* co-culture, but not when grown alone (Fig 4.4C). Further supporting the importance of carbohydrate-rich nutritional context that we identified earlier, when \(\Delta nanA\) *E. coli* was co-cultured in the Carb and Prot media described above, we only observed a significant competitive defect of \(\Delta nanA\) *E. coli* in CarbM media (Supplemental Fig A.4.3 B). There was no significant competitive defect for
ΔnanA in ProtM media, even though this condition contained identical quantities of mucin as a source of sialic acid, which is consistent with the lack of co-culture growth advantage in this media (Fig 4.2). There was furthermore no significant competitive defect for ΔnanA in the absence of Bacteroidales in any media (Supplemental Fig A.4.3 B). We did not observe any measurable competitive defect for ΔnagB and ΔfucI mutants in co-culture (Supplemental Fig A.4.3 C-D), although there was a trend for ΔfucI that may achieve statistical significance with further replicates. Together these data suggest that sialic acid metabolism contributes to E. coli growth in the presence of Bacteroidales, and that this interaction is specifically enhanced in a polysaccharide- and mucin-rich environment.

B. fragilis has been shown to benefit from E. coli during extraintestinal co-infection due to E. coli production of heme237. We found that the growth of B. fragilis was dependent on organic iron (hematin) in the media during monoculture, but not during E. coli co-culture (Supplemental Fig A.4.4 A). Growth was not dependent on the concentration of vitamin B12, another putatively limiting micronutrient (Supplemental Fig A.4.4 B). To test whether organic iron was responsible for the B. fragilis growth advantage in co-culture, we used a B. fragilis ΔfeoAB knockout strain deficient in heme uptake239. As expected, ΔfeoAB did not benefit as strongly from co-culture as wild-type B. fragilis (Fig 4.4D), despite similar growth in monoculture. These data suggest that B. fragilis benefits from E. coli-derived heme.

Notably, both ΔnanA E. coli and ΔfeoAB B. fragilis still benefited significantly from co-culture, suggesting that other unidentified factors also contribute to this growth synergy (Supplemental Fig A.4.4 C-D). Together, these data point to at least two metabolic pathways involved in Bacteroidales-E. coli growth synergy; however, future work is needed to clarify their contributions and to identify additional pathways important for cross-feeding.
Figure 4.4 *E. coli* and *Bacteroidales* exchange soluble metabolites, including sialic acid and heme. (A-B) Growth of *E. coli* (A) and *Bacteroidales* (B) at 24 endpoint, as determined by optical density at 600 nm (O.D.600) in the sterile filtered supernatants of blank media (‘Blank’), a 24h *E. coli* culture (‘E’), or a 24h *Bacteroidales* culture (‘B’). (C) Competitive index of ΔnanA *E. coli*, deficient in sialic acid metabolism, compared to wild-type in the presence or absence of *Bacteroidales*. (D) Competitive index of ΔfeoAB *B. fragilis*, deficient in heme uptake, compared to wild-type in the presence or absence of *E. coli*. Mean +/- SEM are displayed. Significance was determined by Kruskal-Wallis with Dunn’s post-hoc test (A-B) or by Mann-Whiney (C-D). B, *Bacteroidales* mix; E, *E. coli* mix.
4.5 Discussion

Microbial communities are built from complex ecological interactions. Many, if not most, of these interactions are negative: bacteria compete with one another and take advantage of their neighbours in the battle for scarce resources\textsuperscript{81}. This high level of competition within the gastrointestinal microbiota is beneficial to the host, by maintaining relatively stable and diverse communities and by competitively excluding pathogens\textsuperscript{81,241}. In contrast, bacterial cooperation is a relatively unusual ecological interaction with predicted destabilizing effects on microbial communities. By mutually enhancing one another’s growth, cooperative bacteria are expected to ‘boom and bust’ together, leading to unstable and low-diversity communities\textsuperscript{81}. Such low-diversity communities are generally considered to be the hallmark of dysbiosis in gut microbiota research, particularly when the high-abundance taxa are facultatively anaerobic \textit{Proteobacteria}\textsuperscript{75}.

Here we find that a mixture of \textit{Bacteroidales} and \textit{Enterobacteriaceae} are capable of mutually cooperative, synergistic growth, and thus have the potential to destabilize intestinal communities. Although it has been previously shown that certain \textit{Bacteroides} spp. can support the growth of pathogenic \textit{Enterobacteriaceae}\textsuperscript{97,232}, and that certain \textit{E. coli} strains can support \textit{Bacteroides} growth\textsuperscript{237,238}, the possibility for bidirectional feedback has never been explored. In fact, it was explicitly shown that \textit{B. fragilis}, unlike \textit{B. thetaiotaomicron}, did not provide any direct benefit to \textit{E. coli}\textsuperscript{97}. We confirm these results but find that \textit{B. fragilis} can take advantage of a metabolic exchange between \textit{B. ovatus} and \textit{E. coli}. In a mixed community, \textit{B. fragilis} and \textit{E. coli} bloom simultaneously: this mutual advantage is dependent on the presence of other \textit{Bacteroidales} and especially \textit{B. ovatus}, a known ‘cooperator’ which provides a benefit to \textit{E. coli} while being out-competed for the reciprocal advantage. These results define a multi-species interaction network with potential functional relevance in the gut.
Nutritional context was key to the emergence of synergistic growth in these bacteria: coculture synergy required the presence of both host mucin and dietary carbohydrates. Importantly, such favourable growth conditions are more likely to occur in the human gut during protein undernutrition, where there is a paucity of dietary protein compared to plant-based carbohydrates\textsuperscript{1,78}. Bacterial foraging of the mucus layer is also enhanced when dietary nutrient sources are lacking\textsuperscript{87}; furthermore, the availability of mucus itself might be enhanced during intestinal infection and inflammation, since the host generates and sheds additional mucus to try and exclude invading pathogens\textsuperscript{96}. We thus hypothesize that conditions are favourable to bacterial cross-feeding in the undernourished, inflamed guts of children in low-income settings, and that synergistic growth of \textit{Bacteroidales} and \textit{Proteobacteria} under these conditions may help contribute further to intestinal dysbiosis and damage.

An overabundance of intestinal \textit{Proteobacteria} species has been consistently reported in the gut microbiomes of undernourished children and is sometimes accompanied by increases in specific \textit{Bacteroidales}\textsuperscript{58,60,71,72}. Members of the \textit{Bacteroidales} also bloom in mice fed malnourished diets compared to high-fat recovery diets\textsuperscript{57,78}. Remarkably, as noted, human isolates of \textit{Bacteroides} and \textit{E. coli}, including a combination of the strains used in this paper, have been shown to exacerbate intestinal inflammation and growth faltering in undernourished mice, and this phenotype is dependent on the presence of both taxa at once\textsuperscript{59,78}. An intervention trial which administered resistant-starch prebiotics to children in Malawi noted that, in contrast to the expected beneficial effects of the prebiotics, the intervention caused an increase in the fecal inflammatory marker calprotectin, as well as intestinal blooms in fecal \textit{Bacteroidetes}, LPS biosynthesis, and specific \textit{E. coli}-associated enzymes\textsuperscript{125}. Although these results were unexpected and unfortunate, they are consistent with our prediction that an overabundance of fermentable
fiber in malnourished children can exacerbate *Bacteroides*-*E. coli* synergistic growth and lead to intestinal inflammation.

Interestingly, members of the *Bacteroidales* have been the subject of considerable research interest at the opposite end of the nutritional spectrum: their abundance is widely associated with a lean, non-obese phenotype in Western individuals. Specific *Bacteroidales*, including *P. distasonis*, directly protect against obesity and diabetes in mouse models fed high-fat diets\(^\text{242–244}\). This has been linked to decreased total energy harvest by the microbiota in *Bacteroidetes*-rich communities compared to *Firmicutes*-rich communities, as well as to an influence of *Bacteroides* on host appetite and metabolism\(^\text{245}\). There is therefore substantial evidence that *Bacteroidales* can regulate host metabolic health; unfortunately, the very characteristics which make these bacteria useful in combatting obesity and diabetes in the western world could be harmful for undernourished children struggling to gain weight.

In addition to the regulation of host metabolic health, both *Bacteroidales* and *E. coli* have the potential to drive inflammation and intestinal damage. Critically, of all the *Bacteroides*, *B. fragilis* is the species most commonly observed in extraintestinal infections, and is most likely to carry toxins that cause human intestinal damage and diarrhea\(^\text{246}\). It is therefore noteworthy that *B. fragilis* benefitted most significantly from synergistic growth in mixed co-culture. Indeed, enterotoxigenic strains of *B. fragilis* are prevalent in stunted children and cause weight loss in mice; strikingly, Wagner *et al.* showed that weight loss in mice was dependent both on the toxin-producing strain of *B. fragilis* and on the presence of a complex, *Enterobacteriaceae*-containing microbiota from the stunted child\(^\text{63}\).

There is substantial evidence for the role of *Enterobacteriaceae* in promoting intestinal inflammation, dysbiosis, and barrier damage in multiple different contexts; the concept of
Enterobacteriaceae-rich dysbiosis has become well established in microbiome research\textsuperscript{75}. Intestinal inflammation and oxygenated gut environments are broadly associated with Enterobacterial blooms and infections; furthermore, many of these species carry toxins and virulence factors, and pathogenic strains are prevalent in undernourished children even in the absence of overt diarrheal disease\textsuperscript{66,73,171}. As discussed in Chapter 1, intestinal inflammation can contribute to growth faltering by causing intestinal damage and nutrient malabsorption, by consuming energetic resources to sustain an immune response, and by directly altering the IGF-1 growth axis\textsuperscript{131,132,247}.

Taken together, therefore, both Bacteroidetes and Enterobacteriaceae have the potential to exacerbate EED and growth faltering in undernourished children. In this study, we provide proof-of-principle experiments to show that mutual growth synergy occurs between these undernutrition-associated taxa, and furthermore that such synergy is exacerbated under protein-limited, carbohydrate-rich conditions. Although the \textit{in vitro} approach taken in this paper is not sufficient to represent the complexities of the gastrointestinal tract \textit{in vivo}, it allowed us to mechanistically define a complex inter-species network. On the basis of this evidence, we hypothesize that self-perpetuating synergistic growth between Bacteroidales and Enterobacteriaceae on low-protein, carbohydrate-rich diets may drive dysbiotic microbial communities and intestinal inflammation. This specific, testable hypothesis can be applied in future research to unravel the contribution of intestinal microbiota to child undernutrition.
Chapter 5: Conclusion

5.1 Contributions to the field

Microbial contributions to undernutrition are an area of intense research interest given the failure of nutritional and sanitary interventions to reverse child stunting. Acutely undernourished children exhibit dysbiosis of the intestinal microbiome, characterized in part by abundant and IgA-bound *Enterobacteriaceae*, but the causes of this microbial dysbiosis remain unclear. In 2015 when this thesis commenced, it was unknown whether similar microbiota dysbiosis occurred in chronic undernutrition, a condition with long-lasting health consequences. In the last five years, the existence of microbial dysbiosis in chronic undernutrition has received growing attention. The data presented here support the presence of intestinal dysbiosis in chronic undernutrition, and further clarify specific bacterial-bacterial and bacterial-host interactions that may contribute to it.

In Chapter 2 I show that chronic dietary undernutrition in mice directly affects IgA-bacterial interactions, abolishing IgA recognition of commensal *Lactobacillus*. Dietary adaptations in *Lactobacillus* altered its ability to bind host IgA and to colonize the small intestinal mucous layer. *Lactobacillus* species frequently have beneficial effects on host health, in part via their interactions with the mucosal immune system\(^{116,124,133,207,248,249}\). Indeed, *Lactobacillus* is considered a promising probiotic candidate for undernutrition, as discussed in Chapter 1\(^{112,116,117,119}\). Reduced immune recognition and mucosal colonization of this bacterium during undernutrition could thus affect its utility as a probiotic, emphasizing the importance of monitoring the colonization and adaptation of probiotics in target populations\(^{114,119,198}\). These data may also have implications for the use of oral vaccines in undernourished populations\(^{250}\). Despite sufficient mucosal IgA levels, children from low-income regions are more susceptible to
intestinal infections and mount weaker IgA responses to oral vaccines\cite{30,46}; dysregulation of IgA-microbial interactions during undernutrition could help to explain this phenomenon\cite{250}. Future work looking at IgA-microbiota binding and its relation to oral vaccine responses in undernourished children may provide valuable insights.

In Chapter 3 I measure IgA-bacterial interactions in chronically undernourished (stunted) and non-stunted children from Madagascar and CAR through the Afribiota project. I assisted in characterization of the total fecal and duodenal microbiota, showing that enteropathogens and oropharyngeal bacteria were overabundant in the gastrointestinal tracts of stunted children\cite{211}. Recently, a similar microbiota signature was observed in undernourished children in Bangladesh and was causally associated with small intestinal inflammation and damage in gnotobiotic mice\cite{251}. Of the oropharyngeal bacteria identified by these studies, I found that *Haemophilus* in particular was strongly recognized by host IgA in children from Madagascar and CAR\cite{252}. Gastrointestinal *Haemophilus* has been associated with markers of host inflammation and metabolism in other populations\cite{162,223,224,251}; the work presented in Chapter 3 identifies *Haemophilus* as a putative pathobiont in stunted children that interacts strongly with the mucosal immune system.

In Chapter 3, I also showed that stunted children had increased levels of IgA+ fecal bacteria compared to non-stunted children. Increased Ig-binding of the microbiota is seen in IBD patients and is consistent with the pathobiont-rich microbiota we observed. Our work and others’ support the conclusion that although undernutrition can compromise host immune responses, undernourished children and mice display sufficient or even heightened mucosal IgA levels in the absence of overt infection\cite{169,170,253,254}. In the undernourished mucosa, repetitive infection and immune stimulation by pathobionts might enhance chronic immune activity while
simultaneously dampening beneficial responses to commensals, infections, and oral vaccines. Further research in this area is needed to understand this apparent paradox and achieve intestinal immune health in undernourished children.

In addition to improving our understanding of the microbial etiology of child undernutrition, the work presented in Chapters 2 and 3 contributes to our fundamental understanding of IgA-bacterial interactions in the mammalian intestine. The factors shaping IgA-bacterial interactions are poorly understood; however, IgA recognition is known to be highly strain-dependent\textsuperscript{137,162,255}. My data in Chapter 2 show for the first time that strain-specific IgA properties are quickly and dynamically modulated by bacterial adaptation to the environment, independently of host immune factors. These findings support a ‘bottom-up’ modulation of IgA-bacterial binding by intrinsic bacterial properties. This work also addresses a gap in the field by providing a possible mechanistic explanation for altered IgA-recognition of the microbiota during malnutrition, as has been observed during both acute undernutrition and obesity\textsuperscript{209,256,257}.

By thoroughly confirming IgA-SEQ findings through experimental methods in Chapter 2, I have also provided a validation dataset to support the development of alternative IgA-targeting estimations which improve the signal to noise ratio (Jackson M et al., in preparation). Further, I show that even ‘non-canonical’ glycan-glycan binding between antibody and bacteria can be strain-specific, can have a functional impact on bacterial aggregation, and can be rapidly evaded by bacteria. This contributes to a growing body of literature on cross-reactive and glycan-mediated antibody responses to the microbiota, collectively challenging the traditional focus on epitope-specific and T-cell dependent antibody interactions\textsuperscript{201,202,258}. Finally, in addition to diet-dependent changes, in Chapter 3 I show that major differences exist in IgA-bacterial interactions between geographically distinct human populations. These data expand our understanding of
variability between individuals by providing the first evidence for population-level differences, although further research is needed to determine whether such differences are driven by host or environmental factors. In supplementary work attached in Appendix B, I also show that species-specific IgA-targeting patterns are preserved between human fecal donors and recipients in a small cohort of C. difficile patients. Overall, the work presented in this thesis expands our understanding of the determinants of IgA recognition in the mammalian intestinal tract.

In Chapter 4 I show that metabolic cooperation occurs between intestinal pathobionts associated with malnutrition. Proteobacteria species that drive EED features in mice benefit from the presence of Bacteroidales, but only when grown in low-protein, carbohydrate-rich nutritional conditions. These findings provide a plausible mechanism for the existing mouse models of EED, whereby both Bacteroidales and E. coli are required to induce EED features on a background of dietary malnutrition\textsuperscript{59,78}. The growth advantages experienced by pathobionts in protein-limited conditions could also help explain why bacterial overgrowth and Proteobacteria blooms are so prevalent in undernourished children. Further, this work may point to some cautionary considerations when designing prebiotics and micronutrient supplements in vulnerable undernourished populations. In undernourished children, resistant starch supplementation exacerbated inflammation, accompanied by markers of both Bacteroides and E. coli growth\textsuperscript{125}, and iron supplements have been shown to increase infection risk, likely due to their ability to fuel pathogen growth\textsuperscript{259}. It may therefore be prudent to refrain from administering fermentable prebiotics and iron supplements in undernourished populations, as these have the potential to exacerbate microbial dysbiosis. However, this work is experimental proof-of-concept and we emphasize that future research is needed to understand the impact of microbial cross-feeding in human populations.
Taken together, this work contributes to a body of literature on the presence of intestinal dysbiosis in chronic early life undernutrition. Our findings suggest that nutrient limitation directly impacts the growth and function of intestinal bacteria. Dietary context shaped the cross-feeding growth of pathobionts and the IgA-binding ability of commensal *Lactobacillus*, with potential consequences for intestinal dysbiosis and host immune function. Our findings also indicate that child stunting is characterized by a pathobiont-rich and IgA-bound microbiome, indicative of immune-bacterial dysbiosis in human populations. Collectively, this research improves our fundamental understanding of the intestinal microbiota and of the microbial contributions to early life undernutrition.

### 5.3 Limitations and Future Directions

There are several limitations to the work presented in this thesis. One limitation is the lack of direct host impact: I did not directly test the effect of microbial cross-feeding or bacterial IgA-binding on host health, in either preclinical models or in human populations. Thus, although I have speculated on possible health implications of this work, it is important not to infer causality without future research. Leveraging mouse models of EED and human organoid systems would be valuable to test the impact of (a) IgA-bound *Lactobacillus*, (b) IgA-bound *Haemophilus* and (c) *Bacteroidales-Enterobacteriaceae* cross-feeding on intestinal inflammation and linear growth.

In Chapter 2, several other exciting research directions remain unexplored. The pathways by which carbohydrate metabolism and PTS transporters affects surface glycans are unknown; so are the specific glycan-glycan interactions by which *Lactobacillus* interacts with IgA. Future biochemical assays to characterize the surface glycans of high- and low-binding *Lactobacillus*,
for example using mass spectrometry, would be valuable. Furthermore, other commensals were differentially IgA-recognized in undernourished mice, including *Bifidobacterium*, another probiotic candidate; it is unclear whether this is also due to dietary adaptations, and whether nutrition directly affects the IgA-binding capabilities of other commensals. Another outstanding question is the impact that IgA-binding has on bacterial metabolism and gene expression. In the future, it would be interesting to test the direct impact of IgA-binding on *Lactobacillus* transcription, carbohydrate transport, and metabolism.

One broad limitation of this work is the challenge of defining both undernutrition and EED. There are many types of dietary undernutrition, including deficiencies in total calories or in micronutrients, which were not modeled here either *in vivo* or *in vitro*; instead, in experimental work, I consistently relied on isocaloric, low-protein diets. Furthermore, EED biomarkers are not consistent predictors of child growth across studies, and they often do not correlate with one another\(^ {20, 49}\). Although in Chapter 3 we measured several typical EED biomarkers, these might not have been best representative of intestinal function in these populations; caution should furthermore be applied in extending any of these findings to other populations. The microbial ‘dysbiosis’ observed in undernutrition may also be variable across human populations, characterized by dietary differences and by distinct bacteria, viruses, helminths and fungi depending on the regional context. Encouragingly, however, an independent study found a similar duodenal microbiota signature in stunted children from Bangladesh to the one observed in Madagascar and CAR\(^ {211, 251}\). Future work in diverse human populations will be helpful to find consistent and generalizable trends in child undernutrition.

Other limitations to the analysis presented in Chapter 3 were discussed in section 3.5. In particular, I discussed specific statistical limitations, such as the sparseness of the data, the use of
non-parametric methods, and a technical batch effect, which limited our statistical power and may have led us to miss true variation between stunted and non-stunted children. In addition, the use of 2-5 year old children in this study may be past the critical windows for early life growth faltering and immune system development. It is also important to note that defining a child as ‘stunted’ or ‘non-stunted’ in a cross-sectional study design may not accurately reflect the health status or growth trajectory of these children. In the future, clearer differences might be observed by studying children longitudinally during the first two years of life, acknowledging that children may be on a stunting continuum.

Specific experimental gaps remain in Chapter 4, which I intend to address in future experiments before submitting this work for publication. Although mutant strains provide evidence of specific metabolic pathways involved in cross-feeding, we do not currently have direct evidence of the production and consumption of these metabolites; I aim to quantify saccharides, heme, and short-chain fatty acids through targeted metabolomics analysis of bacterial supernatants. I also plan to more fully elucidate the role of B. ovatus in cross-feeding, and predict that inulin-derived fructan metabolites produced by B. ovatus might explain the importance of dietary carbohydrates in the synergistic growth we observed. In addition, although we deliberately took a hypothesis-driven approach in this work, future use of an untargeted approach (ex., RNA-seq) would be valuable to uncover novel or unknown metabolic pathways.

Despite the limitations discussed here, the work in this thesis benefited from a diverse combination of research techniques and model systems, integrating in vitro, mouse, and human systems in an attempt to better understand the role of the intestinal microbiota in early life undernutrition. We hope additional insights may be gained from this work through future research, of which there are many exciting avenues to explore.
5.5 Concluding Remarks

Undernutrition is a serious global health issue with long-term consequences on child health and development. Despite the prioritization of this problem by the World Health Organization, reducing the worldwide burden of undernutrition has proven challenging. It is important to acknowledge that – in addition to the nutritional and microbiological factors discussed in this thesis – a huge range of political, sociological and economic factors contribute to child growth and well-being. While targeted microbiological and nutritional interventions may help to improve existing strategies, tackling undernutrition will also require substantial effort to improve economic, diet and hygiene related circumstances at the population level.

In my doctoral thesis I have taken a microbiological approach to elucidate specific bacterial-bacterial and bacterial-host interactions that may contribute to intestinal health during undernutrition. The findings presented here suggest that nutrient limitation directly impacts the cross-feeding growth of pathobionts and the host interaction of commensal *Lactobacillus*, with potential consequences for intestinal health. Further, our data indicate that child stunting is characterized by a pathobiont-rich and IgA-bound microbiome, indicative of intestinal dysbiosis in human populations. Together, this research improves our understanding of the microbial contributions to early life growth faltering, and may help to inform future work on the etiology, prevention and treatment of child undernutrition.
References


43. Geurts, B. & Van Tiel, B. Febrile illness and pro-inflammatory cytokines are associated with lower neurodevelopmental scores in Bangladeshi infants living in poverty.


119. Castro-Mejía, J. L. et al. Restitution of gut microbiota in Ugandan children administered with probiotics (Lactobacillus rhamnosus GG and Bifidobacterium animalis subsp. lactis


Appendices

Appendix A : Supplementary Figures

A.1 Supplementary Information for Chapter 1

There is no supplementary information for Chapter 1. This section is included only to maintain a logical numbering system for supplementary figures.
A.2 Supplementary Figures and Tables for Chapter 2

Figure A.1.1 Unsorted microbiota of CON, MAL, and MAL-b mice. (A-B) Total fecal (A) and jejunal (B) microbiota. (C-D) Differential taxa by diet, as measured by Linear Discriminant Analysis Effect Size (LEfSe), in (C) feces and (D) jejunum. (E) Representative flow plots of gating strategy used to sort IgA+ and IgA- bacteria.
Figure A.2.2 The development of IgA-bacterial interactions in mice. (A-B) Faith’s alpha diversity index in (A) the IgA- fraction and (B) the IgA+ fraction of CON, MAL and MAL-b feces over time. Wk, Week. (n=6-8, two-way ANOVA with Dunnet’s multiple comparisons test). (C-D) IgA-targeting index to (C) Ruminococcus sp. and (D) Akkermansia muciniphila in CON, MAL and MAL-b feces, as determined by IgA-SEQ (n=8, two-way ANOVA with post-hoc Tukey’s test). For (A-D), data are represented as mean ± SEM and significance is relative to the same group at week 3. (E) Spearman’s correlation between IgA Index and relative abundance in the IgA-SEQ dataset (Spearman’s rho=−0.013, p=0.356). (F) Spearman’s correlation between IgA Index in the feces and IgA Index in the jejunum in the IgA-SEQ dataset (Spearman’s rho=0.1167, p=4.521e-06). (G) PCoA plots displaying Bray Curtis distance and PERMANOVA significance at 4999 permutations for unsorted (Pre), IgA+ and IgA- fractions of the fecal and jejunal microbiota.
Figure A.2.3 The development of IgA-bacterial interactions in mice. IgA-targeting of key taxa in MAL and MAL-b mice. (A-B) IgA-targeting of taxa which were significantly different between diets at week 7 in (A) feces and (B) jejunum. Statistics represent adjusted p-value (two-way ANOVA with post-hoc Tukey’s test) for all comparisons. (C-D) Bacteria from the Bacteroidetes-E. coli gavage are present in MAL-b group (C) but are not IgA-targeted (D). (E-F) IgA Index to lactic acid bacteria (Lactobacillus/Lactococcus) (E) or to Lactobacillus johnsonii/gasseri (F) in jejunum as determined by magnetic-activated cell sorting (MACS) and qPCR (n=5, Kruskal-Wallis). Data are represented as mean ± SEM.
Figure A.2.4 Aggregation and adhesion of Lactobacillus. (A) Light microscopy images of Lactobacillus incubated with or without murine intestinal IgA. Scale bar represents 100 µm. (B-C) Proportion of adherent Lactobacillus (B) and Akkermansia (C) in the jejunum of CON, MAL and MAL-b mice, determined by 16S qPCR in tissue and luminal content. (D) Adhesion of Lactobacillus to HT29-MTX cells in the absence of IgA (un-normalized data). Data are represented as mean ± SEM.
Figure A.2.5 Lactobacillus monocolonization and in vitro evolution reversal. (A-D) Germ-free mice were monocolonized with a high-IgA-binding strain of Lactobacillus and placed on a CON or MAL diet for three weeks. (A) Total Lactobacillus colonization in jejunum (jej) and feces at experimental endpoint (n=6–8, two-way ANOVA with post-hoc Sidak’s test). (B) Proportion of adherent Lactobacillus in the jejunum, as determined by CFU count in tissue and luminal content (Mann-Whitney). (C) IgA-binding ability of Lactobacillus isolates taken from monocolonized mice at endpoint (Kruskal-Wallis with post-hoc Dunn’s test). (D) Total IgA in the luminal content of monocolonized mice (two-way ANOVA with post-hoc Sidak’s test). (E-F) Low-IgA binding MAL-b strains were passaged daily for three weeks in either rich media (CON) or nutrient-deplete media (MAL). (E) IgA-binding ability of Lactobacillus over time. (F) Endpoint IgA-binding ability of evolved Lactobacillus after three weeks of passaging, normalized per experiment to the average O.D. of the CON strains. Each data point represents an individually passaged strain. Results are pooled from two independent experiments starting with two different Lactobacillus isolates. Non-significant by Mann-Whitney. Data are represented as mean ± SEM.
Figure A.2.6 Growth and phylogeny of *Lactobacillus* isolates. (A) Phylogeny of *Lactobacillus* isolates in comparison with NCBI reference genomes, based on all protein-coding genes in the genome. (B-C) Percentage of unique non-synonymous mutations found in (B) transporter families and (C) metabolic enzymes in CON or MAL-b isolates based on cross-sectional analysis (n=5, two-way ANOVA with post-hoc Sidak’s test). (D) Growth of *Lactobacillus* isolates in rich media (unmodified MRS, “CON”) or in a nutrient-restricted media (“MAL”) (n=3; two-way ANOVA with post-hoc Sidak’s test). Data are represented as mean ± SEM. *Lactobacillus* isolates used in this figure are the core isolates designated in Table S3.
Table A.2.1 Recipe for nutrient-rich (CON) and nutrient-poor (MAL) *Lactobacilli* broths used in this study, as adapted from the standard De Man, Rogosa and Sharpe (MRS) media.

<table>
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<th>Rationale</th>
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<th>Mal (g/500 mL)</th>
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<td>0.875</td>
</tr>
<tr>
<td>Beef Extract</td>
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<td>0.875</td>
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<tr>
<td>Yeast Extract</td>
<td>vitamin B</td>
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<td>1.25</td>
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<td>Dextrose</td>
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</tr>
<tr>
<td>Polysorbate 80 (Tween 80)</td>
<td>fatty acids</td>
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<td>0.0875</td>
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<tr>
<td>Ammonium Citrate</td>
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<td>1</td>
</tr>
<tr>
<td>Sodium Acetate</td>
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<td>500 mL</td>
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<tr>
<td>Final pH</td>
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Table A.2.2 Primer sequences used in this study. Related to STAR Methods.
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<td>qPCR</td>
</tr>
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<td>Lactobacillus/Lactococcus 16S</td>
<td>F_AGCAGTAGGGAATCTTCCA R_CACCGCTACACATGGAG</td>
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<td>qPCR</td>
</tr>
<tr>
<td>Lactobacillus johnsonnii/gasseri 16S</td>
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<td>This study</td>
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<td>qPCR</td>
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<td>qPCR</td>
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<td>Eubacteria 16S (8F-926R)</td>
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<td>Sanger sequencing</td>
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</table>
Table A.2.3 Main *Lactobacillus* isolates used in this study. Core experimental strains designate those used regularly to represent a typical high-IgA-binding or low-IgA-binding phenotype.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Source</th>
<th>Experimental cohort</th>
<th>IgA-binding phenotype</th>
<th>Ancestral strain</th>
<th>Genome sequence</th>
<th>Core experimental strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk3C3AF</td>
<td>Mouse feces, regular chow diet, 3 weeks old</td>
<td>Exp-AF, July 2018</td>
<td>High</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>C1AF</td>
<td>Mouse jejunum, CON diet, 7 weeks old</td>
<td>Exp-AF, July 2018</td>
<td>Medium-High</td>
<td>Wk3C3AF</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>WkC5LA</td>
<td>Mouse feces, regular chow diet, 3 weeks old</td>
<td>Exp-LA, June 2018</td>
<td>Low</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>C2LA</td>
<td>Mouse jejunum, CON diet, 7 weeks old</td>
<td>Exp-LA, June 2018</td>
<td>High</td>
<td>Wk3C5LA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>C3RA</td>
<td>Mouse jejunum, CON diet, 11 weeks old</td>
<td>Exp-Rev (cage CON-A), Dec 2018</td>
<td>High</td>
<td>NA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Wk3CRB</td>
<td>Mouse feces, regular chow diet, 3 weeks old</td>
<td>Exp-Rev (cage CON-B), Dec 2018</td>
<td>Medium</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>C4RB</td>
<td>Mouse jejunum, CON diet, 11 weeks old</td>
<td>Exp-Rev (cage CON-B), Dec 2018</td>
<td>High</td>
<td>Wk3CRB</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>C5FI</td>
<td>Mouse jejunum, CON diet, 7 weeks old</td>
<td>Exp-First, Oct 2017</td>
<td>High</td>
<td>NA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Wk3E3AF</td>
<td>Mouse feces, regular chow, 3 weeks old</td>
<td>Exp-AF, July 2018</td>
<td>High</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>E1AF</td>
<td>Mouse jejunum, MAL diet, 7 weeks old</td>
<td>Exp-AF, July 2018</td>
<td>Medium-Low</td>
<td>Wk3E3AF</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Code</td>
<td>Description</td>
<td>Experiment Date</td>
<td>Control Level</td>
<td>NA</td>
<td>Treatment Response</td>
<td>Resistance Response</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------------------</td>
<td>---------------</td>
<td>----</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Wk3E8LA</td>
<td>Mouse feces, regular chow, 3 weeks old</td>
<td>Exp-LA, June 2018</td>
<td>High</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>E2LA</td>
<td>Mouse jejunum, MAL diet, 7 weeks old</td>
<td>Exp-LA, June 2018</td>
<td>Low</td>
<td>Wk3E8LA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>E3RA</td>
<td>Mouse jejunum, MAL diet, 11 weeks old</td>
<td>Exp-Rev (cage MAL-A), Dec 2018</td>
<td>Low</td>
<td>NA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Wk3ERB</td>
<td>Mouse feces, regular chow, 3 weeks old</td>
<td>Exp-Rev (cage MAL-B), Dec 2018</td>
<td>Medium</td>
<td>NA</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>E4RB</td>
<td>Mouse jejunum, MAL diet, 11 weeks old</td>
<td>Exp-Rev (cage MAL-B), Dec 2018</td>
<td>Low</td>
<td>Wk3ERB</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>E5FI</td>
<td>Mouse jejunum, MAL diet, 7 weeks old</td>
<td>Exp-First, Oct 2017</td>
<td>Low</td>
<td>NA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>1CON1</td>
<td>CON media, passage 7</td>
<td>In vitro evolution 1</td>
<td>High</td>
<td>Wk3C3AF</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2CON4</td>
<td>CON media, passage 7</td>
<td>In vitro evolution 2</td>
<td>High</td>
<td>C3RA</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>3CON2</td>
<td>CON media, passage 7</td>
<td>In vitro evolution 3</td>
<td>High</td>
<td>Wk3CRB</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>1MAL1</td>
<td>MAL media, passage 7</td>
<td>In vitro evolution 1</td>
<td>Low</td>
<td>Wk3C3AF</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2MAL4</td>
<td>MAL media, passage 7</td>
<td>In vitro evolution 2</td>
<td>Low</td>
<td>C3RA</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>3MAL2</td>
<td>MAL media, passage 7</td>
<td>In vitro evolution 3</td>
<td>Low</td>
<td>Wk3CRB</td>
<td>Yes</td>
<td>No</td>
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</table>
Figure A.3.1 Subject inclusion/exclusion tree. Mada, Madagascar; CAR, Central African Republic; NS, non-stunted; S, stunted.
**Figure A.3.2 Representative flow cytometry gating.** Fecal samples were processed unstained (A) or were stained with SYTO-Green FITC to detect bacterial DNA (B-C) and with an isotype control PE (B) or an anti-human IgA PE (anti-hIgA) (C). Top panels: bacterially-sized events were selected by forward scatter (FSC) and side scatter (SSC). Bottom panels: events were further gated by FITC and PE to detect IgA+ and IgA- bacterial populations.
Figure A.3.3 IgA-bacterial targeting and stunting, by country and batch. (A-B) The percentage of IgA-positive fecal bacteria by flow cytometry (%IgA+) versus height-for-age z-score (HAZ) (A), and stunting status (B) in the full dataset. (C-D) Trend in stunting status by (C) major sorting batch and (D) daily sorting batch. (E-F) Country effect by (E) major sorting batch and (F) daily sorting batch. (G-H) Total fecal IgA by country (G) and stunting status (H). Mada, Madagascar; CAR, Central African Republic. N=188 total samples. N=93 in Madagascar and N=95 in CAR; N=91 in Batch 2017 and N=88 in Batch 2018. An additional N=9 samples were sorted in a single day in 2019 and are thus included in daily batch effect. Statistical significance was determined by Spearman’s correlation (A) and Wilcoxon Rank Sum test (B, C, E, G, H).

Figure A.3.4 IgA-bacterial targeting by inflammatory markers. Associations between the percentage of IgA-positive fecal bacteria by flow cytometry (%IgA+) and serum C-reactive protein (CRP) (A), fecal alpha-1 anti-trypsin (AAT) (B), and fecal calprotectin (C). N=188. “High” CRP was defined as >10 mg/l. Statistical significance was determined by Wilcoxon Rank Sum test (A) and Spearman’s correlation (B-C).
SimpleASV
Ruminococcaceae NK4A214 group d4e
[Ruminococcus] torques group 915
Phascolarctobacterium 846
Gastranaerophilales e22
Faecalibacterium c3b
Subdoligranulum 97e
Subdoligranulum a18
Streptococcus bd2
Stenotrophomonas 891
Solobacterium 461
Senegalimassilia a68
Ruminococcaceae UCG-008 a81
Ruminococcaceae UCG-003 83b
Roseburia 14f
Rhodoglobus 438
Pseudomonas 402
Prevotella 9 11a
Prevotella 9 9a2
Prevotella 9 a67
Prevotella 9 4b
Prevotella 9 351
Prevotella 9 b2d
Prevotella 2 715
Prevotella 2 364
Prevotella 2 28f
Prevotella 2 2b4
Prevotella 2 1bb
Prevotella 2 1be
Prevotella 2 1ge
Phascolarctobacterium 846
Palagibacterium 708
Parabacteroides 14f
NA e80
NA e52
NA 51d
NA 348
Lachnospiraceae UCG-010 c83
Lachnospiraceae UCG-010 c76
Lachnospiraceae UCG-004 130
Lachnospiraceae UCG-004 c54
Lachnospiraceae NK4A136 group 470
Lachnospiraceae FC5020 group 760
Lachnosclostridium 84d
Holdemanella 105
Haemophilus e27
GCA-900066575 58c
Gastranaerophilales e22
Fusugtenibacter dsc
Fournierella e 7a
Faecalibacterium c3b
Faecalibacterium 83d
Faecalibacterium 78s
Faecalibacterium 23f
Borea afd
Dorea 90 a
Dialister d69
Coprococcus 3 7ab
Butyricicoccus aad
Butyricicoccus 407
Brevundimonas 760
Blautia e2
Blautia c1d
Blautia 708
Biophila eca
Bifidobacterium 83d
Bacteroides 668
Bacteroides 51 e
Aerostipes c8a
Alistipes e3a
Acidaminobacter a20
Agathobacter 2d3
Ruminococcus [torques group 915]
[Ruminococcus] torques group 694
[Ruminococcus] torques group 694
[Eubacterium] ruminantium group c3e
[Eubacterium] coprostanoligenes group aa0
[Eubacterium] coprostanoligenes group a07

Fig S2
Figure A.3.5 Highly IgA-targeted taxa at amplicon sequence variant (ASV) level. (A) IgA-targeting profiles at ASV level in the full dataset (All), Madagascar (Mada) and Central African Republic (CAR). ASVs are included if the IgA Index was significantly different from zero by a one-sided Wilcoxon (FDR-adjusted p < 0.05) in at least one subset. Colour of circles indicates the direction of IgA-targeting (red=positively targeted, blue=negatively targeted), and saturation of the colour represents FDR-corrected statistical significance. The size of the circle indicates overall effect size as measured by average IgA Index. (B-D) Most highly IgA-targeted taxa in (B) the full dataset, (C) Madagascar and (D) CAR, as defined by a median IgA Index greater than zero with FDR-adjusted p < 0.05. N=138 total; N=78 in Madagascar and N=60 in CAR.
Figure A.3.6 Abundance and IgA Index correlations in main targeted taxa. (A) Correlation between IgA-targeting and unsorted relative abundance at genus level. The most- and least-targeted taxa, as defined by median IgA Index in Fig 2 and Table S1, are shown. Colour represents Spearman’s rho. A star (*) represents significant correlation at FDR-corrected p<0.05. (B-C) IgA Index of Haemophilus and Campylobacter by stunting status in Madagascar (Mada) and Central African Republic (CAR). (D-E) IgA Index of Haemophilus and Campylobacter by sequencing batch. Statistical significance determined by Wilcoxon Rank Sum test (B-E). N=138 total; N=78 in Madagascar and N=60 in CAR; N=53 in Batch 1 and N=85 in Batch 2.
Figure A.3.7 IgA-targeting of Haemophilus and Campylobacter does not correlate with inflammatory markers. Serum C-reactive protein (CRP) levels (A, D), fecal calprotectin (B, E) and fecal alpha-1-antitrypsin (AAT) (C, F) by the IgA Index of Haemophilus (A-C) and Campylobacter (D-F). Statistical significance was determined by Wilcoxon Rank Sum Test (A, D) or Spearman’s correlation (B, C, E, F). “High” CRP was defined as >10 mg/l. N=138.
Figure A.3.8 Distribution of IgA-targeting by study metadata according to batch and country. (A) Summary of PERMANOVA analysis of the IgA Index in the full dataset when permutations were constrained by either sequencing batch (All ~Batch) or country (All ~Country) using the ‘strata’ parameter, or when analysis was performed in each batch individually (Batch1 and Batch2). Analysis is based on taxa maintained at the ASV level. (B) Summary of PERMANOVA analysis of the IgA Index in the full dataset and in each batch and country.
individually, based on taxa binned at the genus level. Starred variables are significant with an FDR-corrected p <0.05. Each variable was tested individually in the PERMANOVA without other co-variates. (C-F) IgA Index of taxa that differ significantly by country in IgA-targeting. Statistical significance was determined by FDR-corrected linear models that incorporated sequencing depth, batch effect, age and sex. (G-J) Unsorted relative abundance of these same taxa. Numbers and letters following a genus indicate the first digits of the qiime2 feature code for a unique ASV. CAR, Central African Republic; Mada, Madagascar; Coef, the coefficient of variance by PERMANOVA; whz_cont, weight-for-height z-score; haz, height-for-age z-score; crp, serum c-reactive protein; aat, fecal alpha-1 anti-trypsin. N=138 total; N=78 in Madagascar and N=60 in CAR; N=53 in Batch 1 and N=85 in Batch 2.

Figure A.3.9 IgA-targeting of taxa that correlated with age, breastfeeding or HAZ. Taxa in (A-C) were selected by linear models which incorporated country (A-B only), age, sex, batch and sequencing depth as co-variates, at an FDR-corrected p<0.1. Statistical significance and rho as presented in the plots derive from uncorrected Spearman’s correlation. All, full dataset; Mada, Madagascar; Lachno., Lachnospiraceae. N=138 in full dataset (A-B); N=78 in Madagascar (C).
### Table A.3.1 Demographics of children with valid IgA-seq data (n=138) by country.

Values are presented as the group median (continuous variable) or as counts (categorical variable) with missing values excluded. Significance was determined by Wilcoxon Rank Sum test (continuous variable) or Fisher’s exact test (categorical variable). Hemoglobin was adjusted by altitude (-0.2 g/100 mL in Madagascar to account for the height above sea level). Anemia was defined as an adjusted hemoglobin level below 11 g/100 mL. Elevated CRP was defined as >10 mg/l serum. Breastfeeding duration represents the total number of months a child was previously breastfed for; all but four children had been weaned by the time of sampling.

<table>
<thead>
<tr>
<th>Description</th>
<th>Madagascar (N=78)</th>
<th>CAR (N=60)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37/78 (47.4%)</td>
<td>27/60 (45.0%)</td>
<td>0.8636</td>
</tr>
<tr>
<td>Female</td>
<td>41/78 (52.6%)</td>
<td>33/60 (55.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (months)</td>
<td>42.6</td>
<td>38.0</td>
<td>0.6770</td>
</tr>
<tr>
<td>2-3 years</td>
<td>28/78 (35.9%)</td>
<td>26/60 (43.3%)</td>
<td></td>
</tr>
<tr>
<td>3-4 years</td>
<td>23/78 (29.5%)</td>
<td>12/60 (20.0%)</td>
<td></td>
</tr>
<tr>
<td>4-5+ years</td>
<td>27/78 (34.6%)</td>
<td>21/60 (35.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Nutritional status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median HAZ score</td>
<td>-2.12</td>
<td>-1.90</td>
<td>0.7360</td>
</tr>
<tr>
<td>Median WHZ score</td>
<td>-0.40</td>
<td>-0.50</td>
<td>0.8839</td>
</tr>
<tr>
<td>Median hemoglobin (g/100 mL serum)</td>
<td>11.5</td>
<td>10.7</td>
<td>0.0014</td>
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<tr>
<td>Presence of anemia</td>
<td>22/77 (28.6%)</td>
<td>23/53 (43.4%)</td>
<td>0.0019</td>
</tr>
<tr>
<td><strong>Breastfeeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median duration (months)</td>
<td>24</td>
<td>18</td>
<td>1.3e-06</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median AAT (mg/g dry fecal weight)</td>
<td>49.5</td>
<td>37.0</td>
<td>0.0809</td>
</tr>
<tr>
<td>Median calprotectin (µg/g dry fecal weight)</td>
<td>502</td>
<td>296</td>
<td>0.0009</td>
</tr>
<tr>
<td>Elevated CRP (&gt; 10 mg/l serum)</td>
<td>7/77 (9.1%)</td>
<td>10/50 (20.0%)</td>
<td>0.1088</td>
</tr>
<tr>
<td><strong>Parasites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of helminths</td>
<td>69/78 (88.5%)</td>
<td>1/47 (2.1%)</td>
<td>2.2e-16</td>
</tr>
<tr>
<td>Presence of <em>Giardia</em></td>
<td>19/78 (24.4%)</td>
<td>9/47 (19.1%)</td>
<td>0.6584</td>
</tr>
<tr>
<td><strong>Sequencing Batch</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 1</td>
<td>40/78 (51.2%)</td>
<td>13/60 (21.7%)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Batch 2</td>
<td>38/78 (48.7%)</td>
<td>47/60 (78.3%)</td>
<td></td>
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</table>
Table A.3.2 Least IgA-targeted taxa at genus level, as defined by a median IgA Index <0 and an FDR-adjusted \( p \) <0.05 by one-sided Wilcoxon test. Mada, Madagascar; CAR, Central African Republic.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Phylum</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>All, Mada, CAR</td>
<td>Firmicutes</td>
<td>Streptococcus</td>
</tr>
<tr>
<td>All, Mada</td>
<td>Firmicutes</td>
<td>Dialister</td>
</tr>
<tr>
<td>All, CAR</td>
<td>Bacteroidetes</td>
<td>Parabacteroides</td>
</tr>
<tr>
<td>All, CAR</td>
<td>Bacteroidetes</td>
<td>Bacteroides</td>
</tr>
<tr>
<td>All, Mada, CAR</td>
<td>Proteobacteria</td>
<td>Pseudomonas</td>
</tr>
<tr>
<td>All, Mada</td>
<td>Proteobacteria</td>
<td>Stenotrophomonas</td>
</tr>
<tr>
<td>All, Mada, CAR</td>
<td>Proteobacteria</td>
<td>Brevundimonas</td>
</tr>
<tr>
<td>All, Mada</td>
<td>Proteobacteria</td>
<td>Pelagibacterium</td>
</tr>
<tr>
<td>All</td>
<td>Proteobacteria</td>
<td>Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium</td>
</tr>
<tr>
<td>All, Mada</td>
<td>Firmicutes</td>
<td>Roseburia</td>
</tr>
<tr>
<td>All, Mada</td>
<td>Firmicutes</td>
<td>Agathobacter</td>
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<td>All, Mada, CAR</td>
<td>Firmicutes</td>
<td>Butyricoccus</td>
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<td>All, Mada, CAR</td>
<td>Firmicutes</td>
<td>Faecalibacterium</td>
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<tr>
<td>All, Mada</td>
<td>Firmicutes</td>
<td>Blautia</td>
</tr>
<tr>
<td>All, Mada, CAR</td>
<td>Firmicutes</td>
<td>Lachnospiraceae uncultured</td>
</tr>
<tr>
<td>All, Mada</td>
<td>Bacteroidetes</td>
<td>Prevotella 9</td>
</tr>
<tr>
<td>Mada</td>
<td>Firmicutes</td>
<td>Christensenellaceae R-7 group</td>
</tr>
<tr>
<td>CAR</td>
<td>Firmicutes</td>
<td>Ruminococcus 1</td>
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</table>
Table A.3.3 Batch-associated taxa. Taxa which correlate significantly with flow cytometry sorting batch (sort) or 16S rDNA sequencing batch (seq). Batch(es) indicates which type of batch the taxon was significant in, according to Wilcoxon Rank Sum test with an FDR-corrected p<0.05. Taxa in bold were also significantly un-targeted by IgA.

<table>
<thead>
<tr>
<th>Batch(es)</th>
<th>Phylum</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sort, Seq</td>
<td>Proteobacteria</td>
<td>Pseudomonas</td>
</tr>
<tr>
<td>Sort, Seq</td>
<td>Proteobacteria</td>
<td>Brevundimonas</td>
</tr>
<tr>
<td>Sort, Seq</td>
<td>Proteobacteria</td>
<td>Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium</td>
</tr>
<tr>
<td>Seq</td>
<td>Bacteroidetes</td>
<td>Prevotella 9</td>
</tr>
<tr>
<td>Sort</td>
<td>Firmicutes</td>
<td>Ruminococcaceae UCG-005</td>
</tr>
</tbody>
</table>
Table A.3.4 Characteristics of children selected for IgG-SEQ analysis. HAZ, height-for-age z-score; WHZ, weight-for-age z-score; AAT, fecal alpha-1-antitrypsin (mg/g dry fecal weight); Calpro, fecal calprotectin (µg/g dry fecal weight); CRP, serum C-reactive protein (mg/l serum).

<table>
<thead>
<tr>
<th>ChildID</th>
<th>Age (months)</th>
<th>Sex</th>
<th>HAZ</th>
<th>WHZ</th>
<th>History of diarrhea</th>
<th>AAT</th>
<th>Calpro</th>
<th>CRP</th>
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<tbody>
<tr>
<td>MHG1</td>
<td>31.3</td>
<td>M</td>
<td>-4.31</td>
<td>-0.73</td>
<td>No</td>
<td>&gt;mean</td>
<td>&lt;mean</td>
<td>&lt;=10</td>
</tr>
<tr>
<td>MHG2</td>
<td>54.1</td>
<td>F</td>
<td>-3.33</td>
<td>-1.54</td>
<td>No</td>
<td>&lt;mean</td>
<td>&lt;mean</td>
<td>&lt;=10</td>
</tr>
<tr>
<td>MHG3</td>
<td>32.4</td>
<td>M</td>
<td>-1.78</td>
<td>0.53</td>
<td>Yes</td>
<td>&lt;mean</td>
<td>&gt;mean</td>
<td>&lt;=10</td>
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<tr>
<td>MHG4</td>
<td>44.5</td>
<td>M</td>
<td>-3.29</td>
<td>-0.21</td>
<td>Yes</td>
<td>&gt;mean</td>
<td>&lt;mean</td>
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<tr>
<td>MHG5</td>
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<td>-3.38</td>
<td>-0.55</td>
<td>No</td>
<td>&gt;mean</td>
<td>&gt;mean</td>
<td>&lt;=10</td>
</tr>
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</table>
A.4 Supplementary Figures and Tables for Chapter 4

Figure A.4.1 Synergistic growth of Bacteroidetes and Enterobacteriaceae is enhanced in mucin- and carbohydrate-rich complex media. (A) Media macronutrient compositions. After a 24h culture, bacterial endpoint growth was determined for (B) the total community, (C) E. coli and (D) Bacteroidetes. Mean +/- SEM are displayed. Significance was determined by two-way ANOVA with Tukey’s post-hoc test. B, Bacteroidetes mix; E, E. coli mix; CFU, colony forming units; Carb, carbohydrate media; CarbM, carbohydrate plus mucus media; Prot, protein media; ProtM, protein plus mucus media.
Figure A.4.2 *B. fragilis* takes advantage of *B. ovatus*-*E. coli* synergy. (A) Relative abundance of *Bacteroidetes* and *E. coli* communities at 24 endpoint. (B-D) Total growth of *B. dorei* (B), *B. vulgatus* (C) and *P. distasonis* (D) over time in a mixed community with or without *E. coli*. Mean $\pm$ SEM.
**Figure A.4.3 Role of mucin-derived sugars in *E. coli* growth during co-culture.** (A) Growth of *E. coli* at 24 endpoint with or without the *Bacteroidetes* mix or with *B. thetaiotaomicron* (*B. theta*). (B) Competitive index of *ΔnanA* *E. coli* compared to wild-type in the presence or absence of *Bacteroidetes*, across four different media compositions. (C) Competitive index of *ΔfucI* *E. coli* compared to wild-type in the presence or absence of *Bacteroidetes*, across four different media compositions. (D) Competitive index of *ΔnagE* *E. coli* compared to wild-type in the presence or absence of *Bacteroidetes*, across four different media compositions. Mean +/- SEM are displayed. Significance was determined by Kruskal-Wallis with post-hoc Dunn’s test (A) or by two-way ANOVA with post-hoc Sidak’s test (B). B, *Bacteroidetes* mix; E, *E. coli* mix; CFU, colony forming units; Carb, carbohydrate media; CarbM, carbohydrate plus mucus media; Prot, protein media; ProtM, protein plus mucus media.
Figure A.4.4 Metabolite dependency of *B. fragilis* and *E. coli* growth during co-culture. (A) Growth of *B. fragilis* at 24 endpoint with or without the *E. coli* in regular IMM media (1X hematin) or in media with additional organic iron (10X hematin). (B) Growth of *B. fragilis* at 24 endpoint with or without the *E. coli* in regular IMM media (1X vitamin B12) or in media with additional (10X) vitamin B12. (C) Total growth of wild-type (WT) and ∆nanA *E. coli* in the presence or absence of *Bacteroidetes*. (D) Total growth of wild-type (WT) and ∆feoAB *B. fragilis* (BF) in the presence or absence of *E. coli*. Mean +/- SEM are displayed. Significance was determined by two-way ANOVA with post-hoc Tukey’s test (C-D). B, *Bacteroidetes* mix; BF, *B. fragilis*; E, *E. coli* mix.
Table A.4.1 Bacterial strains used in Chapter 4.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source / Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. fragilis</em> 3/1/12</td>
<td>Human fecal isolate</td>
<td>Brown et. al (2015)</td>
</tr>
<tr>
<td><em>P. distasonis</em> 2/1/33B</td>
<td>Human fecal isolate</td>
<td>Brown et. al (2015)</td>
</tr>
<tr>
<td><em>B. fragilis</em> 638R WT</td>
<td>Clinical isolate, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Rocha et. al (2018)</td>
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<td><em>B. fragilis</em> BER-51 ΔfeoAB</td>
<td>638R ΔfeoAB::tetQ, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Rocha et. al (2018)</td>
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<td><em>B. thetaiotaomicron</em></td>
<td>Human fecal isolate</td>
<td>ATCC 29148</td>
</tr>
<tr>
<td><em>E. coli</em> 4/1/47</td>
<td>Human fecal isolate</td>
<td>Brown et. al (2015)</td>
</tr>
<tr>
<td><em>E. coli</em> 3/1/53 ΔnanA</td>
<td>ΔnanA::kanR, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> 4/1/47 ΔnanA</td>
<td>ΔnanA::kanR, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td><em>E. coli</em> 3/1/53 ΔfucI</td>
<td>ΔfucI::kanR, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td><em>E. coli</em> 4/1/47 ΔfucI</td>
<td>ΔfucI::kanR, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td>This study</td>
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<td>ΔnagE::kanR, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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Table A.4.2 Macronutrient composition of complex rich medias.

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<th>CON-M</th>
<th>MAL</th>
<th>MAL-M</th>
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<td>1.70</td>
<td>3.00</td>
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<tr>
<td>Cellulose (g/L)</td>
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</tr>
<tr>
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<td>0.12</td>
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<td>0.15</td>
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<tr>
<td>Casein (g/L)</td>
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<td>1.00</td>
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<td>0.30</td>
</tr>
<tr>
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</tr>
<tr>
<td>Mucus (g/L)</td>
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<tr>
<td>Ratio (carbs/protein)</td>
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<td>1.84</td>
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Table A.4.3 Macronutrient composition of protein- and carbohydrate-only medias.

<table>
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<th>ProtM</th>
<th>Carb</th>
<th>CarbM</th>
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<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>(g/L) Cellulose</td>
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<td>0</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Inulin (g/L)</td>
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<td>0</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Casein (g/L)</td>
<td>3.40</td>
<td>3.40</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>L-cysteine (g/L)</td>
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<tr>
<td>Mucus (g/L)</td>
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Appendix B : Changes in IgA-targeted microbiota following fecal transplantation for recurrent *Clostridioides difficile* infection

B.1 Synopsis

Secretory immunoglobulin A (IgA) interacts with intestinal microbiota and promotes mucosal homeostasis. IgA-bacteria interactions are often strain specific, but how these interactions are shaped by bacterial, host and environmental factors remains unclear. In this study, we utilized IgA-SEQ to profile IgA-bound fecal bacteria in 48 recurrent *Clostridioides difficile* patients before and after successful fecal microbiota transplantation (FMT) to gain further insight. Prior to FMT, patients harboured a low-diversity microbiota characterized by abundant and IgA-targeted *Escherichia coli*; following FMT, the microbiota more closely resembled that of the donors, with colonization and IgA recognition of multiple *Firmicutes*. Post-FMT IgA-targeting was unaffected by the route of FMT delivery (colonoscopy versus capsule), suggesting that both methods lead to the establishment of healthy immune-bacterial interactions in the gut. Bacterial taxa generally maintained consistent IgA-targeting status across donors and recipients, supporting the concept that intrinsic bacterial properties drive IgA recognition in the human intestine. Indeed, fecal donor identity was significantly associated with IgA-targeting patterns in FMT recipients, although this factor was also confounded by sex matching of the donor-patient pairs. Machine learning models were poorly able to predict variation in IgA-targeting using available host or microbiota data, indicating that many factors influencing IgA-targeting in the human gut remain unknown. This study improves our understanding of IgA-bacterial interactions in the human microbiota and suggests that normal IgA-bacterial interactions quickly re-establish in *C. difficile* patients who receive FMT.
B.2 Introduction

The human intestine harbours a complex microbiota which influences health and disease\textsuperscript{261}. This intestinal microbiota provides ‘colonization resistance’, excluding pathogens from invading the gut, which helps prevent infection\textsuperscript{81}. When the microbial community is disrupted, for example due to repeated antibiotics exposure, the gut environment becomes susceptible to \textit{Clostridioides difficile}, a major cause of hospital-acquired and recurrent infections in North America and Europe\textsuperscript{262,263}. Fecal microbiota transplant (FMT) from healthy donors is the most effective treatment for recurrent \textit{C. difficile} infection (rCDI), as it restores a diverse microbial community and associated colonization resistance\textsuperscript{264}. FMT has also allowed researchers to study the establishment of intestinal microbial communities in humans. Interestingly, the microbiota of FMT recipients is an emergent mixture of donor strains and pre-existing patient strains, and its composition can be successfully predicted by machine learning techniques\textsuperscript{265,266}.

The mucosal antibody immunoglobulin A (IgA) interacts closely with the intestinal microbiota and is an integral part of intestinal homeostasis. IgA excludes pathogens, promotes the adhesion of commensals, and alters microbial gene expression, shaping both the composition and the function of the microbiota\textsuperscript{152}. However, the factors which determine IgA-bacterial interactions in the human gut remain poorly understood. IgA-bacterial interactions are often highly strain-specific, differentiating between closely related amplicon sequence variants (ASVs); paradoxically, however, IgA clones frequently display cross-reactivity to multiple bacterial species, or even polyreactivity to multiple distinct antigens, and are capable of binding to microbes via antigen-independent glycan-glycan interactions\textsuperscript{152}. Further, environmental
factors such as host nutrition have been recently shown to influence IgA-bacterial binding in mice. The extent to which bacterial properties, host immunity, and environmental context contribute to IgA-bacterial specificity in the human intestine is unknown.

FMT provides a unique opportunity to study the establishment of IgA-microbiota interactions in the human intestinal tract. Further, immune-bacterial interactions are known to be disrupted during rCDI; a better understanding of these interactions and their recovery after FMT could help improve the rational design of microbial therapies. Here we characterize the IgA-targeted microbiota before and after FMT from 48 of 116 patients who participated in a clinical trial and were randomized to either colonoscopy or oral capsule delivered FMT for rCDI. We evaluate the influence of delivery method, donor and pre-transplant microbiota, host anthropometry and systemic metabolites on post-FMT IgA-bacterial targeting.

B.3 Materials & Methods

B.3.1 Study participants and sample collection

Adult patients with at least 2 CDI recurrences were recruited in Alberta between 2014-2016 as previously described. Each patient was maintained on vancomycin suppression till 24 hours prior to the assigned FMT treatment. Stool samples were collected at screening (pre-FMT), and at 4 weeks after FMT (post-FMT), and were stored at -80 C as previously reported.

B.3.2 Ig-sorting

IgA-sequencing was performed as described previously. Approximately 50 mg of each fecal sample (+/- 10 mg) was homogenized in 1 mL of phosphate buffered saline (PBS; HyClone DPBS-/-, SH30028.02) and spun gently to settle debris; intestinal bacteria were then filtered
through a 0.7 µm filter. A volume of suspension equal to 5 mg of sample was washed in FACS buffer (PBS containing 1% bovine serum albumin) and blocked for 20 minutes in FACS buffer containing 10% fetal bovine serum. Samples were then stained with anti-human IgA-PE (Miltenyl 130-093-128) or an isotype control (eBioscience, 12-4714-42) at 1:25 dilution for 30 minutes in the dark. Samples were washed twice more and fixed overnight in 2% paraformaldehyde (PFA) at 4°C in the dark without shaking. The next day, the PFA was washed off and samples were stained with SYTO-BC (1:4000 dilution) for bacterial DNA, washed again and sorted by flow cytometry into IgA-positive (FITC+PE+) and IgA-negative (FITC+PE-) populations. A minimum of 50 000 events are collected in the IgA+ and IgA- fraction and frozen at -20°C for further analysis. Each sample was stained with both an anti-human IgA antibody and an isotype control, and the final percentage of IgA-positive bacteria was reported after subtraction of the isotype-positive population. Samples in which the isotype and antibody-specific populations could not be distinguished were excluded from further sequencing analysis.

B.3.4 16S Library preparation

Sorted bacterial suspensions were boiled for 15 min at 100°C and 2 µL of lysate was used as template for 16S PCR, using Illumina-tagged and barcoded primers specific for the 16S V4 region. PCR was performed with Phusion polymerase under the following cycling conditions: 5 minutes initial denaturation at 98°C, 30 cycles of 20 seconds at 98°C, 15 seconds at 55°C, 30 seconds at 72°C, and 10 minutes final extension at 72°C. Reactions were run on a gel to ensure successful amplification, and were purified and normalized using the 96well Sequel-Prep kit (ThermoFisher A1051001). All reactions were subsequently pooled and gel extracted (GeneJet K0692) to remover primer-dimers. Sequencing was performed on an Illumina MiSeq using a v2
kit for 2x250 bp reads with 30% PhiX at the Biomedical Research Centre (BRC) Sequencing Core of the University of British Columbia.

**B.3.5 Bioinformatics analysis of 16S rRNA data**

Demultiplexed forward reads were analyzed in QIIME2 (https://qiime2.org)\(^{184}\), using the Dada2 option\(^{215}\) for sequence quality control and trimming to 250 bp. Taxonomic assignment was performed using the SILVA database\(^{216}\). Further filtering was then performed in R using phyloseq\(^{187}\). Filtering included removal of unintended targets (archaea, mitochondria and chloroplast) as well as common contaminants of Ig-SEQ datasets (*Alphaproteobacteria*)\(^{138,252}\), removal of singleton taxa, and rarefaction to 5000 reads. Due to the paired nature of the data, if a single sorted fraction had low sequencing depth (<5000 reads), we also excluded all matched samples (e.g. if the IgA- fraction had low sequencing depth pre-FMT, we also excluded the IgA+ fraction pre-FMT and both fractions post-FMT). A log-adjusted IgA index was calculated as described previously\(^{59,212}\) after adding a pseudocount of 0.0000001 relative abundance to allow for zero values. Taxa were maintained at either the ASV level or the genus level for calculation of the IgA Index. IgA Index data was further filtered for prevalence within each pre-FMT and post-FMT dataset, by excluding taxa in which ≥75% of samples had zero values. Relative abundance was estimated by summing the IgA-positive and IgA-negative relative abundance. We did not find any evidence of a sorting batch effect on the IgA Index. Raw sequencing data has been deposited to the Sequence Read Archive (SRA) under BioProject PRJNA650203, and is available at the following reviewer-access link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA650203?reviewer=cpreio670f9k8k497vkr27hcf9
B.3.6. Detection and quantification of short chain fatty acids (SCFAs) in feces and serum

Targeted gas chromatography-mass spectrometry (GC-MS) for SCFA detection, identification and quantification was performed using adaptation of previously described protocols for the analysis of samples of stool and serum. Samples analysis was performed on an Agilent 7890B GC system coupled to an Agilent 5977A mass selective detector (Agilent, USA). Analysis of data was performed using MassHunter software (Agilent), with SCFA concentrations being integrated from a freshly prepared calibration curve for each standard.

B.3.7 Biometal profiling

Selected trace metal (iron, selenium, zinc, cadmium, cobalt, copper, magnesium, manganese, nickel and lead) concentrations in plasma were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analytical technique, using the ICPMS-2030 spectrometer (Shimadzu, Japan). Details regarding ICP-MS measurement conditions and parameters are described in previous studies. Briefly, 100 µL serum was digested in a combination of 300 µL nitric acid (70% purified by redistillation, Sigma-Aldrich), 100 µL hydrogen peroxide solution (25-35% for ultratrace analysis, Sigma Aldrich) and 100 µL hydrochloric acid (30% suprapure, Merck). Serial dilutions of ICP-Multi-element standard solution IV (Merck, Certipur) and ICP-MS selenium single standard solution (Sigma-Aldrich, TraceCERT) were used for calibration of all the biometals analytes. A certified reference material, BCR 637 (Institute for Reference Materials and Measurements) as well as reference material ERM-DA120 (European Reference Materials) were analysed to validate the calibration. A solution containing scandium, yttrium, terbium, tellurium and rhodium (Sigma-Aldrich, TraceCERT) in 1% nitric acid was used as an internal standard (automatically added during analysis through T-piece).
B.3.8 Serum N-glycome analysis

Analysis of total serum N-glycome was performed as previously described\textsuperscript{274}. Briefly, serum N-glycans were enzymatically released from proteins by PNGase F, fluorescently labelled with 2-aminobenzamide and cleaned-up from the excess of reagents by hydrophilic interaction liquid chromatography solid phase extraction (HILIC-SPE), as previously described. Fluorescently labelled and purified N-glycans were separated by HILIC on a Waters BEH Glycan chromatography column, 150 × 2.1 mm i.d., 1.7 μm BEH particles, installed on an Acquity ultra-performance liquid chromatography (UPLC) H-class system (Waters), consisting of a quaternary solvent manager, sample manager and a fluorescence detector set with excitation and emission wavelengths of 250 nm and 428 nm, respectively. Obtained chromatograms were separated into 39 peaks. The amount of N-glycans in each chromatographic peak was expressed as a percentage of total integrated area. From 39 directly measured glycan peaks we calculated 12 derived traits which average particular glycosylation traits like galactosylation, sialylation and branching across different individual glycan structures and are, consequently, more closely related to individual enzymatic activities and underlying genetic polymorphisms. Derived traits used were: the proportion of low branching (LB) and high branching (HB) N-glycans, the proportion of a-, mono-, di-, tri- and tetra-galactosylated N-glycans (G0, G1, G2, G3 and G4, respectively), and a-, mono-, di-, tri- and tetra-sialylated N-glycans (S0, S1, S2, S3 and S4, respectively).

B.3.9 Statistical analysis

All statistical analysis was performed in R studio using the phyloseq and ggplot2 packages. Data structure of the IgA Index was explored using a Principle Component Analysis
based on Euclidean distance. A PERMANOVA analysis was iteratively applied to each variable of interest to determine its contribution to the distribution of the IgA Index. Non-parametric Wilcoxon rank sum tests were used to compare data with two groups, and data were paired by patient for pre-FMT versus post-FMT comparisons. A one-sided Wilcoxon rank sum test was used to identify taxa with an IgA Index significantly different from zero. Spearman’s correlation was applied for continuous variables, including serum metabolites. Unless otherwise indicated, multiple correction of statistical tests was applied using the False Discovery Rate (FDR).

B.4 Results and Discussion

B.4.1 IgA responses before and after FMT

Fecal samples from 48 randomly selected patients recruited from Edmonton and cured of rCDI were obtained before and after FMT. Fecal bacteria were sorted into IgA-positive and IgA-negative populations by flow cytometry; the sorted fractions were subsequently characterized by 16S rDNA amplicon sequencing to identify IgA-targeted bacteria, a technique known as “IgA-SEQ”\textsuperscript{59,212}. After filtering and rarefaction, a total of 36 patients (mean [SD] age, 57.9 [18.5] years; 22 women [61%]; 17 [47%] colonoscopy delivered FMT; 1 patient had underlying ulcerative colitis and 1 had Crohn’s disease) had high-quality 16S data in both sorted fractions pre- and post-FMT, allowing for calculation of a log-adjusted IgA Index and an estimate of relative abundance in these paired samples.

The proportion of fecal bacteria bound by IgA was not different between donors and pre- or post-transplant patients (Fig B.4.1A). However, the IgA+ microbiota of rCDI patients pre-FMT was extremely low-diversity, while alpha diversity was restored in FMT recipients to more closely resemble that of the fecal donors (Fig B.4.1B). This diversity pattern was also seen in the
IgA- fraction of the microbiota (data not shown). Broad changes in IgA-targeted taxa, as measured by PERMANOVA analysis of the IgA Index, were apparent before and after FMT (Fig B.4.1C). Thus, the IgA+ microbiota mirrors trends established in the unsorted microbiota of this cohort and others, with dramatic differences in composition and diversity before and after FMT.

Prior to FMT, *Escherichia coli* was the most abundant and the most highly IgA-targeted member of the fecal microbiota (Fig B.4.1D). We did not detect *C. difficile* in the pre-FMT gut, likely due to vancomycin suppression all patients received before transplantation. However, *Enterobacteriaceae* are known to be common and abundant during intestinal dysbiosis, due in part to their ability to tolerate oxygen; *E. coli* is also a frequent target of IgA in inflammatory conditions. In comparison, *E. coli* was significantly less abundant and less IgA-targeted in the healthy fecal donors (Fig B.4.1D, p<0.05 by Kruskal Wallis with post-hoc Dunn’s test for Donor versus Pre-FMT samples). Although the relative abundance of *E. coli* decreased in FMT recipients, it remained relatively IgA-positive in these patients (Fig B.4.1D). This could indicate a sustained immune response to *E. coli*, or persistence of immunogenic strains from the pre-FMT microbiota.

Following FMT, microbiota diversity was restored (Fig B.4.1B, B) and a larger number of taxa were abundant and IgA-targeted, including multiple *Firmicutes* species (Fig B.4.1D). These taxa are often IgA-positive in healthy adults. Notably, for all of these newly established ASV, the direction of IgA-targeting was consistent between donors and recipients; for example, *[Ruminococcus]* ASV e59 was IgA-positive in both donors and recipients, while *Ruminococcus* ASV 98f was IgA-negative in both donors and recipients (Fig B.4.1D). Overall, the consistency of IgA responses to the same ASV across FMT agrees with IgA-targeting
patterns in gnotobiotic mouse models\textsuperscript{59,137,162,255} and supports the concept that specific bacterial properties determine IgA-coating status in the human gut, independently of host variation.
B.4.2 IgA response by FMT delivery

FMT was delivered in this study via oral capsule or colonoscopy, which we previously showed did not have an impact on success of FMT or resultant composition of the microbiota\textsuperscript{269}. However, colonic and small intestinal IgA responses differ greatly; route of delivery could thus conceivably alter the initiation of an immune response. Here we confirm that there were no differences in IgA-bacterial interactions by delivery method. Both capsule and colonoscopy recipients had comparable IgA-coating levels of the microbiota (Fig B.4.2A), and alpha-diversity of the IgA-positive microbiota (Fig B.4.2B). There was no difference in distribution of the IgA Index by delivery method according to PERMANOVA analysis (Fig B.4.2C; \( p>0.05 \)). Further, no ASV differed in IgA-targeting by Wilcoxon Rank Sum Test at FDR<0.1 (Fig B.4.2D). These data suggest that the route of delivery does not influence fecal IgA-bacterial interactions; both methods lead to an IgA-targeted community resembling that of the healthy donor gut.
Fig 2. Differences in IgA-targeted microbiota post-FMT by delivery method.

(A) %IgA+ Bacteria

(B) IgA+ alpha diversity

(C) PCA of IgAIndex (Post)

(D) Abundance of IgA Index

- Veillonella d32
- Ruminococcus ee9
- Ruminococcus ee9
- Ruminococcus 16c
- Ruminococcus e59
- Lachnospiraceae e80
- Parabacteroides 14f
- Oscillospira 7c9
- Lachnospiraceae d5c
- Lachnospiraceae 694
- Faecalibacterium e55
- Escherichia 1b1
- Erysipelotrichaceae 216
- Dorea a0b
- Coprococcus cd2
- Blautia dc2
- Blautia a0a
- Blautia 9f8
- Bifidobacterium 7b2
- Bacteroides 668
- [Ruminococcus] e59
- [Ruminococcus] cc2
- [Ruminococcus] 90a

Abundance of IgA Index

- Capsule
- Colonoscopy

IgA Index

Abundance

PC1 (8.4%)

PC2 (7.2%)

Groups
- Capsule
- Colon

Donor

Pre

Post

Simple ASV

- Abundance

- IgA Index

- Veillonella d32
- Ruminococcus ee9
- Ruminococcus 16c
- Ruminococcus e59
- Parabacteroides 14f
- Oscillospira 7c9
- Lachnospiraceae e80
- Lachnospiraceae d5c
- Lachnospiraceae 694
- Faecalibacterium e55
- Escherichia 1b1
- Erysipelotrichaceae 216
- Dorea a0b
- Coprococcus cd2
- Blautia dc2
- Blautia a0a
- Blautia 9f8
- Bifidobacterium 7b2
- Bacteroides 668
- [Ruminococcus] e59
- [Ruminococcus] cc2
- [Ruminococcus] 90a
**Figure B.4.2 IgA-targeting of microbiota post-FMT by delivery method.** (A) Percentage of IgA-positive (%IgA+) fecal bacteria in 48 FMT recipients (n=23 capsule delivery and n=25 colonoscopy delivery). Significance determined by Wilcoxon rank sum test. (B) Alpha-diversity of the IgA+ fecal microbiota. Significance determined by Wilcoxon Rank Sum test. (C) Principal Coordinate Analysis based on Euclidean distance of the IgA Index. Non-significant by PERMANOVA (p>0.1). (D) Heatmap depicting average relative abundance and average IgA Index in post-transplant patients by delivery method. No taxa were significantly different between capsule and colonoscopy recipients, as determined by Wilcoxon rank sum test with FDR-corrected p-value <0.05. Letters and numbers after the genus designate the first characters of a unique ASV code used by qiime2. For (B-D), n=36 FMT recipients (n=19 capsule delivery and n=17 colonoscopy delivery).

### B.4.3 IgA responses by fecal donor identity

The microbiota composition of FMT recipients has been shown to involve a mixture of newly engrafted donor strains and pre-existing patient strains\(^{265}\). Given the importance of strain identity in determining IgA responses\(^{162,212,255}\), we next examined whether IgA-targeting of the post-FMT microbiome was associated with IgA-targeting of the individual donor microbiome. For this analysis, we excluded one fecal donor and associated recipients due to insufficient sample size (n=2 recipients of this donor with %IgA-coating data and n=1 with valid IgA Index data); this left three donor-recipient groups for analysis (n=14, n=12 and n=9 FMT recipients with valid IgA Index data for donors 1, 2 and 3 respectively).

The overall proportion of IgA-bound microbes did not differ significantly in FMT recipients by donor identity (Fig B.4.3A). However, there was a modest difference in the alpha-diversity of the IgA-positive microbiota by donor identity (Fig B.4.3B, p=0.038 by Kruskal-Wallis); this trend was also reflected in the IgA-negative microbiota, albeit less significantly (Fig B.4.4A, p=0.107 by Kruskal-Wallis), suggesting a more general change in community diversity by donor. Furthermore, there was a significant separation of samples by donor identity according
to principle coordinate analysis of the IgA Index (Fig A.4.3C; p=0.01 by PERMANOVA
analysis).

As expected, multiple taxa differed significantly in abundance between the recipients of
the three main donors post-FMT; in total, 39 taxa were differentially abundant, including both
Firmicutes and Bacteroidetes species (Fig B.4.3D, FDR-adjusted p<0.05 by Kruskal-Wallis). In
contrast, only four taxa showed significantly different IgA-targeting patterns in the recipients of
the different fecal donors (Fig B.4.3D, FDR-adjusted p<0.05 by Kruskal-Wallis). All of these
taxa were Firmicutes species; only one, Ruminococcus ASV 16c, was differentially abundant in
the same patients (Fig B.4.3D). The IgA-targeting of donors and their respective recipients was
often concordant (i.e. both negative or both positive); it is notable however that in several cases,
including the significant hit Oscillospira ASV 680, donor and recipient targeting was discordant
(Fig B.4.3D). It remains unclear why IgA-targeting of these particular strains differed between
recipients; it is possible that these ASV represent new colonizers or expanded strains from the
patient microbiota, or, alternatively, that there were host differences in immune recognition
between FMT recipients.

Importantly, since there was sex-matching between stool donor and recipient, testing for
sex differences in post-FMT patients also yields similar taxa (Fig B.4.4B-E). We cannot,
therefore, exclude the possibility that sex is a driver of IgA-targeting post-FMT, particularly as
immune function is known to differ significantly between males and females. However, we
did not see any differences in microbiota or IgA-targeting by sex in the pre-FMT microbiota
(data not shown).

Collectively, we find that the microbiota of FMT recipients differs by donor stool in both
alpha-diversity and in the engraftment of specific ASV. IgA-targeting patterns also differ
significantly by donor identity, but are overall more conserved across donors and recipients. The factors driving these IgA-targeting differences post-FMT remain unclear.

![Diagram](image-url)
Figure B.4.3 IgA-targeting of microbiota post-FMT by donor identity. (A) Percentage of IgA-positive (\%IgA+) fecal bacteria in 46 FMT recipients (n=15 of donor 1; n=17 of donor 2; n=14 of donor 3). Significance determined by Kruskal-Wallis. (B) Alpha-diversity of the IgA+ fecal microbiota. Significance determined by Kruskal-Wallis with post-hoc Dunn’s test. (C) Heatmap depicting average relative abundance and average IgA Index in post-FMT recipients by donor identity. Donor value itself is shown for comparison. D, Donor, R, Recipient; 1-3 designate different FMT donors. Star (*) indicate taxa that were different between recipients of different donors, as determined by Kruskal-Wallis with FDR-corrected p-value <0.05. Letters and numbers after the genus designate the first characters of a unique ASV code used by qiime2. For (B-D), n=35 FMT recipients (n=14 of donor 1; n=12 of donor 2; n=9 of donor 3). FMT recipients from a fourth donor were excluded as the sample size was too small for statistical comparison (n=2 recipients with \%IgA+ data and n=1 recipient with IgA Index data).
Figure B.4.4 IgA-targeting of microbiota post-FMT by donor identity and patient sex. (A) Alpha-diversity of the IgA-negative (IgA-) fecal microbiota in 46 FMT recipients (n=15 of donor 1; n=17 of donor 2; n=14 of donor 3). Significance determined by Kruskal-Wallis. (B) Principal Coordinate Analysis based on Euclidean distance of the IgA Index post-FMT, by donor identity and by sex of patient. (C-E) IgA Index of taxa which different significantly by both sex and by donor identity, by Wilcoxon Rank Sum test with FDR-corrected p<0.05. For (C-E), n=35 FMT recipients (n=14 of donor 1; n=12 of donor 2; n=9 of donor 3). FMT recipients from a fourth donor were excluded for donor analysis, as the sample size was too small for statistical comparison (n=2 recipients with %IgA+ data and n=1 recipient with IgA Index data) and were therefore also excluded from sex analysis for comparison.
B.4.4 Variability and Predictability of IgA-targeted microbiota

To identify other factors which might explain variation in IgA-targeting across samples, we performed a PERMANOVA analysis of the IgA Index across multiple host and metabolic variables (Fig B.4.5A). As shown above, sample type (pre-FMT, post-FMT or donor) contributed significantly to variation by PERMANOVA in the full dataset, while sex of the patient (confounded with donor identity) contributed significantly in the post-FMT dataset. Other host variables available such as patient age; weight (body mass index prior to FMT, and weight loss following FMT); and underlying IBD, did not influence the IgA Index in this cohort. Technical variation (batch effect and sequencing depth prior to rarefaction) also did not have an important influence by PERMANOVA (Fig B.4.5A).

Restoration of the fecal microbiota correlates with systemic metabolite changes, including increases in circulating SCFA, decreases in complexity of serum N-glycan structures, and decreases in serum selenium and copper levels264,271,274,276,277. Since SCFA, glycosylation and nutrition are known to impact IgA responses to the microbiota, we also looked for the impact of these metabolites on IgA-targeting (Fig B.4.5A). Total serum SCFA levels contributed significantly to variation in the full dataset according to PERMANOVA analysis (Fig B.4.5A, FDR-adjusted p<0.05) but not in the pre- or post-FMT datasets alone; this result may be confounded by significant changes in SCFA levels by FMT status itself. No other metabolite reached statistical significance by PERMANOVA after FDR correction. We did not find the IgA-targeting of any individual taxon to correlate significantly with systemic metabolites, with the exception of a positive correlation between Pseudomonas veronii and nickel concentrations in post-FMT patients (Spearman’s rho=−0.83, FDR-adjusted p=0.002). There were also several
positive correlations between SCFA levels and the IgA-targeting of *Firmicutes* species post-FMT, but these did not pass FDR correction.

To further understand the determinants of microbiota composition and IgA-targeting post-FMT, we built machine learning models to predict post-FMT relative abundance and IgA Index. We based our approach on the Random Forest models described by Smillie *et al.*\textsuperscript{265}, incorporating microbiota information from donors and from pre-FMT patients. Models were trained on information from 28 out of the 36 patients, with 8 left out for a validation set. Encouragingly, as reported previously\textsuperscript{265}, we were able to predict the relative abundance of taxa post-FMT based solely on pre-FMT and donor microbiota information. This model explained 32.9% of the variation post-FMT and was significant by permutation test. Further, when the model was applied to the 8 remaining validation patients, the predicted relative abundance values correlated significantly with real values (Fig B.4.5B: Spearman’s rho=0.38, p<2.2e-16; root mean square error=4.6). The most important variables in this model were abundance of the taxon in the donor and in the pre-FMT stool samples (Fig B.4.5C), similar to the model previously reported\textsuperscript{265}.

In contrast, using pre-FMT and donor IgA Index data, we were less able to predict post-FMT IgA Index of the recipients (accounting for only 2.4% of the variation post-FMT). The correlation of predicted and real values in the remaining validation patients was significant, but at a low coefficient of correlation (Fig B.4.5D; Spearman’s rho=0.16, p=4.5e-10; root mean square error=0.17). The most important features in this model were the IgA Index in the donor stool samples, whereas the pre-FMT IgA Index was relatively less important (Fig B.4.5E). To be consistent between approaches, both abundance and IgA Index are presented on a log scale, with a pseudocount added to account for zero values (see Methods); these pseudo-zero values appear
to be poorly predicted (Fig B.4.5 B,D), however, we felt that removing all sparse taxa would introduce additional bias and therefore retained the zero count data. Incorporating additional information such as delivery method, technical variation (sorting batch and sequencing depth), the overall percentage of IgA+ bacteria, and host factors such as age, sex, and BMI, improved the abundance model slightly but did not improve the IgA-Index model (37.5% of variance explained in relative abundance versus 1.9% of variance explained in IgA Index). Adding serum metabolite data also did not help improve the model, but was limited by the smaller sample size for these features (data not shown). Thus, the microbiota composition post-FMT can be predicted using information from the pre-FMT and donor microbiota; in contrast, the ability to predict IgA-targeting of this microbiota remains more challenging.
Figure B.4.5 Variability and predictability of IgA-targeted microbiota. (A) PERMANOVA analysis, based on Euclidean distance of the IgA Index, was performed on the full dataset (n=77, including donor samples), or on pre- and post-transplant patients (n=36). A star (*) indicates significance at FDR-correction <0.05. Colour represents raw p value, and coef is the coefficient of variance. Permutations were constrained by day of sorting to account for a possible batch effect. (B) Comparison of measured versus predicted relative abundance of taxa in the post-FMT microbiota, on a test set from n=8 patients. P-value and rho determined by Spearman’s correlation. (C) Features used in the predictive abundance model, ranked by importance. (D) Comparison of measured versus predicted IgA Index of taxa in the post-FMT microbiota, on a test set from n=8 patients. P-value and rho determined by Spearman’s correlation. (E) Features used in the predictive IgA Index model, ranked by importance.
B.4.5 Conclusions and future directions

This is the first study to examine the changing patterns of IgA-targeted microbiota in rCDI patients before and after FMT. These findings confirm that in addition to broad changes in the total microbiota, immune-bacterial interactions are profoundly altered by FMT in rCDI patients. Mode of delivery does not alter the establishment of these interactions, supporting the use of either colonoscopy or capsule as a viable FMT delivery method. Since IgA recognizes a subset of the total microbiota and has been shown to preferentially target small intestinal and mucosal bacteria, our data may also point to changes in mucosal populations pre- and post-FMT, although biopsy samples would be needed to confirm this. Dysbiosis in the interactions of mucosal bacteria with the immune system might perpetuate immune dysregulation, contributing to C. difficile persistence and proliferation; indeed, low IgA levels in infants are associated with increased colonization of C. difficile278. Together, therefore, these findings have implications for the etiology and treatment of C. difficile.

Importantly, this study also furthers our fundamental understanding of IgA-microbiota interactions in the human intestine. We show that IgA responses to specific bacteria are relatively well conserved among healthy donors and among FMT recipients, supporting the concept that specific bacterial properties determine IgA recognition independently of host variation. Nevertheless, there was inter-individual variability in IgA responses, even in recipients of the same fecal donor, which is poorly explained by available host, microbiota and metabolite data or by sources of technical variability. It is possible that strain-level differences not captured by 16S amplicon data could contribute to this variability between recipients; alternatively, host genetic and immune differences – for example, BCR repertoire and memory based on previous microbial exposures – may play a role. In the future, resolving strain-level differences through
deep metagenomic sequencing, and accounting for host genetic and immune variation, may help to better explain IgA-targeting variation.

This study has several limitations. The study cohort consisted of a small sample size from a single center. Additionally, all the donors were all drawn from a healthy Western population and often showed similar IgA-coating patterns; in contrast, important differences in both strain identity and IgA-targeting may exist between geographically distinct populations and by disease state\textsuperscript{252}. Caution should therefore be applied in generalizing these findings to other populations. Nevertheless, this study is the first to track IgA-targeting by FMT status in a human population, and therefore provides valuable insights both into rCDI etiology and into our fundamental understanding of IgA-microbial homeostasis.