Early-life exposure to multiple micronutrient deficiencies induces clinically-relevant antibiotic resistance in a pre-clinical murine model

Paula Littlejohn (paula.littlejohn@msl.ubc.ca)
University of British Columbia https://orcid.org/0000-0003-3252-7862
Avril Metcalfe-Roach
University of British Columbia https://orcid.org/0000-0002-1016-3765
Ravi Holani
University of British Columbia
Haggai Bar-Yoseph
University of British Columbia
Yiyun Fan
University of British Columbia https://orcid.org/0000-0002-4614-665X
B Finlay
https://orcid.org/0000-0001-5303-6128

Article

Keywords:

Posted Date: September 27th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2056188/v1

Early-life exposure to multiple micronutrient deficiencies induces clinically-relevant antibiotic resistance in a pre-clinical murine model

Paula T. Littlejohn[a,b], Avril Metcalfe-Roach [a,b], Ravi Holani[a], Haggai Bar-Yoseph[a], Yiyun M. Fan,[c] B. Brett Finlay [a,b,d]

a Michael Smith Laboratories, University of British Columbia, Vancouver, British Columbia, Canada

b Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

c Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, British Columbia, Canada

d Biochemistry and Molecular Biology Department, University of British Columbia, Vancouver, Canada

*B. Brett Finlay, corresponding author
bfinlay@interchange.ubc.ca

Abstract:

Globally, ~340 million children (<5 years) suffer from multiple micronutrient deficiencies, accompanied by high pathogenic burden and death due to multi-drug resistant bacteria. The role of early-life micronutrient deficiencies in mitigating antibiotic resistance within the host microbiota remains unexplored. We developed a postnatal preclinical murine model deficient in multiple micronutrients: zinc, folate, iron, vitamin A, and vitamin B12. Fecal metagenomic sequencing after dietary exposure revealed enrichment in the relative abundance of pathobionts and antibiotic resistance mechanisms including efflux pumps, target drug site alteration, antibiotic degrading enzymes, permeability, and acquisition of antibiotic resistance genes (ARGs). Despite being antibiotic naïve, we observed increased abundance of genes encoding resistance to several antibiotic classes: tetracycline, vancomycin, methicillin, fosfomycin, aminoglycosides and glycopeptides. Moreover, in vitro antibiotic susceptibility testing revealed clinically-relevant resistance to multiple antibiotic classes, namely vancomycin, methicillin, amoxicillin and erythromycin in mice fed the low-micronutrient diet. These novel findings present an unappreciated and profound role of cooccurring multiple micronutrient deficiencies in contributing to the global burden of antibiotic resistance, with significant clinical implications for undernourished children living in low-to-mid income countries.

Introduction

Micronutrients (vitamins and minerals required in small amounts) play an important role in health and disease.[1] Globally, an estimated 2 billion people suffer from micronutrient deficiencies; of this, 340 million are children, amounting to half of all children under the age of five. [2–5] Approximately 50% of all childhood deaths are attributed to undernutrition.[6] Indeed, young children typically die not from starvation, but from treatable childhood diseases, such as infections, to which micronutrient deficiency substantially contributes.[7] While no country is immune, the burden of micronutrient deficiency lies primarily in low-to-mid-income countries (LMIC).[4,8] More importantly, deficiencies that occur within the first 1000 days confer immediate and lifelong health deficits.

Compelling evidence also shows that the first 1000 days are a critical period for the assembly and maturation of the intestinal microbiome, and perturbations have immediate and long-term consequences. The microbiome within the protein undernourished gut has been well characterized, and an altered and immature microbiota is a characteristic feature of the protein deficient host.[9–14] Researchers from the Afribiotia project have also found significant decompartmentalization of the gut microbiome in stunted children, characterized by a higher abundance of oropharyngeal bacteria along the gastrointestinal tract.[15] Germ-free animal models have further demonstrated that the microbiome plays a causal role in malnutrition.[11] A nebulous element of the intestinal environment, however, is the gut resistome: the collection of the full complement of antibiotic resistance genes ubiquitously expressed primarily by commensals and pathogens within the gut microbiome habitat.[16–18] Studies show that the resistome is highly dynamic over the first few months of life as the commensal community assembles.[17] Antibiotic resistance is a global public health threat which disproportionately affects individuals living in LMICs.[19] In 2019 an estimated 1.27 million deaths were attributable to bacterial antimicrobial resistance (AMR). In the same year, an estimated 1 in 5 deaths in children under five were due to antibiotic resistant bacteria.[19] Yet, while pathogens, pathobionts, and even commensals have received considerable attention in the field of undernutrition, very little is known about the resistome within the undernourished gut.

Presently, a paucity of data exists on the characterization of the gut resistome in undernourished children with and without antibiotic exposure.[20,21] Even less is known about the impact of co-occurring micronutrient deficiencies on the developing gut microbiome, and no data currently exist on their impact on the resistome early in life. Thus, our understanding of the undernourished microbiome remains incomplete, and strategies for intervention are potentially misguided. We have developed, to our knowledge, the first postnatal preclinical murine model to investigate the impact of multiple micronutrient deficiencies on the maturing gut microbiome and resistome. Here, we show that micronutrient deficiencies significantly alter the gut microbiome composition, and we describe for the first time how micronutrients shape the antibiotic resistome.

Results

Micronutrient deficiency increases the relative and absolute abundance of Enterobacteriaceae

Weanling C57BL/6N male mice received an isocaloric experimental control or multiple micronutrient deficient (i.e., low zinc, folate, iron, vitamin A and vitamin B12) diet for 28 days (i.e., 4 weeks). We used whole genome shotgun metagenomic sequencing to characterize the gut microbiome and resistome before and after dietary treatment. Our results showed a striking expansion of Proteobacteria in mice fed the low-micronutrient diet on Day 28, while Firmicutes expanded in the CON group (FIG.1.a). At the genus level we observed higher relative abundance of *Klebsiella, Faecalibaculum, Erysipelatoclostridium, Parabacteroides, Enterocloster,* and *Blautia* and loss of *Lachnoclostridium, Muribaculum, and Ligilactobacillus* in the LM mice on Day 28 (FIG.1.b). We also saw a relative expansion of the genus *Faecalibaculum* and loss of *Ligilactobacillus* and *Lachnoclostridium* in CON mice; conversely, however, *Klebsiella*, *Enterocloster*, *Muribaculum*, and *Blautia* were reduced. Species-level resolution revealed increased abundance of *Faecalibaculum rodentium* and loss of *Ligilactobacillus murinus, Muribaculum gordoncarteri,* and *Lachnoclostridium sp*. YL32 in both groups. Enrichment of *Erysipelatoclostridium* [clostridium] *innocuum, Enterocloster bolteae, Parabacteroides goldsteinii,* and *Blautia producta* were also apparent in LM, as well as loss of *Enterocloster boltae* in the CON mice (FIG.1.c). At the end of the experiment an absolute increase in *Enterobacteriaceae* ($>10^{10}$ CFU) was seen in the LM group. No difference was found between the groups at Day 0 (FIG.1.d).

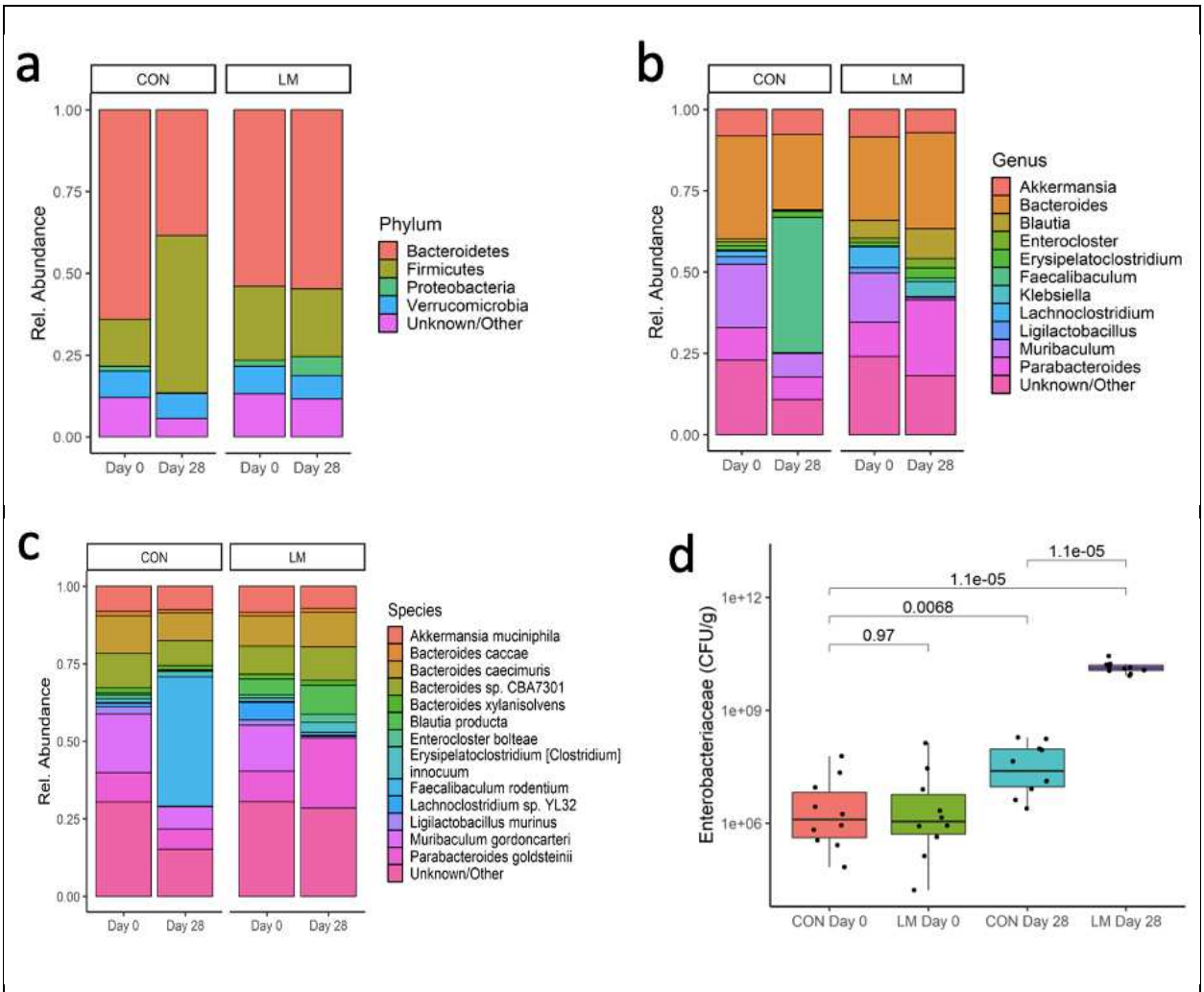


Figure 1: Taxonomic bar plots (a-c) represent differences in mean relative abundance between LM and CON at (a) phylum, (b) genus and (c) species levels from Day 0 to Day 28. Only taxa with >1% mean abundance across all samples were plotted; all others were grouped in 'Unknown/Other'. (d) Difference in absolute abundance in colony forming units (CFU) (MacConkey agar) on Day 0 and Day 28 between LM and CON. Data analyzed using Wilcoxon rank-sum tests.

Gut microbiome alteration in micronutrient deficient mice is driven by Klebsiella and Enterobacter species.

Indicator species analyses identified microbes that were highly prevalent and specific to the treatment groups. We found a total of 14 indicator species associated with baseline or diet treated CON and LM microbiomes: *Pseudoalteromonas spongiae, Klebsiella* sp. P1CD1*, Klebsiella* sp. BDA-134-6, *Klebsiella quasivariicola, Klebsiella grimontii, Kaistella jeonii, Enterbacter cloacae complex* sp. FDA-CDC_AR_0164*, Corynebacterium stationiis, Comamonas aquatica,*

Candidatus synthrocurvum alkaliphilum, Candidatus arthromitus sp. SFB-mouse-NL*, Candidatus arthromitus* sp. SFB-mouse, *Bacillus* sp. 1 NLA3E*, and Acinetobacter equine*. Low-micronutrient mice experienced a significant loss in the entire *Corynebacterium* genus (P = 0.00013) at Day 28 compared to CON (FIG.2.b.). While, *Klebsiella* (P < 0.0001) and *Enterobacter* (P < 0.0001) genera characterized the gut microbiome of the mice fed the LM diet at Day 28 (FIG.2.c, d). A general loss in mouse-associated segmented filamentous bacteria *Candidatus arthromitus* genus was also observed in both CON and LM groups at Day 28.

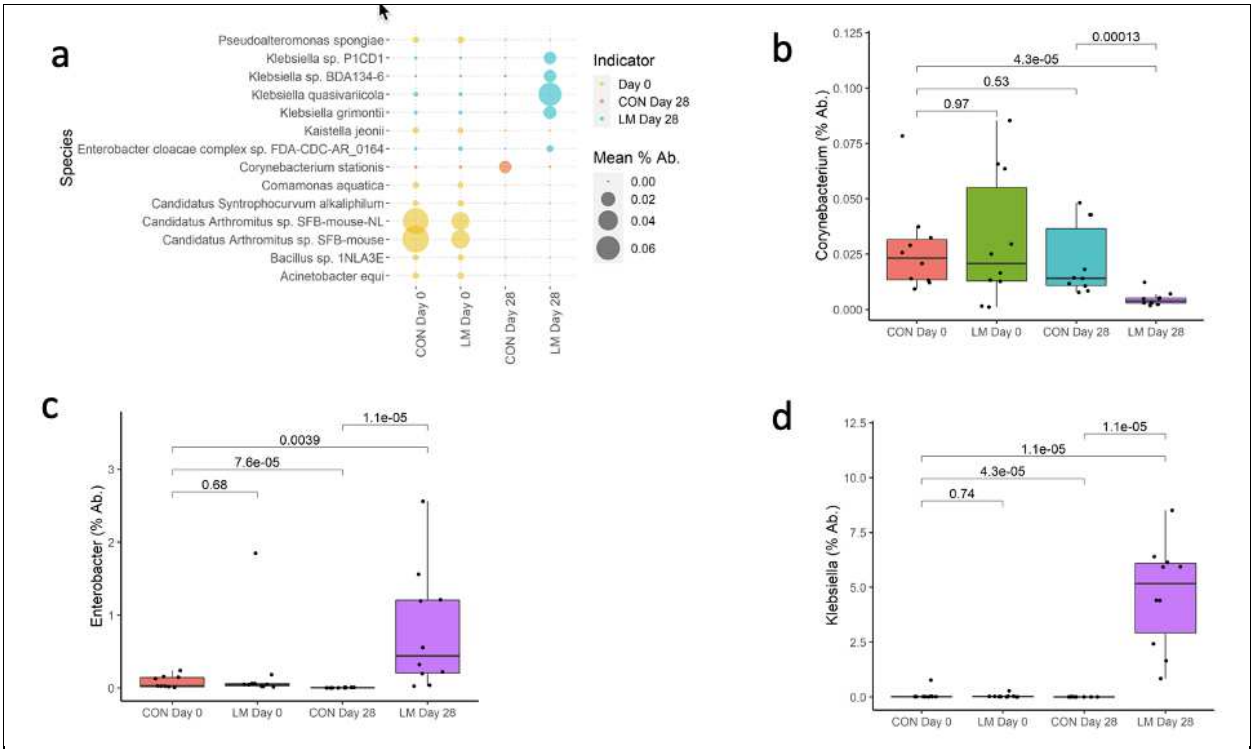


Figure 2: Indicator species contributing to microbiome compositional change. In (a), color represents the group associated with each indicator and size represents mean percent abundance. Plots (b-d) show genus-level abundances of the indicator species associated with Day 28 groups.

Micronutrient deficiency induces the expansion of pathobionts and reshapes the gut community

Expansion of pathobionts (opportunistic commensal bacteria) is a common phenotype of the undernourished gut in children.[9,15,22–25] Thus, we aimed to phenotype the pathobionts within our model compared to children. A total of eleven known undernutrition-associated pathobionts were queried at the genus or species level: *Bacteroides, Campylobacter, Enterococcus, Escherichia, Haemophilus, Klebsiella, Phocaeicola vulgatus*, *Prevotella copri, Shigella, Staphylococcus,* and *Streptococcus*. Our results showed an overall expansion in relative abundance of total pathobionts in the mice fed the low-micronutrient deficient diet compared to the experimental controls on Day 28 ($p < 0.0001$) (FIG.3.a). Of the 11 taxa, seven were significantly increased in relative abundance in the LM group at Day 28 (Wilcoxon rank-sum test, FDR-corrected values): *Klebsiella* ($P < 0.001$)*, Enterococcus* ($P < 0.05$), *Phocaeicola vulgatus* ($P < 0.05$), *Escherichia* ($P < 0.001$)*, Campylobacter* ($P < 0.01$)*, Haemophilus* ($P < 0.001$) and *Shigella* ($P < 0.01$) (FIG.3.b-c). Gut microbiome community composition was also reshaped by the significant loss of the genus *Streptococcus* ($P < 0.001$), *Staphylococcus* ($P < 0.001$) and *Prevotella copri* ($P < 0.05$) (FIG.3.d). *Bacteroides fragilis* was also increased ($P = 0.08$). The most significant loss was within the genus *Streptococcus*. No microbes significantly increased in the CON treated group.

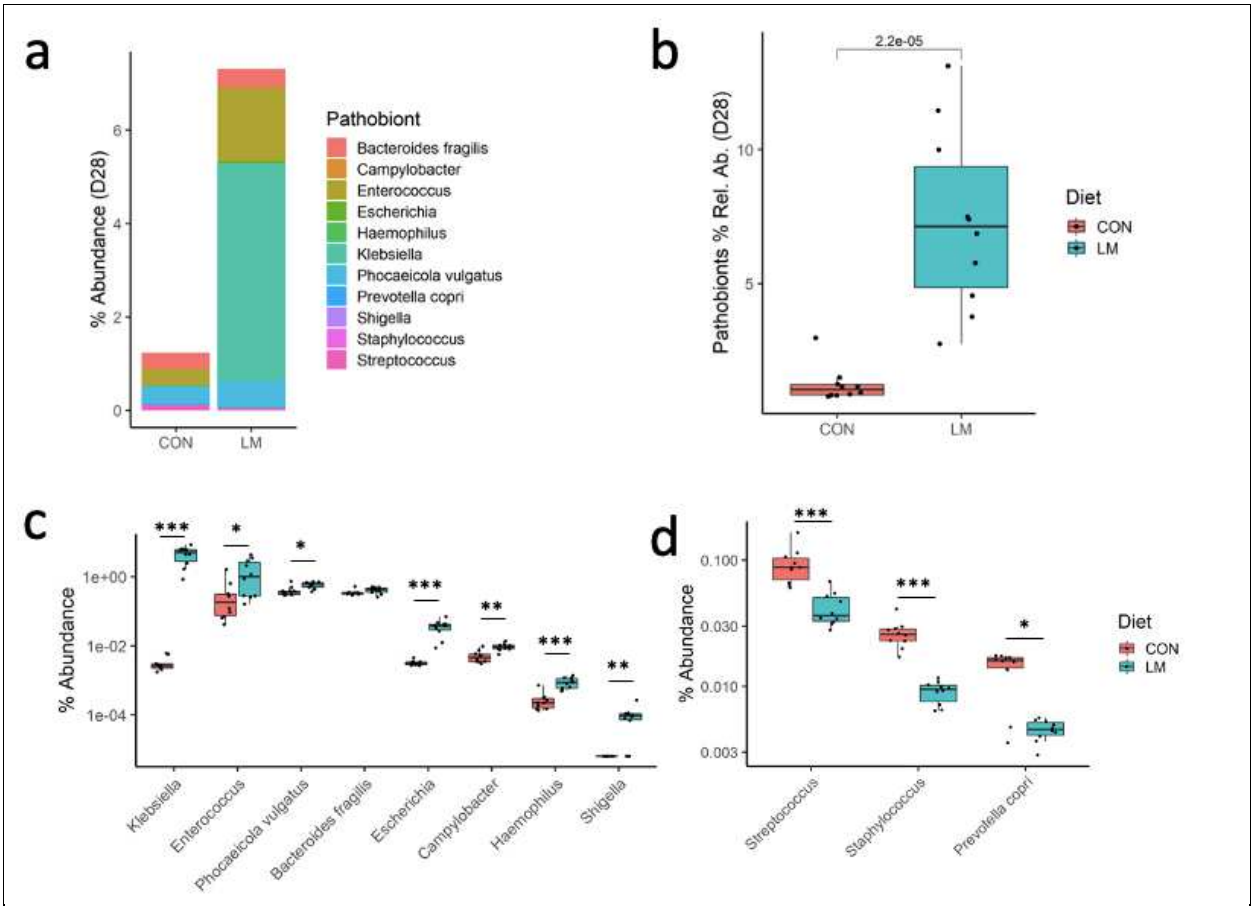


Figure 3: Total pathobionts increased in the LM treated mice. Analysis reveals 8 LM positively associated pathobionts and three negatively-associated species. Corrected P values: (n/s) P > 0.05, *P < 0.05, **P < 0.01, *** P < 0.001.

Postnatal micronutrient deficiency induces multiple antibiotic resistance mechanisms

Profiling of the gut resistome revealed increased relative abundance in total ARGs within the LM group at Day 28 (FIG.4.a). Conversely, we saw a significant decrease in ARGs within the control mice which is consistent with maturation of the resistome (Supplemental FIG 1). Our data also revealed significant increased abundance of genes representing all five antibiotic resistance mechanisms shown in (FIG.4.b-c). We saw greater abundance of efflux pumps genes, namely RND (resistance-nodulation division) ($p < 0.01$), multi-drug resistance (*MDR*) ($p < 0.0001$), and ABC (ATP-binding cassettes) ($p < 0.01$). Genes encoding beta-lactamase ($p < 0.0001$) - a key enzyme that confers beta-lactam resistance and the beta-lactamase receptor-1 (BlaR1) ($p < 0.01$) - were also significantly increased in the low-micronutrient mice. Our data also showed a

significant increase in genes encoding lysozyme resistance ($p < 0.0001$), an intrinsic outer-membrane permeability mechanism. There was an overall increase in the relative abundance of ARGs to several antibiotic classes including tetracycline (*tetM*) ($p < 0.0001$), a ribosomal protection protein (RPP) that modifies target site binding, and resistance genes fosfomycin ($p < 0.0001$), methicillin ($p < 0.001$), vancomycin ($p < 0.01$) aminoglycosides ($p < 0.05$) and glycopeptides ($p < 0.05$) in the low-micronutrient mice on Day 28. Conversely, the abundance of genes related to fluoroquinolone and tetracycline q (*tetQ*) antibiotic classes were significantly decreased in the low-micronutrient mice. No difference was found in other tetracycline (tetO), (tetP) and erythromycin resistance genes. Although many core ARGs were retained at some detectable level between all groups and timepoints the Venn diagram showed that the groups appear to diverge and the LM group developed more detectable ARGs than the CON group by Day 28 (FIG.4.d)

We further examined if pathobionts correlated with specific ARG compounds (FIG.4.g-l). Within the low-micronutrient mice, we found a positive relationship between the relative abundance of *Klebsiella* and lysozyme ($P = 0.0001$, FDR = 0.01) and *Streptococcus* and beta-lactamase ($P = 0.0003$, FDR = 0.02). Conversely, we saw a negative association between the relative abundance of *Haemophilus* and *BlaR1* ($P = 0.0004$, FDR = 0.03). Within the control mice a positive association was found between relative abundance of *Escherichia* and beta-lactamase ($P = 0.0004$, FDR = 0.02), *Phocaeicola vulgatus* and *BlaR1* ($P = 0.0005$, FDR = 0.03) and *Enterococcus* and *tetM* ($P = 0.0006$, FDR = 0.03). Of the pathobionts identified in the low-micronutrient group, only *Enterococcus* showed any correlation after FDR correction, albeit negatively, with total ARG abundance ($P = 0.005$, FDR < 0.05) (Supplemental FIG 1). We observed only a negative trend, but no strong relationship between total relative abundance of pathobionts and total abundance of ARGs ($P = 0.09$) in the low-micronutrient group (Supplemental Figure 1). When we combined the dietary groups, we found that ARGs correlated significantly with the Bacteroidetes and Firmicutes phyla on Day 28. No difference was observed in the phylum Verrucomicrobia and a positive but not significant trend in Proteobacteria (Supplemental Figure 1).

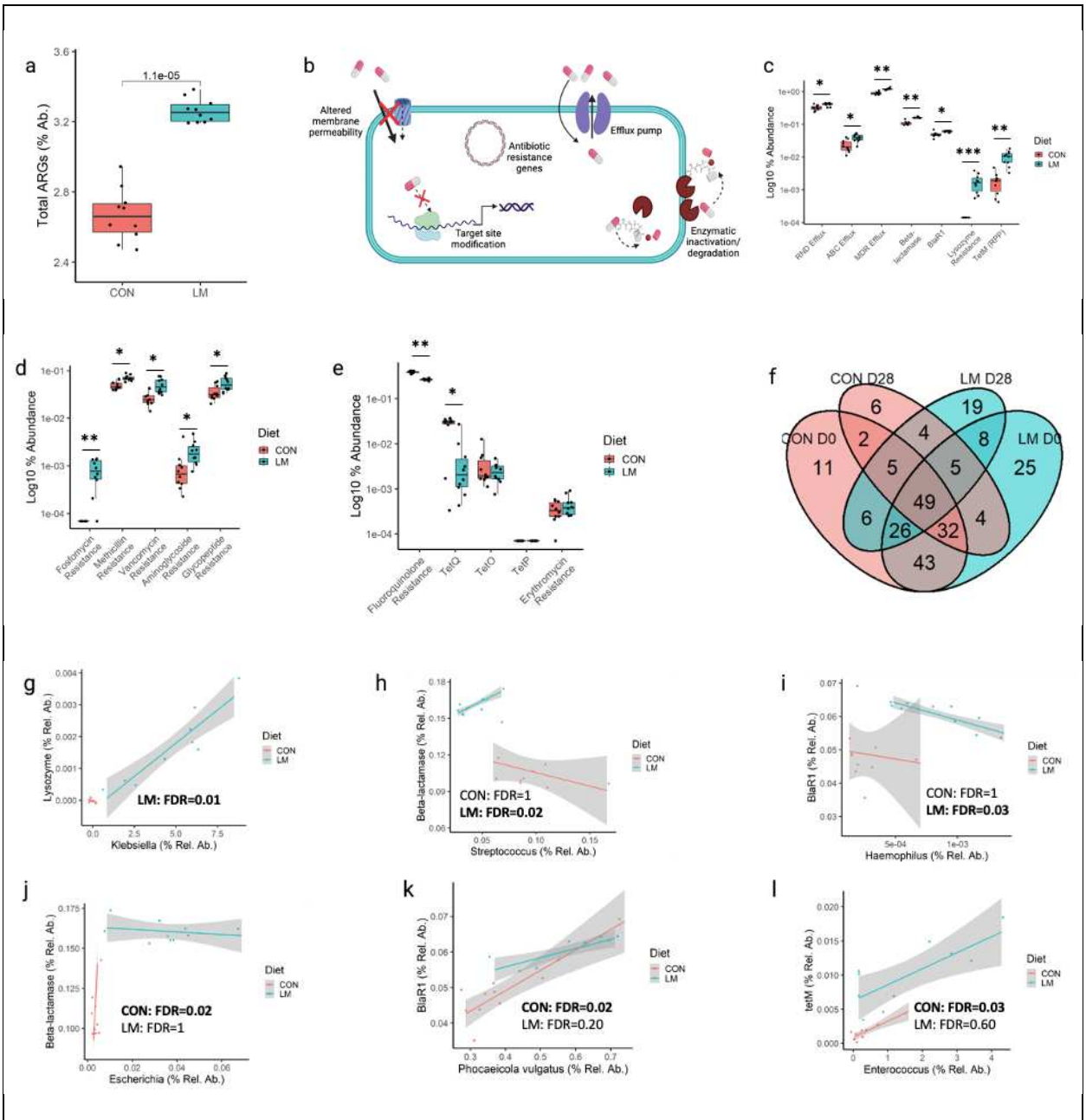
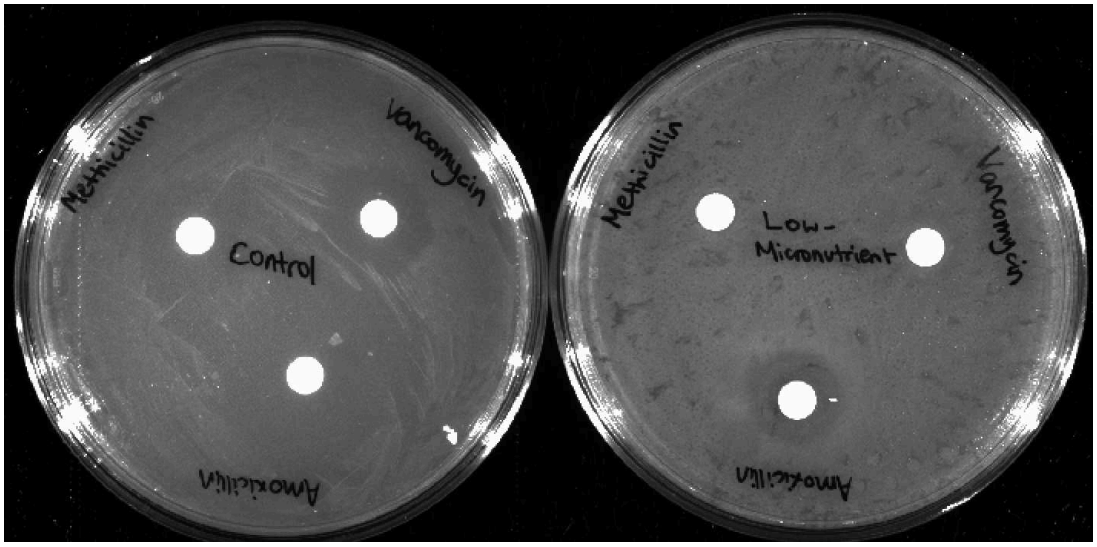


Figure 4: Total relative abundance of ARGs increased in LM mice on Day 28 (a). Antibiotic resistance mechanism enriched in LM mice (b-e). Venn diagram showing shared and unique genes by treatment (f). Positive and negative correlations between pathobionts and ARGs (g-l). Corrected P values: * = p<0.05, ** = p<0.01, *** = p<0.001.

Early life exposure to multiple micronutrient deficiencies confers clinical resistance to antibiotic drug classes despite non exposure

We next investigated whether the metagenomic analysis accurately predicted a biological

resistance phenotype. Bacterial samples were subjected to antibiotic susceptibility testing by exposure to nine antibiotics; namely, vancomycin, amoxicillin, methicillin, tetracycline, erythromycin, ciprofloxacin, ofloxacin, levofloxacin (fluoroquinolones) and gentamicin (aminoglycoside) antibiotics. Strikingly, of the nine examined, we found total resistance to four within the low-micronutrient group. Consistent with the metagenomics data, the low-micronutrient microbiome showed resistance to vancomycin and methicillin (FIG 5a-b). No resistance was found to the fluoroquinolones which was also consistent with gene capacity in the metagenome. Our metagenome showed increased gene abundance of tet(M) and not the other tet proteins which may not have been sufficient to confer a biological phenotype. However, there was increased resistance to erythromycin and no resistance to gentamicin in the low-micronutrient group which is contradictory to our metagenomics results. Intriguingly, we also found resistance to amoxicillin which was not annotated in our metagenome analysis.



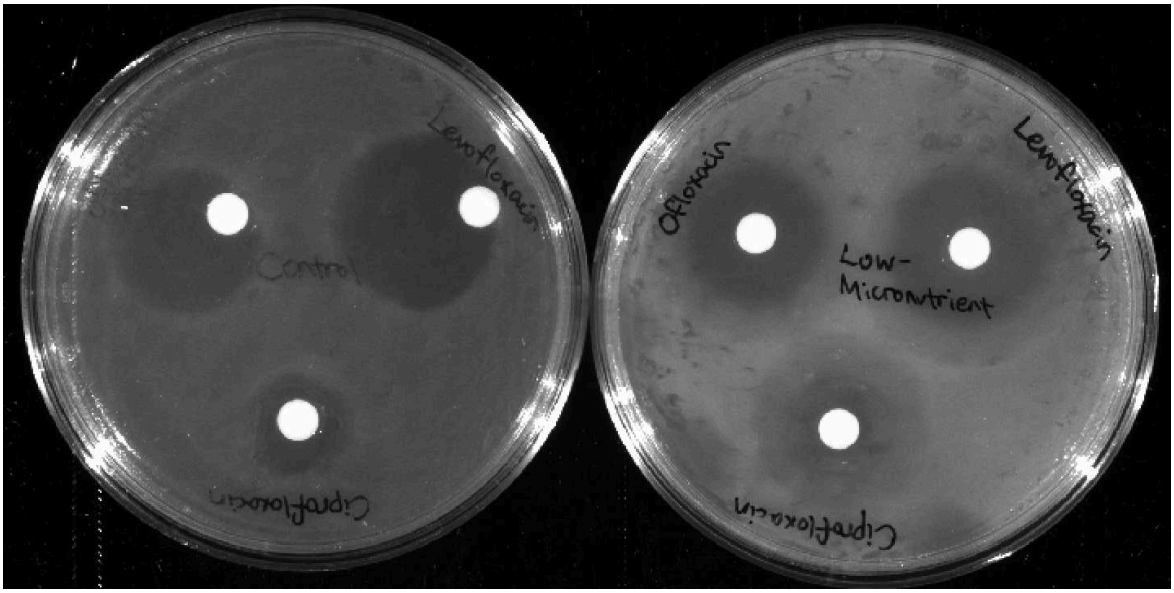


Figure 5: Antibiotic resistance susceptibility testing. Fecal microbiome exposed to antibiotics showed resistance to vancomycin, methicillin and amoxicillin in mice fed the low-micronutrient diet. No difference in resistance to fluroquinolones.

Multiple micronutrient deficiencies induce increased transposable elements (TE) and stress response

Dissemination of ARGs within commensals can occur via transposable elements, [26–28] and the *Bacteroidales* order has been shown to contribute to the dissemination of antibiotic resistance by HGT through their vast repertoire of conjugative transposons (CTns).[18,29] Total TE abundance was markedly increased in the LM group ($P = 0.001$), however, no correlation between total ARGs and TEs in the LM mice ($P = 0.43$) was found (FIG.6.a-b). Our data revealed increased overall abundance of *Bacteriodales* conjugative transposons (TraG, TraN, etc.) within the LM sample ($P = 0.001$). Another form of TE, class 1 (Intl1) and class 2 (Intl2) integrases, have been well studied for their role in antibiotic resistance dissemination.[30] We found increased enrichment of both Intl1 ($P < 0.0001$) and Intl2 ($P = 0.035$) in the LM group (FIG.6.c-e).

Environmental stressors such as nutrient deprivation and antibiotic exposure can trigger stress responses in bacteria that can induce antibiotic resistant mechanisms as a survival strategy.[18,31,32] Paradoxically, bacteria can also induce reactive oxygen species (ROS) to self-destruct in the presence of lethal stress.[33] Given our nutrient-deprived gut environment, we further examined

various stress response mechanisms, (i.e., alarmones) within our sample. We found genes encoding oxidative stress and oxidative stress response to be more abundant in the LM group (FIG.6.f).

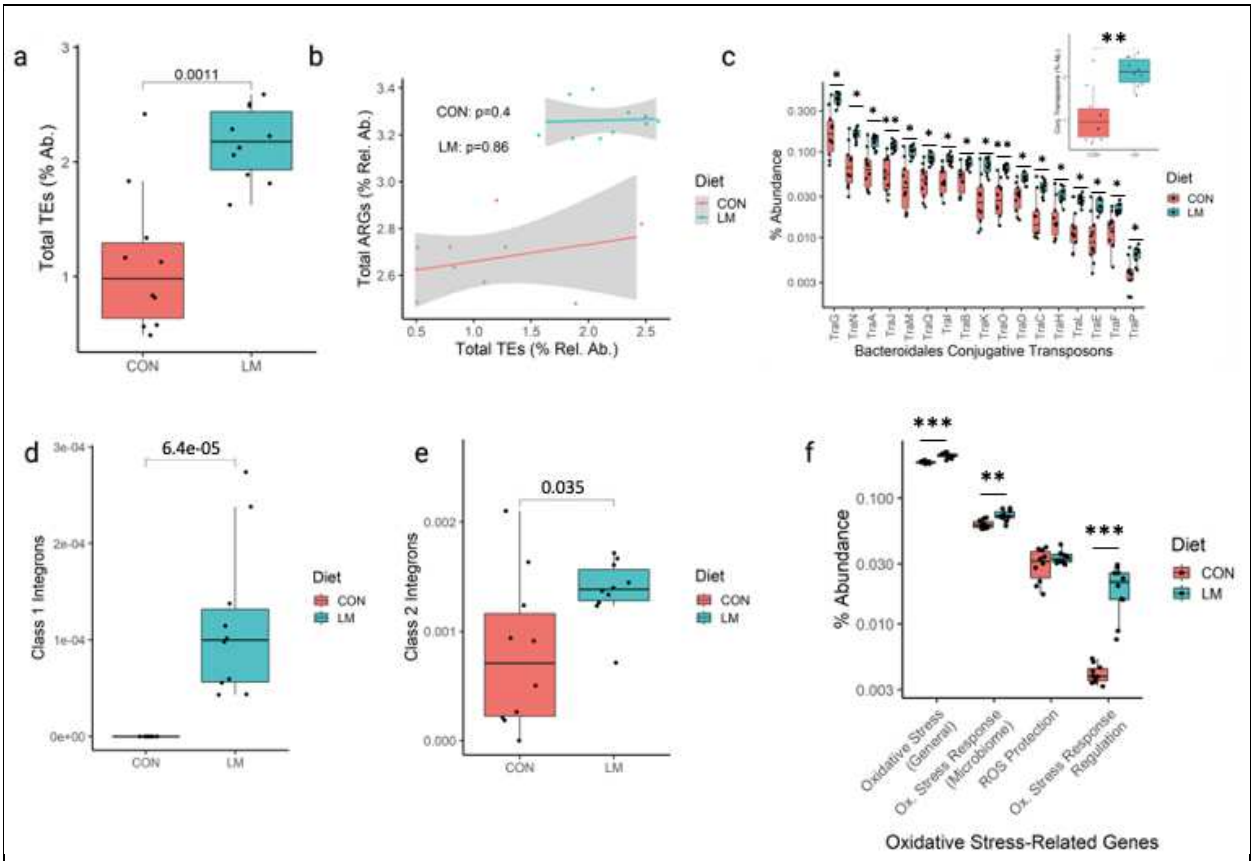


Figure 6: Transposable elements are highly prevalent in mice fed a low micronutrient deficient diet. Transposable elements (TE) (a) were enriched in the LM mice, but no correlation with ARGs (b). Bacteroidales conjugative transposons and integrons genes enriched in LM mice (c-e). SOS response measures were dysregulated in LM mice (f). All values represent the percent abundance of genes at Day 28. Corrected P values: *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion

Micronutrient deficiencies are a major public health crisis and a neglected area of research. Vulnerability to micronutrient deficiency remains throughout the life course. However, nutritional deficiencies that occur during infancy and childhood, or the first 1000 days, are of particular concern.[34] To date, very little work has been done to understand the role of early life multiple micronutrient deficiencies in shaping the gut microbiome and resistome. Animal studies

of single micronutrients deficiency demonstrate an altered microbial composition. Reed *et al*. showed that chicks deficient in zinc have increased abundance of Proteobacteria and decreased abundance of Firmicutes.[35] Hibberd and colleagues showed that vitamin A deficiency significantly altered the fitness, genome, and function of *B. vulgatus* in adult mice.[13] However, there remains a considerable gap in the literature on multiple deficiencies that recapitulate real life scenarios commonly seen in undernourished children.

Metagenomic sequencing and colony forming unit (CFU) count revealed strikingly increased relative and absolute abundance of *Enterobacteriaceae,* respectively. We also reported an increase in several pathobionts (i.e., opportunistic commensal bacteria), *Klebsiella*, *Escherichia, Shigella, Haemophilus, Enterococcus, Campylobacter, Enterobacter, Phocaeicola vulgatus* (i.e., *B. vulgatus*), and *Bacteroides fragilis* as an altered signature in mice postnatally exposed to co-occurring micronutrient deficiencies. These species commonly associate with multiple diseases and are highly prevalent in protein malnourished children.[15,23,25] Highlighting how these opportunistic pathogens gain fitness in the presence of multiple micronutrient deficiencies further underscores the urgent need for research on micronutrient deficiencies and the microbiome. A recent study by Popovic *et al*. using samples collected from a cluster randomized control trial (ClinicalTrials.gov, number NCT00705445) in a Malawian cohort found that multiple micronutrient powder (MNP) without zinc was associated with increased protozoan and fungal communities in the gut microbiome. *Escherichia-Shigella* were also enriched in 12-month old infants who received the (MNP) with and without zinc supplementation.[36] This study highlights the importance of micronutrients in shaping the composition of the gut microbial community, including eukaryotes, in young undernourished children. Data from the original trial showed infants presented with multiple micronutrient deficiencies at the start of the trial, specifically iron, zinc and retinol (i.e., vitamin A) at baseline.[37] *Campylobacter, Shigella* and other enteropathogens were also represented in all study cohorts prior to MNP intervention.[37] Given that our preclinical mouse model showed similar microbial alterations to those observed in these studies (i.e., increased *Campylobacter, Shigella, Escherichia*) our model serves as a promising tool for exploring how micronutrients in early life shape the composition and structure of the gut microbiome. Moreover, our model includes an experimental control group, thus providing a robust means of validating clinical trial results, exploring mechanisms, and designing

targeted therapies. Our study also has potential implications for public health policies and practices surrounding the prophylactic use of antibiotics as treatment in children with uncomplicated malnutrition.

Antibiotic resistance (ABR) continues to be a global public health threat and the gut microbiome is the epicentre of ARGs (i.e., resistome).[30] Indeed, non-pathogenic anaerobic commensal bacteria possess the largest reservoir of ARGs.[18] Although ARGs transfer from commensals to opportunistic pathogens such as *Enterobacteriaceae* appears to be low, their presence still poses a potential risk.[16,18] A recent study by Montassier et al. suggests that probiotics might unintentionally influence ARG expansion following antibiotic treatment by promoting the growth of surviving ARG-carrying species. The authors also noted that some commercial probiotics may also carry ARGs, however, more research is needed to understand whether they contribute to the resistome repertoire.[38] The authors also noted that some commercial probiotics may also carry ARGs, however, more research is needed to understand whether they contribute to the resistome repertoire. For the vast majority of bacteria, however, ARG acquisition occurs between closely related taxa, namely *Bacteriodales,* and resistance can be mediated by integrative and conjugative elements, plasmids, transposons, integrons, and bacteriophages.[16,18,28,39] These mobile genetic elements are highly promiscuous and play a crucial role in the dissemination and acquisition of ARGs.[16,28,31] Nonetheless, although this an active area of research, the gut resistome in undernourished populations remains largely undescribed. Several studies have shown that the reservoir of antibiotic resistance genes can confer clinically-relevant resistance. Li *et al*. found > 400 genes that predicted resistance to more than 30 classes of antibiotics in the resistome of healthy infants. Of this, 167 conferred clinically-relevant resistance to multiple antibiotics.[40] Interestingly, the infants' resistomes included resistance against multiple antibiotics to which the infants had not been previously exposed.[40]

Bacteria mainly gain resistance through several mechanisms: enzymatic degradation or inactivation of the antibiotic, modification or mutation of the drug target site, reduced cell wall permeability, activation of efflux pumps, and acquisition or transfer of antibiotic resistance genes through transduction, transformation and conjugation between bacterial species. Most of these mechanisms are mediated through HGT or transposable elements.[18,28,41–43] Our data revealed

enrichment of genes involved in each of these antibiotic resistance mechanisms despite the lack of antibiotic exposure. An overall higher abundance of ARGs was observed in mice fed the low-micronutrient diet that predicted several classes of antibiotics. We saw an enrichment in genes encoding resistance to tetracycline, methicillin, fosfomycin, aminoglycosides, glycopeptides and vancomycin. Most significantly, we showed that early life exposure to multiple micronutrient deficiencies conferred multi-drug antibiotic resistance within the gut resistome in an antibiotic susceptibility test. This has important clinical implications for undernourished children who commonly present with bacteremia caused by multidrug-resistant bacteria.[44] Our metagenomics analysis accurately predicted all but one of the biologically observed phenotypes, showing its utility as a valuable tool for potential antibiotic resistance screening. Moreover, this is, to our knowledge, the first demonstration of this micronutrient-gut resistome relationship. We also found that genes encoding transposable elements, namely *Bacteroidales* conjugative transposons, a particular group of transposable elements (TE) and class 1 and 2 integron integrases, both largely responsible for dissemination of antibiotic resistance through horizontal gene transfer, were increased.[29] Our findings strongly support a role for micronutrient deficiency in shaping the early life gut resistome which we propose may play a crucial role in early life infection and antibiotic treatment response.

Both nutritional interventions and antibiotic treatment of infections in children in LMICs experience a high failure rate and together may inadvertently induce adverse effects on the microbiome, resistome and host health.[45] In addition, the current routine use of antibiotics in children with uncomplicated malnutrition has been highly contested due to its lack of efficacy, which in some cases is due to its role in propagating resistance.[46,47] Calls for better surveillance and judicious antibiotic use have been issued, yet still, only marginal adjustments to these practices have been made, and prophylactic use remains prevalent.[46,47] Our results suggest that our understanding of the undernourished phenome and how it assembles is incomplete and may affect treatment response. Moreover, we confirm that the ecological niche of the micronutrient deficient gut is indeed primed for greater antibiotic resistance which may further complicate both nutritional and infection-related interventions within LMICS. Consequently, future strategies aimed at addressing undernutrition and antibiotic resistance in children living in LMICs should also prioritize the underlying resistome. Furthermore, our work provides an exciting opportunity

to examine the "nutrition-gut-resistome axis" and to validate our findings in children in a clinical setting.

Additionally, our model complements and confirms findings in children, such as those reported by Osakunor et al.[20] In this study, Osakunor and colleagues found several ARGs present within mildly undernourished Zimbabwean children, the most abundant being *cfxA6, tet(Q), tet(W), sul(2), erm(F)* and *nim(E)* in descending order. Resistance to classes of antibiotics included tetracycline, followed closely by beta-lactam, macrolide, and sulfonamide. Increased abundance of Bacteroidetes, Firmicutes and Proteobacteria in that order was also found within the cohort fecal samples. No correlations between ARGs and sample metadata (ex. antibiotic use, malnutrition, etc.) were found, although more than 50% of the children had been previously exposed to antibiotics. Additionally, only 18.4% of the participants presented with a form of malnutrition (3.7% malnourished and 14.7% stunted); therefore, full characterization of ARGs in the undernourished resistome cannot be ascertained from this cohort. Nevertheless, the study highlights that even within mildly malnourished populations, consideration of the composition of the gut microbiome and relative abundance of ARGs within the resistome may prove useful in selecting appropriate antibiotic therapies for children in LMICs.

Finally, our findings challenge the current paradigm situated around the rules of engagement of antibiotic resistance emergence and propagation and we show a previously unidentified role for co-occurring multiple micronutrient deficiencies in the global conversation on antibiotic resistance. Examination of these conditions concurrently may yield greater strategies to combat both. Indeed, as eloquently stated by Singh and colleagues, "*studying the reservoirs of antibiotic resistance and elucidating the factors affecting the abundance and transmission of AMR genes is the need of the hour"* [16]—to which we agree.

Materials and Methods

Animal experiments

Animal experiments were approved and conducted in accordance with the University of British Columbia's Animal Care Committee (ACC) and Canadian Council on Animal Care (CCAC) ethics guidelines and standard operating procedures (SOPs) for research, training, and housing.

Weanling 3-week-old C57Bl/6N male mice were purchased from Charles Rivers (Kingston, NY, USA). Upon arrival (Day 0), pups were randomized equally by weight and housed 5 per cage in a 12-hour light/dark cycle barrier animal vivarium. At the end of the experiment (Day 28), mice were sacrificed, and appropriate samples were harvested for downstream analysis.

Diet and experimental timeline

One group of mice (n=10) received an experimental control diet (CON) and the other a low-micronutrient (LM) treatment diet deficient in vitamin A, B12, B9, zinc, and iron. Diets were isocaloric, comprising similar macronutrients (protein 20%, carbohydrates 65%, fat 15%) ([D18062501I and D19041709I, respectively], Research Diets Inc., New Brunswick, NJ, USA). All diets were irradiated prior to shipment and stored at -20^{o}C upon arrival. Chow was allowed to reach room temperature before use, and mice were given ad libitum access throughout the experiment. All mice remained on their respective diets for 28 days +/- 2 (i.e., 4 weeks).

Fecal bacterial DNA extraction and library preparation

Mice are coprophagic; therefore, we developed a cage swapping method (e.g., CON mice from cage 1 were swapped with CON mice in cage 2 and vice versa) was used to prevent cage effects on the gut microbiome. At the end of the experiment, a total of 40 samples were sent to Microbiome Insights (Vancouver, BC) for DNA extraction and metagenomic sequencing. Paired fecal samples were taken at two timepoints, Day 0 and Day 28, for a total of 40 samples or data points. Upon collection, pellets were immediately placed on dry ice and transferred to -80°C freezer until further processing. Extraction, processing, and analysis were performed by Microbiome Insights using the following procedures. DNA was extracted using the Qiagen MagAttract PowerSoil DNA KF kit (Formerly MOBio PowerSoil DNA Kit, Qiagen, USA) using a KingFisher robot (Thermo-Fischer Scientific, City Waltham, MA, USA). DNA quality was evaluated visually via gel electrophoresis and quantified using a Qubit 3.0 fluorometer (Thermo-

Fischer, Waltham, MA, USA). Libraries were prepared using an Illumina Nextera library preparation kit (Illumina, San Diego, CA, USA) with an in-house protocol (Microbiome Insights, Vancouver, BC, Canada).

Whole genome shotgun metagenomics sequencing and functional analysis

Paired-end sequencing (150 bp x 2) was done on a NextSeq 500 in medium-output mode yielding 5-6.5 million reads and 0.8-0.9 Gbases per sample. Shotgun metagenomic sequence reads were processed with the Sunbeam pipeline. Initial quality evaluation was done using FastQC v0.11.5.[48] Processing took part in four steps: adapter removal, read trimming, low-complexity-reads removal, and host-sequence removals. Adapter removal was done using cutadapt v2.6.[49] Trimming was done with Trimmomatic v0.36[50] using custom parameters (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36). Low-complexity sequences were detected with Komplexity v0.3.6 .[51]

High-quality reads were next mapped to the human genome (Genome Reference Consortium Human Reference 37), and those that mapped to it were with at least 50% similarity across 60% of the read length were removed. The remaining reads were taxonomically classified using Kraken2 (https://ccb.jhu.edu/software/kraken2/) with the PlusPF database from 2021-05-17 using the protocol by Wood *et al*.[52] For functional profiling, high-quality (filtered) reads were aligned against the SEED database via translated homology search and annotated to Subsystems, or functional levels, 1-3 using Super-Focus. We used the SUbsytems Profile by databasE Reduction using SUPER-FOCUS tool for functional analysis.[53]

Culture-based quantification of Enterobacteriaceae

Fresh fecal pellets were obtained on days 0 and 28 and immediately placed in sterile 1ml $PBS^{-/-}$ on ice to quantify Enterobacteriaceae. Pellets were homogenized for 5 minutes at 25 frequency/sec (Mixer Mill 400, Retsch, Clifton, NJ, USA). The slurry was then plated on MacConkey agar (B212123, BD Biosciences, Sparks, MD, USA) in dilutions and cultured overnight in a 37ºC incubator. Colony-forming units (CFU) per gram of feces were calculated.

Bioinformatics (General):

All bioinformatic analyses were performed in R (version 4.1.0), and general microbiome data wrangling was performed using Phyloseq v1.36.0. All binary comparisons were calculated using Wilcoxon rank-sum or signed-rank tests unless otherwise indicated, and p values were corrected using the Benjamini-Hochberg method (FDR) where applicable. (Corrected) p values were considered significant when below 0.05.

Bioinformatics (Taxonomy):

Taxonomy barplots were made by first merging OTUs to the taxonomy level of interest using *tax_glom()*, and then sampled were merged by Day/Diet groupings using *merge_samples()*. After merging, all taxa with an average abundance of <1% were combined with unknown reads to improve plot readability. Values were then plotted as relative abundances, where each stacked bar sums to 1.

Indicator species analysis was performed using the indicspecies R package, v1.7.9. Taxa were aggregated to the species level as described, and all species with a mean relative abundance above 0.001% were retained. All Day 0 samples were pooled, and group-specific indicator taxa were then identified via the *multipatt()* function using 999 permutations and the default *IndVal.g* method. Significant indicators were defined as stat>0.7 and $p<0.05$, where higher stat values indicate a stronger association with a given subset of the input groups across all permutations. Indicators representing multiple groups were discarded. Mean abundances of each indicator species were then calculated per group and plotted using *geom_point()*, where the size of the point represented the mean abundance and colours represented the associated group. Day 0 groups were separated when plotting to demonstrate their similarity at baseline.

Pathobionts determined *a priori* based on their common association with malnourished children were characterized at the genus and species level. Mean taxon-specific abundances were calculated per Day 28 group and plotted as a stacked bar plot. Total and individual pathobiont abundances per sample were also plotted for both timepoints and Day 28, respectively.

Bioinformatics (Function):

Functional information was annotated from metagenomic sequencing data as described above, resulting in four levels of hierarchical information where all values are in percent abundance (/100%). For any functional tests involving higher-level functional annotations, the abundances of all subannotations were summed. For total ARG abundance, annotations under 'Resistance to antibiotics and toxic compounds' (Subsystem Level 2) were summed. In order to visualize the number and overlap of individual ARGs across all groups, a Venn diagram was prepared using the R package VennDiagram v1.7.3. ARGs were considered to be present in a given group if they were detected in at least one sample.

Individual ARGs were manually categorized by compound, and compounds categorized by type. Abundances of each compound and type were compared between Day 28 CON and LM groups using *kruskal.test()*, and p values were adjusted using the Benjamini-Hochberg method (FDR). Significant results (FDR<0.05) were plotted on a log10 scale after the addition of a pseudocount of 0.001%. Individual conjugative transposons (Tra_ proteins) were tested in an identical manner; however, no pseudocount was added before plotting as all values were non-zero.

All continuous correlations between two variables were assessed using the *glm()* linear regression command, and were univariate unless otherwise noted. P values were corrected per timepoint/dietary group (as applicable) whenever multiple tests of the same nature were performed (ex. the correlation of total ARGs with individual phyla). For multiple testing involving individual pathobionts and/or ARGs, only elements that had been identified as differentially abundant between Day 28 CON and LM groups were assessed.

Correlations between total ARGs and individual phyla only included phyla with a mean abundance of >1%: Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia. In order to assess whether phyla associated with ARGs in a manner independent of diet, dietary groups were pooled at each timepoint and diet was included as a covariable in the linear regressions.

Culturing fecal microbiome

Flash frozen fecal samples were placed in 1ml of sterile $PBS^{-/-}$ and homogenized by pipetting. A sterile loop was used to streak bacteria unto a fastidious anaerobic agar plate (FAA) which were

incubated for 18-24 hours in an anaerobic canister at 37 °C. Bacteria were then stored in aliquots in fastidious anaerobic broth (FAB) supplemented with 10% glycerol at -80 degrees Celsius.

Antibiotic susceptibility testing

Antibiotics were prepared by mixing 10 mg of antibiotics in 1 ml sterile $PBS^{-/-}$ (Gibco, ThermoFisher, Canada) in 2 ml Eppendorf tubes. Plates were labeled by group (i.e., control and low-micronutrient) and three antibiotic per plate for a total of nine. One-hundred microliters of bacteria from glycerol stock was plated and lawned on Mueller-Hinton agar (Mueller-Hinton Broth, and Agar, BD BioSciences, Mississauga, ON, Canada). Blank sterile antibiotic discs were carefully positioned onto the plates by hand using sterile high precision tweezers. Exactly 10 µl of each antibiotic was pipetted in the center of the individual discs and allowed to sit for 5-10 minutes to ensure disc was firmly in place. Plates were then incubated in an anaerobic gas canister for 18 to 24 hours at 37 °C. Antibiotic zones were measured using standard ruler and comparison of resistance determined by bacterial growth between the control and the low-micronutrient. Images were obtained using a Bio-Rad ChemiDoc Universal Hood III Imager (Hercules, CA, USA).

Antibiotics used in the susceptibility assay: vancomycin hydrochloride (CAT # V-200-25, Gold Biotechnology, St. Louis, MO, USA), gentamicin (CAT # G-400-5, Gold Biotechnology, St. Louis, MO, USA), erythromycin (#16486, Cayman Chemicals, Ann Arbor, MI, USA), tetracycline hydrochloride (Product # T7660 Sigma-Aldrich Canada, Oakville, ON), amoxicillin (Product # A8523, Sigma-Aldrich Canada, Oakville, ON), ciprofloxacin (Lot #17850, Sigma-Aldrich Canada, Oakville, ON), ofloxacin (Product #08757, Sigma-Aldrich Canada, Oakville, ON), levofloxacin (Product # 28266, Sigma-Aldrich Canada, Oakville, ON) and methicillin sodium salt (LOT Sigma-Aldrich Canada, Oakville, ON).

ACKNOWLEDGEMENTS

This work was supported by research grants from the Canadian Institutes of Health Research (CIHR) (B.B.F.) [FDN-159935]. B.B.F. is a University of British Columbia Peter Wall Distinguished Professor. We are grateful to Dr. Kelsey Jones for his contribution to conceptualization of this model, Tahereh Bozorgmehr for her assistance with animal experiments

and Dr. Wanyin Deng for his insights into this manuscript and to the Finlay Lab overall for their support and feedback. We are grateful for the insightful input and feedback from Dr. Seth Bloom, MD, PhD on the metagenomics data and antibiotic susceptibility testing. We would also like to thank Dr. Ariangela Kozik, Dr. Edward Cunningham-Oakes and Dr. Ruiari Robertson for their critical feedback on the data. Finlay lab members Sarah Woodward and Mihai Cirstea thank you for the resources and Manjeet Bains (Robert Hancock Lab, University of British Columbia) for the fluroquinolone and methicillin antibiotics.

Author Contributions

P.T.L. conceptualized, designed the study, draft figures and interpreted data. A.M.R. performed all bioinformatics analysis, produced figures, interpreted data edited manuscript. H.B.Y performed microbial culturing. R.H. assisted with experiments, paper structure and plate imaging. Y.M.F. assisted with antibiotic susceptibility testing procedures. P.T.L. prepared the draft manuscript and all authors were involved in review and editing. P.T.L., A.MR. and B.B.F. approved of the final version of the manuscript.

Competing interests

The authors declare no competing interests.

References:

1. Han X, Ding S, Lu J, Li Y. Global, regional, and national burdens of common micronutrient deficiencies from 1990 to 2019: A secondary trend analysis based on the Global Burden of Disease 2019 study. *eClinicalMedicine*. 2022;44:101299. doi:10.1016/j.eclinm.2022.101299

2. Research Institute (IFPRI) IFP. Global Nutrition Report 2016 From Promise to Impact Ending Malnutrition by 2030 Summary. Published online 2016. doi:10.2499/9780896299948

3. GNP. Global Nutrition Report 2017: Nourishing the SDGs. *Global Nutrition Report 2017*. Published online 2017:115.

4. Bailey RL, West KP, Black RE. The epidemiology of global micronutrient deficiencies. *Annals of Nutrition and Metabolism*. Published online 2015. doi:10.1159/000371618

5. *UNICEF (2019). The State of the World's Children 2019. Children, Food and Nutrition: Growing Well in a Changing World.* UNICEF; 2019.

6. Ahmed T, Hossain M, Sanin KI. Global Burden of Maternal and Child Undernutrition and Micronutrient Deficiencies. *Ann Nutr Metab*. 2012;61(s1):8-17. doi:10.1159/000345165

7. Children: improving survival and well-being. Accessed November 6, 2021. https://www.who.int/news-room/fact-sheets/detail/children-reducing-mortality

8. Black R. Micronutrient deficiency--an underlying cause of morbidity and mortality. *Bull World Health Organ*. 2003;81(2):79. Accessed January 13, 2022. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2572405/

9. Brown EM, Wlodarska M, Willing BP, et al. Diet and specific microbial exposure trigger features of environmental enteropathy in a novel murine model. *Nature Communications*. Published online 2015. doi:10.1038/ncomms8806

10. Reinert P. Infections in malnourished infants and children. *Developpement et sante : revue de perfectionnement medical et sanitaire en pays tropical*. Published online 1993.

11. Subramanian S, Huq S, Yatsunenko T, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature*. Published online 2014. doi:10.1038/nature13421

12. Blanton L V., Charbonneau MR, Salih T, et al. Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science*. Published online 2016. doi:10.1126/science.aad3311

13. Hibberd MC, Wu M, Rodionov DA, et al. The effects of micronutrient deficiencies on bacterial species from the human gut microbiota. 2017;4069(May). doi:10.1126/scitranslmed.aal4069.The

14. Robertson RC, Manges AR, Finlay BB, Prendergast AJ. The Human Microbiome and Child Growth – First 1000 Days and Beyond. *Trends in Microbiology*. Published online 2019. doi:10.1016/j.tim.2018.09.008

15. Vonaesch P, Morien E, Andrianonimiadana L, et al. Stunted childhood growth is associated with decompartmentalization of the gastrointestinal tract and overgrowth of oropharyngeal taxa. *Proc Natl Acad Sci U S A*. 2018;115(36):E8489-E8498. doi:10.1073/pnas.1806573115

16. Singh S, Verma N, Taneja N. The human gut resistome: Current concepts & future prospects. *Indian J Med Res*. 2019;150(4):345-358. doi:10.4103/ijmr.IJMR_1979_17

17. von Wintersdorff CJH, Wolffs PFG, Savelkoul PHM, et al. The gut resistome is highly dynamic during the first months of life. *Future Microbiol*. 2016;11(4):501-510. doi:10.2217/fmb.15.154

18. van Schaik W. The human gut resistome. *Philos Trans R Soc Lond B Biol Sci*. 2015;370(1670):20140087. doi:10.1098/rstb.2014.0087

19. Murray CJ, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*. 2022;399(10325):629-655. doi:10.1016/S0140-6736(21)02724-0

20. Osakunor DNM, Munk P, Mduluza T, et al. The gut microbiome but not the resistome is associated with urogenital schistosomiasis in preschool-aged children. *Commun Biol*. 2020;3(1):1-11. doi:10.1038/s42003-020-0859-7

21. Oldenburg CE, Hinterwirth A, Ourohiré M, et al. Gut Resistome after Antibiotics among Children with Uncomplicated Severe Acute Malnutrition: A Randomized Controlled Trial. *Am J Trop Med Hyg*. 2022;107(1):59-64. doi:10.4269/ajtmh.22-0007

22. Forgie AJ, Drall KM, Bourque SL, Field CJ, Kozyrskyj AL, Willing BP. The impact of maternal and early life malnutrition on health: a diet-microbe perspective. *BMC Med*. 2020;18(1):135. doi:10.1186/s12916-020-01584-z

23. Huus KE, Rodriguez-Pozo A, Kapel N, et al. Immunoglobulin recognition of fecal bacteria in stunted and non-stunted children: findings from the Afribiota study. *Microbiome*. 2020;8(1):113. doi:10.1186/s40168-020-00890-1

24. Wagner VE, Dey N, Guruge J, et al. Effects of a gut pathobiont in a gnotobiotic mouse model of childhood undernutrition. *Sci Transl Med*. 2016;8(366):366ra164. doi:10.1126/scitranslmed.aah4669

25. Robertson RC. The Gut Microbiome in Child Malnutrition. *Nestle Nutr Inst Workshop Ser*. 2020;93:133-144. doi:10.1159/000503352

26. Shoemaker NB, Vlamakis H, Hayes K, Salyers AA. Evidence for Extensive Resistance Gene Transfer among Bacteroides spp. and among Bacteroides and Other Genera in the Human Colon. *Appl Environ Microbiol*. 2001;67(2):561-568. doi:10.1128/AEM.67.2.561-568.2001

27. Quesada-Gómez C. Bacteroides mobilizable and conjugative genetic elements: antibiotic resistance among clinical isolates. *Rev Esp Quimioter*. 2011;24(4):184-190.

28. Sultan I, Rahman S, Jan AT, Siddiqui MT, Mondal AH, Haq QMR. Antibiotics, Resistome and Resistance Mechanisms: A Bacterial Perspective. *Frontiers in Microbiology*. 2018;9. Accessed March 31, 2022. https://www.frontiersin.org/article/10.3389/fmicb.2018.02066

29. Whittle G, Shoemaker NB, Salyers AA. The role of Bacteroides conjugative transposons in the dissemination of antibiotic resistance genes. *Cell Mol Life Sci*. 2002;59(12):2044-2054. doi:10.1007/s000180200004

30. Deng Y, Bao X, Ji L, et al. Resistance integrons: class 1, 2 and 3 integrons. *Ann Clin Microbiol Antimicrob*. 2015;14:45. doi:10.1186/s12941-015-0100-6

31. Beaber JW, Hochhut B, Waldor MK. SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature*. 2004;427(6969):72-74. doi:10.1038/nature02241

32. Dam S, Pagès JM, Masi M. Stress responses, outer membrane permeability control and antimicrobial resistance in Enterobacteriaceae. *Microbiology (Reading)*. 2018;164(3):260-267. doi:10.1099/mic.0.000613

33. Zhao X, Drlica K. Reactive oxygen species and the bacterial response to lethal stress. *Curr Opin Microbiol*. 2014;0:1-6. doi:10.1016/j.mib.2014.06.008

34. Agosti M, Tandoi F, Morlacchi L, Bossi A. Nutritional and metabolic programming during the first thousand days of life. *Pediatr Med Chir*. 2017;39(2):157. doi:10.4081/pmc.2017.157

35. Reed S, Neuman H, Moscovich S, Glahn R, Koren O, Tako E. Chronic Zinc Deficiency Alters Chick Gut Microbiota Composition and Function. *Nutrients*. 2015;7(12):9768-9784. doi:10.3390/nu7125497

36. Popovic A, Bourdon C, Wang PW, et al. Micronutrient supplements can promote disruptive protozoan and fungal communities in the developing infant gut. *Nat Commun*. 2021;12:6729. doi:10.1038/s41467-021-27010-3

37. Soofi S, Cousens S, Iqbal SP, et al. Effect of provision of daily zinc and iron with several micronutrients on growth and morbidity among young children in Pakistan: a cluster-randomised trial. *Lancet*. 2013;382(9886):29-40. doi:10.1016/S0140-6736(13)60437-7

38. Montassier E, Valdés-Mas R, Batard E, et al. Probiotics impact the antibiotic resistance gene reservoir along the human GI tract in a person-specific and antibiotic-dependent manner. *Nat Microbiol*. 2021;6(8):1043-1054. doi:10.1038/s41564-021-00920-0

39. Frost LS, Leplae R, Summers AO, Toussaint A. Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol*. 2005;3(9):722-732. doi:10.1038/nrmicro1235

40. Li X, Stokholm J, Brejnrod A, et al. The infant gut resistome associates with E. coli, environmental exposures, gut microbiome maturity, and asthma-associated bacterial composition. *Cell Host & Microbe*. 2021;29(6):975-987.e4. doi:10.1016/j.chom.2021.03.017

41. Kashef N, Hamblin MR. Can microbial cells develop resistance to oxidative stress in antimicrobial photodynamic inactivation? *Drug Resist Updat*. 2017;31:31-42. doi:10.1016/j.drup.2017.07.003

42. Sun D, Jeannot K, Xiao Y, Knapp CW. Editorial: Horizontal Gene Transfer Mediated Bacterial Antibiotic Resistance. *Front Microbiol*. 2019;10:1933. doi:10.3389/fmicb.2019.01933

43. Reygaert WC. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol*. 2018;4(3):482-501. doi:10.3934/microbiol.2018.3.482

44. Ahmed M, Mirambo MM, Mushi MF, Hokororo A, Mshana SE. Bacteremia caused by multidrug-resistant bacteria among hospitalized malnourished children in Mwanza, Tanzania: a cross sectional study. *BMC Res Notes*. 2017;10(1):62. doi:10.1186/s13104-017-2389-z

45. ReActGroup.ORG. Antibiotic use, resistance and the link to nutrition. February 2018. ReAct. Accessed July 11, 2022. https://www.reactgroup.org/toolbox/

46. Alcoba G, Kerac M, Breysse S, et al. Do Children with Uncomplicated Severe Acute Malnutrition Need Antibiotics? A Systematic Review and Meta-Analysis. *PLOS ONE*. 2013;8(1):e53184. doi:10.1371/journal.pone.0053184

47. Isanaka S, Langendorf C, Berthé F, et al. Routine Amoxicillin for Uncomplicated Severe Acute Malnutrition in Children. *N Engl J Med*. 2016;374(5):444-453. doi:10.1056/NEJMoa1507024

48. Bioinformatics Group at the Babraham Institute. Bioinformatics Group at the Babraham Institute. Software available at:https://www.bioinformatics.babraham.ac.uk/. Accessed March 22, 2022. https://www.bioinformatics.babraham.ac.uk/

49. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*. 2011;17(1):10-12. doi:10.14806/ej.17.1.200

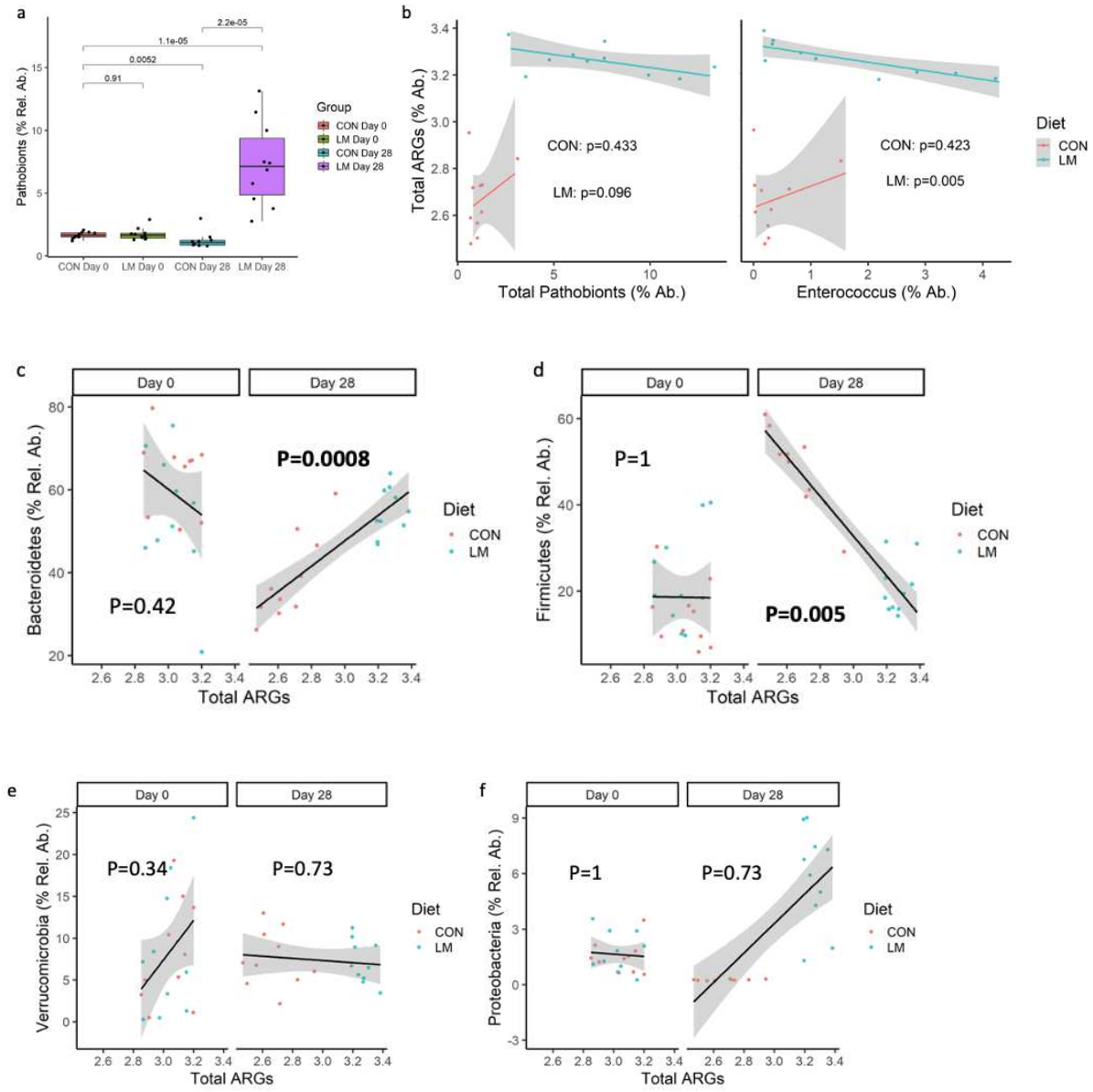
50. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30(15):2114-2120. doi:10.1093/bioinformatics/btu170

51. Clarke EL, Taylor LJ, Zhao C, et al. Sunbeam: an extensible pipeline for analyzing metagenomic sequencing experiments. *Microbiome*. 2019;7(1):46. doi:10.1186/s40168-019-0658-x

52. Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. *Genome Biol*. 2019;20(1):257. doi:10.1186/s13059-019-1891-0

53. Silva GGZ, Green KT, Dutilh BE, Edwards RA. SUPER-FOCUS: a tool for agile functional analysis of shotgun metagenomic data. *Bioinformatics*. 2016;32(3):354-361. doi:10.1093/bioinformatics/btv584

Supplemental Figures



Supplemental Figure 1: (a) relative abundance of pathobionts from Day 0 to Day 28 (b) correlation of total ARG with total pathobionts and Enterococcus.