Biogeography of the relationship between the child gut microbiome and innate immune system

Nelly Amenyogbea,b+, Pedro Dimitriuc+, Kinga Smolena, Eric M. Brownd, Casey P. Shannone, Scott J. Tebbutte,f, Phillip J Cooperg,h, Arnaud Marchanti, Tessa Goetghebuerj, Monika Esserk, Brett B. Finlayd, Tobias R. Kollmannl,b++, William W. Mohnc++#

aDepartment of Experimental Medicine, University of British Columbia, Vancouver, Canada

bTelethon Kids Institute, Perth, Australia

cDepartment of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, Canada

dMichael Smith Laboratories, University of British Columbia, Vancouver, Canada

ePROOF Centre or Excellence, St. Pauls’s Hospital, University of British Columbia, Vancouver, Canada

fCentre for Lung Innovation, Department of Medicine, Division of Respiratory Medicine, University of British Columbia, Vancouver, Canada

gFacultad de Ciencias Medicas, de la Salud y la Vida, Universidad Internacional del Ecuador, Quito, Ecuador

hInstitute of Infection and Immunity, St. George’s University of London, London, UK

iInstitut d’Immunologie Médicale, Université libre de Bruxelles, Charleroi, Belgium

jDépartement de Pédiatrie, Centre Hospitalier Universitaire St Pierre, Université libre de Bruxelles, Belgium

kthe Immunology Unit, Division of Medical Microbiology, Department of Pathology, NHLS and Stellenbosch University, Matieland

lDepartment of Pediatrics, Division of Infectious Diseases, University of British Columbia, Vancouver, Canada

Running Head: Child gut microbiomes correlate to systemic immunity

# Address correspondence to William W. Mohn, [wmohn@mail.ubc.ca](mailto:wmohn@mail.ubc.ca)

+Nelly Amenyogbe and Pedro Dimitriu contributed equally to this work.

++Tobias R. Kollmann and William W. Mohn contributed equally to this work.

Author order was determined on the basis of contribution.

**Abstract**

The gut microbiome is a well-recognized modulator of host immunity, and its composition differs between geographically separated human populations. Systemic innate immune responses to microbial derivatives also differ between geographically distinct human populations. However, the potential role of the microbiome in mediating geographically-varying immune responses is unexplored. We here applied 16S amplicon sequencing to profile the stool microbiome and in parallel measured whole blood innate immune cytokine responses to several Pattern Recognition Receptors (PRRs) agonists among 2-year-old children across biogeographically diverse settings. Microbiomes differed mainly between high- and low-resource environments and were not strongly associated with other demographic factors. We found strong correlations between responses to Toll-like receptor (TLR) 2 and relative abundances of *Bacteroides* and *Prevotella* populations, sharedamong Canadian and Ecuadorean children together. Additional correlations between responses to TLR2 and bacterial populations were specific to individual geographic cohorts. As a proof of concept, we gavaged germ-free mice with human donor stools and found murine splenocyte responses to TLR stimulation were consistent with responses of the corresponding human donor populations. This study identified differences in immune responses correlating to gut microbiomes across biogeographically diverse settings and evaluated biological plausibility using a mouse model. This insight paves the way to guide optimization of population-specific interventions aimed to improve child health outcomes.

**Importance**

Both the gut microbiome, and innate immunity, are known to differ across biogeographically diverse human populations. The gut microbiome has been shown to directly influence systemic immunity in animal models. With this, modulating the gut microbiome represents an attractive avenue to improve child health outcomes associated with altered immunity using population-specific approaches. However, there are very scare data available to determine which members of the gut microbiome are associated with specific immune responses, and how this differs around the world, creating a substantial barrier to rationally designing such interventions. This study addressed this knowledge gap by identifying relationships between distinct bacterial taxa and cytokine responses to specific microbial agonists across highly diverse settings. Further, we provide evidence that immunomodulatory effects of region-specific stool microbiomes can be partially recapitulated in germ-free mice. This is an important contribution towards improving global child health by targeting the gut microbiome.

**Introduction**

The gut microbiome is a well-recognized modulator of host systemic immunity throughout life1. Its composition differs between geographically separated human populations2-4. Systemic innate immune responses to microbes are largely driven by stimulation of pattern recognition receptors (PRR), i.e. microbially-derived agonists, triggering production of a range of cytokines. These immune responses have also been shown to differ between geographically distinct populations5, 6. However, the specific role of the microbiome composition and function in mediating these differing immune responses across geographical regions remains unknown. Given the ability of the host microbiome to modulate systemic innate immunity, and the known geographical differences between both gut microbiomes and innate immune phenotypes, what is missing is a mechanistic understanding of how the distinct microbiomes likely contribute to immune differences. Robust correlations from human studies are therefore needed to inform mechanistic work using animal models.

Previous studies have found associations between systemic immunity and host microbiome within single cohorts, finding that relative abundance of microbial taxa or their genes could be correlated with select cytokine responses to TLR stimulation7, 8. These studies relied on univariate statistics of relative abundance data to find a small subset of microbiome-immune correlations. Components of human microbiomes have also been shown to modulate immune phenotypes *in vitro9*. However, univariate statistical methods may often lead to spurious results as the independence assumption between predictor variables is not met. Further, by considering only one-to-one associations, univariate approaches test each OTU individually and disregard interactions or correlations among OTUs, providing a limited insight to the system10.

Direct comparisons of innate immune responses across different locations is hampered by the need to standardize and control all aspects of immune assessment to avoid technical artefacts. We previously applied a rigorously standardized approach to quantify cytokine responses to a panel of TLR agonists among 2-year-old children recruited in Brussels, (Belgium), Cape Town (South Africa), Quininde (Ecuador), and Vancouver (Canada)6. These four biogeographically distinct settings differ in many ways that can potentially modulate both systemic immunity and the gut microbiome - resource availability, ancestry of the human populations, diet, climate, vaccination schedules, and cultural practices, being examples. Hence, these sites were chosen to test the hypothesis that systemic innate immunity differs among diverse child populations. Recently, we found that differences in gut microbiome and immune phenotype between HIV-exposed and healthy HIV-unexposed children were specific to each cohort.11. Here, we extended this work by integrating the stool microbiomes of healthy children measured via sequencing of the 16S rRNA genes (V6 region) to innate immune responsiveness measured around the time of stool sample collection. This further allowed us to test the hypothesis that regionally distinct gut microbiomes are associated with differential development of systemic immunity. To do so, we employed a sparse Partial Least Squares (sPLS) integrative approach12 to extract correlations between microbiome and immune phenotype.. We also assessed causality, whether a specific microbiome can drive development of particular immune phenotype, via human fecal transplantation into germ-free mice. Taken together, this study provided evidence that differences in systemic innate immunity across biogeographically diverse populations correlate with differences in the gut microbiome.

**Methods**

**Ethics Statement**

All research involving humans was done according to principles in the Declaration of Helsinki and approved by the University of British Columbia Ethics Board under protocol number H11-01423. Each study site obtained ethical approval separately with their research institutions. Informed consent was obtained from primary guardians for children involved in this study. Research involving animals was conducted under ethical approval from the University of British Columbia Animal Care Committee under protocol number A13-0265.

**Recruitment of Study Participants**

The recruitment of the four cohorts of children of approximately two years of age was previously described6. Study participants were recruited from ongoing collaborative studies or healthy child cohorts at each of the four sites; Canadian children were recruited at the BC Children’s Hospital in Vancouver13, Belgian children were enrolled in a birth cohort enrolling St Pierre Hospital in Brussels, and included mostly healthy male children presenting for a routine circumcision. Ecuadorean children were enrolled in the ECUAVIDA birth cohort in Quininde14, and South African children were enrolled in a prospective birth cohort at the Tygerberg Academic Hospital in Cape Town15, 16. Participants were only included in the study if the child was considered healthy based on medical history, and they were excluded if they met one or more of the following criteria: significant chronic medical condition, immune deficiency, immunosuppression by disease or medication, cancer, bone marrow or organ transplantation, receipt of blood products within 3 months, bleeding disorder, major congenital malformation, genetic disorder, or born to HIV-positive mothers.

**Innate Immune Phenotyping**

Innate immune phenotyping for these cohorts was previously described and published6. Briefly, 3-5 ml peripheral blood was drawn per participant. Whole blood was then stimulated with the following PRR agonists: PAM3CYSK4 (PAM, stimulates TLR2), polyinosinic-polycytidylic acid (Poly I:C, stimulates TLR3), Lipopolysaccharide (LPS, stimulates TLR4), resiquimod (R848, stimulates TLR7:8), Peptidoglycan (PGN, stimulates both TLR2 and nucleotide-binding oligomerization domain-containing protein 1/2 [NOD1/2]), and media alone. Whole blood was stimulated for 24 hours, and supernatants analyzed for the following cytokines measured using the Luminex multiplex assay (Luminex, Upstate/Millipore “Flex Kit” system): IFN-α2, IFN-γ, CXCL10, IL-12p70, IL-12p40, IL-6, TNF-α, IL-1β, CXCL8, CCL3, CCL4, and IL-10.

**Child fecal microbiome analysis**

Human stool microbiome composition was determined using amplicon sequencing targeting the V6 region of the 16S rRNA gene. Stool samples were collected within the same month as blood samples for each child, stored at -80oC, and transported to the Vancouver laboratory on dry ice. Total DNA was extracted from all samples within one month of arrival to the laboratory using the Qiagen QIAamp DNA stool mini kit (Qiagen Cat. No. 51504). PCR and DNA sequencing closely followed previously described protocols and rationale for amplicon sequencing targeting the V6 region of the 16S rRNA gene17. Further details on microbiome analysis can be found in the supplement.

**Germ-free mouse model of human fecal transplantation.** To test whether divergent immune phenotypes of South African children could be causally linked to their gut microbiomes, we performed a proof of principle experiment whereby male germ-free Swiss-Webster mice were gavaged with stools from either Canadian or South African male children. Stool samples from only male children were selected to match the sex of experimental animals, of which only males were available. Three weeks after gavage, we compared the splenocyte cytokine responses to TLR stimulation and assessed their gut barrier integrity, using the lactulose:mannitol test. Mouse fecal and intestinal microbiomes at the end of the experiment were measured via 16S amplicon sequencing. DNA from the donor human stools was re-extracted, PCR amplified, and sequenced on the same sequencing run as the mouse samples. The experimental design is shown in Figure 5A, and further experimental details are found in the supplement.

**Statistical Analyses**

**Microbiome analysis.** Briefly, we assessed differences in gut microbiomes of children across study sites using measures of alpha diversity (observed richness and Shannon index) and beta diversity. Differences in microbiome community composition were further explored by identifying discriminatory OTUs among cohorts using univariate (DeSeq218) and multivariate (sparse Partial Least Squares Discriminant Analysis, sPLS-DA19) approaches. We also conducted exploratory analyses to determine whether host factors captured in our study (sex, delivery mode, anthropometric measurements, gestational age, birthweight, and maternal age) were associated with differences in either alpha or beta diversity of the gut microbiome. Further details can be found in the supplement.

**Cytokine response signatures among cohorts.** We used sPLS-DA to identify cytokine signatures that distinguish Belgian, Canadian, and Ecuadorean children in a multivariate space and calculated classification accuracy for each site.

**sPLS Integration of cytokine, microbiome, and demographic data** To uncover potential gut microbiome–host immune interactions, we examined the joint multivariate structure of gut microbiota composition and host innate immune responses via sPLS analysis, a method that incorporates variable selection, making it particularly suitable to high-dimensional data-sets19. This analysis was performed to identify both interactions that were robust among all children and interactions specific to individual cohorts. To this end, separate analyses were done for all cohorts combined and for each cohort separately. Further details can be found in the supplement.

**Data Availability.**

All sequencing data presented in this manuscript has been deposited at the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA). Sequencing data from child stool samples is accessible via the following URLs: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA660015> for human stool microbiome and <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA662365> for germ-free mouse microbiome datasets.

Data presented in this manuscript and accompanying scripts are publicly accessible via the following URL: <https://github.com/nelly-amenyogbe/Global_cohort_microbiome_immun>

**Results**

**Cohort characteristics**

Study participants were recruited between May 2011 and January 2012. Cohort characteristics for the children included in the immune analysis were previously described6. Stool samples for microbiome analysis were collected from a subset of these children, including 17 Belgian, 32 Canadian, 42 Ecuadorean, and 8 South African children. Blood samples were collected within 10 days of stool samples (Supl. Figure 1). Baseline characteristics differed between the two sites (Table 1). Notably among the Belgians, only one of 17 subjects was female, while sex was more balanced in the other regional cohorts. Belgians were almost exclusively, and South Africans were exclusively, vaginally delivered, whereas Caesarean delivery was more common in the Canadian and Ecuadorean cohorts. Other differences included anthropometric measurements and the younger average age of recruited Canadians (mean 1.65 years). Ethnic heterogeneity also varied (Table 2). All children were born and raised at the sites of sample collection, except for a subset of the Belgian children (7%) who were born in African countries, or in one case, Germany. Immune data for these children born outside of Belgium were not available.

**Child stool microbiome was strongly impacted by country of birth**

The 16S amplicon libraries yielded a total of 4030 OTUs after quality filtering and binning at a 97% similarity threshold. Sequencing depth did not differ significantly among cohorts (data not shown).

**Alpha Diversity.** We first sought to understand whether there was a difference in alpha diversity between the four cohorts by calculating the observed richness or the Shannon index to estimate diversity. The Canadian children harbored significantly fewer species compared to Ecuadorean children (Figure 1A). Shannon diversity of fecal microbiota did not differ among cohorts (Figure 1B). Based on linear regression, host demographic factors did not correlate with Shannon diversity across the four cohorts, but there were some correlations within individual cohorts. Delivery mode correlated with diversity in the Canadian cohort (Supl. Figure 2A). Maternal age correlated with diversity in Ecuadorean and Canadian cohorts (Supl. Figure 2B). Because Canadian mothers who delivered by C-section were older than those that delivered vaginally, we used multiple linear regression, which showed that maternal age remained significantly associated with diversity while delivery mode did not. Interestingly, maternal age was positively correlated with diversity in Ecuadoreans, but negatively correlated in Canadians. When data from both cohorts were combined, the younger maternal age range of Ecuadoreans and older range of the Canadians revealed a significant quadratic relationship (method; p < 0.05, R2 = 0.09), with both the youngest and oldest mothers having children with lower diversity (Supl. Figure 2C).

**Beta Diversity.** We found that the microbiomes differed substantially between Canadian versus Ecuadorian and South African populations (Figure 1C). Of note, Belgian microbiomes were distributed across both groups, with the Belgian African-born sub-group (comprised of subjects born in different African countries) more often clustering with South Africans and Ecuadorians (Figure 1D).

Forward selection-based analysis to determine the contribution of demographic variables (sex, delivery mode, gestational age, maternal age, and anthropometric measurements) to the explanation of community composition resulted in cohort (country of origin) as the sole variable of importance (adjusted-R2 = 0.11, p = 0.002), with no other demographic variables contributing significantly to community composition. Because the effect of cohort could potentially mask effects of demographic variables within each cohort, we performed distance-based redundancy analysis (dbRDA) and *ordiR2step* for these variables in each cohort separately. We found that weight-for-length Z-score at the time of sampling was significant in explaining community composition in Canadian (adjusted-R2 = 0.031, p = 0.031) and South African (adjusted-R2 = 0.17, p = 0.020) children. No other host factors were significant for any other cohort. Thus, the demographic variables measured did not have major associations with microbiome composition.

**Microbiome taxonomic composition.** Taxonomic composition of microbiomes reflected commonly identified human taxa, with *Prevotella, Bacteroides, Faecalibacteria, Lachnospira,* and *Dialister* being the top 5 most abundant genera (Figure 2A). Most individuals were dominated by either *Prevotellaceae* or *Bacteroidaceae*.

We tested for associations between abundances of individual OTUs and cohort membership with the DESeq2 likelihood ratio test and found 442 OTUs differentially abundant among the cohorts. These OTUs were ranked for their capacity to discriminate cohorts using PLS-DA. The top 50 OTUs selected by PLS-DA discriminated Canadians and Belgian-born Belgians versus Ecuadoreans, South Africans, and African-born Belgians (Figure 2B). However, these 50 OTUs did not discriminate South Africans from Ecuadoreans or discriminate Belgians from any other cohort. Selected OTUs enriched in the Ecuadorean and South African clusters were almost exclusively members of *Prevotella*. A smaller subset of OTUs enriched in the Canadian cluster included a diverse range of genera, mostly belonging to the *Firmicutes* (including *Ruminococcus, Clostridia,* and unclassified *Firmicutes). Prevotella* OTUs were very rare in Canadians, present at high abundances in only 3 of 32 children.

**PRR ligand-specific responses associated with each cohort.**

Cytokine responses to PRR stimulation were published previously6, most notably showing that children from South Africa were distinct in profoundly under-responding to every PRR agonist except for PGN (of TLR2 and NOD12), based on univariate tests and principal components analysis (PCA). However, additional, albeit more subtle differences existed among the other cohorts. sPLS-DA analysis identified discriminatory cytokine responses among Canadian, Belgian, and Ecuadorean children. Canadians were classified largely by lower responses to PAM (TLR2) stimulation compared to Ecuadoreans and Belgians (Figure 3A-B), while Belgians were classified largely by lower responses to endosomal PRR agonists, Poly I:C (TLR3) and R848 (TLR7:8) (Figure 3C-D). Ecuadoreans did not have lower or higher responses to any PRR stimulation and were thus classified by their exclusion from the other two cohorts. The cytokine response signatures allowed us to classify each cohort with an error rate of 25% or less with only two sPLS-DA components (Figure 3E-F).

**TLR-responsiveness correlated with distinct microbiome features**

**Findings across cohorts.** Integration analysis was performed using study participants with both microbiome and complete immune data available; 8 Belgian, 19 Canadian, 41 Ecuadorean, and 8 South African children.Initial integration of OTU and cytokine data using all available subjects yielded a very poor correlation structure between the two datasets. Because South African immune profiles were highly distinct from those of all other cohorts, we hypothesized that the extreme phenotype of this cohort correlated poorly with those of the other cohorts. To this end, we performed sPLS integration using data from Belgian, Canadian, and Ecuadorean children only. The features selected whether South African children were included or not overlapped substantially. However, the co-variance of selected features was weaker when South African children were included (Supl. Figure 4).

The sPLS model including Belgian, Canadian, and Ecuadorean children was thus utilized for further analyses. Co-varying OTUs and cytokines were selected along the first sPLS component (Figure 4A). The selected features from both datasets were dominated by negative correlations between *Bacteroides* OTUs and cytokine responses to PAM (TLR2) stimulation, and positive correlations between *Prevotella* OTUs and the same responses (Figure 4A-C). These associations were also significant in the Ecuadorean cohort alone (Figure 4D). IL-6, IL-8, and IP-10 responses to PGN (TLR2, NOD1/2) and MIP-1α and MIP-1β responses to endosomal TLR stimulation followed the same pattern. IL-23 responses to both PGN and LPS were selected for their distinct relationships to the selected OTUs, correlating negatively with *Prevotella* but not with *Bacteroides*.

**Findings within cohorts.** Unique OTU-cytokine correlations were additionally identified within individual cohorts (Figure 5; Supl. Figure 4). Cytokine responses to PAM (TLR2 stimulation) were over-represented features in sPLS models for Belgium, Canada, and Ecuador separately and combined (Figure 5E). Cytokine over-representation in models were rare, and only included MIP-1α and MIP-1β among Ecuadoreans, and IL-8 and IL-12p40 among South Africans (Figure 5F). The only bacterial family over-represented in any cohort model was the *Prevotellaceae* for both the three combined cohorts and Ecuador separately.

Among the Belgians, cytokine responses to PAM stimulation and to R848 (TLR7:8) stimulation all negatively correlated with *Firmicutes,* including *Lachnospiraceae* and *Oscillospira,* and cytokine responses to LPS positively correlated with Firmicutes*,* including *Clostridia* and *Ruminococcus* (Figure 5A, Supl. Figure 4A).

The Canadian cohort was dominated by pro-inflammatory and Th17-supporting cytokine responses to LPS (including IL-1β, TNF-α, IL-23 and IL-12p40) and Th1 responses to R848 (including IFN-γ, IL-12p40, and IL-12p70), correlated with several *Lachnospiraceae* and *Bacteroides* OTUs (Figure 5B, Supl. Figure*.* 4B). Additionally, cytokine responses to PAM positively correlated with a diverse subset of Firmicutes.

Among Ecuadoreans, cytokine responses to both PAM and PGN stimulation were over-represented and correlated with multiple *Prevotella* and *Bacteroides* OTUs (Figure 5C, Supl. Figure 4C). Also, production of MIP-1α and MIP-1β correlated with diverse bacterial taxa.

South African children were the only ones for which responses to PAM were not over-represented. However, responses to PGN were over-represented, as were cytokines IL-12p40 and IL-8 in response to multiple ligands. These correlated almost exclusively to *Firmicutes.* (Figure 5D, Supl. Figure 4D).

**Host factors did not associate with host microbiome-immune correlations.** At the time of enrolment, seven Canadian children and one Ecuadorean child included in microbiome-immune integration were still being breastfed (Supl. Figure 5A). We identified a negative relationship between duration since weaning, and OTUs belonging to the *Lachnospiraceae* family and *Roseburia* genus (Supl. Figure 5B). These associations were not present among Ecuadorian children (Supl. Figure 5C-D). No correlations were identified between any breast feeding factors and immune responses either in block sPLS, or in univariate assessment of each stimulus-cytokine pair individually. The remainder of host factors demonstrated sparse relationships either OTUs or cytokines unique to either Canadian or Ecuadorean children, including associations between delivery mode, sex, weight for length Z-scores (WLZ), and weight for age Z-scores (WAZ) among Canadian children (Supl. Figure 6 A-B, examples in E-G) and between delivery mode and maternal age for Ecuadorean children (Supl. Figure 6C-D, examples in H-I).

Among Canadian and Ecuadorean children together, maternal age, height for age Z-scores (HAZ), WAZ, and time since breast feeding were found to co-vary with *Bacteroides, Prevotella,* and responses to TLR2 stimulation (Supl. Figure 7A). Given that the host factors showing strongest associations also differed between these cohorts, we determined the correlation strength of these relationships among Canadian and Ecuadorean children separately, finding that globally, these associations were no longer significant (Supl. Figure 7B-C, with specific examples in D-E). Thus, demographic factors, stool microbiome composition, and innate immunity did not correlate across multiple cohorts.

**Fecal transplant dictates immune phenotype of germ-free mice**

Mouse models of human fecal transplantation are a potentially useful tool to dissect host-microbiome relationships *in vivo*20, 21*.* In a proof-of-principle experiment, we directly tested whether human gut microbiota used to colonize germ-free mice could induce differences in systemic immune phenotypes similar to those observed in the human donors. We compared South African versus Canadian microbiomes for their potential effects on splenocyte responses to TLR stimulation in germ-free mice (Figure 6A). Principal Component Analysis of recipient mouse cytokine responses to PRR stimulation demonstrated that the type of stimulus primarily determined the response (Principal Component 1), as was observed within the human data6. However, within each stimulus, cohort-specific clustering was evident for responses to both R848 (PC1 vs. PC2; Figure 6B) and LPS (PC1 vs. PC3; Figure 6C). In mice inoculated with South African feces versus Canadian feces, IFN-γ and IL-10 responses to LPS and IL-10 and IL-6 response to R848 were significantly suppressed (Figure 6D). Responses to pro-inflammatory cytokines TNF-α and MIP-1β were similar between the groups, while IL-23 and IFN-α2 were not produced in this assay (Supl. Figure 8A). Overall, mice inoculated with South African feces mounted lower cytokine responses than those inoculated with Canadian feces, as was observed in the corresponding donor children.

We assessed the intestinal and fecal microbiomes of recipient mice at the end of the experiment alongside re-extracted DNA from human donor stools (Figure 6E). The microbiomes of the mice were distinct from their respective source feces. However, within each tissue and the feces, the microbiomes were distinct between mice with the different fecal sources (Figure 6F). Further, the small intestinal barrier integrity was significantly lower between mice inoculated with South African feces versus those inoculated with Canadian feces (Figure 6G). A subset of the differentially abundant OTUs in the original human samples were also found in the mice (Supl. Figure 8B-D). Of the OTUs differentially abundant in both the human donors and the mice, six consistently distinguished the treatment groups in the ileum, jejunum or feces. Among these, four OTUs, belonging to the genera, *Alistipes, Odoribacter,* and *Prevotella,* and to the family, *Rikenellaceae,* were enriched in the South African human donor stools and in the mice inoculated with those stools (Supl. Figure 8E). And, the remaining two OTUs, belonging to the genus, *Clostridium,* were enriched in the Canadian human donor stools and in the mice inoculated with those stools (Supl. Figure 8F).

**Discussion**

Given the ability of the host microbiome to modulate innate immunity, and the known geographical variability of both gut microbiomes and innate immune phenotypes, it is surprising that a correlation between the two has not previously been assessed. Also missing is a mechanistic understanding of how the geographically distinct microbiomes contribute to host immune differences. Robust correlations from human studies are therefore needed to inform mechanistic work using animal models. Here we provide evidence from humans on the biogeography of the relationship between host microbiome and systemic immunity and a proof of concept supporting the existence of a causal relationship between them.

While this study did not incorporate data pertaining to diet and lifestyle, the observed differences in stool microbiome composition are consistent with surveys of the gut microbiome in similar environments in terms of resource availability and diet. For example, children living in a rural environment in Burkina Faso, where diets are rich in complex carbohydrates and soluble fiber but low in animal fats and proteins were colonized by *Prevotella* while children living in westernized environments such as Italy and the USA where animal products are a major part of the diet were dominated by *Bacteroides*2, 3. Some of these differences may be driven by lifestyles associated with urbanization. Urban dwellers in Nigeria and Burkina-Faso both have microbiomes more closely resembling those of residents of industrialized urban centers compared to rural dwellers from the same countries22, 23, again with greater relative abundance of *Bacteroides* to *Prevotella* ratio in the urban dwellers. However, the Belgian children included in this study all lived in an urban environment, yet stool microbiomes of some Belgians with African heritage were dominated by *Prevotella* and clustered with Ecuadorean and South African children. Relative abundance of *Bacteroides* among Ecuadoreans was variable, and did not correlate to breastfeeding. Hence, diet alone is unlikely to explain all global patterns of stool microbiome composition we observed. Notably, *Bacteroides* were well-represented within the Ecuadorean children, even in individuals where *Prevotella* abundance was high. However, *Prevotella* were not detected in Canadian children dominated by *Bacteroides*, suggesting that those environments may have been unfavorable for *Prevotella* colonization.

We found that the demographic factors we measured did not have strong associations with the composition of the children’s microbiomes. In the Canadian and Ecuadorean cohorts, we did find a quadratic relationship between maternal age and microbial diversity, with lower diversity associated with both younger and older mothers. The lack of this association within the Belgian and South African cohorts may reflect the lower sample sizes in these cohorts, although it is possible that it reflects a region-specific effect of maternal age on child microbiomes. Both extremes of maternal age have been associated with increased risk for adverse birth outcomes24, stunting at two years of age, and altered glucose metabolism in adulthood25. The possibility that the microbiome is involved in such outcomes associated with maternal age warrants further investigation.

Using multi-omic integration, we identified correlations between the host microbiome and systemic immune responses, both within and across cohorts. Most notably, we found higher cytokine responses to TLR2 were associated with a greater relative abundance of *Prevotella* and a lower relative abundance of *Bacteroides* in the Belgian, Canadian, and Ecuadorean cohorts. We did not provide direct evidence that *Prevotella* modulates TLR2 responsiveness, or identify any correlations to health outcomes. *Bacteroides* and *Prevotella* have been shown to modulate mucosal immune responses through TLR2. *Prevotella copri* induces a more robust pro-inflammatory cytokine response from human dendritic cells in a TLR2-dependent manner26, 27. These effects on dendritic cells link *Prevotella*-rich gut dysbioses in humans to Rheumatoid Arthritis28, 29 and periodontal disease30. Conversely, *Bacteroides fragilis* sphingolipid Polysaccharide A (PSA) also signals through TLR2, stimulating dendritic cells to produce IL-10, contributing to an anti-inflammatory environment, systemically31 and in the mucosa32, 33. *Prevotella* species have also been associated with positive health outcomes. Research has revealed substantial diversity among the *Prevotella* genus34, and many of the OTUs identified as *Prevotella* in our data were not classified at the Species level. Gut microbiomes dominated by *Prevotella* have been associated with increased levels of short-chain fatty acids (SCFA) in a rural African setting2, and with high-fibre diets among adults living in Westernized nations35, 36.

Our study revealed closer associations with the TLR2 response pathway with the host microbiome than other PRRs. The selection of microbial taxa other than *Bacteroides* and *Prevotella* in cohort-specific networks suggest other immunomodulatory relationships that have not yet been experimentally evaluated. Finally, not all cohort-specific immune responses correlated to fecal microbiomes. For example, we did not find any relationships between increased IL-10 responsiveness among the Belgian children and their gut microbiomes. Thus, there are likely additional environmental or genetic determinants of systemic innate immune phenotypes that we did not capture.

Breast milk modulates the gut microbiome through several mechanisms, including variation in human milk oligosaccharide (HMO) composition37. HMO composition varies by geographical region and lifestyle factor38, 39. Among microbial taxa selected by microbiome-immune integration, *Bifidobacteria*, *Prevotella*, and *Bacteroides* are able to use HMOs for growth40, 41. However, we were not able to identify effects of time since breast feeding on immune responsiveness in either Canadian or Ecuadorean children, and among Canadian children time since breast feeding was only associated with levels of *Lachnospira* and *Rothia*, but not with cytokine responses, even though seven of nineteen children were still breast feeding. With this, breast feeding was not a likely contributor to the host immune-microbiome correlations we observed. However, given the small sample size applied to this analysis, and that these data were only available for Canadian and Ecuadorean children, identifying more subtle relationships between breast feeding and immune responsiveness or gut microbiota composition was not possible, and these findings do not preclude the existence of such relationships.

Innate immune phenotypes of South African children were previously described by us to be highly distinct from those of the other cohorts6, while our present study found their gut microbiomes to be indistinguishable from Ecuadorean children. Notably, fecal gavage of germ-free mice resulted in mouse immune phenotypes consistent with those of the respective donor children. South African donors induced strikingly lower cytokine responses favoring Th1 and Th17 development. However, pro-inflammatory cytokine responses were unaffected. Thus, the microbiome-induced mouse phenotypes were partly, but not completely, in agreement with those previously reported for the children6. Importantly, we did not identify a causal mechanism, or a specific component of the microbiome responsible for the observed effects on immune phenotype in germ-free mouse recipients. Hence, these results provide biological plausibility that must be further explored using animal models and validated in human cohorts. While this extreme-phenotype approach likely did not capture all relevant interactions, this *in vivo* data supports the existence of a causal relationship between human gut microbiomes and systemic immune function.

There are limitations to this study worth noting. The major limitation of this study was sample size, and statistical power to detect associations between host microbiome and immune phenotypes. Similar studies that identified robust associations between gut microbiome, immune phenotype, and host demographic factors among adults included over 500 adults8. This limitation does not negate the associations that were identified but may have caused us to overlook weaker associations. This study also suffered from unequal sample numbers among the four cohorts. With this, the absence of significant findings in some instances, especially associations with host factors, may have been due to lack of statistical power to detect more subtle relationships. Belgian children, uniquely, were almost all male, while child sex was more balanced for the other three cohorts. However, given that child sex was not a significant contributor to microbiome community composition at the other three sites, child sex was not found to contribute to cohort-specific cytokine responses in our previous findings6, it is unlikely that the sex bias of the Belgian cohort influenced the integration results. We limited the number of OTUs retained for integrated analysis to roughly 5% of those identified among the four cohorts. Also, especially for Ecuadorean children, differences in time between blood sample and stool sample collection may have influenced results. We also did not analyze the fecal metagenome, which has been shown to associate with systemic immunity in previous work8. While the cohorts are referred to by their countries of recruitment, the enrolled subjects are not always representative of the overall populations or resource availability in those countries. The germ-free mouse experiments were not conducted in a germ-free facility, which may have contributed to the divergence of engrafted mouse microbiomes from the original inocula.

**Conclusion**

This study provided supporting evidence to the link geographically distinct immune phenotypes to gut microbiomes, and identified a predominant association between systemic cytokine responses to TLR2 stimulation and stool microbiome composition. We also provide supporting evidence via human fecal transplantation in germ-free mice showing that the human host microbiome can induce changes to systemic immunity. Monitoring gut microbiome and immune system ontogeny along with well-defined clinical outcomes (e.g., infections or vaccine responses) in larger cohorts will further understanding of geographic differences in those clinical outcomes.

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**Figure Legends**

**Figure 1. Alpha and beta diversity of child fecal microbiomes.**  A-B. Alpha diversity in cohorts from each country determined using observed richness (A) or Shannon Diversity (B). Statistics: Kruskal-Wallis test with Dunn’s post-test, p-values adjusted with the Benjamini-Hochberg method. \* adjusted p-value < 0.05. C. Beta diversity based on NMDS scaling coded according to country of residence. D. Beta diversity based on NMDS scaling with Belgian children coded according to country of birth.

**Figure 2. Taxonomic composition of child fecal microbiomes.** A. Top 10 most abundant genera in members of cohorts from each country ordered by relative abundance of *Bacteroides*. B**.** Heatmap showing normalized abundance of the top 50 taxa differentially abundant across all cohorts determined via DeSeq2 analysis (adjusted p < 0.01) and further selected by multivariate PLS-DA analysis. Hierarchal clustering of subjects according to abundance profiles of taxa (top) and of taxa according to their abundance profiles across subjects (left).

**Figure 3. Discriminatory cytokines for Canadian and Belgian children selected by sPLS-DA.** A-B. Discriminatory features in component 1 with lower or higher responses in Canadians. C-D. Discriminatory features in component 2 with lower or higher responses in Belgians. E. sPLS-DA ordination of children based on cohort-defining cytokines. F. Maximum error rates overall and per class show that BLG, CAD, and ECD are classified with an error rate of 18%, 10%, and 0% respectively, with a minimum overall error rate of 6.8%.

**Figure 4. Cytokine-microbe correlations across multiple cohorts.** A. Heatmap showing correlations between selected OTUs and cytokines, with hierarchical clustering of OTUs based on cytokine correlation profiles (left) and hierarchical clustering of cytokines based on OTU correlation profiles (top). B. Correlation circle plot and C. Network showing correlation structure among selected OTUs and cytokines. In circle plot, circles indicate OTUs and triangles indicate cytokines. Network edge colors denote Pearson correlation strength and nodes are sized according to number of connections. D. Selected correlations between Bacteroides OTU\_1 and cytokine responses to PAM3Cys stimulation (IL-10 and IP-10) and positive correlations between Prevotella OTU\_2215 and the same responses, as observed across Belgian, Canadian and Ecuadorian cohorts (B) and in only the Ecuadorian cohort (C).

**Figure 5. Cohort-specific integrations selected distinct cytokine-OTU relationships.** Heatmaps show Pearson correlations between sPLS-selected OTUs and cytokines in cohorts from Belgium (A), Canada (B), Ecuador (C), and South Africa (D). Hierachical clustering as in Figure 4. OTU legend colors are assigned at the Family level for the top-8 represented families (or highest level of classification) in all analyses combined. The remaining OTUs are colored according to Phylum. E. Over-represented TLR ligand responses among all samples and within each cohort separately. F. Over-represented cytokine responses within cohorts from Ecuador and South Africa.

**Figure 6. Human fecal transplantation into germ-free mice recapitulated immune signatures in human donors.** A.Schematic of experimental design. B-C. Principal Components Analysis of cytokine responses by murine splenocytes: Principal Component 1 vs 2 (B), and 1 vs 3 (C) demonstrating clustering by stimulus, and by transplant donor cohort. D.Cytokine responses to TLR stimulation that significantly differed between mice gavaged with Canadian versus South African child stools. Statistical Analyses: Wilcoxon Rank-Sum test, p-values adjusted via Benjamini-Hochberg method with q < 0.1 considered significant; \*q < 0.05, + q < 0.1 (all nominally significant); boxplots indicate medians with first and third quartiles (25% to 75%); whiskers extend no further than 1.5\*IQR from the hinge. E.NMDS of Bray-Curtis distance showing similarity of microbiomes in mouse feces, ileum, and jenunum and human feces. F.NMDS of Bray-Curtis distance showing community composition of microbiomes in mouse feces, Ileum, and jejunum separately, with statistics indicating variance contributed by stool donor cohort. G.Lactulose-mannitol ratios of mice given SAF vs CAD child stools; the groups are significantly different (p < 0.01, student’s t-test).

**Supplementary Information**

**Supplementary Methods.** The supplement includes detailed information for human stool sample collection, DNA extraction, and V6-16S amplicon sequencing (library preparation and sequencing). For germ-free mouse experiments, supplementary methods include further details describing mice used for the experiment, human fecal transplantation, and measuring the lactulose to mannitol ratio in mouse urine samples. For the immunological experiments performed for germ-free mice, further details of splenocyte stimulation with TLR agonists and measurements of supernatant cytokines is supplied. Further details are also given for V4-16S amplicon sequencing of germ-free mouse intestinal and fecal samples. For statistical analysis, steps taken to prepare amplicon sequencing and immune data for analysis, and detail for all analyses performed throughout the manuscript are supplied.

**Supplementary Figures**

**Supplementary Figure 1. Time between blood draw and stool sample collection.** Days between blood and stool sample collection for Belgian, Canadian, Ecuadorean, and South African children for whom both immune and microbiome data were available. Negative values denote stool sample collected prior to blood sample. Missing data: Belgium n = 6, no missing data points for Canadian, Ecuadorean, or South African cohorts.

**Supplementary Figure 2. Interactions between host factors and fecal microbiome alpha diversity.** A. Relationship of diversity and delivery mode in Canadian and Ecuadorian cohorts. B. Correlation of diversity and maternal age in Canadian and Ecuadorian cohorts. C. Quadratic relationship between diversity and maternal age in combined Canadian and Ecuadorian cohorts.

**Supplementary Figure 3. sPLS integration performed with all children or only Belgian, Canadian and Ecuadorean children.** A-B. Percent of significantly co-varying cytokines to OTUs selected (A) or OTUs significantly co-varying with selected cytokines (B) by both models. Lines denote 30% cutoff for selected feature retention among all children (blue) and BLG,CAD,ECD children (red).C. Heatmap showing correlations between selected OTUs and cytokines that correlate with at least 30% of features of the complimentary data type D. Network of cytokine-OTU correlations between selected cytokines and OTUs. Edge colors denote Pearson correlation strengths. E-F. Correlations between Bacteroides OTUs and cytokine responses to PAM among all children (E) and BCG,CAD,ECD children only (F). r statistics and p-values computed via Pearson correlation.

**Supplementary Figure 4A. Belgian cohort-specific integrations.** A. Correlation circle plot and B. Network showing correlation structure between selected OTUs and cytokines. Network edge colors represent Pearson correlation strength. C. Examples of sPLS-selected OTUs correlating to cytokines in response to PAM (TLR2:6 stimulation). Statistics: Pearson correlation strength and significance.

**Supplementary Figure 4B. Canadian cohort-specific integrations.** A. Correlation circle plot and B. Network showing correlation structure between selected OTUs and cytokines. Network edge colors represent Pearson correlation strength. C. Examples of sPLS-selected OTUs correlating to cytokines in response to PAM (TLR2:6 stimulation). Statistics: Pearson correlation strength and significance.

**Supplementary Figure 4C. Ecuadorean cohort-specific integrations.** A. Correlation circle plot and B. Network showing correlation structure between selected OTUs and cytokines. Network edge colors represent Pearson correlation strength. C. Examples of sPLS-selected OTUs correlating to cytokines in response to PGN (TLR2:6 and NOD stimulation). Statistics: Pearson correlation strength and significance.

**Supplementary Figure 4D. South African cohort-specific integrations.** A-B. Correlation circle plots and C. Network showing correlation structure between selected OTUs and cytokines. Network edge colors represent Pearson correlation strength. D. Examples of sPLS-selected OTUs correlating to cytokines in response to PGN (TLR2:6 and NOD stimulation). Statistics: Pearson correlation strength and significance.

**Supplementary Figure 5. Associations between breast feeding and the microbiome.** A. Duration of breast feeding for Canadian and Ecuadorean children. B. Heat map indicating significant indicating significant correlations between time since breast feeding, breast feeding duration, and microbes along the second model component. C-D. Correlations between time since breasteeding and Roseburia (C) and Lachnospiraceae (D) among Canadian and Ecuadorean children.

**Supplementary Figure 6. Block-sPLS integration of microbiome, immune, and microbiome data among Canadian and Ecuadorean children separately.** A-D. Heat map indicating significant correlations between host factors, OTUs, and cytokines for the first and third model components for Canadian children (A-B) and between delivery mode along the first, and maternal age along the second model components for Ecuadorean children (C-D). E-G. Boxplots indicating significant correlations between delivery mode (E), child sex (F), and WAZ, WLZ scores (G) and sPLS-selected OTUs and cytokines among Canadian children. H-I. Boxplots indicating significant correlations between delivery mode, OTUs, and responses to R848 stimulation (H) and plots indicating negative correlations between maternal age and *Prevotella* OTUs (I) among Ecuadorean children.

**Supplementary Figure 7. Block-sPLS integration of microbiome, immune, and microbiome data among Canadian and Ecuadorean children.** A-C. Heatmaps depicting correlation strength between demographic factors and host features selected by sPLS among both cohorts (A), Canadian children only (B), and Ecuadorean children only (C). D-E: plots depicting significant correlations between maternal age, HAZ, Bacteroides, and Prevotella OTUs among all children (D), but not significant among Canadian or Ecuadorean children separately (D-E).

**Supplementary Figure 8**. A. Cytokine responses to TLR stimulation in germ free mice inoculated with Canadian or South African feces. Statistical analysis: Wilcoxon Rank-Sum test, p-values adjusted using the Benjamini-Hochberg method. \*q <= 0.05, + q <= 0.1, ns q > 0.1. OTUs differentially abundant between mice inoculated with CAD versus SAF child fecal microbiomes. A-C. Differentially abundant OTUs in the jejunim (A), ileum (B), and feces (C). D. Four OTUs enriched in SAF inoculated mice that were enriched in South African donor stools. E. Two OTUs enriched in CAD inoculated mice that were enriched in Canadian donor stools. All OTUs presented in D and E were significant after correction using the DeSeq2 test. Boxplots indicate medians with first and third quartiles (25% to 75%). Whiskers extend no further than 1.5\*IQR from the hinge.

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