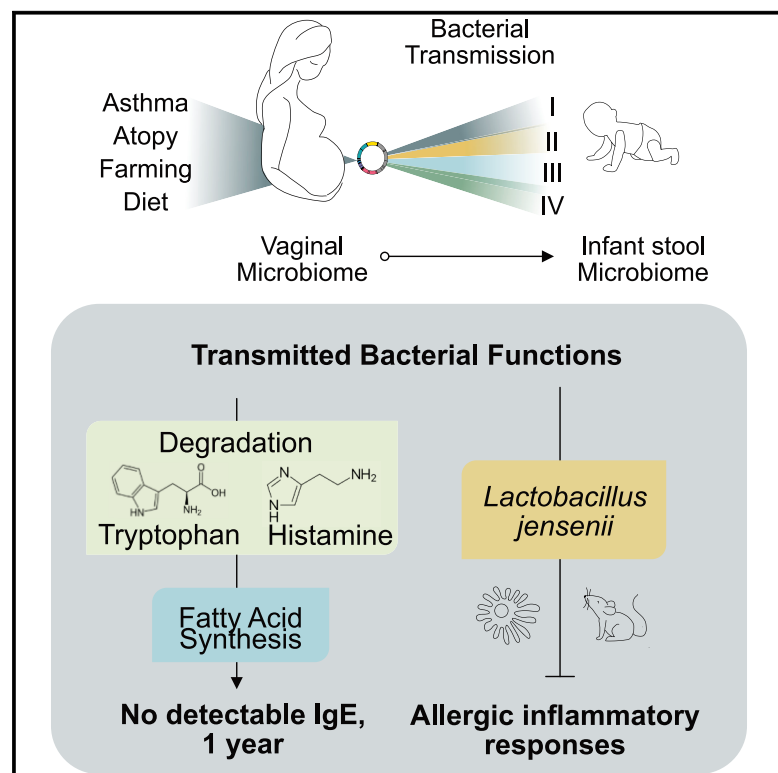Article

Heritable vaginal bacteria influence immune tolerance and relate to early-life markers of allergic sensitization in infancy

Graphical abstract

Highlights

- Distinct prenatal vaginal microbiotas relate to maternal health and exposures

- Variance in heritable bacteria and functions relate to infant markers of allergy

- Fetal *Lactobacillus jensenii* inhibits primary human antigen-presenting cell activation

- Vertically transmitted bacteria suppress airway allergic responses *in vivo*

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In brief

McCauley and Rackaityte et al. examine a large cohort of paired maternal-infant vaginal and stool samples and identify shared bacteria and microbial gene functions that relate to early-life features of atopy. They also demonstrate the allergy-preventative capacity of vertically transmitted Lactobacilli. The data suggest a microbial component of intergenerational allergy transmission.

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Heritable vaginal bacteria influence immune tolerance and relate to early-life markers of allergic sensitization in infancy

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https://doi.org/10.1016/j.xcrm.2022.100713

SUMMARY

Maternal asthma status, prenatal exposures, and infant gut microbiota perturbation are associated with heightened risk of atopy and asthma risk in childhood, observations hypothetically linked by intergenerational microbial transmission. Using maternal vaginal (n = 184) and paired infant stool (n = 172) samples, we identify four compositionally and functionally distinct *Lactobacillus*-dominated vaginal microbiota clusters (VCs) that relate to prenatal maternal health and exposures and infant serum immunoglobulin E (IgE) status at 1 year. Variance in bacteria shared between mother and infant pairs relate to VCs, maternal allergy/asthma status, and infant IgE levels. Heritable bacterial gene pathways associated with infant IgE include fatty acid synthesis and histamine and tryptophan degradation. *In vitro*, vertically transmitted *Lactobacillus jensenii* strains induce immunosuppressive phenotypes on human antigen-presenting cells. Murine supplementation with *L. jensenii* reduces lung eosinophils, neutrophilic expansion, and the proportion of interleukin-4 (IL-4)$^+$ CD4$^+$ T cells. Thus, bacterial and atopy heritability are intimately linked, suggesting a microbial component of intergenerational disease transmission.

INTRODUCTION

Neonatal microbiomes are simple and primarily comprised of species sourced from maternal skin, gut, and vaginal microbiomes.[1] Although very-early-life gut microbial species may ultimately be outcompeted by later colonizers, their presence and bioactive products shape seminal microbial co-associations and early-life immune training and function and relate to risk of atopy and asthma in childhood.[2-4] Several studies have now described infant fecal microbiomes depleted of key immunomodulatory bacterial species and anti-inflammatory metabolites to be associated with increased risk of atopy and asthma in childhood.[2-4] High-risk gut microbiomes exhibit increased concentrations of specific microbially derived metabolites with the capacity to promote canonical features of allergic immune dysfunction *in vitro* and *in vivo*.[5] For example, at 1 month of age, fecal concentrations of 12,13 di-

HOME and copy number of bacterial genes encoding epoxide hydrolases responsible for its production are elevated in infants at significantly higher risk of atopy or asthma development in childhood. Introduction of these bacterial epoxide hydrolases into the gut microbiome of mice is sufficient to significantly increase circulating 12,13 diHOME concentrations and exacerbate airway allergic inflammation in these animals.[5] Thus, the species, genes, and products of the very-early-life gut microbiome relate to immune cell functions relevant to allergy and asthma and to the risk of developing these conditions in childhood.

Support for a heritable component of childhood asthma stems primarily from the nearly universal epidemiologic finding of an association between maternal asthma and risk of asthma in offspring.[6] However, genes related to increased risk of allergy and asthma are only partially responsible for development of these pathologies,[7] suggesting that additional factors or interactions

account for disease heritability. Intergenerational microbial transmission could have inflated family-based estimates of asthma heritability because meconium microbiomes of neonates at high risk for asthma (having at least one parent diagnosed with asthma) are compositionally distinct compared with infants born to parents without asthma.[2] These high-risk infants develop a distinct gut microbiota over the first year of life and by 6 months of age exhibit bacterial depletion and metabolic dysfunction consistently associated with childhood asthma in independent human cohorts[3,4,8] and in mouse models of allergic airway sensitization.[9]

Microbial transmission may occur horizontally[1] or vertically; indeed, viable bacterial species have been reproducibly isolated from subsets of human fetal intestinal specimens.[10,11] Human fetal meconium isolates of *Micrococcus luteus* exhibited the capacity to survive in fetal antigen-presenting cells, grow to low cell density on estradiol and progesterone (detected at high concentrations *in utero*), and reduce fetal memory T cell production of interferon-gamma (IFNγ). The presence of *M. luteus in utero* also correlated with differential programs of epithelial gene expression, including increased expression of antimicrobial genes and those that regulate T cell responses.[11] Fetal isolates of *Lactobacillus* and *Staphylococcus* induce expansion of intestinal memory T cells, pointing to the capacity of these vertically transmitted bacteria to modulate long-lived immune responses.[10] Thus, vertically transmitted microbes accumulated *in utero* or in the early post-natal period may influence gut microbiome and immune developmental trajectories in infancy and later clinical outcomes in childhood.

The vaginal microbiome, which represents a reservoir for microbial species found in the infant gut,[1,12,13] undergoes a program of restructuring in early pregnancy, resulting in *Lactobacillus* domination at the expense of other species.[14,15] Vertically transmitted vaginal bacteria, particularly species of the highly studied *Lactobacillus*, play important functional roles in mucosal and immune development, including promotion of barrier function,[16–18] displacement of opportunistic pathogens,[18] and production of lactic acid, which influences intestinal pH, a strong selective pressure on gut microbiome membership and productivity.[19] Because early life represents an inflection point in human microbial development and is primarily sourced from maternal microbiomes, we investigated relationships between maternal vaginal (n = 184) and paired infant gut microbiotas (n = 172) in two birth cohorts focused on early-life exposures and allergy-related outcomes. We hypothesized that maternal environmental exposures known to modulate asthma risk (e.g., farming environment, maternal asthma, and elevated stress) relate to maternal prenatal vaginal microbiome composition and that the bacterial types and encoded functions shared between the maternal vaginal and infant gut microbiotas shape immune function and relate to markers of allergic disease development in the offspring.

RESULTS

Maternal diet and farming exposures relate to the maternal vaginal microbiota

An initial comparative analysis of the microbes, asthma, allergy and pets (MAAP) and Wisconsin Infant Study (WISC) cohorts indicated that the prevalence of maternal asthma and allergy did not

significantly differ between cohorts (p > 0.2) but factors such as race, a child's mode of nutrition (breastfeeding, formula feeding, or both), and mode of delivery did (Table S1). Using a harmonized MAAP and WISC vaginal 16S rRNA dataset (Figures 1A, S1A, and S1B; n = 184; n = 147 WISC, n = 37 MAAP), we next identified factors that explained variance in vaginal microbiota composition. Unsurprisingly, given the differences in environment and demographics, vaginal microbiota composition differed between MAAP and WISC mothers, but cohort explained only a small proportion (3%) of the observed variance (n = 184, R-squared [R^2] = 0.03, p = 0.006; Table S2). Additional factors that explained microbiota variability included specific maternal dietary exposures, maternal Hispanic ethnicity, and cord blood and early-life infant immunoglobulin G (IgE) levels, but these observations also explained only a small portion of variability and tended to be cohort specific (Table S2). Selected findings with unequal sample sizes per group underwent additional sensitivity analyses and were found to be robust to type I error (more information can be found in the STAR Methods).

Consistent with a previous report,[15] dominant bacterial sequence variant (SV) explained the greatest proportion of vaginal microbiota variability in our cohort [weighted UniFrac; n = 184; permutational analysis of variance (PERMANOVA) R^2 = 0.62, p = 0.001; Table S2], suggesting that a limited number of discrete prenatal vaginal microbiota structures existed in our dataset. Using hierarchical clustering, we identified four vaginal microbiota clusters (VC I–IV) (n = 184; Figures 1B and S1), three of which were dominated by distinct *Lactobacillus* variants (VC I, SV 5; VC II, SV 3; VC III, SV 15), with VC IV dominated by *Lactobacillus* (SV 22) or *Gardnerella* (SV 25 and SV 72; VC IV; Figure 1C). VC frequency did not differ by cohort (chi-square test, n = 184, p = 0.911; Table 1), suggesting that these four distinct VCs represent relatively conserved structures across this population of pregnant women irrespective of inter-cohort differences. Indeed, similar vaginal microbiota structures have been repeatedly described across large populations of women.[20–22]

We next asked whether these four VCs related to maternal environmental exposures and health status and serum IgE concentrations and sensitization in their offspring. Contrary to our expectations, no significant associations between VCs and maternal eczema or asthma were identified (chi-square test, all p > 0.2; Table 1). However, several maternal environmental factors, such as the number of previous children, as well as several cohort-specific factors, including maternal stress and depression, dietary exposures, and farming exposures, in particular working on a farm (n = 147, chi-square test, p = 0.006), differed significantly across the four VCs (Table 1). The frequency of children with multiple sensitizations and milk allergy also significantly differed based on VC, but it should be noted that these were observations with small sample sizes. These data indicate that the distinct vaginal microbiotas evident in the latter stages of pregnancy relate to maternal lifestyle and environmental exposures and to markers of allergic sensitization in their offspring.

Variance in heritable bacteria relates to maternal asthma, VC, and elevated IgE in offspring

Maternal asthma is an established risk factor for asthma development in offspring,[23] indicating a heritable component of the
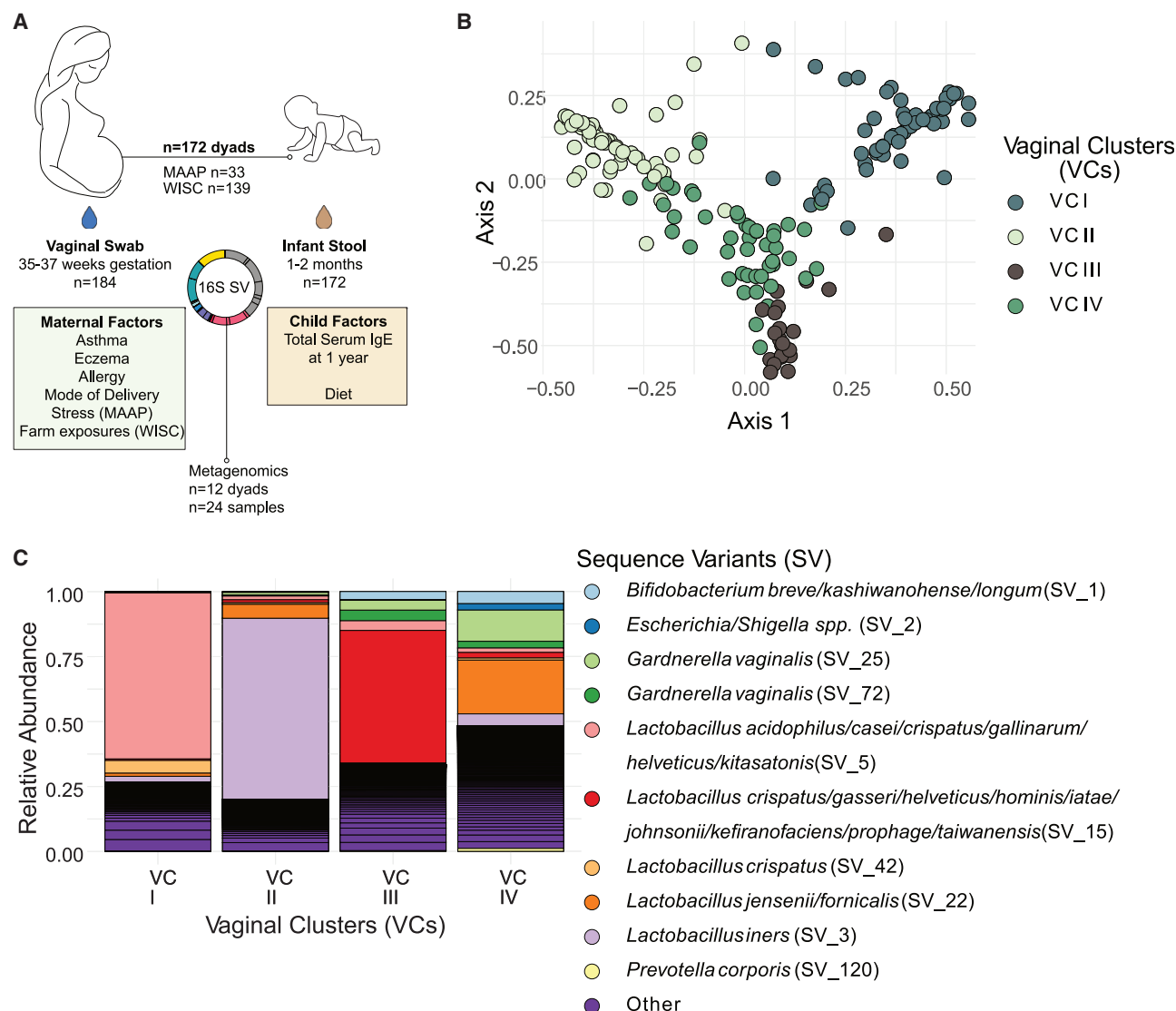
Figure 1. Maternal vaginal microbiota during pregnancy stratifies into four distinct clusters

(A) Schematic of the study design, harmonizing biospecimens, and metadata from the MAAP and WISC cohorts.

(B) Hierarchical clustering identifies four compositionally distinct vaginal microbiota clusters (VCs I–IV; PERMANOVA R^2 = 0.347, p = 0.001). Shown is a principal coordinates analysis (PCoA) plot of Bray-Curtis distance using 16S rRNA gene sequence variants (SVs); each point represents a maternal vaginal profile.

(C) Mean relative abundance of maternal vaginal bacteria in each VC derived from 16S rRNA amplicon sequence variant analysis. 184 biological replicates are shown. See also Figure S1 and Tables S1 and S2.

disease. We considered that heritable vaginal bacteria may, in part, explain this risk. We defined heritable bacteria for this study as those 16S rRNA SVs shared between paired maternal vaginal and infant gut samples. We next determined whether the frequency and types of heritable bacterial phylotypes differed across VCs and were influenced by early-life exposures or related to maternal asthma during pregnancy or elevated infant serum IgE at 12 months. All available dyads (individuals for whom a paired maternal vaginal and infant stool sample was available) at 1 month post-partum in MAAP (n = 33) or 2 months in WISC (n = 139, total = 172) were used for this analysis. A relatively small number (n = 137) of heritable bacterial SVs were identified. Of

these, the most commonly detected SVs belonged to *Bifidobacterium*, *Bacteroides*, *Finegoldia*, *Lactobacillus*, and *Staphylococcus*. The number of heritable bacteria did not differ between cohorts (negative binomial, p = 0.237) or across VCs (p = 0.557), nor was it related to infant IgE (p = 0.278), maternal asthma during pregnancy (p = 0.117), or a child's early-life mode of nutrition (p = 0.317), indicating a relatively consistent frequency of intergenerational bacterial inheritance across dyads in our study.

We next examined variance in the types of shared bacterial SVs among the subset of dyads that shared at least one SV (n = 137). These differed significantly based on VC (unweighted UniFrac PERMANOVA; MAAP, n = 23; WISC, n = 114; R^2 = 0.046;

Table 1. Maternal and infant features that relate to VCs.

	N	VC-I	VC-II	VC-III	VC-IV	Overall p value[a]	FDR p value
Cohort (WISC/MAAP)	184	42/11	51/13	18/3	36/10	0.911	1
Maternal factors							
Eczema ever, yes/total (%)	184	13/53 (24%)	12/64 (19%)	4/21 (19%)	7/46 (15%)	0.708	0.967
Eczema, pregnancy, yes/total (%)	184	5/53 (9%)	8/64 (12%)	2/21 (9%)	1/46 (2%)	0.303	0.932
Asthma, ever, yes/total (%)	183	13/52 (25%)	14/64 (22%)	6/21 (29%)	7/46 (15%)	0.572	0.952
Asthma, pregnancy, yes/total (%)	182	7/51 (14%)	7/64 (11%)	2/21 (9%)	2/46 (4%)	0.488	0.932
Total previous children, mean ± SD	183	0.8 ± 1.1[b]	1.3 ± 1.2	1.7 ± 1.2	1.7 ± 1.6[b]	0.002	0.061
Work on a farm,[c] yes/yotal (%)	147	13/42 (31%)	18/51 (35.3%)	13/18 (72.2%)	9/36 (25%)	0.006	0.086
Feed grain exposure,[c,d] mean ± SD	147	1.5 ± 0.8	1.47 ± 0.7	2.2 ± 0.9[b]	1.3 ± 0.7[b]	0.001	0.038
Hay exposure,[c,d] mean ± SD	147	1.5 ± 0.8	1.5 ± 0.8	2.2 ± 0.9[b]	1.4 ± 0.7	0.05	0.087
Manure exposure,[c,d] mean ± SD	147	1.4 ± 0.7	1.3 ± 0.6	1.6 ± 0.9	1.1 ± 0.2[b]	0.35	0.432
Pig exposure,[c] yes/total (%)	147	7/42 (16.7%)[b]	4/51 (7.8%)	3/18 (16.7%)	0/36 (0%)[b]	0.046	0.465
Child factors							
Multiple sensitizations,[e] yes/total (%)	31	0/9 (0%)	0/11 (0%)	0/3 (0%)	3/8 (38%)f	0.023	0.445
Milk specific IgE positivity,[c] yes/total (%)	100	1/28 (4%)	6/36 (17%)[b]	0/13 (0%)	0/23 (0%)	0.038	0.432

[a]ANOVA for continuous variables and chi-square test for categorical variables; table includes all significant variables and selected non-significant variables.
[b]Relationship is significant compared with all other clusters using generalized linear models (p < 0.05).
[c]Variable only available from WISC.
[d]Variable reflects frequency of exposure (1, never/almost never; 2, weekly; 3, daily).
[e]Variable only available from MAAP.

p = 0.007; Figures 2A and 2B). This finding was consistent after multivariate adjustment for potential confounding factors (cohort, mode of delivery, and mode of early-life nutrition; n = 111; R^2 = 0.06; p = 0.002). Several additional factors related to variance in the types of shared bacteria, including maternal asthma (Figure 2C) and eczema during pregnancy, mode of delivery, birthweight of the child, total IgE (among WISC participants), maternal multiple sensitizations during pregnancy (in the MAAP cohort), and maternal BMI before pregnancy (Table S3). These data indicate that the early-life gut microbiota of infants exhibits shared bacterial phylogeny with the maternal vaginal tract and that the types of heritable vaginal bacterial phylotypes found in the infant gut microbiota relate to maternal asthma and atopy and to early-life markers of allergic sensitization in infancy.

Heritable bacterial functions persist in the infant gut microbiome and relate to elevated IgE in later infancy
To confirm that heritable bacteria identified by 16S rRNA SV analysis represented shared species between maternal vaginal and infant stool samples, metagenomic analysis of 24 samples from 12 mother-infant dyads from the WISC cohort with sufficient remaining DNA for analysis was performed (Table S4). The functional gene capacity of maternal vaginal microbiomes co-varied with VC, indicating that VCs encode distinct microbial functional features (PERMANOVA Bray-Curtis, n = 12, R^2 = 0.52, p = 0.017). Infant gut microbiome function was related to age (n = 12, R^2 = 0.237, p = 0.019) and total IgE detection at 1 year of age (PERMANOVA Bray Curtis, n = 7, R^2 = 0.364, p = 0.029) following adjustment for mode of delivery. Using assembly-based analysis,[24] several bacterial species were identified in

samples, including species of *Bifidobacterium* (*Bifidobacterium longum*, *Bifidobacterium breve*, and *B. bifidum*), *Lactobacillus* (*Lactobacillus jensenii*, *Lactobacillus iners*, and *Lactobacillus gasseri*), and *Gardnerella vaginalis* (Figure S2). Aligning assembled contigs from paired maternal vaginal and infant stool samples revealed high sequence identity (>97%) along large contigs (in some cases ≥50 kb), providing evidence that these likely represent vaginally sourced heritable bacteria that are maintained over the first months of life in the infant gut (Figure S3). Bacteria such as *Staphylococcus* and *Finegoldia*, found to be frequently shared by 16S rRNA SV analysis, were not detected by shotgun metagenomics, perhaps reflective of their presence at lower abundance in the infant gut compared with the maternal vaginal microbiota during pregnancy.

Our data suggested that heritable vaginal microbes may confer distinct functional features to the nascent infant gut microbiome that relate to serum IgE levels in later infancy. To further investigate this, vaginal and infant gut metagenomic reads were aligned; 2.5%–15% of infant gut bacterial reads were also identified in paired vaginal microbiomes. Although the proportion of shared reads did not differ by a child's IgE or mode of delivery (Welch's t test, p > 0.2), variance in the genes encoded by these shared reads was related to detectable total IgE at 1 year of age (PERMANOVA binary distance, n = 7, R^2 = 0.219, p = 0.027), mode of delivery (n = 12, R^2 = 0.128, p = 0.045), and maternal BMI (n = 9, R^2 = 0.172, p = 0.014). IgE-discriminatory pathways were identified using machine learning algorithms and included enrichment of glyceraldehyde-3-phosphate dehydrogenase and streptomycin synthesis as well as tryptophan and histamine metabolism pathways in children

A

B

C
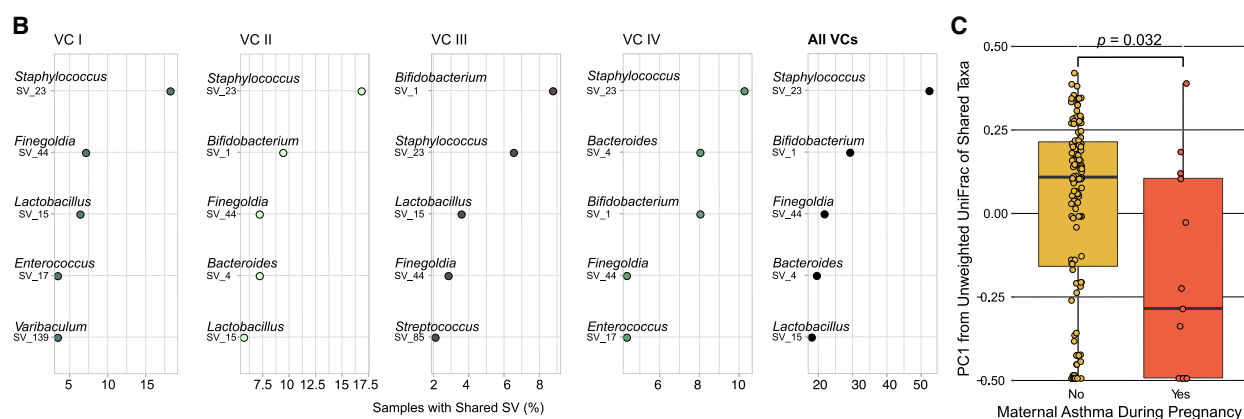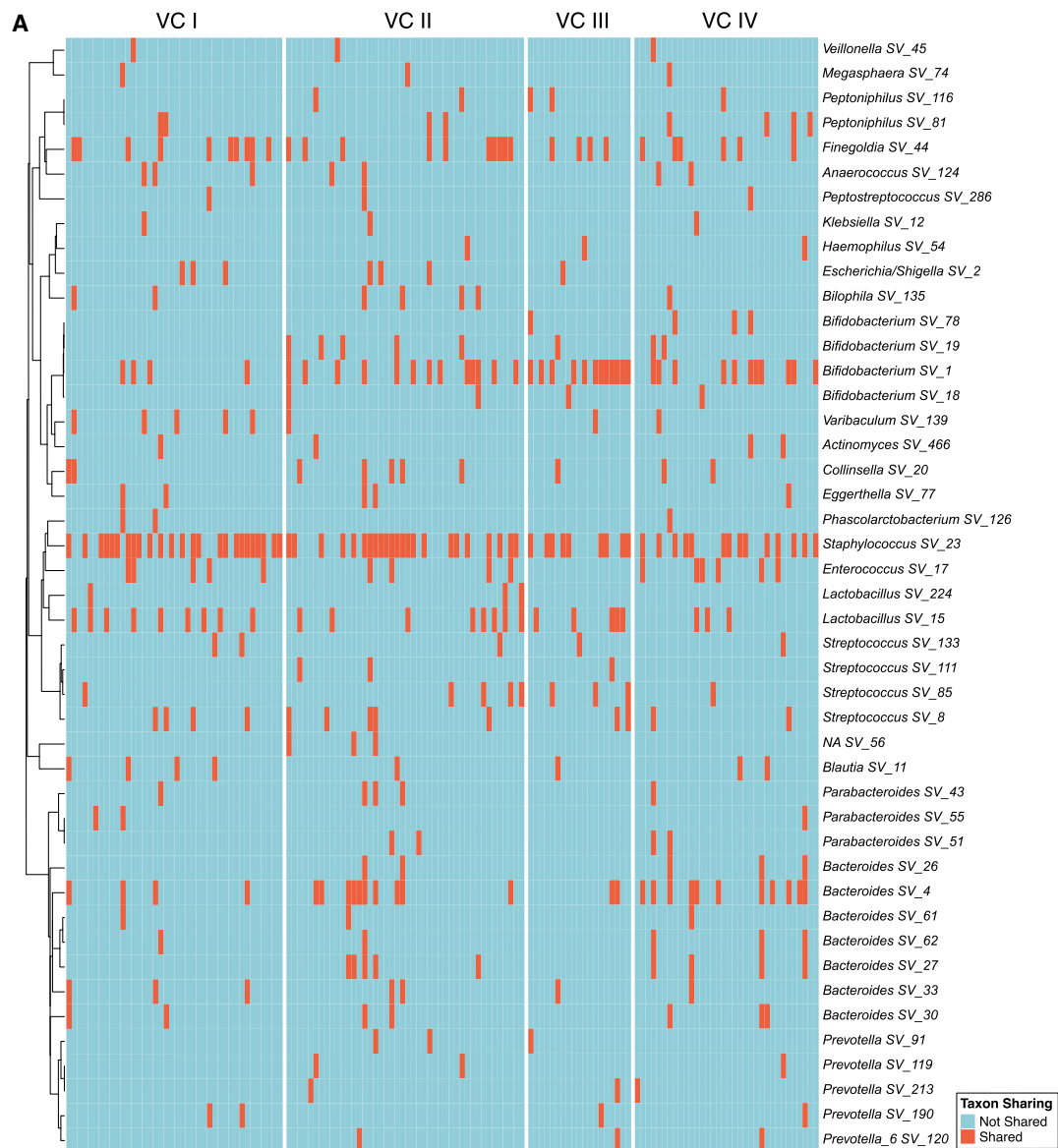
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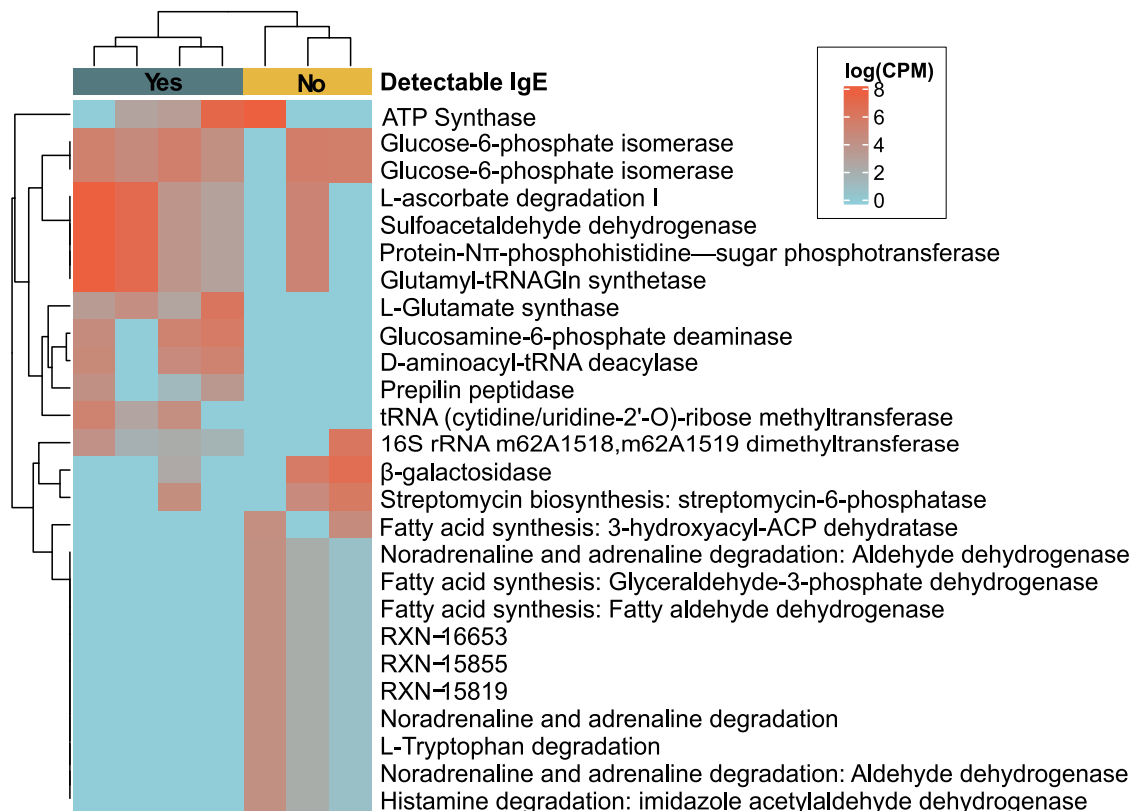
Figure 3. Functional capacity of shared bacteria is distinct in infants with detectable IgE at 1 year
Heatmap of machine learning-selected pathways among shared metagenomic reads that discriminate infants with or without detectable IgE at 1 year of age. Shared metagenomic reads were identified by requiring identical sequence alignment between mother and infant metagenomic datasets, shown in log-transformed copies per million for each. MetaCyc annotated reactions are described by their catalyzing enzyme. 7 biological replicates are shown.
See also Figures S2 and S3 and Table S4.

with no detectable serum IgE, whereas bacterial pathways for 16S rRNA dimethyltransferase, glutamate synthesis, and glucose-6-phosphate isomerase were enriched in children with detectable IgE (Figure 3). These data indicate that heritable microbial strains, and in particular their encoded metabolic functions, remain present until at least 2 months of age in the infant gut microbiome and relate to detectable IgE in later infancy.

Vertically transmitted fetal *Lactobacillus* promotes immune tolerance in the context of allergic inflammation
The association of heritable microbes with early-life markers of allergy in our human cohort suggested that inter-generationally transmitted microbes may influence allergic inflammatory dis-

ease. Given that the fetal intestine contains viable bacteria with immunomodulatory properties,[10,11] we turned to a bank of fetal small intestine contents as a source of vertically inherited bacterial strains to test this hypothesis. Using culture conditions that mimicked the *in utero* hormonal milieu (Table S5), two *Lactobacillus* isolates (L01 and L02) were obtained from two independent meconium specimens from a cryopreserved human fetal meconium bank that was unrelated to the WISC/MAAP cohorts. Whole-genome sequencing of L01 and L02 (Table S6) indicated that they exhibited greatest similarity to *L. jensenii* (99.86% average nucleotide identity [ANI] for both; Figure 4A; Table S7). Analysis of shared single-copy genes (bootstrap value = 1 for relevant clade; Figure 4B) confirmed that both isolates were *L. jensenii* strains. We next asked whether these fetal isolates

Figure 2. Shared bacterial SVs associate with VC and maternal asthma during pregnancy
(A) Shared SVs, identified as those with more than 10 reads in maternal vaginal and infant stool microbiota within dyads are indicated in red; SVs not shared between mother-infant pairs are indicated in blue. Each column represents SVs shared within a mother-infant dyad. SVs are ordered by phylogeny and were included when they were shared in at least three dyads.
(B) Top five shared taxa for each VC or across all clusters (right).
(C) Maternal asthma status significantly relates to principal coordinate 1 (PC1; unweighted UniFrac) of shared SVs; t test for significance. 172 biological replicates are shown.
See also Figure S2 and Table S3.
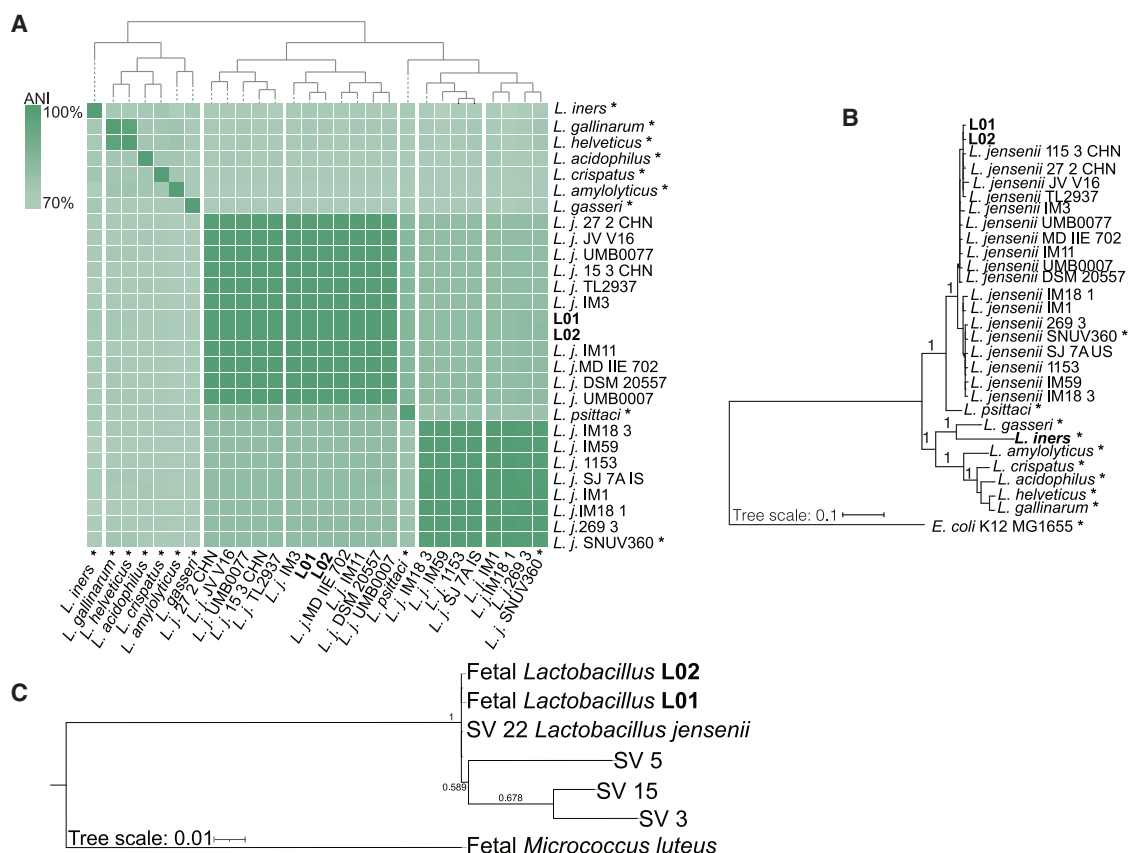
Figure 4. Comparative genomics of fetal *L. jensenii* isolates

(A) Hierarchical clustering of whole-genome average nucleotide identity (ANI) of all available genomes of *L. jensenii*, several reference *Lactobacillus* genomes, and fetal meconium isolates L01 and L02 indicates greatest homology with *L. jensenii*.

(B) Phylogenetic trees of single-copy conserved genes across select publicly available genomes within *Lactobacillus* and fetal meconium isolates L01 and L02 confirm greatest similarity with *L. jensenii* strains.

(C) 16S rRNA V4 region of fetal meconium isolates L01 and L02 compared with SVs in our study indicate greatest homology with SV22 detected in maternal vaginal samples.

When available strain origin is represented, hierarchical clustering was performed on ANI; an asterisk indicates a reference or a representative genome. *Escherichia coli* K12 and fetal *M. luteus* were used as outgroups in (B) and (C), respectively. See also Figure S4 and Tables S5, S6, and S7.

bore sequence similarity to those found in our maternal prenatal vaginal and paired infant stool samples. The V4 region of the 16S rRNA gene of these fetal *Lactobacillus* isolates aligned with SVs identified in MAAP and WISC samples and was most similar to SV22 (bootstrap value = 1 for relevant clade; Figure 4C). *L. jensenii* was confirmed in maternal vaginal and infant stool samples by assembly-based metagenomic sequencing (Figure S2). Using our shotgun metagenomics data, 16 of 24 vaginal and infant stool samples exhibited more than 50% coverage of each of the fetal *L. jensenii* strain's genome (Figure S4). The finding that highly related *L. jensenii* strains exist in paired maternal prenatal vaginal microbiome and post-natal infant stool and in unrelated human fetal meconium samples suggested that this species may represent an important inter-generationally transmitted, immunomodulatory species.

We next assessed the capacity of these meconium-derived *Lactobacillus* isolates to modulate immunity *in vitro* and *in vivo*, hypothesizing that vertically transmitted *Lactobacillus* species

promote protection against allergic sensitization. Antigen-presenting cells (APCs) are critical mediators of tolerance in the context of allergic sensitization. Co-stimulatory molecules CD86 and CD83, expressed on HLA-DR^{+} APCs promote efficient human T cell priming,[25] whereas CD11c^{+} dendritic cells (DCs) expressing CD103 are required to promote regulatory T cell responses to inhaled allergens.[26] To investigate the role of these *Lactobacillus* isolates in early-life cell populations, primary human fetal splenic cells were treated with L01 and L02, and APC phenotypes were assessed. An additional fetal strain of *M. luteus* (Micro36), also isolated from fetal meconium,[11] and non-fetal bacterial American Type Culture Collection (ATCC) strains of *L. iners* (isolated from the vaginal tract) or *M. luteus* were also used for comparison. Exposure of fetal splenic HLA-DR[+] APCs to fetal strains (L01, L02, and Micro36) and *L. iners* significantly reduced their co-expression of CD86 and CD83 whereas non-fetal *M. luteus* did not (Figure 5A). The greatest reduction in APC activation was achieved by L01 and L02, which

A

B
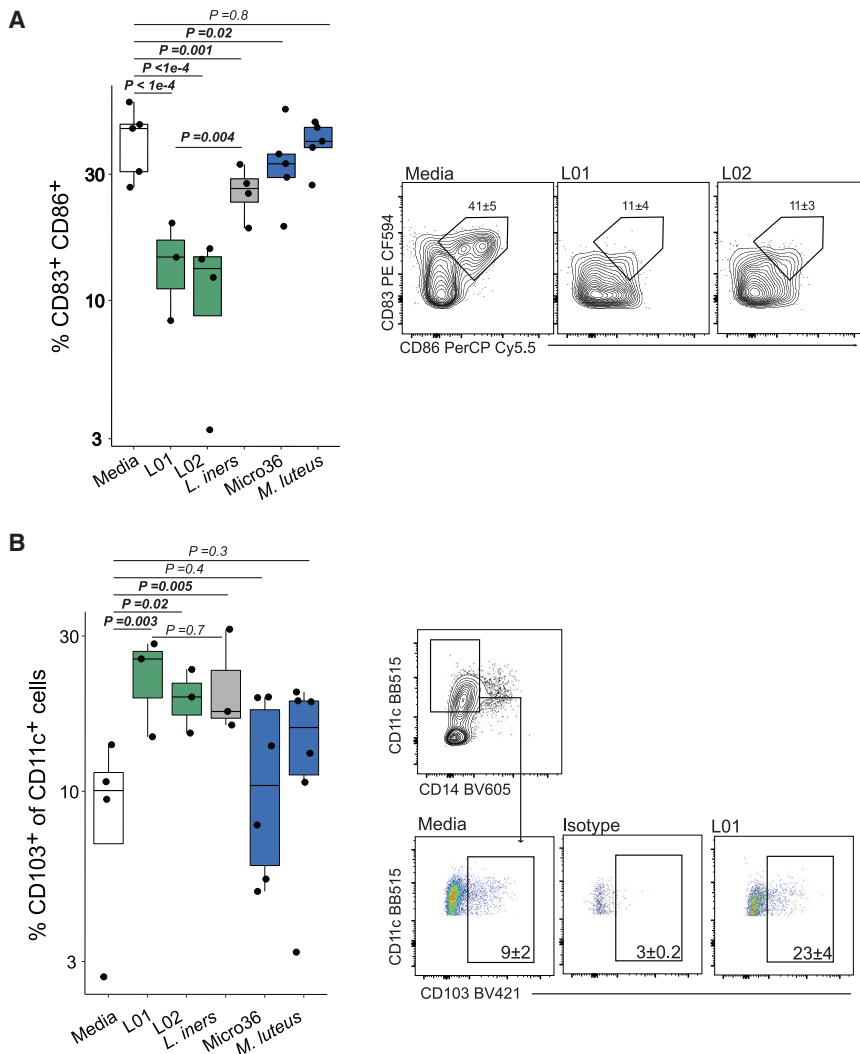
Figure 5. Vertically transmitted *Lactobacillus* isolates promote tolerance in primary human APCs

(A and B) CD83 and CD86 expression is reduced (A) and CD103 expression increased (B) among live CD45$^+$ HLA-DR$^+$ fetal splenocytes after 4 h of treatment with fetal isolates L01 or L02 compared with the medium control and additional fetal strain Micro36 as well as non-fetal strains of *L. iners* and *M. luteus*. Representative flow cytometry plots with median ± SEM are shown on the left. Two-sided Satterthwaite's method on the linear mixed effects (LME) model was used to test for significance, controlling for repeated measures of cell donor across treatments. Box plots indicate quartiles of the data distribution. Each dot represents a biological replicate, with at least n ≥ 3 per treatment.

induced significantly lower activation compared with the non-fetal *L. iners* strain. All strains of *Lactobacillus,* but not *Micrococcus,* induced CD103 expression on CD11c$^+$ DCs (Figure 5B), indicating a genus-specific effect on fetal immunity. These data suggest that certain vertically transmitted *Lactobacillus* are capable of poising primary human cells for immunotolerance.

To investigate whether immunosuppression by vertically transmitted fetal bacterial isolates can confer immune tolerance in the context of allergic inflammation *in vivo*, we next supplemented mice with L01 or Micro36 in an adult house dust mite (HDM) airway allergic sensitization model (Figure 6A). Mice were orally gavaged with PBS or 10^7 colony-forming units (CFUs) L01 or Micro36 and subsequently subjected to intra-tracheal sensitization and challenge with HDM allergen; a control (baseline) group was treated with PBS intra-tracheally and orally (Figure 6A). HDM challenge induced eosinophilia and reduction of neutrophils in the lungs, consistent with reported phenotypes of this model[27] (Figures 6B, 6C, and S5A). Oral gavage with L01 significantly reversed these effects, whereas Micro36 only ameliorated neutrophil accumulation (Figures 6B and 6C). Neither fetal strain reduced

CD4$^+$ T cell infiltration into the lungs (Figure 6D), but both significantly altered their function in the lung-draining lymph node (mediastinal lymph node [medLN]). L01 and Micro36 reduced the proportion of inflammatory interleukin-4 (IL-4)$^+$ and IL-17A$^+$ CD4$^+$ T cells to baseline levels (Figures 6E–6G and S5B) but did not affect IFNγ$^+$ CD4$^+$ T cell proportions (Figure 6H). Because the mice were supplemented orally with fetal strains, we investigated T cell function in the intestinal-draining lymph node. Intra-tracheal HDM treatment did not influence inflammatory T cell subsets (i.e., Th1, Th2, and Th17) in the intestinal-draining lymph node compared with baseline (mesenteric lymph node [MLN]; Figures 6F–6H). However, L01 supplementation resulted in a striking reduction in the proportion of Th1, Th2, and Th17 cells in this lymph node below baseline levels (Figures 6F–6H), whereas Micro36 effects localized only to the lung draining lymph node. This suggests that L01 induces potent intestinal tolerance effects and that this immunosuppressive capacity extends to distal sites of allergic inflammation in the airways. Thus, certain vertically transmitted fetal bacterial strains reduce allergic inflammation *in vivo* and restore immune tolerance in adult murine models of allergy.

DISCUSSION

This study was prompted by mounting evidence that environmental exposures[28] and maternal asthma status during pregnancy[29] relate to risk of atopic sensitization in offspring. Children born by cesarian section exhibit distinct gut, skin, and oral microbiomes in very early life[30,31] and are at significantly increased risk of allergic sensitization and asthma development in childhood,[32] suggesting that maternal microbiomes during pregnancy and their transmission to the developing infant may etiologically contribute to this relationship. The maternal vaginal microbiome,
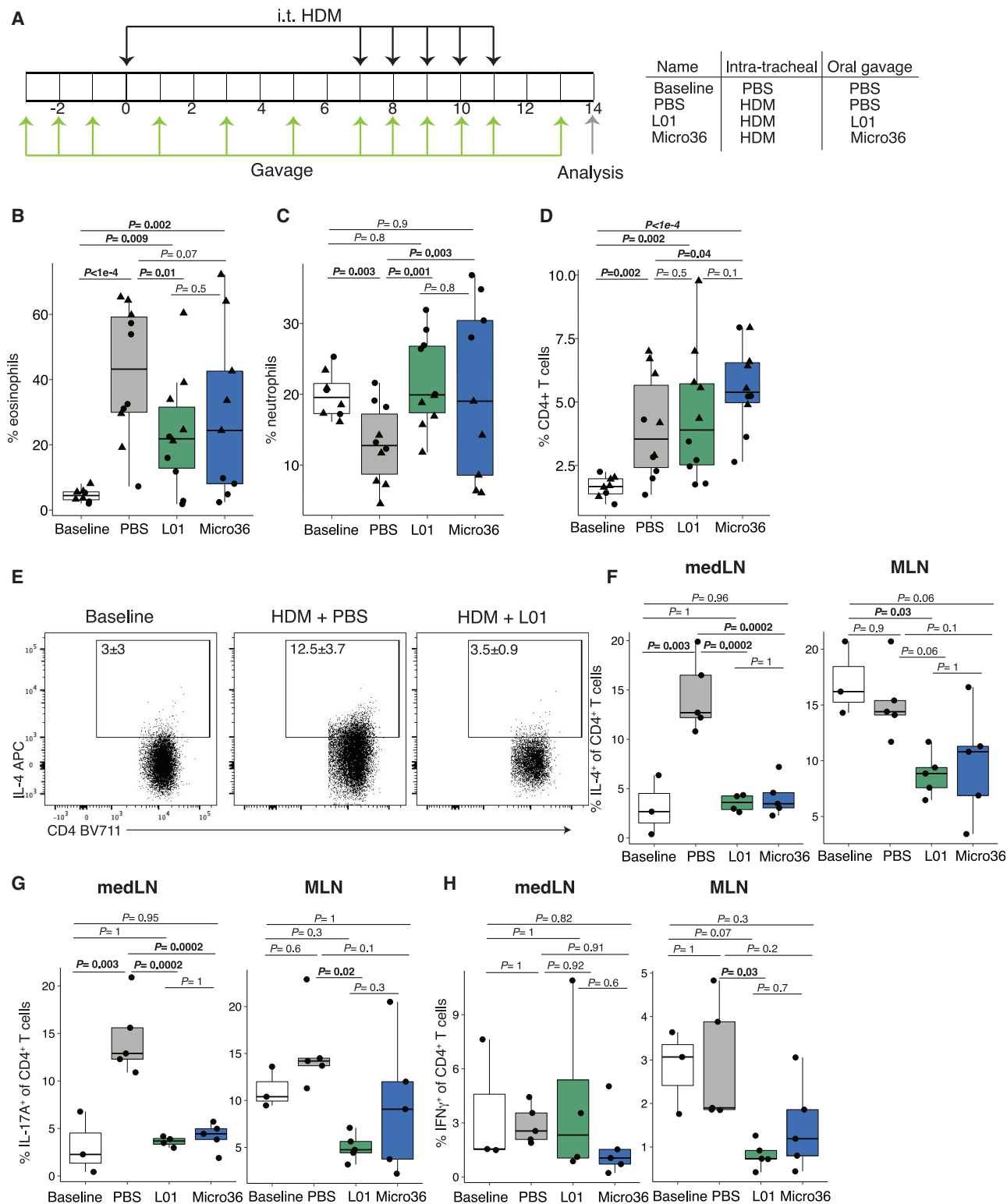
Figure 6. Vertically transmitted *Lactobacillus* L01 and Micro36 strains ameliorate airway allergic sensitization *in vivo*

(A) Murine HDM intra-tracheal sensitization and challenge (itHDM) scheme in animals orally gavaged with meconium-isolate *L. jensenii* L01 or *M. luteus* Micro36 or PBS.

(legend continued on next page)

together with microbiomes at other body sites, is a key source of vertically transmitted microbes.[1] Thus, heritable microbes may be key to establishment of infant microbiomes whose presence and products shape immune cellular training, function, and memory as well as microbial development in early life. Some of these earliest colonizers have also been shown to persist into adulthood, suggesting that, under the correct conditions, they may be lifelong microbial colonizers[33] that contribute to immune homeostasis and protection against allergic disease.

We took advantage of a large collection of maternal vaginal samples during pregnancy and paired infant gut samples in two birth cohort studies spanning a range of environments from urban to rural farming communities as well as an independent cryobank of human fetal intestinal meconium samples to examine heritable bacteria and their relationship with allergy. Consistent with previous reports,[15,34] we identified several distinct late-third-trimester VCs, each dominated to various degrees by distinct *Lactobacillus* species and exhibiting significant relationships with environmental exposures. These four VCs corroborate several previous studies of the vaginal microbiome,[14,34] which have identifed between five and seven distinct vaginal microbiota structures, each dominated by distinct *Lactobacillus* species, including those described in our prenatal cohorts.

Farm exposures are known to reduce risk of allergic asthma in offspring.[35] In our study, WISC mothers with reduced farm-related exposures more frequently possessed a vaginal microbiota (VC-IV) dominated by *Lactobacillus fornicalis* or *G. vaginalis*. In the MAAP cohort, this VC was associated with increased numbers of sensitizations in the offspring. Based on genotyping and soluble protein profiles, the relatively recently described *L. fornicalis* forms a *Lactobacillus* clade[36] distinct from *Lactobacillus* species dominating other VCs in this study, while *G. vaginalis* is an established pathogen in bacterial vaginosis[37,38] and is implicated in pre-term birth.[39] These data suggest that reduced exposure to asthma-protective environmental exposures such as farming during pregnancy may influence the vaginal microbiome and that putative pathogen domination of this niche is linked to increased risk of sensitization in children born to mothers with this VC.

Although the vaginal microbiota as a whole did not directly relate to maternal asthma or early-life markers of allergy and asthma in the children, the heritable bacterial component of the vaginal microbiome was highly related to maternal asthma. This suggests that immune activation associated with maternal asthma may influence which vaginal microbes are successfully transmitted from mother to infant, a possibility recently corroborated in mouse models showing that human vaginal bacteria influence murine offspring health.[40] Significant relationships were observed when the composition, rather than the frequency, of heritable bacteria was considered and indicated that the types of bacteria shared between mothers and their offspring relate to maternal asthma.

Early life nutrition and environmental exposures influence infant microbiomes,[2,19] plausibly contributing to whether maternally sourced bacteria are sustained or lost from the infant gut microbiome. We recognize that our cross-sectional study sampling infants at 1 or 2 months of age is limited in that it cannot assess microbes that are vertically transmitted but lost in early infancy, a phenomenon that may be equally important for childhood atopy development. By 1 or 2 months of age, vaginally sourced bacteria represent a very small proportion of the total infant stool microbiome,[41] a finding corroborated by our metagenomic analysis. Detection of distinct heritable vaginal bacteria and functional genes at 2 months of age that relate to maternal asthma status and elevated IgE levels in infancy suggests that at least a subset of maternally sourced vaginal bacteria are sustained throughout early infancy, which may functionally contribute to heritability of or protection against atopy development. For example, infants with detectable IgE at 12 months inherited bacteria encoding glutamate synthesis and glucose-6-phosphate isomerase. The former, a neurotransmitter, has more recently been shown to activate T cells and promote their adhesion, migration, and cytokine secretion, whereas auto-antibodies against glucose-6-phosphate isomerase are a hallmark of rheumatoid arthritis,[42] raising the possibility that inherited bacterial activity may promote early-life inflammation in these infants. These infants were also enriched for 16S rRNA dimethyltransferase, which confers aminoglycoside resistance, providing evidence for intergenerational transmission of antimicrobial resistance. Infants without detectable IgE at 12 months possessed microbiomes encoding glyceraldehyde-3-phosphate dehydrogenase, which increases *Lactobacillus* adhesion to host intestinal mucins,[43] and streptomycin biosynthesis, a microbially derived aminoglycoside active against aerobic pathogenic bacteria,[44] suggesting that heritable vaginal bacteria associated with atopy protection may colonize and shape microbial colonization in the developing gut microbiome. These infants also housed heritable bacteria capable of histamine and tryptophan degradation. Histamine, a canonical feature of allergic response, can be bound and depleted by lactic acid bacteria, including *Lactobacillus* strains.[45,46] Tryptophan has been shown to be elevated in asthmatic children[47]; its degradation through the mammalian indoleamine 2,3-dioxygenase (IDO) is known to promote resolution of chronic inflammation.[48] These data point to the underexplored role of heritable microbes in assembly of the early-life gut microbiome and their functional features that program immune function in early life.

(B–D) Percentage of (B) lung eosinophils, (C) neutrophils, and (D) CD4$^+$ T cells among CD3$^+$ live cells in lungs of animals after HDM sensitization across treatment groups. Two-sided Satterthwaite's method of the linear mixed effects (LME) model test for significance, with random effect attributed to experimental repeat.
(E) Representative flow plots of intracellular IL-4 production in CD4 T cells within the mediastinal lymph node (medLN).
(F–H) Percentage of (F) IL-4$^+$ (G) IL-17A$^+$, and (H) IFNγ^+ T cells in the medLN (left) and mesenteric lymph node (MLN; right) in animals after allergic sensitization. ANOVA test for significance.
.Shapes (triangles and circles) indicate mice from two independent experiments. Each treatment group had 5 mice as biological replicates, and the experiment was repeated independently twice.
See also Figure S5.

Our primary human cell and murine studies further confirm the capacity of certain vertically transmitted *Lactobacillus* strains to promote atopy-protective effects. *Lactobacillus* strains isolated from a bank of human fetal ileal meconium samples provide evidence of viable, vertically transmitted bacteria *in utero*.[10,11] Fetal isolates limited activation and induced CD103 expression on primary human DCs *in vitro*. CD103-expressing DCs can also produce high levels of IDO, contribute to tryptophan degradation, and are implicated in resolution of allergic asthma in mice.[48] Thus, certain heritable *Lactobacillus* species may synergistically modulate tryptophan concentrations to promote resolution of allergic inflammation. CD103+ DCs are also key suppressors of Th2 and Th17 responses,[49] which were dampened *in vivo* in animals orally supplemented with L01. These anti-inflammatory effects were potently induced not only at the site of allergen challenge in the lungs but also in the intestine, indicating that vertically transmitted *Lactobacillus* species may promote global dampening of allergic inflammation. Our data suggest that failure to transmit or sustain such strains may lead to opportunistic microbial pathogen colonization and a relative absence of the normal bacterial functional capacity to regulate allergic inflammation. Further study of the heritability of these strains *in vivo* as well as neonatal allergic challenge models in animals would bolster these conclusions.

This study provides evidence of a heritable microbial component of asthma and demonstrates that functional genes encoded by these bacteria include pathways plausibly linked to loss of immunomodulatory capacity. Further studies of larger cohorts, including those with additional assessments of allergy at later stages of development, will allow deeper investigation of these microbiological relationships within mother-infant dyads. Infants in our two birth cohorts with distinct exposures are still young, and additional follow-up will determine whether these infants ultimately develop clinically relevant allergy beyond our 1-year IgE measurements. We also acknowledge that shared 16S rRNA fragments are insufficient to assess heritable bacteria and that these microbes may also be shared through other means of contact (skin, orally) especially in cesarean-section-delivered infants or via vertical transmission *in utero*. Our study attempts to address these limitations through shotgun metagenomic sequencing from a subset of mother-infant dyads to provide more robust evidence of inherited vaginal bacteria and their relationship with immunoglobulin markers of allergy in later infancy.

Our study demonstrates that the phylogeny of shared microbes in paired maternal vaginal and infant stool were related to allergic outcomes in the child, and microbial functional genes associated with infant serum IgE levels provide additional insights into plausible mechanisms by which inherited microbes contribute to or protect against atopy development. Isolated, vertically transmitted fetal *Lactobacillus* strains with a high degree of similarity to those detected in our birth cohort exhibited immunosuppressive, atopy-protective capacity *in vitro* and *in vivo,* further supporting a role for heritable bacteria in protection against atopy. These data suggest that strategies to influence very-early-life gut microbiome development may offer the opportunity for allergic disease prevention or treatment.

Limitations of the study

To fully demonstrate heritability of strains and identify their immunomodulatory properties, isolation of identical strains from maternal vaginal tract and infant stool in larger cohorts is warranted. Performing assembly-based metagenomics using these strains as scaffolds would provide additional evidence of strain transmission. Unfortunately, the paired mother-infant samples in our study were not preserved in a manner that would support such isolation efforts. We therefore turned to a recently developed independent bank of cryopreserved fetal meconium collected at mid-gestation, which, by definition, represents vertically transmitted material. *Lactobacilli* isolated from this fetal meconium are highly similar to strains detected by shotgun metagenomics in paired mother-infant dyad samples in our study and are thus relevant for study. If possible, future fetal meconium biobanking should consider collecting data on maternal atopy/asthma status, the lack of which we recognize as a limitation of our study.

To address our hypothesis, we focused on transmission from the maternal vaginal tract to the developing infant gut, but we recognize that there are additional maternal sites that contribute microbes to the developing infant gut microbiome. Interrogation of these sites (e.g., maternal gut, skin, placenta, uterine, breastmilk, as well as the maternal built environment) would greatly add to future studies. Our human and murine studies suggest that fetal microbes exert a strong program of tolerance locally in the intestine and distally in areas of allergic inflammation. A limitation of our interpretation is that introduction of these strains along the course of development may have differential effects on immune responses. Future work will investigate the relevant timing and dosage necessary to direct lifelong immune protection from chronic inflammatory disease.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100713.

AUTHOR CONTRIBUTIONS

K.E.M., E.R., C.C.J., C.M.S., J.E.G., and S.V.L. designed the study. C.B., K.B., H.K., and K.J. coordinated sample collection within each study site. E.R., B.L., D.W.F., K.E.F., A.R.P., and K.V.L. performed sample extraction and 16S rRNA sequencing. K.E.M. and E.R. performed statistical analyses. S.V.L. and E.R. conceptualized and designed microbiological, immunological, and murine studies. E.R., J.H., V.F.M., and T.D.B. assisted with human sample collection. E.R. performed microbiological, immunological, and murine studies, A.R.P. and D.L.L. assisted with murine studies. K.E.M., E.R., and S.V.L. wrote the manuscript. D.R.O., C.C.J., C.M.S., H.A.B., and J.E.G. reviewed the manuscript and provided significant contributions toward its direction.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
L. jensenii L01	This study	L01
L. jensenii L02	This study	L02
L. iners	ATCC	[ATCC]: 55195
M. luteus Micro36	(Rackaityte et al., 2020)	Micro36
M. luteus	ATCC	[ATCC]: 4695
Biological samples		
Maternal vaginal samples at 36-week gestation	(Elisa et al., 2021; Seroogy et al., 2019)	N/A
Infant stool samples at 1 month (MAAP)	(Elisa et al., 2021)	N/A
Infant stool samples at 2 months (WISC)	(Seroogy et al., 2019)	N/A
Fetal meconium	(Rackaityte et al, 2020)	N/A
Chemicals, peptides, and recombinant proteins		
Human Fc blocking antibody	STEMCELL Technologies	Cat# 60012; RRID: AB_2722545
Foxp3/Transcription Factor Staining Buffer set	eBioscience	Cat# 00-5523-00
CD45 APC Mouse anti-human	Tonbo	Clone HI30; Cat# 20-0459; RRID: AB_2621579
HLA-DR APC-R700 Mouse anti-human	BD Biosciences	Clone G46-6; Cat# 565127; RRID: AB_2732055
CD3 biotin Mouse anti-human	eBioscience	Clone OKT3; Cat# 13-0037-82
CD19 biotin Mouse anti-human	BioLegend	Clone HIB19; Cat# 302204; RRID: AB_314234
CD20 biotin Mouse anti-human	Thermo Fisher Scientific	Clone 2H7; Cat# 13-0209-82; RRID: AB_657690
CD56 biotin Mouse anti-human	BD Biosciences	Clone NCAM16.2; Cat# 555515; RRID: AB_395905
Streptavidin conjugated to BV421	BD Biosciences	Cat# 563262; RRID: AB_2869478
Aqua LIVE/DEAD Fixable Dead Cell Stain Kit	Invitrogen	Cat# L34957
CD11c FITC Hamster anti-mouse	ThermoFisher	Clone N418; Cat# 11-0114-82; RRID: AB_464940
SiglecF PERCP-CY5.5 Rat anti-mouse	BD Biosciences	Clone E50-2440; Cat# 565526; RRID: AB_2739281
FoxP3 PE Rat anti-mouse	BD Biosciences	Clone MF23; Cat# 560414; RRID: AB_1645252
Ly6C PE-Cy7 Rat anti-mouse	BD Biosciences	Clone AL-21; Cat# 560593; RRID: AB_1727557
Ly6G APC-Cy7 Rat anti-mouse	BioLegend	Clone 1A8; Cat# 127624; RRID: AB_10640819
CD25 APC Rat anti-mouse	BD Biosciences	Clone PC61; Cat# 557192; RRID: AB_398623
CD19 Biotin Rat anti-mouse	ThermoFisher Scientific	Clone eBio1D3; Cat# 13-0193-82; RRID: AB_657656
CD8a Biotin Rat anti-mouse	BD Biosciences	Clone 53-6.7; Cat# 553028; RRID: AB_394566
CD11b BV605 Rat anti-mouse	BioLegend	Clone M1/70 Cat# 101257; RRID: AB_2565431
F4/80 BV650 Rat anti-mouse	BD Biosciences	Clone 6F12; Cat# 744338; RRID: AB_2742165
CD4 BV711 Rat anti-mouse	BD Biosciences	Clone RM4-5; Cat# 563726; RRID: AB_2738389
CD3 BUV395 Rat anti-mouse	BD Biosciences	Clone 17A2; Cat# 740268; RRID: AB_2687927
Mouse Fc blocking antibody	BD Biosciences	Cat# 553142; RRID: AB_394657
IL5 FITC Rat anti-mouse	Leinco	Clone TRFK5; Cat# I-1061; RRID: AB_2830394
TCRb PERCP-Cy5.5 Hamster anti-mouse	BioLegend	Clone H57-597; Cat# 109228; RRID: AB_1575173
IL-13 PE Rat anti-mouse	Thermo Fisher Scientific	Clone ebio 13A; Cat# 12-7133-41; RRID: AB_10852712
IFNg PE Rat anti-mouse	BD Biosciences	Clone XMG1.2; Cat# 557649; RRID: AB_396766
IL-4 APC Rat anti-mouse	BioLegend	Clone 11B11; Cat# 504106; RRID: AB_315320
IL17A PacBlue Rat anti-mouse	BioLegend	Clone TC11-18H10.1; Cat# 506926; RRID: AB_2632611
CD8 BV605 Rat anti-mouse	BD Biosciences	Clone 53-6.7; Cat# 563152; RRID: AB_2738030
Liquid brain heart infusion	TekNova	Cat# LM0028
Progesterone	Tocris Bioscience	Cat# 2835
Beta-estradiol	Tocris Bioscience	Cat# 2824

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Hexadecyltrimethylammonium bromide	Sigma-Aldrich	Cat# 52365
Polyethylene glycol 6000	Sigma-Aldrich	Cat# 81260-1KG
Lysis Matrix E tubes	MB Biomedicals	Cat# 6914-100
Qubit dsDNA HS Assay Kit	ThermoFisher Scientific, MA	Cat# Q32854
SequalPrep Normalization Plate Kit	Invitrogen	Cat# A1051001
Agencourt AMPure XP system	Beckman-Coulter	Cat# 75803-122
KAPA Library Quantification Kit	Kapa Biosystems	Cat# KK4873
NextSeq 500/550 High Output Reagent Kit (300 Cycles)	Illumina	Cat# FC-404-1004
PhiX Control v3 Library	Illumina	Cat# FC-110-3001
Punch Biopsy w/Plunger 4MM	Integra Miltex	Cat# 95039-102
Deposited data		
16S rRNA sequencing data	European Nucleotide Archive	[ENA]: PRJEB46659
Shotgun metagenomics data	European Nucleotide Archive	[ENA]: PRJEB46659
Isolate Genomes	NCBI	L01 under [NCBI]: PRJNA498338, L02 under [NCBI]: PRJNA498340
Code for Statistical Analysis	Zenodo	[Zenodo]: https://doi:10.5281/zenodo.5176780
Experimental models: Organisms/strains		
C57BL/6 Mice	Jackson Laboratories	Stock# 000664
House Dust Mite extract	Greer Laboratories	Cat# NC9756554
Oligonucleotides		
16S rRNA primer pair 515F/806R	ID Technology	Cat# 515F, 806R
16S rRNA primer pair 27F/1492R	ID Technology	Cat# 27F, 1492R
Software and algorithms		
R Console 3.6.2	(R Core Team, 2018)	https://www.r-project.org/
Divisive Amplicon Denoising Algorithm 2 (DADA2) v1.16 protocol	(Callahan et al., 2016)	https://benjjneb.github.io/dada2/index.html
SILVA v132	(Quast et al., 2013)	https://www.arb-silva.de/
Phangorn package	(Schliep, 2011)	https://cran.r-project.org/web/packages/phangorn/index.html
DECIPHER package	(Wright, 2016)	https://www.bioconductor.org/packages/release/bioc/html/DECIPHER.html
FASTQC	(Andrews, 2010)	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
bbTools v. 38.73	(Bushnell, 2019)	https://sourceforge.net/projects/bbmap/
GRCh38 reference genome	(Schneider et al., 2016)	https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.39
IDseq platform	(Kalantar et al., 2020)	https://idseq.net/
HUMAnN 3.0	(Beghini et al., 2021)	https://huttenhower.sph.harvard.edu/humann/
UniRef90 (January 2019)	(Suzek et al., 2015)	http://huttenhower.sph.harvard.edu/humann_data/
MetaPhlan (January 2019)	(Beghini et al., 2021)	http://huttenhower.sph.harvard.edu/humann_data/
Chocophlan (v296)	(Franzosa et al., 2019)	http://huttenhower.sph.harvard.edu/humann_data/
MetaCyc	(Caspi et al., 2018)	https://metacyc.org/
bowtie2 v2.4.2	(Langmead and Salzberg, 2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
DESeq2 package	(Love et al., 2014)	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
phyloseq package	(McMurdie and Holmes, 2013)	https://joey711.github.io/phyloseq/
ape v5.3 package	(Paradis and Schliep, 2019)	https://cran.r-project.org/web/packages/ape/index.html
vegan package	(Oksanen et al., 2016)	https://cran.r-project.org/web/packages/vegan/index.html
cluster v2.1.0 package	(Maechler et al., 2019)	https://cran.r-project.org/web/packages/cluster/index.html
RandomForests package v4.6.14	(Liaw and Wiener, 2002)	https://cran.r-project.org/web/packages/randomForest/index.html
ComplexHeatmap package (v2.2.0)	(Gu et al., 2016)	https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html
Clustal Omega	(Sievers et al., 2011)	http://www.clustal.org/omega/
SINA	(Pruesse et al., 2012)	https://www.arb-silva.de/aligner/sina-download/
MUMmer	(Kurtz et al., 2004)	http://mummer.sourceforge.net/
MetaSPAdes	(Nurk et al., 2017)	https://cab.spbu.ru/software/meta-spades/
TrimGalore	(Krueger et al., 2021)	https://github.com/FelixKrueger/TrimGalore
FLASh	(Magoč and Salzberg, 2011)	https://ccb.jhu.edu/software/FLASH/
SPAdes	(Bankevich et al., 2012)	https://cab.spbu.ru/software/spades/
QUAST	(Gurevich et al., 2013)	http://quast.sourceforge.net/quast
anvi'o	(Eren et al., 2015)	https://merenlab.org/software/anvio/
NCBI genome download tool	github.com/kblin/ncbi-genome-download	N/A
PyANI	(Pritchard et al., 2015)	widdowquinn.github.io/pyani/
MUSCLE	(Edgar, 2004)	https://www.drive5.com/muscle/
FastTree2	(Price et al., 2010)	http://www.microbesonline.org/fasttree/
iTOL	(Letunic and Bork, 2016)	https://itol.embl.de/
FACS Diva software	BD Biosciences	N/A

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Susan Lynch (susan.lynch@ucsf.edu).

Materials availability
Isolated strains are available from the lead contact, Susan Lynch (susan.lynch@ucsf.edu) with a materials transfer agreement through UCSF.

Data and code availability
- Raw amplicon and shotgun metagenomic sequences are available from the European Nucleotide Archive, and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. The participant data reported in this study cannot be deposited in a public repository as the studies are still ongoing. Access to confidential Children's Respiratory and Environmental Workgroup (CREW) data requires written authorization from the CREW study sponsor, The National Institutes of Health Environmental Influences on Child Health Outcomes Program, and a data request submitted to the lead contact.
- All original code is available on Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

Vaginal swabs collected during the 36-week Group-B *Streptococcus* screening clinical visit and infant stool sampled at 1 or 2 months of age were obtained from mother-infant dyads in two birth cohorts participating in CREW.[50] Stool samples from participants were balanced with respect to participant gender, with 93 males and 91 females. The Microbes, Allergy, Asthma, and Pets (MAAP) birth cohort recruited pregnant women from two hospital systems in Michigan and their offspring, half of whom lived in households with dogs and half of whom life in pet-free households. The study was established to understand how dogs in early life modify the child's risk for asthma.[51] The Wisconsin Infant Study Cohort (WISC) was established in rural Wisconsin and developed to understand the effects of farm exposure on viral respiratory infection and allergic disease. The study includes two arms: farm exposed versus non-farm exposed.[52] A previously established human fetal meconium cryobank was also utilized[11]; no human patient information is associated with these specimens.

The study protocol was approved by the local institutional review board of each birth cohort. Written informed consent or parent's/guardian's permission was obtained along with child assent as appropriate, for CREW Protocol participation and for participation in specific cohorts.

Animals

UCSF IACUC approved and provided ethical oversight for *in vivo* murine studies under protocol number AN171803-02. Six-week-old female C57BL/6 mice were obtained from Jackson Laboratories and were cohoused for 2–3 weeks before treatment to minimize cage effects. Mice were randomly assigned to treatment groups, and all experiments were performed with 3–5 mice per cage. To avoid genetic drift, mice were not bred at our facility, obtained directly from the supplier for each experiment. Mice were purchased for this experiment only and were not pretreated or used for any other purpose. Treatment groups were housed in independent cages to mitigate potential effects of cohousing and coprophagy, and cages were kept in the same rack and handled identically to minimize microbial variation.[53]

Microbe strains

Twenty-four-hour cultures of single colonies (L01, L02, and *L. iners*) were grown in MRS liquid media at 37°C in microaerophilic conditions. Strains of *Micrococcus luteus* (ATCC 4698 and Micro36) were grown in BHI liquid media at 37°C in microaerophilic conditions.

METHOD DETAILS

Sample collection

Maternal vaginal samples were collected from WISC (n = 147) and MAAP (n = 37) participants during routine Group B *Streptococcus* (GBS) screening clinical visits at 35-37 weeks of gestation by trained Obstetrics and Gynecology physicians. In MAAP, obstetricians collected samples using standard procedures for GBS screening, involving swabbing of both the vaginal tract and rectum. After collection, swabs were placed directly into RNAlater and kept at 4°C for 24 hours, to allow nucleic acid stabilization. Samples were then stored in a −80°C freezer until shipment to the University of California, San Francisco (UCSF) for sequence-based analyses. In WISC, vaginal swabs were collected using a Copan ESwab (Copan Diagnostics, Corona, CA) and similar technique. Immediately after collection, swabs were placed directly into Liquid Ames media, pressing the swab against the tube wall multiple times for 20 seconds. The tube was stored at room temperature until pickup by the research coordinator, within 2 hours of collection. After pickup, samples were catalogued and stored at −80°C.

Stool samples from WISC infants were collected at the 2-month home visit. Samples were obtained by the caregiver less than 24 hours before the arrival of the research coordinator for the home visit. Samples were collected by placing sterile 4" x 4" gauze pads into the diaper; once a bowel movement occurred, caregivers were instructed to store the soiled diaper and gauze in a refrigerator until the arrival of the study coordinator. In MAAP, stool samples were collected at one month of age. Caregivers were provided with 2 biohazard bags, and an ice pack, instructed to double-bag the soiled diaper and to place it with the frozen ice pack inside an insulated mailer, which was then sent via overnight delivery to the study center. Upon arrival, samples were catalogued, and stored at −80°C until shipped to UCSF for processing.

Donated human fetal tissues were obtained under the auspices of the UCSF Committee on Human Research–approved protocols after written informed consent from the Department of Obstetrics, Gynecology and Reproductive Science. All sample collection methods comply with the Helsinki Declaration principles, and no patient health information is associated with these specimens. Samples were transported in medium on ice and processed within 2 h after collection. A previously established fetal meconium bank was utilized and no new meconium specimens were obtained for this study: briefly, internal contents of human fetal intestines were aseptically collected and cryopreserved.[11] Cells isolated from spleens were used for *in vitro* studies, as previously described.[11]

Isolation of Lactobacillus *from fetal meconium*

Punch biopsies were taken from three samples of cryopreserved human fetal meconium using a sterile surgical punch biopsy tool (Integra Miltex) in a biosafety class II cabinet. Two independent fetal meconium samples were used for bacterial isolation. Punch biopsies were incubated in pre-reduced liquid brain heart infusion (BHI, TekNova) with 5% defibrinated sheep blood supplemented with 1×10^{-5} M progesterone and 1×10^{-6} M 17β-estradiol (Tocris Bioscience; reconstituted in ethanol) for 48 h at 37°C in an anaerobic chamber under stationary culture conditions. Liquid cultures were streaked onto BHI agar plates with 5% defibrinated sheep blood agar plates to permit individual colonies to be picked and sequenced to assign identity. Colony sequencing (Quintara Biosciences) was performed using the full-length 16S rRNA gene using primer pairs 27 F and 1492 R.[54] The full-length 16S rRNA gene was assembled using Clustal Omega, and taxonomy was determined by SINA[55] against the curated SILVA database. Additional control strains utilized in our studies were obtained from ATCC (vaginally isolated *Lactobacillus iners* ATCC# 55195 and *Micrococcus luteus* ATCC# 4698) or isolated from our previous studies of fetal meconium (*M. luteus* Micro36 [11]).

DNA extraction

Vaginal and stool samples were processed at UCSF. Two sub-samples (approximately 0.25 g each) per frozen stool (maintained on dry ice during sub-sampling) were obtained under aseptic conditions in a biosafety cabinet using a sterile punch biopsy prior to being pooled and added to 500 μL of cetyltrimethylammonium bromide (CTAB) extraction buffer (5% CTAB in 0.25 M phosphate buffer and 1M NaCl). Vaginal samples were vortexed with the swab remaining in the tube; 500 μL of transport medium was withdrawn from the tube and added to 500 uL of CTAB extraction buffer. DNA from all samples and several extraction blanks were extracted using a modified CTAB–polyethylene glycol (PEG) protocol as previously described.[4,56] Briefly, cells were lysed by bead-beating in Lysis Matrix E tubes (MB Biomedicals) at 5.5 m/s for 30 seconds, phases were separated by centrifugation at 16,000 *g* for 5 min, and the aqueous layer transferred to a new tube. To improve extraction efficiency, a secondary extraction was performed via addition of 500 μL CTAB to the original extraction tube, bead-beating and phase separation, after which the aqueous layer was mixed with that from the previous extraction. To remove excess phenol, the pooled aqueous layers from both extractions were extracted by vortexing in an equal volume of chloroform followed by centrifugation at 16,000 *g* for 5 min. The resulting aqueous phase was added to 1mL of PEG precipitating solution (30% PEG 6000 in 1.6M NaCl) and stored overnight at 4°C. Precipitated DNA was recovered by centrifugation for 60 min at 3,000*g*. DNA pellets were washed twice with 300μL 70% ethanol, air-dried for 10 minutes in a biosafety cabinet, and resuspended in 50 μL of molecular grade water. DNA concentrations were quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, MA), diluted to 10 ng/μL^{-1} and stored at −20°C.

16S rRNA V4 amplicon library preparation and sequencing

The variable region 4 (V4) of the 16S rRNA gene was amplified in triplicate reactions per sample using 515F/806R primers and PCR conditions previously described.[57] Pooled amplicon reactions with $\geq$ 5 ng were purified using the SequalPrep Normalization Plate Kit (ThermoFisher Scientific, MA) according to the manufacturer's specifications, quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, MA), and pooled at equimolar concentrations. The amplicon library was concentrated using the Agencourt AMPure XP system (Beckman-Coulter), quantified using the KAPA Library Quantification Kit (APA Biosystems), and diluted to 2 nM. Equimolar PhiX was added at 40% final volume to the amplicon library followed by sequencing on the Illumina NextSeq 500 Plat-form employing a 2 × 150 bp sequencing run.

WGS of fetal Lactobacillus *isolates*

For whole-genome sequencing, 24-hour cultures of single colonies (L01 and L02) were grown in MRS liquid media at 37°C in micro-aerophilic conditions, and DNA was extracted using the CTAB-based protocol described above. gDNA was fragmented and Illumina adaptors were ligated using a Nextera XT (Illumina) kit following manufacturer's instructions. gDNA library quality was verified by Bioanalyzer (Agilent) and was sequenced on an Illumina MiSeq using a MiSeq Reagent kit v3 (Illumina) with 300 × 300 bp paired-end reads.

Metagenomic sample preparation

DNA remaining from the initial extraction protocol was quantified and provided to Omega Bioservices (Norcross, GA) for library preparation and shotgun metagenomic sequencing. In total, 24 samples from 12 dyads had more than 100 ng of high-quality DNA remaining. These samples underwent sequencing with a target read depth of 50M reads per sample.

In vitro APC activation with bacterial isolates

Human fetal spleen cells were isolated by a 30-min digestion with freshly prepared medium in 1 mg mL^{-1} collagenase IV (Gibco) and 10 mg mL^{-1} DNAse (Roche) in complete RPMI [RPMI medium (GIBCO) without antibiotics, 10% FBS (GIBCO), 1 mM sodium pyruvate (Life Technologies), 2 mM L-glutamine (Life Technologies), 1× nonessential amino acids (Life Technologies) and 10 mM HEPES (Life Technologies)].

Digested splenic cells were gently pressed through a 70-μm strainer, and cells were separated in a 20%:40%:80% Percoll density gradient at 400g for 40 min; APCs were recovered at the 20–40% interface. All cells were washed twice with cRPMI medium. Viability was measured with Aqua dye (Invitrogen) using flow cytometry. APCs from human fetal spleen were enriched by positive selection

using Easy Step Human Biotin Isolation kit (STEMCELL Technologies) and the biotinylated human HLA-DR monoclonal antibody. Cells were seeded into 96-well plates and incubated with a multiplicity of infection (MOI) of 10 bacterial cells in antibiotic-free cRPMI for 4 h at 37°C with 5% CO_2 and 4% O_2 to mimic hypoxic conditions in the fetal intestine[58] and normalize for bacterial growth. Cells were harvested by fixation and stained for flow cytometry as described below.

Animal model of allergic airway sensitization

Mice were orally gavaged daily three days prior to house dust mite sensitization with either sterile PBS or 10^7 CFU of the *Lactobacillus jensenii* L01 or *M. luteus* Micro36 fetal meconium isolates from overnight liquid cultures prepared as described above. Oral gavage of PBS or 10^7 CFU of L01 or Micro36 was continued on days 1, 3, 5, 7–11 and 13 throughout the course of house dust mite sensitization and challenge. Mice were intratracheally sensitized and challenged with house dust mite as previously described.[59] Briefly, on days 0 and 7–11, naïve mice were anesthetized with isoflurane and administered 10 μg HDM extract *(Dermatophagoides pteronyssinus*, Greer Laboratories) by oropharyngeal aspiration. On day 14, mice were euthanized, and lungs, mediastinal lymph nodes (medLNs), spleen, and mesenteric lymph nodes (MLN) were collected for flow cytometry.

Antibodies and flow cytometry

Extracellular staining of isolated cells was performed in 2% FBS in PBS with 1mM EDTA (staining buffer) with human Fc blocking antibody (STEMCELL Technologies) and with fluorochrome-conjugated antibodies, as previously described.[11] Intracellular proteins were detected in fixed, permeabilized cells using the Foxp3/Transcription Factor Staining Buffer set (Tonbo Biosciences). Mouse anti-human monoclonal antibodies used in this study: CD45 APC (Clone HI30, Tonbo Cat. No. 20-0459, 1:100), HLA-DR APC-R700 (Clone G46-6, BD Cat. No. 565127, dilution 1:100), CD3 biotin (Clone OKT3, eBioscience Cat. No. 13-0037-82, dilution 1:100), CD19 biotin (Clone HIB19, BioLegend Cat. No. 203304, dilution 1:100), CD20 biotin (Clone 2H7, eBioscience Cat. No. 13-0209-82, dilution 1:100), CD56 biotin (Clone NCAM16.2, BD Cat. No. 555515, dilution 1:100). Rat anti-mouse, mouse anti-human, and hamster anti-mouse antibodies are listed in the key resources table. Biotin antibodies were detected with streptavidin conjugated to BV421 (BD Biosciences Cat. No. 563262, 1:200). Dead cells were excluded from analysis using Aqua LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) stain. All data were acquired with BD LSR/Fortessa Dual SORP using FACS Diva software (BD Biosciences) and analyzed with FlowJo (TreeStar) software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Immunoglobulin E (IgE) quantification

Infant serum total IgE levels were generated within the WISC[52] and MAAP[60] studies as previously described. Specific IgE for foods was available from WISC participants (milk, egg, peanut). Too few participants (n = 3) had assessments for aeroallergens (cat, dog, *Alternaria*, and dust mite), thus these measurements were not included in the analysis.

16S rRNA V4 sequence data processing and quality control

For-ward and reverse paired-end reads were demultiplexed using QIIME1 v1.9.1.[61] Samples from both studies were sequenced across several runs, and initial steps were completed on each sequencing run individually, as recommended by the Divisive Amplicon Denoising Algorithm 2 (DADA2) v1.16 protocol[62] in R with the following modifications: Reads were maintained if they exhibited a maximum expected error of 2 and a read length of at least 150 bp using the *filterAndTrim* function in the *dada2* package.[62] Reads were dereplicated and errors were learned on 1×10^8 reads, from samples chosen at random. Finally, chimeras were identified using the "consensus" method. Paired reads were merged with a minimum overlap of 25 bp, and reads were aggregated into a table of counts. Any V4 sequences abnormally short or long (±5 bp from the most frequently observed bp length; here: 253 bp) were also removed. We assigned taxonomic classifications to Sequence Variants (SVs) using *assignTaxonomy* in the *dada2* package and an 80% bootstrap cutoff, utilizing the SILVA v132 database,[63] and species identification with *assignSpecies* at 100% identity. All species achieving an exact match were recorded, and the first in the list was used for descriptive purposes. Once these steps were completed for each run, all runs were combined into a complete SV table. A phylogenetic tree was constructed using *phangorn*[64,65] and *DECIPHER* packages.[66]

The SV table was then filtered only to variants belonging to the kingdom Bacteria. Variants were also removed if they were present in less than 0.001% of the total number of observed sequence reads. Next, we employed methods to remove potential contaminants based on SVs present in negative controls. Specifically, SVs were removed if they were present in greater than 15% of the negative controls and less than 15% of the samples (primarily *Pseudomonas* SVs). For the remaining sequence variants in negative controls, the mean of the read count for each was calculated, rounded upward to the nearest whole number and subtracted for each of these SVs in the dataset.

Metagenomic sequence data processing

Raw metagenomic sequences across multiple lanes were merged into a concatenated file. Raw FASTQ files underwent FASTQC[67] and quality and contaminant filtering using *bbTools* v38.73.[68] Specifically, *bbduk* trimmed Illumina adapters, removed any PhiX contamination, filtered low-quality sequences, and employed trimming after a Q score less than 15 from both the 3 and 5′ directions.

Finally, *bbmap* removed reads mapping to the human genome using GRCh38[69] as the reference database. In parallel, raw sequence reads were analyzed using the open source, cloud-based IDseq platform, as previously described.[24]

Conserved functional feature profiling
After quality control using *bbtools*, HUMAnN 3.0[70] was used to identify functional features of the maternal vaginal and infant stool microbiota, using the January 2019 release of UniRef90, MetaPhlan, and Chocophlan (v296). Once UniRef labels were assigned, data was normalized to copies per million and UniRef90 IDs were converted into MetaCyc reactions. Pathway information was obtained manually from MetaCyc (metacyc.org).

Metagenomic shared read identification
In a dyad-specific manner, sequence reads from the vaginal sample were used as a reference to which the child's reads were mapped using *bowtie2*[71] v2.4.2 with no mis-matches (−N 0). Subsequently, sequence reads from infant stool samples that represented an exact match to one or more maternal vaginal reads were then processed using HUMAnN 3.0 as described above.

De novo *contig assembly*
Quality- and human-filtered reads underwent sample-specific assembly using the metaSPAdes metagenome assembler,[72] and genome assembly quality was ascertained though QUAST.[73] In order to identify regions of contigs that overlapped between mother-infant dyad paired samples, contig similarity was calculated using MUMmer.[74] Regions of overlapping contigs were retained if the overlap was at least 1 kb and they possessed at least 97% identity to each other. Resulting regions were visualized using Complex Heatmap.[75]

Assembly of fetal *Lactobacillus* isolate genomes
Reads were removed from adaptors and quality filtered using TrimGalore. When possible, paired-end reads were assembled using FLASh[76] for use as a single-ended library for assembly using SPAdes[77] genome assembler. Genome assembly quality was determined by QUAST[73] and genomes were submitted to the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline. Annotation was performed locally using NCBI COG database in the *anvi'o* package.[78]

Comparative genomics
Lactobacillus genomes were downloaded from NCBI using the NCBI genome download tool (github.com/kblin/ncbi-genome-download) and imported into anvi'o pangenome analysis environment. Contigs from mother-infant dyad metagenomic data were generated using IDseq. Average nucleotide identity and coverage was calculated using ANIb within the *pyani* package[79] (widdowquinn.github.io/pyani/). Single-copy genes[80] identified for all relevant genomes within anvi'o environment, aligned using MUSCLE.[81] Phylogenetic trees were constructed using FastTree2[82] and visualized in iTOL.[83]

Statistical analysis
Individual-level epidemiologic variables from MAAP and WISC were harmonized by the CREW Informatics Core (Madison, WI). These data included environmental exposures such as livestock/pet ownership, mode of delivery, maternal health status (including maternal self-reported allergic sensitization and doctor-diagnosed asthma status), as well as infant demographic and health data (race, early life diet, IgE levels and early life parental report of wheeze).

All statistical analyses were completed in R v3.6.2. Following negative control filtering, the SV table was normalized using the Variance Stabilized Transformation[84] through the *DESeq2* package[85] before being used for downstream analyses. Maternal vaginal microbiota clusters (VCs) were classified based on the SV with the greatest proportion of reads within each sample (dominant sequence variant). Several distance matrices [Weighted and Unweighted UniFrac,[86] Bray-Curtis, and Canberra] were generated using the *distance* function from *phyloseq* v1.30.0[87] and ordinated into two-dimensional space using the *pcoa* function from the *ape* v5.3 package.[88] Permutational Analysis of Variance tests (PERMANOVA; R^2 and p values) were generated using *adonis2* from the *vegan* package v2.5-6.[89] Hierarchical clustering of the Bray-Curtis distance matrix using the *hclust* function and the *Ward.D2* method was used to determine if compositionally distinct vaginal microbiota existed. The average silhouette statistic was calculated with the *silhouette* function from the *cluster* v2.1.0 package[90] for between 2 and 18 possible clusters. The number of VCs achieving the highest silhouette statistic was used for downstream analysis.

We note that *adonis2* from the *vegan* package is more sensitive to differences in dispersion than unequal sample sizes. Dispersion estimates were calculated using the *betadisper* function in *vegan* for selected variables, and no significant differences were noted (Pdisper >0.4). To test the sensitivity of *adonis2*'s implementation of PERMANOVA, we focused on unbalanced variables and randomly sampled values without replacement 100 times and recalculated the test statistic and p-value. If the results were sensitive to the unequal sample sizes, we would expect to see findings as significant as our test statistic at least 5% of the time in a simulation where no significance is expected. For all variables tested (cohort ID, child's IgE in MAAP, infant milk IgE positivity, and maternal consumption of hot dogs), only 1% of test statistics from randomized values were as significant as our reported p-value, suggesting that these PERMANOVA results are robust to the unequal sample sizes.

To relate VCs with environmental exposures, chi-square tests were used for factor variables and generalized linear models were used to determine differences across clusters, using a binomial model for two-level variables and a Gaussian model for continuous variables. All variables with at least 5 observations per group or 5 unique values were included. False-discovery corrections were made using the Benjamini-Hochberg method.

Variants were determined to be shared between maternal vaginal samples and infant stool if they were present with greater than 10 VST-transformed reads in both sample types for each dyad. Relationships between phylogenetically related microbiota and environmental features were determined using an unweighted UniFrac distance matrix of mother-infant dyads' shared microbiota and PERMANOVA using *adonis2* and the by = "margin" option, indicating a Type III Sum of Squares–like test.

Metagenomic profiles were normalized by copies per million, and Bray-Curtis and Binary (presence/absence) distance matrices were calculated using *phyloseq*. Variables were related to this functional data using *adonis2* and a P-value threshold of 0.05. For analysis of metagenomic data, total IgE was dichotomized into below normal range (<2 kU/L) and detectable (range: 4.5–14 kU/L), as previously described[91] due to sample size limitations (n = 12 dyads). Random forests analysis from the *RandomForests* package (v4.6.14) was used to identify IgE-discriminatory MetaCyc reactions using classification. Reactions with a Mean Decrease in Accuracy greater than 1 were extracted and plotted in a heatmap using the *ComplexHeatmap* package (v2.2.0).