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POPULATION STUDY ARTICLE

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A prospective study of the infant gut microbiome in relation to vaccine response

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BACKGROUND: The establishment of the gut microbiome plays a key symbiotic role in the developing immune system; however, its influence on vaccine response is yet uncertain. We prospectively investigated the composition and diversity of the early-life gut microbiome in relation to infant antibody response to two routinely administered vaccines.
METHODS: Eighty-three infants enrolled in the New Hampshire Birth Cohort Study were included in the analysis. We collected blood samples at 12 months of age and assayed the isolated serum to quantify total IgG and measured antibody to pneumococcal capsular polysaccharide and tetanus toxoid. Stool samples were collected from infants at 6 weeks of age and sequenced using 16S rRNA, and a subset of 61 samples were sequenced using shotgun metagenomics sequencing.
RESULTS: We observed differences in beta diversity for 16S 6-week stool microbiota and pneumococcal and tetanus IgG antibody responses. Metagenomics analyses identified species and metabolic pathways in 6-week stool associated with tetanus antibody response, in particular, negative associations with the relative abundance of *Aeriscardovia aeriphila* species and positive associations with the relative abundance of species associated with CDP-diacylglycerol biosynthesis pathways.
CONCLUSIONS: The early gut microbiome composition may influence an infant's vaccine response.

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IMPACT:

- Early intestinal microbiome acquisition plays a critical role in immune maturation and in both adaptive and innate immune response in infancy.
- We identified associations between early life microbiome composition and response to two routinely administered vaccines—pneumococcal capsular polysaccharide and tetanus toxoid—measured at approximately 1 year of age.
- Our findings highlight the potential impact of the gut microbiome on infant immune response that may open up opportunities for future interventions.

INTRODUCTION

Vaccines reduce infant mortality and morbidity from infections worldwide.[1] The World Health Organization and the US American Association of Pediatrics guidelines outline a standard schedule of immunizations for infants to provide protection against potentially fatal infectious diseases. Rates of vaccine administration vary globally.[2] Among children born in the US in 2016 and 2017, approximately 99% of infants received vaccines, with only 1.2% not receiving any vaccines by 24 months of age.[3] However, immune responses to vaccinations vary by host with reported factors being sociodemographic characteristics, perinatal exposures, breast or formula feeding, antibiotic use, and variation in timing and other characteristics of the vaccination itself.[4]

The early establishment of infant gut microbiome is now known to play an essential role in the development of the immune system.[5,6] Growing evidence points to the impact of the gut microbiome on immune response to vaccination, including among infants treated with antibiotics.[7–11] For example, recent studies examined the relation between infant gut microbiota and response to oral poliovirus, bacille Calmette–Guérin, tetanus toxoid (TT), hepatitis B virus, and rotavirus vaccines among infants living in Bangladesh, Ghana, and Pakistan.[12–14] These studies identified specific bacterial taxa in the intestinal microbiome associated with differential vaccine response and microbial compositions in high vaccine responders to be similar to those of healthy infants from high-income countries.[13,14] Prospective studies are lacking in common vaccine response in relationship to the very earliest development of the intestinal microbiome. To gain a better understanding of the role of the infant gut microbiome on vaccine response, we investigated the association between the early infant gut microbiome composition and antibody response to two common vaccines administered in the

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first year of life—pneumococcal capsid protein and TT—at 1 year of age in the New Hampshire Birth Cohort Study (NHBCS).

METHOD

Study population

The current study included participants in the NHBCS who had stool samples collected at approximately 6 weeks of age for microbiome analyses and infant blood samples at approximately 12 months of age for vaccine response assays. The NHBCS comprised of pregnant women aged 18–45 with a singleton pregnancy who received care at prenatal clinics in New Hampshire, USA as described previously.[15] We collected longitudinal survey data for maternal and infant lifestyles, and we ascertained infant birth characteristics and vaccine information from delivery and pediatric medical records. The Committee for the Protection of Human Subjects at Dartmouth College approved all protocols, and informed consent was obtained from all participants upon enrollment.

Blood sample collection, serum isolation, and assaying

Infant blood samples were collected from well-child visits scheduled at approximately 12 months of age post-partum. Serum was isolated from the blood samples, frozen at −80 °C, and shipped to Stanford University in Palo Alto, California for serological analysis. Antibody responses to pneumococcus capsule-based vaccines were measured using VaccZyme Anti-PCP IgG Enzyme Immunoassay kit (Binding Site, Birmingham, UK). Antibody responses to TT-based vaccines were measured using Tetanus Toxoid IgG ELISA (Genway Biotech, San Diego, CA).

Stool sample collection, DNA extraction, sequencing, and profiling

Infant stools samples were collected at approximately 6 weeks of age as described previously.[16,17] These samples were aliquoted and frozen at −80 °C, and DNA was extracted from thawed samples using Zymo DNA extraction kit (Zymo Research, Irvine, CA). OD260/280 nanodrop was used to measure sample quality and purity. Samples were sent to Marine Biological Laboratory in Woods Hole, MA for bacterial 16S rRNA gene sequencing of the V4–V5 hypervariable region using Illumina MiSeq (Illumina, San Diego, CA). We conducted quality-control measures internally by amplifying in triplicate with one negative control as described previously.[17] We inferred amplicon sequence variants (ASVs) using DADA2[18] and assigned taxonomies using the SILVA database.[19] A subset of stool samples also underwent shotgun metagenomics sequencing using Illumina NextSeq (Illumina, San Diego, CA) as previously described.[20] DNA samples were extracted and sheared to a mean insert size of 400 bp using a Covaris S220 focused ultrasonicator, and sequencing libraries were constructed with Nugen's Ovation Ultralow V2 protocol. We merged and trimmed DNA reads using KneadData,[21] inferred taxonomic profiles at the species-level using Metaphlan3,[22] and profiled metabolic pathways using HUMAnN3.0.[22]

Statistical analysis

We examined the association between the stool microbiome and antibody response to pneumococcal capsular polysaccharide (PCP) and TT. Using the 16S data, we aggregated ASVs to genera and calculated beta diversity using Bray–Curtis dissimilarity. We used permutational multivariate analysis of variance (PERMANOVA) to test the differences between groups. Using the metagenomics data, we $\log_2$-transformed the relative abundance of bacterial species and metabolic pathways. We removed species that were present in <10% of subjects and performed linear regression on all remaining taxa. We further investigated metabolic pathways by removing pathways that were present in <10% of subjects and standardizing the relative abundance of pathways. We used elastic net to select pathways with possible associations with vaccine response. We then conducted linear regression on each elastic-net selected pathway with false discovery rate (FDR) correction.

We applied a FDR threshold of 0.1 to adjust for multiple testing. Factors associated with both the gut microbiome and vaccine response were considered potential confounders and included in all analyses, including infant birth weight (g), breast feeding at 6 weeks (exclusively breast fed/ever formula fed), and maternal pre-pregnancy body mass index (BMI; kg/m^2). Since delivery mode is not associated with vaccine response, it was not considered a potential confounder. For missing confounding data, we assumed entries were missing at random and used multiple imputation by chained equations and the predictive mean matching method to impute missing data in a randomly chosen iteration. All analyses were performed in R version 3.4.3, using functions *diversity*, *vegdist*, *ade4*, *adonis*, *mice*, and *cv.glmnet* in packages "vegan," "mice," and "glmnet."

We also performed a sensitivity analysis of 65 infants in whom we verified that they had received at least one dose of pneumococcal conjugate vaccine (PCV) 13 or diphtheria, tetanus, and acellular pertussis vaccine (DTaP) in their pediatric medical records.

Table 1. Selected baseline characteristics of infants and mothers in the New Hampshire Birth Cohort Study ($N = 83$).

Variable	Sample size	No. (%) or mean (SD)
Infant characteristics		
Sex, no. (%)	83	
Female		38 (45.8%)
Male		48 (54.2%)
Delivery mode, no. (%)	83	
Vaginal		65 (78.3)
Cesarean		18 (21.7)
Breast feeding at 6 weeks, no. (%)	73	
Exclusively breast fed		39 (53.4)
Fed any formula		34 (46.6)
Breast feeding at 1 year, no. (%)	73	
Exclusively breast fed		19 (26.0)
Fed any formula		54 (74.0)
Daycare (ever in first year of life), no. (%)	63	
Yes		35 (55.6)
No		28 (44.4)
Birth weight (g), mean (SD)	82	3342 (558)
Received PCV13 vaccine, no. (%)	66	
At least one dose recorded in medical record		65 (98.5)
None		1 (1.5)
Received DTaP vaccine, no. (%)	66	
At least one dose recorded in medical record		65 (98.5)
None		1 (1.5)
PCP vaccine response (mg/L), mean (SD)	80	30.4 (22.5)
TT vaccine response (IU/mL), mean (SD)	68	0.932 (0.759)
Maternal characteristics		
Maternal pre-pregnancy BMI (kg/m^2), mean (SD)	83	26.1 (5.7)
Parity, No. (%)	83	
0		44 (53.0)
1+		39 (47.0)

RESULTS

Baseline characteristics

We measured vaccine response in 155 study infants for PCP and 133 subjects for TT. Of those with PCP vaccine response data, 80 stool samples were analyzed by 16S and 59 by metagenomics sequencing. For TT response, 68 infants had 16S data and 53 had metagenomics sequencing data. Our study group had a roughly

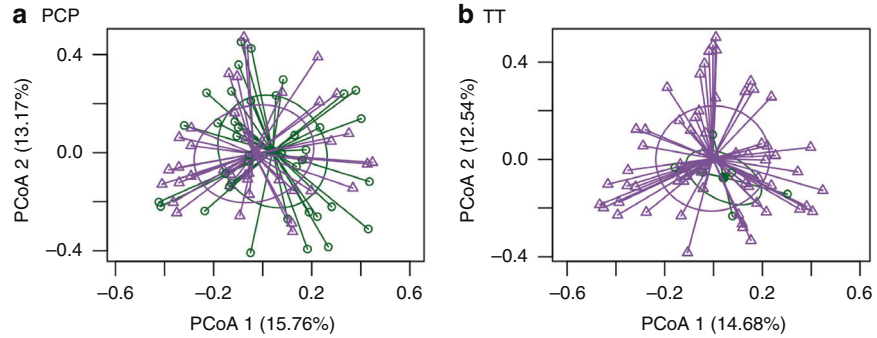

Fig. 1 PCoA plots of bacterial 16S V4-V5 rRNA sequencing Bray–Curtis dissimilarity for PCP and TT. Percentages on the *X* and *Y* axis of plots represent percentage of variance explained by first two eigenvectors. **a** Differences between PCP groups assigned by median PCP IgG concentration threshold. **b** Differences between TT groups assgined by preferred protection threshold of 0.1 IU/mL.

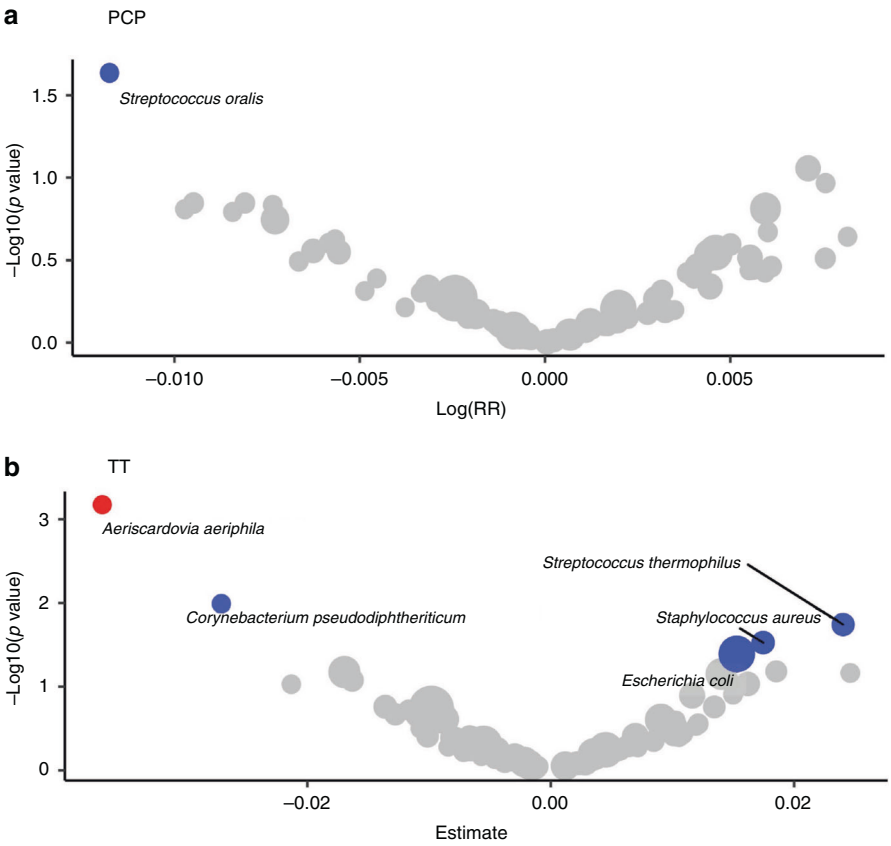

Fig. 2 Associations between metagenomics bacterial species and vaccine response. Dots indicate bacterial species, and size of dots vary by mean abundance. Blue indicates species with *p* value <0.05. Red indicates species with *p* values <0.05 and meet FDR correction. **a** Volcano plot of associations between species and PCP response. **b** Volcano plot of associations between species and TT response.

equal distribution of male (54.2%) and female (45.8%) infants (Table 1). Nearly 80% of subjects were delivered vaginally, and roughly half (53.4%) had been exclusively breast fed at 6 weeks of life (Table 1). Mean infant birth weight was 3342 g and mean maternal pre-pregnancy BMI was 26.1 (Table 1). The mean PCP antibody response was 30.4 mg/L (SD = 22.5), and the mean TT antibody response was 0.932 IU/mL (SD = 0.759). The 80 infants in our study who had both pneumococcal vaccine response measurements and 16S sequence data all had pneumococcal vaccine response measurements above the preferred PCP protection threshold of 0.2 mg/L.[23] Of the 68 infants who had tetanus vaccine response measurements and 16S data, 6 subjects (8.8%) had measurements below the preferred TT protection threshold of 0.1 IU/mL (Supplementary Fig. 1).[24]

16S V4-V5 rRNA gene: beta diversity

In PERMANOVA analyses of pair-wise community composition, we observed a weak association between PCP antibody response at or below versus above the median (PERMANOVA $p = 0.112$; Fig. 1a). We further observed a borderline statistically significant difference in beta diversity for TT antibody response at or below the TT protection threshold and above the threshold (PERMANOVA $p = 0.065$; Fig. 1b).

Metagenomics: species and pathways

Streptococcus oralis species was inversely associated with PCP antibody response although the association did not meet FDR threshold (Fig. 2). *Aeriscardovia aeriphila* was inversely associated with TT antibody response, whereas *Staphylococcus aureus*,

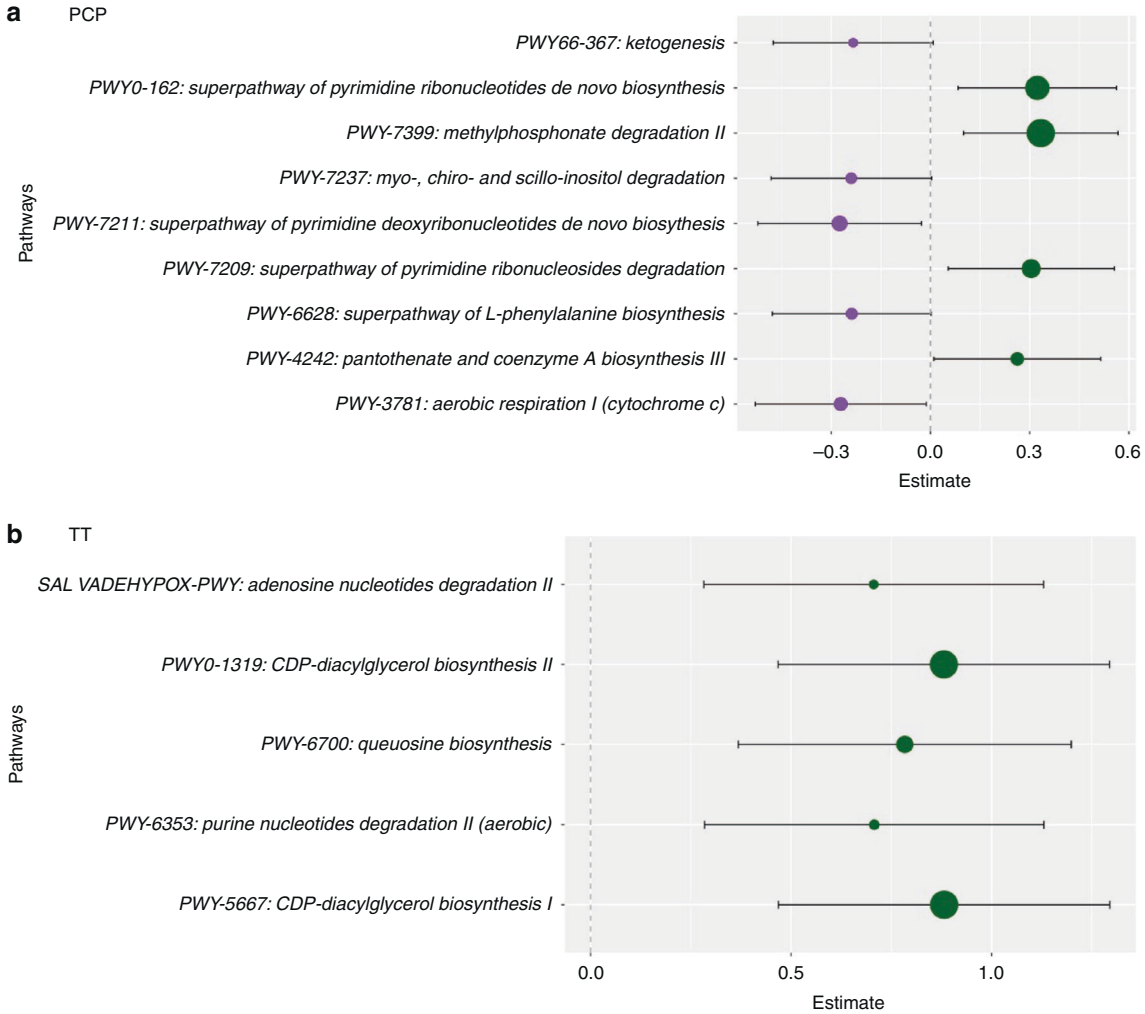

Fig. 3 Associations between elastic-net and metabolic pathways and vaccine response. Dots indicate effect size, and horizontal bands indicate 95% CI. Green dots represent positive association, while purple dots represent negative association. Size of dots vary by *p* value: larger dot indicates smaller *p* value. Only pathways selected by elastic net and FDR correction shown here. **a** Metabolic pathways associated with PCP response. **b** Metabolic pathways associated with TT response.

Escherichia coli, *Streptococcus thermophilus*, and *Anaerococcus vaginalis* were positively associated with TT antibody response. Of these, only *Aeriscardovia aeriphila* remained statistically significant after FDR correction (Fig. 2 and Supplementary Table 1).

Several metabolic pathways were found to be associated with vaccine response. The nine pathways associated with lower PCP vaccine response included higher abundance of taxa related to aerobic respiration I (cytochrome c), superpathway of L-phenylalanine biosynthesis, superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis, myo-, chiro-, and scillo-inositol degradation, and ketogenesis (Fig. 3a and Supplementary Table 2). In contrast, higher abundance of taxa related to pantothenate and coenzyme A biosynthesis III, superpathway of pyrimidine ribonucleosides degradation, methylphosphonate degradation II, and superpathway of pyrimidine ribonucleotides de novo biosynthesis were associated with higher PCP antibody response (Fig. 3a and Supplementary Table 2). Five pathways were positively associated with TT antibody response, including CDP-diacylglycerol biosynthesis I, purine nucleotides degradation II (aerobic), queuosine biosynthesis, CDP-diacylglycerol biosynthesis II, and adenosine nucleotides degradation II (Fig. 3b and Supplementary Table 2). No inverse associations were observed for TT.

Our sensitivity analyses of infants with medical record confirmation of vaccination revealed similar results. Results are provided in Supplementary Figs. 2–4 for beta diversity, bacterial species, and metabolic pathways, respectively.

DISCUSSION

In a prospective study of the infant gut microbiome using both 16S and metagenomic sequencing, we observed differences in infant gut microbiome composition at 6 weeks of age in relation to pneumococcal conjugate and TT vaccines at 1 year of age. Microbial community structure (beta diversity) was associated with both PCP and TT antibody response although with limited statistical power. In analyses of individual bacterial species, associations were observed with decreased antibody response to TT including *Aeriscardovia aeriphila* after correction for multiple comparisons. Pathway analyses indicated several potential mechanisms by which microbial metabolites might influence vaccine response especially those related to CDP-diacylglycerol biosynthesis.

Guidelines by the AAP in the US state that children under 2 years of age should receive three doses of PCV13 that provide protection against 13 strains of *Streptococcus pneumoniae*.[25] These microbes are responsible for the majority of invasive bacterial infections in infants and young children, including otitis media infections, resulting in significant morbidity, antibiotic exposure,

and at times difficulty with hearing.[26] Streptococcus also results in pneumonia, "pink eye," and other infectious illnesses in children 2 months and older, hindering daycare attendance, and with consequent financial loss to working parents. Following PCV, antibodies to PCP antigens conjugated to a carrier protein are induced by both T cell and B cell responses.[27] Tetanus is a life-threatening infection that is caused by *Clostridium tetani* and is preventable by a toxoid vaccine. It is part of several combination vaccines including DTaP.[25] DTaP primarily induces T cell response.[28] By age 1 year, most infants in the US would have received three doses of PCV13 and DTaP.[25]

Using 16S V4-5 rRNA sequencing data, we found marginally statistically significant between-group (beta diversity) differences in PCP and TT antibody response. One prior study of Chinese infants observed differences in beta diversity among those with and without a positive oral poliovirus IgA vaccine response based on 16S rRNA analyses of stool collected the day of vaccination. However, many prior studies have not detected such differences with either oral poliovirus[29] or rotavirus vaccination.[30,31]

The association between specific microbial taxa and infant vaccine response has been examined in only a few prospective studies of infants. In a study of 48 Bangladeshi infants, positive associations were found for *Corynebacterium* and *Bifidobacterium* and negative associations for *Escherichia/Shigella* and *Acinetobacter* and T cell proliferation response to TT vaccine among young infants (measured at 15 weeks of life).[12] In this study, a positive association for *Actinomyces* and a negative association for *Staphylococcus* with IgG response to TT vaccine also were observed. Further, a positive association was identified between *Bifidobacteriaceae* and TT T cell proliferation response. This contrasts with our finding of an inverse association with *Aeriscardovia aeriphila*, a bacterial species of the family *Bifidobacteriaceae*. A later publication from the Bangladesh cohort of infants found a positive association between *Bifidobacterium* and CD4 and IgG response to vaccines, including to the TT vaccine at age 2 years.[32] Other studies have focused on vaccine response to oral live attenuated rotavirus. For instance, a case–control study of 78 Ghanaian infants found negative correlations between rotavirus vaccine response in serum collected 4 weeks after the last vaccine dose and *Bacteroides* and *Prevotella* species, and positive correlations with bacteria in the *Bacilli* phylum in 6-week stool samples collected before vaccination.[13] Similarly, in a study performed in Pakistan, a case–control study found positive correlation between rotavirus vaccine response 28 days after the last vaccine dose and relative abundance of *Clostridium cluster XI* and Proteobacteria, including *Serratia* and *Escherichia coli* in the pre-vaccination stool of 20 infants.[14] A randomized control trial found correlations between *Bifidobacteria* and antipoliovirus IgA response in 30 French infants.[33] Another study of polio vaccine in 107 Chinese infants found increased *Firmicutes* and decreased *Actinobacteria* in stool collected the day of the last oral poliovirus vaccine dose among infants with negative IgA response.[29] Many of these studies investigated response to oral vaccines, and gut microbiota may interact differently with oral vaccines compared to intramuscular vaccines due to direct contact between oral vaccines and the gut. Thus, further studies are needed in diverse study populations and with response to multiple types of vaccines.

External factors that influence both the gut microbiome and vaccine response highlight opportunities for recommendations and interventions to improve vaccine response. Germ-free mice treated with antibiotics and mice deficient in toll-like receptor 5 expression have demonstrated lower responses to influenza vaccine, but not other vaccines.[11] Diminished vaccine response was restored after reconstituting their gut microbiome with flagellated *E. coli*. In another mouse experiment, lower antibody response after ovalbumin and Freund's adjuvant immunization was observed in infant mice whose mothers were treated with antibiotics during pregnancy.[9] The study also observed that germ-free mice with deficient antibody response can increase their response after introduction to normal gut flora, raising the possibility that interventions may be able to enhance immune response. A similar study in mice found associations between maternal and early-life antibiotic exposure and gut microbiome dysbiosis and lower IgG responses for several vaccines, including PCV13 and Hexa, which produces antibodies to tetanus.[10] Further, impaired PCV13 response in mice treated with antibiotics was not observed if they received fecal transplant from age-matched untreated mice, while impaired response remained in mice treated with antibiotics and fecal transplant from antibiotic-treated mice. Further experimental studies and mediation analyses will help establish the potential for microbiome-directed interventions to bolster immune response to vaccines.

Further evidence of the importance of the microbiome on vaccine response comes from studies on beneficial effects of probiotics. A study of 20 French infants found those given *Bifidobacterium breve* strain C50 and *Streptococcus thermophilus* supplementation in the first 4 months of life had higher antipoliovirus IgA response at 4 months.[33] Interestingly, in our analyses, we observed a positive association between *Streptococcus thermophilus* and TT antibody response, although this was no longer statistically significant after FDR correction. Prenatal and early-life supplementation with *Lactobacillus rhamnosus GG*, *L. rhamnosus LC705*, *Bifidobacterium breve Bbi99*, and *Propionibacterium freudenreichii* ssp. *shermanii* in the first 6 months of life was associated with higher IgG antibody response to *Haemophilus influenzae* type b at 6 months of age in a randomized controlled study of 61 allergy-prone Finnish infants.[34] A randomized control study of 61 mothers and their infants found maternal *Lactobacillus rhamnosus GG* supplementation starting at 36 weeks gestation was associated with lower IgG response of TT, *Haemophilus influenzae* type b, and several serotypes of PCV7 vaccines in infants at 12 months of age.[35] Thus, further research on antibiotic use and prenatal and early-life probiotic supplementation is warranted to elucidate immunomodulation by gut microbiota.

Based on metagenomic analysis, we found several metabolic pathways associated with PCP and TT vaccine response. Nucleotides including purine and pyrimidine have an important role in the immune system. Several pathways involving pyrimidine were associated with differential response to PCP. A link between pyrimidine biosynthesis and the immune system has been observed.[36,37] Furthermore, the purine nucleotide degradation II pathway was observed to be positively associated with TT response in our study. CDP-diacylglycerol biosynthesis I and II were positively associated with TT response in our study. CDP-diacylglycerol has an important function in lipid metabolism, which could affect the immune system.[38]

The main strength of our study is the prospective analyses of early-life infant gut microbiome and vaccine response. However, there also were limitations. Due to the high dimensional nature of microbiome data and available sample size, our analyses had limited statistical power. Therefore, we applied a filtering process as well as regularization techniques for dimension reduction and variable selection. Our study population was from rural northern New England; therefore, our results may not be generalizable to other study populations. Delivery mode was not related to vaccine response and therefore not included as a covariate in our analyses. Several potential confounders, such as breast feeding, were adjusted for in our analyses; however, residual confounding remains a possibility. Further work is warranted to investigate the gut microbiome as a possible mediator or effect modifier on the relationships between exposures and vaccine response. Delivery mode and breast feeding are two such exposures associated with relative abundance of gut bacteria,[39] and associations with these

exposures have been observed in our cohort.[16,40] Breast feeding may influence a child's vaccine response.[4] Analyses with larger sample sizes may be able to identify mediating or modifying effects as well as to corroborate or refute associations lacking statistical significance after correction for multiple hypotheses found in our species and pathway analyses.

In conclusion, we found patterns of the developing gut microbiome captured at a very early time point in development associated with differential response to vaccines administered in the first year of life. The microbes and pathways associated with antibody response to PCP and TT may offer clues to the critical role the developing microbiome plays in shaping the immune system as measured by vaccine response. Our findings provide insight into possible interventions to optimize antibody response and improve vaccine efficacy during a critical time in early immune maturation and when susceptibility to infection is at its highest.

DATA AVAILABILITY

The microbiome data used in this study can be found at http://www.ncbi.nlm.nih.gov/sra under accession number PRJNA296814.

CODE AVAILABILITY

Code is available upon request.

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AUTHOR CONTRIBUTIONS

Y.M., M.R.K., and J.M. contributed to conception of the study. M.R.K., J.M., K.C.N., and H.G.M. contributed to data acquisition and processing. Y.M. and J.G. contributed to statistical analyses. Y.M., M.R.K., J.M., and K.C.N. interpreted results. Y.M. wrote the first manuscript draft. Y.M., M.R.K., J.M., K.C.N., H.G.M., and J.G. reviewed and edited the manuscript.

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COMPETING INTERESTS

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Committee for the Protection of Human Subjects at Dartmouth College approved all protocols, and we provided written informed consent to all participants upon enrollment to this study.

ADDITIONAL INFORMATION

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