Stool Microbiota and Vaccine Responses of Infants

WHAT'S KNOWN ON THIS SUBJECT: Oral vaccine responses are low in children from less-developed countries perhaps as a result of intestinal dysbiosis. New high-throughput DNA-based methods allow characterization of intestinal microbiota as a predictor of vaccine responses.

WHAT THIS STUDY ADDS: High abundance of stool *Actinobacteria*, including *Bifidobacterium*, was associated with higher responses to oral and parenteral vaccines and a larger thymus in Bangladeshi infants. Conversely, high abundance of *Clostridiales*, *Enterobacteriales*, and *Pseudomonadales* was associated with neutrophilia and lower vaccine responses.

abstract

OBJECTIVE: Oral vaccine efficacy is low in less-developed countries, perhaps due to intestinal dysbiosis. This study determined if stool microbiota composition predicted infant oral and parenteral vaccine responses.

METHODS: The stool microbiota of 48 Bangladeshi infants was characterized at 6, 11, and 15 weeks of age by amplification and sequencing of the 16S ribosomal RNA gene V4 region and by *Bifidobacterium*-specific, quantitative polymerase chain reaction. Responses to oral polio virus (OPV), bacille Calmette-Guérin (BCG), tetanus toxoid (TT), and hepatitis B virus vaccines were measured at 15 weeks by using vaccine-specific T-cell proliferation for all vaccines, the delayed-type hypersensitivity skin-test response for BCG, and immunoglobulin G responses using the antibody in lymphocyte supernatant method for OPV, TT, and hepatitis B virus. Thymic index (TI) was measured by ultrasound.

RESULTS: *Actinobacteria* (predominantly *Bifidobacterium longum* subspecies *infantis*) dominated the stool microbiota, with *Proteobacteria* and *Bacteroidetes* increasing by 15 weeks. *Actinobacteria* abundance was positively associated with T-cell responses to BCG, OPV, and TT; with the delayed-type hypersensitivity response; with immunoglobulin G responses; and with TI. *B longum* subspecies *infantis* correlated positively with TI and several vaccine responses. Bacterial diversity and abundance of *Enterobacteriales*, *Pseudomonadales*, and *Clostridiales* were associated with neutrophilia and lower vaccine responses.

CONCLUSIONS: *Bifidobacterium* predominance may enhance thymic development and responses to both oral and parenteral vaccines early in infancy, whereas deviation from this pattern, resulting in greater bacterial diversity, may cause systemic inflammation (neutrophilia) and lower vaccine responses. Vaccine responsiveness may be improved by promoting intestinal bifidobacteria and minimizing dysbiosis early in infancy. *Pediatrics* 2014;134:e362–e372

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KEY WORDS

vaccine, intestinal, microbiota, *Bifidobacterium*, *Actinobacteria*, *Proteobacteria*, Bangladesh, T lymphocyte, antibody, polio, tetanus, tuberculosis, hepatitis

ABBREVIATIONS

BCG—bacille Calmette-Guérin
DTH—delayed-type hypersensitivity
HBV—hepatitis B virus
Ig—immunoglobulin
OPV—oral polio virus
PCR—polymerase chain reaction
PPD—purified protein derivative of *Mycobacterium bovis*
qPCR—quantitative polymerase chain reaction
rRNA—ribosomal RNA
SEB—*Staphylococcus* enterotoxin B
SI—stimulation index
T-RFLP—terminal restriction fragment length polymorphism
TI—thymus index
TT—tetanus toxoid

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The composition of the community of microbes that inhabits the gastrointestinal tract (the gut microbiota) has a profound influence on the developing infant.[1] Dysbiosis, defined as deviation from an optimal, health-promoting microbial community,[2] may cause necrotizing enterocolitis and sepsis in premature infants[3] and allergic disease in term infants.[4] A gut microbiota dominated by appropriate commensal bacteria promotes infant health by a variety of mechanisms,[5,6] including appropriate development of the immune system.[7,8]

Infant immunization is an important measure for decreasing morbidity and mortality from infectious diseases.[9] However, oral vaccines are often less effective than expected when used in developing countries, as described in a recent review,[10] perhaps as a result of malnutrition, intestinal dysbiosis, or the presence of other inhibitory factors related to the local environment. Evidence that dysbiosis may influence vaccine responses include direct effects of intestinal bacteria on the ability of polio virus to infect target cells in the intestine[11] and direct impairment by dysbiosis of the host immune response.[12] Studies showing that probiotic interventions increase immune responses to oral vaccines in adults[13] and animals[14] support this hypothesis, although results in infants and children are equivocal.[15,16]

New DNA-based methods that use direct sequencing of short regions of the bacterial 16S ribosomal RNA (rRNA) gene produce a detailed picture of the gut microbiota, providing the relative abundance of taxa at multiple phylogenetic levels (ie, phylum, class, order, family, and genus).[17] The intestinal microbiota of term infants is dominated by 4 major phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. The relative abundance of taxa within these phyla is influenced early in infancy by mode of delivery,[18] gestational age,[19] type of feeding,[20] and locality.[21,22] Early colonizers typically include commensal facultative anaerobes such as *Escherichia coli* and other *Enterobacteriaceae* (phylum *Proteobacteria*), followed by an increased relative abundance of strict anaerobes including *Bifidobacterium* (phylum *Actinobacteria*), *Bacteroides* (phylum *Bacteroidetes*), and *Clostridium* (phylum *Firmicutes*).[1] *Firmicutes* includes several genera of importance to infant health including *Streptococcus*, *Staphylococcus*, and *Lactobacillus*, whereas the phylum *Proteobacteria* includes pathogens from the genera *Escherichia*, *Shigella*, and *Campylobacter*. The relative abundance of these taxa is used to define the gut microbiota.

In the current study we hypothesized that the composition of the gut microbiota would affect responses to oral and perhaps parenteral vaccines. To test this hypothesis, we characterized the microbiota from the stool of 48 Bangladeshi infants and measured responses to 4 vaccines: oral polio virus (OPV), bacille Calmette-Guérin (BCG; given to protect against tuberculosis), tetanus toxoid (TT), and hepatitis B virus (HBV) by using 2 methods for each vaccine. Vaccine-specific T-cell proliferation was measured for all vaccines; the delayed-type hypersensitivity (DTH) skin test to purified protein derivative (PPD) of *Mycobacterium tuberculosis* was measured as a second, functional indicator of response to BCG vaccination; and vaccine-specific immunoglobulin (Ig) G levels were measured for OPV, TT, and HBV using the antibody in lymphocyte supernatant assay[23] as an index of the memory B-cell response.

METHODS

Subjects

The 48 infants in this study were the first to be recruited in a larger trial (clinicaltrials.gov identifier: NCT01583972) and were included here because a relatively complete set of immunologic data were available when funding became available to analyze stool microbiota. Procedures are summarized in Supplemental Table 3. Parents of infants born at the Maternal and Child Health Training Institute in Dhaka, Bangladesh, were approached during the third trimester and informed consent was obtained within 48 hours of birth. The study was approved by the Ethical Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) and by the Human Studies Committee of the World Health Organization.

Immune Function

Thymic index (TI), blood leukocytes, vaccine-specific T-cell stimulation index (SI), vaccine-specific IgG responses using the antibody in lymphocyte supernatant assay, and the PPD-DTH skin test were measured as described in the Supplemental Information.

Bacterial DNA Methods

The relative abundance of stool bacteria at the phylum, class, order, family, and genus levels was characterized by sequence analysis of the V4 segment of the 16S rRNA gene, as described in the Supplemental Information. In addition, 3 methods were used to identify members of the genus *Bifidobacterium*: (1) a terminal restriction fragment length polymorphism (T-RFLP) assay[24] was used to determine the relative abundance of *Bifidobacterium* species, (2) *Bifidobacterium* 16S rDNA copy numbers (per gram of stool) were measured by quantitative polymerase chain reaction (qPCR), and (3) the *Bifidobacterium longum* subspecies *infantis* and *longum* were identified by polymerase chain reaction (PCR). Methods are described in the Supplemental Information and Supplemental Table 4.

Statistical Analysis

Spearman rank-order correlation and Wilcoxon rank-sum analyses were used to identify associations between microbiota and immune variables. Correlation was used when ≥75% of samples had nonzero results, whereas the Wilcoxon test (using a normal adjustment) was used when ≥25% of samples had nonzero results to compare "high" and "low" responders defined as those above or below the median value for each of the immune variables. A P value of $<.05$ was considered statistically significant for all analyses. Data are presented as means ± SDs or as medians (interquartile range) unless otherwise indicated. Statistical analysis was performed by using SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

Subjects

Of the 48 infants studied, 28 (58%) were male, 16 (33%) had a birth weight <2500 g, and 40 (83%) were born by cesarian delivery. The prevalence of moderate wasting malnutrition (>2 *z* scores below the reference mean weight-for-length) was 4% to 8% depending on age, and prevalence of moderate stunting was 10% to 12% (Table 1). All infants were breastfed with some receiving supplemental foods (Table 2). The median (25th–75th percentiles) years of education for mothers and fathers were 9 (7–10) and 9 (5–10) years, respectively. Seventy-one percent of mothers (34 of 48) had completed at least some secondary education. All 48 households had electricity, 98% had paved flooring, 44% had glass in the windows, 92% cooked with gas (rather than wood), 75% had drinking water piped into the house, and 38% had flush toilets available.

Actinobacteria, Particularly *Bifidobacterium*, Dominated the Stool Microbiota

DNA sequence analysis of the V4 region of the 16S rRNA gene was used to characterize the relative abundance of bacteria from the phylum through the genus level in stool samples at 6, 11, and 15 weeks of age. *Actinobacteria* was the most abundant phylum at all ages, followed by *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* (Fig 1; Supplemental Tables 5 and 6). The relative abundance of *Proteobacteria* and *Bacteroidetes* increased with age, resulting in greater bacterial diversity by 15 weeks (Supplemental Table 7). Within these 4 phyla, a total of 24 common genera (seen in at least 25% of infants) were identified at 15 weeks of age (Supplemental Table 8): 5 from *Actinobacteria* (*Bifidobacterium* being the most abundant), 3 from *Bacteroidetes* (*Bacteroides*, *Parabacteroides*, and *Prevotella*), 13 from *Firmicutes* (*Streptococcus* and *Lactobacillus* being the most abundant but also including *Enterococcus*, *Staphylococcus*, and *Clostridium*), and 3 from *Proteobacteria* (*Campylobacter*, *Escherichia/Shigella*, and *Acinetobacter*). Although diversity increased by 15 weeks, the median relative abundance of *Actinobacteria* remained quite high at 0.823 (0.329), with the median relative abundance of *Bifidobacterium* being 0.675 (0.282).

Bifidobacterium-specific PCR amplification and T-RFLP analysis were used as additional methods to independently quantify *Bifidobacterium* levels and to identify and quantify *Bifidobacterium* species and subspecies. By using these methods, the median *Bifidobacterium* concentration in stool at 15 weeks was found to be 3.08×10^{10}/g (2.74×10^{10}). Several *Bifidobacterium* species were identified by T-RFLP (Supplemental Fig 8), including *Bifidobacterium adolescentis*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium breve*, and *Bifidobacterium bifidum*, as expected,[1] but in these infants *B longum* was found to be by far the most abundant species, with a median relative abundance of 96.4% (8.9%) within the genus. Within *B longum*, the *infantis* subspecies predominated, at 68.1% (31.3%), with the *longum* subspecies contributing 31.9% (31.3%).

TABLE 1 Nutritional Status of Subjects by Age

	6 Weeks	10 Weeks	15 Weeks	*P*
Weight-for-length				
z Score, mean ± SD.	−0.314 ± 1.044	−0.256 ± 1.051	−0.317 ± 1.221	.29[a]
< −2 *z* scores, *n* (%)	2 (4.2)	4 (8.3)	4 (8.3)	.65[b]
Length-for-age				
z Score,mean ± SD.	−0.991 ± 1.091	−0.914 ± 0.988	−0.975 ± 0.869	.62[c]
< −2 *z* scores, *n* (%)	7 (14.6)	7 (14.6)	5 (10.4)	.78[b]
Weigh-for-age				
z Score,Mean ± SD.	−1.139 ± 0.970	−0.981 ± 0.977	−0.963 ± 1.004	.16[a]
< −2 *z* scores, *n* (%)	5 (10.4)	5 (10.4)	6 (12.5)	.93[b]

$N = 48$.
[a] Repeated-measures analysis of variance of ranks.
[b] χ^2 Test.
[c] Repeated-measures analysis of variance.

TABLE 2 Number (%) of infants by breastfeed status at 6, 11 and 15 wk of age.

Breastfeeding Status	6 Weeks	11 Weeks	15 Weeks
Exclusive breastfeeding[a]	38 (79.2)	29 (60.4)	22 (45.8)
Breastfeeding + water or fruit juice	3 (6.2)	4 (8.3)	11 (22.9)
Breastfeeding + non–human milk or other liquid food	7 (14.6)	15 (31.2)	12 (25)
Breastfeeding + any solid food	0 (0)	0 (0)	3 (6.2)
Total	48 (100)	48 (100)	48 (100)

Data are presented as *n* (%).
[a] Allows use of oral medications, vitamin supplements, and oral rehydration therapy, as needed.

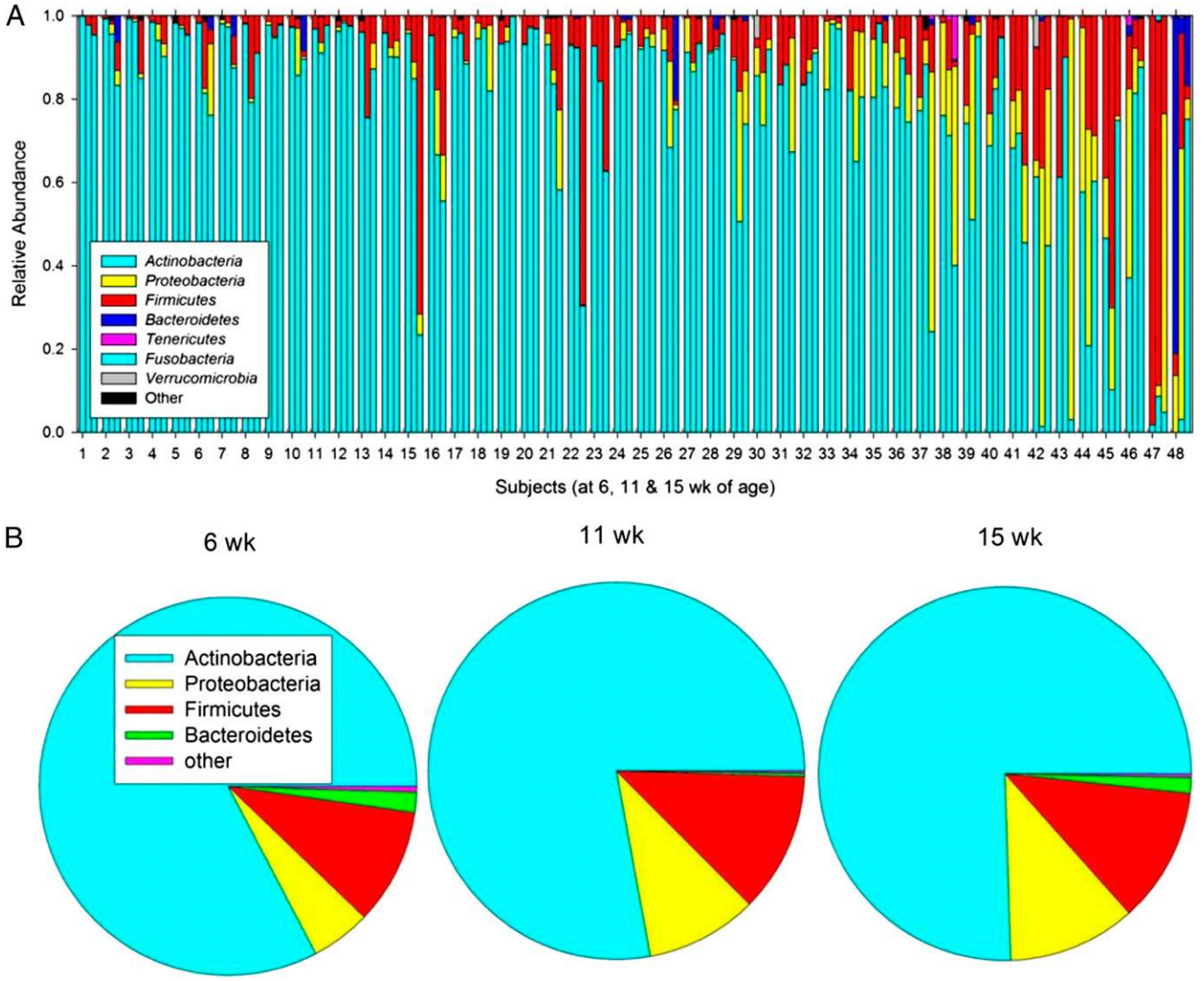

FIGURE 1

A, Relative abundance of microbiota by 16S rRNA gene sequence analysis in each of the 48 subjects (numbered from highest to lowest abundance of *Actinobacteria* at 6 weeks) at all time points. For each subject, bars are ordered by age (6, 11, and 15 weeks) from left to right. B, Mean relative abundance at 6, 11, and 15 weeks of age.

Environmental Factors and Stool Microbiota Composition at 15 Weeks of Age

The relative abundance of stool microbiota often differs by mode of delivery (vaginal versus cesarean),[18] but in the current study no such differences were seen at 15 weeks (data not shown). Breastfeeding status also strongly affects stool microbiota[20]; and in these infants, who were all predominantly breastfed, more extensive breastfeeding was associated with a higher abundance of *B longum*, as might be expected, but few other differences were seen (Supplemental Table 9). Nutritional status (as measured by length-for-age) was also found to be positively associated with *B longum* and negatively associated with *Escherichia/Shigella* (Supplemental Table 10).

Stool *Actinobacteria* and Vaccine Responses

Vaccine responses are characterized in the Supplemental Information (including Supplemental Fig 9). Both OPV-specific T-cell proliferation (Supplemental Figs 10–12) and OPV-specific IgG responses (Supplemental Figs 13 and 14) were positively associated with members of the phylum *Actinobacteria*. For example, infants with a "high" CD4 T-cell response to OPV vaccination (ie, those above the median response) had a significantly greater relative abundance of *Actinobacteria* in 15-week stool samples than did infants with a "low" (ie, below the median) OPV-CD4 response (Fig 2A). At the family level, high OPV-CD4 responders also had a higher ($P = .024$) median relative abundance of *Coriobacteriaceae* (1.76×10^{-4} [1.77×10^{-2}]) than did low OPV-CD4 responders (0.00 [6.18×10^{-5}]). By using *Bifidobacterium*-specific PCR,

high OPV-CD4 responders were found to have higher *B longum* subspecies *infantis* levels than low responders (Fig 3A) and high OPV-CD8 responders had higher *Bifidobacterium*, *B longum*, and *B longum* subspecies *longum* levels than did low OPV-CD8 responders (Fig 3B). A significant, positive correlation was also seen between *Bifidobacterium* relative abundance (assessed by 16S rRNA gene sequencing) and the OPV-IgG response (Fig 4D).

Positive associations were also seen between *Actinobacteria*, including *Bifidobacterium*, and BCG vaccine responses, including CD4 T-cell proliferation to PPD antigen and the skin-test response to PPD. High PPD-CD4 vaccine responders had significantly higher *Actinobacteria* (Fig 2B) and *Bifidobacteriales* (Fig 5D) relative abundance in stool than did low responders, and a similar association of marginal statistical significance (P = .055) was also seen for *Bifidobacterium*, with the relative abundance in high and low responders being 0.720 (0.128) and 0.619 (0.350), respectively. By using *Bifidobacterium*-specific PCR, *B longum* subspecies *infantis* levels were found to be higher in high versus low PPD-CD4 responders (Fig 3C). The PPD-DTH skin-test response also revealed a marginally significant positive correlation with *Actinobacteria* relative abundance (R = 0.284, P = .053) as well as significant, positive associations with *Bifidobacteriales* (Fig 5A) and *Bifidobacterium* (Fig 4C). Positive associations were also seen between the PPD-DTH response and another *Actinobacteria* order, *Actinomycetales* (Fig 5B), and genus, *Rothia* (high PPD-DTH responders: 2.48×10^{-3} [2.55×10^{-3}]; low responders: 9.14×10^{-4} [1.19×10^{-3}]; P = .034).

The TT-CD4 response was positively associated with *Actinobacteria* from the phylum (Fig 2C) through the genus level, including both *Bifidobacterium* (Fig 4A) and *Corynebacterium* (R = 0.325, P = .041; n = 40). *Bifidobacterium*-specific PCR also showed a positive correlation between the TT-CD4 response and *B longum* subspecies *infantis* (R = 0.319, P = .048; n = 39).

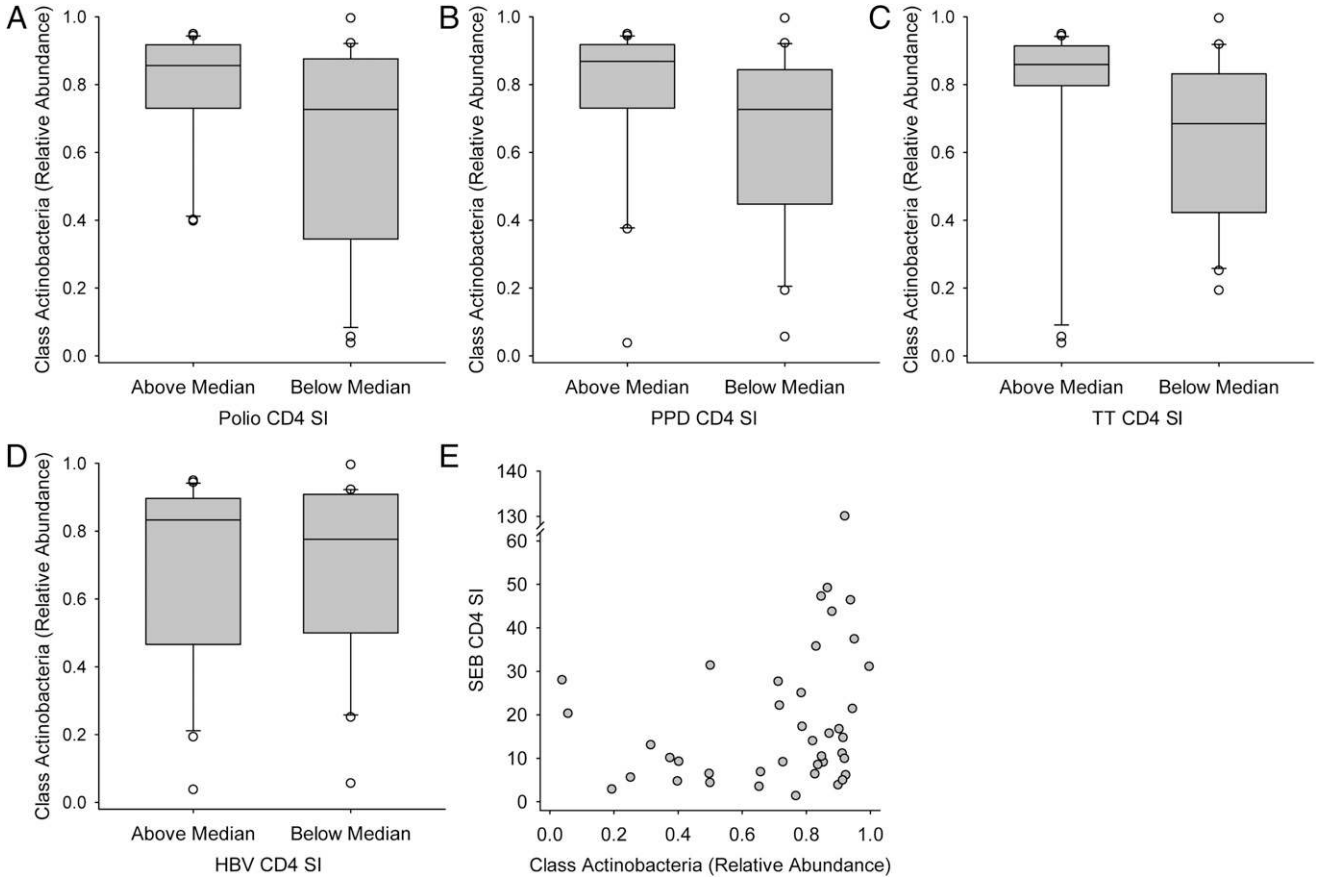

FIGURE 2

Positive association of vaccine responses with stool *Actinobacteria* at 15 weeks of age. The relative abundance of stool microbiota was determined by sequence analysis of the 16S rRNA gene. Vaccine responses were characterized by the CD4 T-cell SI in response to vaccine antigens for OPV vaccine (A), BCG vaccine using PPD antigen (B), TT vaccine (C), HBV vaccine (D), and SEB (E), the positive control for T-cell stimulation. Box plots (A–D) show the median; 10th, 25th, 75th, and 90th percentiles; and individual outliers. P values (Wilcoxon rank-sum test) for comparisons between groups were as follows: A, .046; B, .036; C, .019; and D, 0.78. E, The scatterplot association was determined by Spearman correlation (R): R = 0.331, P = .034.

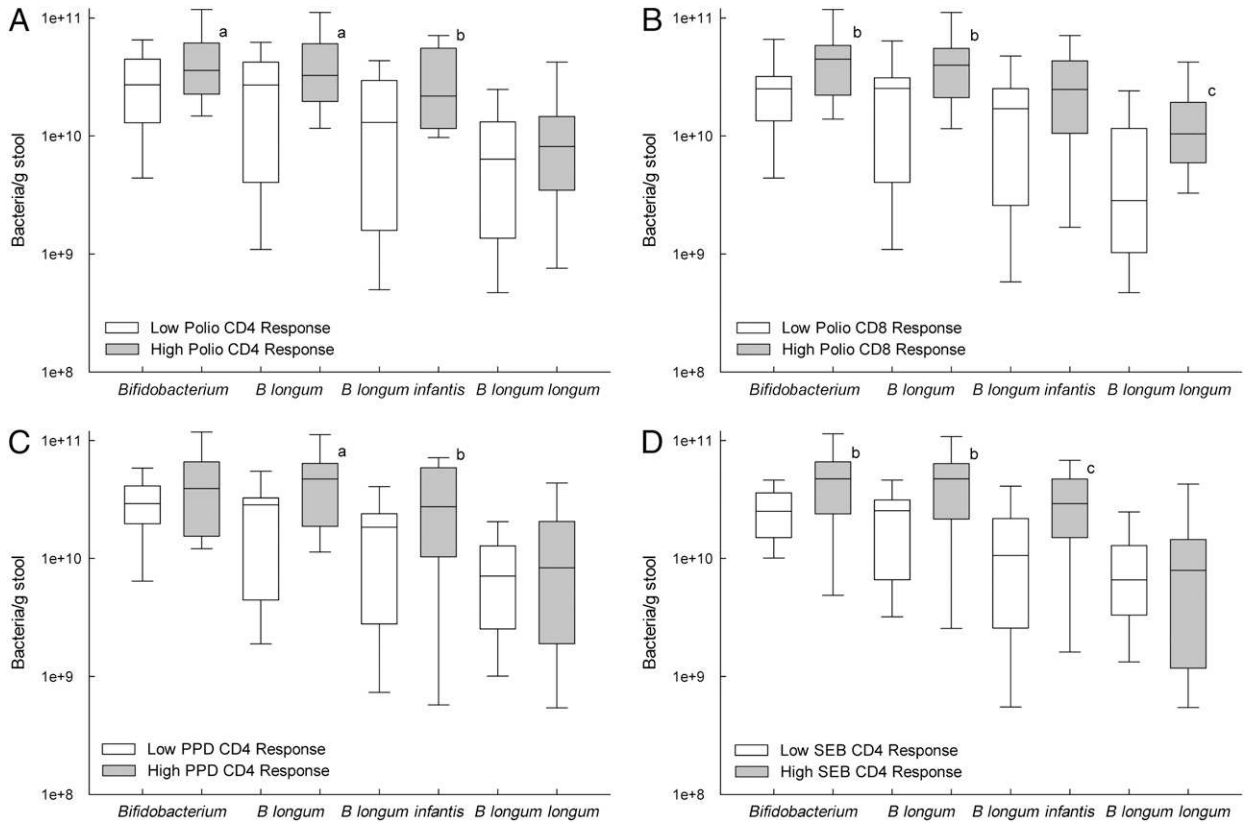

FIGURE 3

Positive association of vaccine responses with stool *Bifidobacterium*, *B longum*, *B longum* subspecies *infantis*, and *B longum* subspecies *longum* at 15 weeks of age. Infant vaccine responses were characterized as being above ("high") or below ("low") the median vaccine responses for the CD4 SI for OPV (A), the CD8 SI for OPV (B), the CD4 SI for PPD as an index of response to the BCG vaccine (C), and for the positive control for polyclonal T-cell proliferation, the CD4 SI for SEB (D). Differences between those above and below the median were identified by the Wilcoxon rank-sum test as indicated by the superscript letters: $^{a}P < .10$, $^{b}P < .05$, $^{c}P < .01$. The *Bifidobacterium* genus was identified by PCR, *B longum* species by T-RFLP, and *B longum* subspecies *infantis* and *B longum* subspecies *longum* as described in Methods.

Although the TT-IgG response was not associated with *Bifidobacterium*, it was positively associated with the *Actinobacteria* order *Actinomycetales* (Fig 5C). Thus, the response to TT immunization showed a consistent, positive association of *Actinobacteria* with both T-cell and IgG responses.

When the HBV-CD4 responses were evaluated, positive associations were not seen with *Actinobacteria* (Fig 2D, Supplemental Fig 10) or with other stool microbiota, although a positive association was seen between the HBV-IgG response and the *Actinobacteria* genus *Rothia* ($R = 0.406$, $P = .029$; $n = 29$).

Three of the four vaccines (OPV, BCG, and TT) showed positive associations between vaccine-specific T-cell proliferative and the relative abundance of taxa from the phylum *Actinobacteria*, particularly the genus *Bifidobacterium*. This positive association was also seen for the *Staphylococcus* enterotoxin B (SEB)–stimulated cultures, which were included as a positive control for polyclonal T-cell proliferation. Positive associations were seen from the phylum (Fig 2E) through the *Bifidobacterium* genus (Fig 4B) level and, using *Bifidobacterium*-specific PCR, again at the genus level but also at the species and subspecies level for *B longum* and *B longum* subspecies *infantis* (Fig 3D). Thus, *Bifidobacterium* relative abundance is also associated with polyclonal T-cell proliferation, in addition to vaccine-specific responses.

Gammaproteobacteria, Bacterial Diversity and Vaccine Responses

Several taxa of *Gammaproteobacteria* had negative associations with vaccine responses. *Pseudomonadales* and its members showed negative associations with T-cell SI responses for TT (Fig 6A), OPV, PPD, HBV, and SEB as well as a negative association with the OPV-IgG response (Supplemental Figs 10 and 13), whereas the order *Enterobacteriales* showed a negative association with the TT-CD4 SI response (Fig 6B) as well as with the PPD-DTH skin-test response (Supplemental Fig 13). Similar but less consistent associations (across phylogenetic levels) were seen with *Firmicutes* and *Bacteroidetes* (Supplemental Figs 10 and 13). High

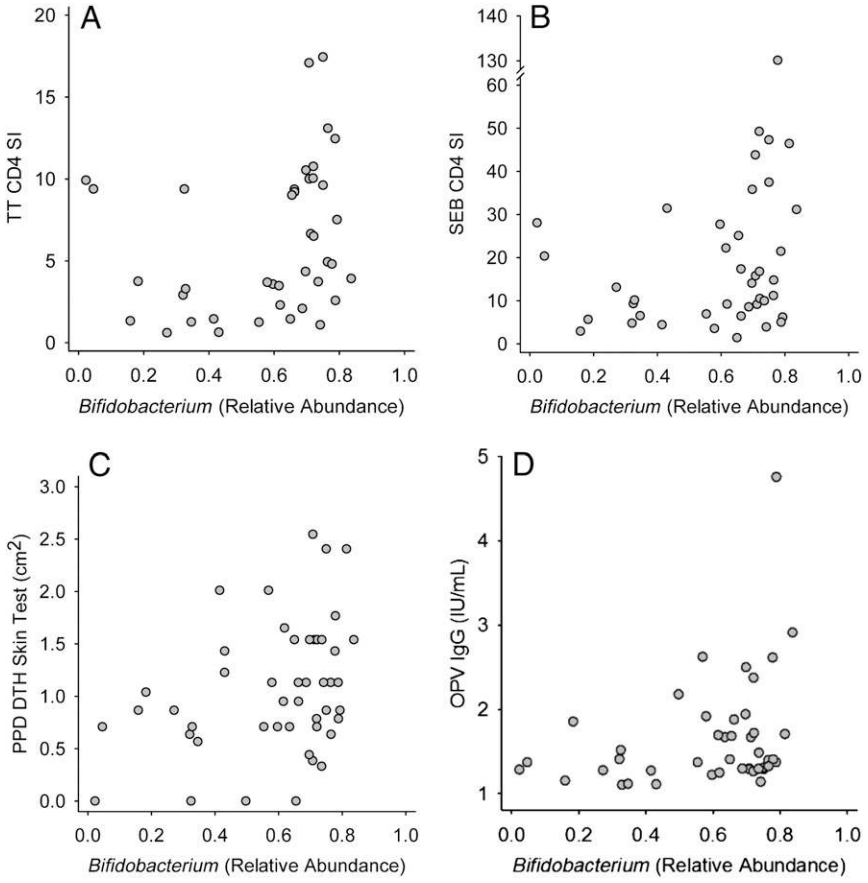

FIGURE 4

Positive association of vaccine responses with relative abundance of stool *Bifidobacterium* determined by sequence analysis of the 16S rRNA gene. Vaccine responses were characterized by CD4 stimulation index response to TT (A), CD4 stimulation index in response to the polyclonal T-cell mitogen SEB (B), the DTH skin-test response to PPD antigen as an index of the response to BCG immunization (C), and the IgG response to the OPV vaccine (D). Spearman correlation coefficients (R) and P values were as follows: A, $R = 0.389$, $P = .013$; B, $R = 0.315$, $P = .045$; C, $R = 0.337$, $P = .020$; and D, $R = 0.301$, $P = .047$.

diversity was also negatively associated with the CD4 TT SI response (Fig 6C) and with the CD4-SEB response ($R = -0.361$, $P = .021$).

Possible Mediators of Microbiota Associations With Vaccine Response

Gut microbiota may affect vaccine responses indirectly by affecting development of T cells. To examine this possibility, TI and peripheral blood T-cell concentrations were correlated with vaccine responses; positive associations were found between TI and OPV-CD4 ($R = 0.377$, $P = .015$), OPV-CD8 ($R = 0.336$, $P = .032$), and SEB-CD4 ($R = 0.396$, $P = .010$), but no associations were found with T-cell concentrations. In addition, systemic inflammation caused by enteric bacteria might disrupt a vaccine response. Peripheral blood neutrophil, eosinophil, and monocyte levels were used as indicators of such inflammation and correlated with vaccine responses: neutrophils correlated negatively with PPD-CD8 ($R = -0.334$, $P = .023$) and TT-CD4 ($R = -0.358$, $P = .028$). Given these results, we searched for associations between TI, neutrophil levels, and microbiota taxa associated with vaccine responses.

Among taxa that were positively associated with vaccine responses, positive associations were also found with TI but no associations were found with neutrophils. Specifically, the relative abundance of *Bifidobacterium* (from 16S rRNA gene sequencing) was positively correlated with TI ($R = 0.293$, $P = .046$). In addition, stool concentrations of *Bifidobacterium* ($R = 0.388$, $P = .0070$), *B longum* ($R = 0.394$, $P = .0075$), and *B longum* subspecies *infantis* (Fig 7A) were positively associated with TI, and these associations were shown to be independent of length-for-age (Supplemental Table 11), which also correlated with TI.

Among taxa that were negatively associated with vaccine responses, only 1 association was seen with TI (a negative association of *Lactococcus*: $R = -0.389$, $P = .006$), whereas multiple positive associations were seen with neutrophils in phylum *Firmicutes*, including *Clostridia* and *Clostridiales* ($R = 0.418$ and $P = .0038$ for both), and phylum *Proteobacteria*, including *Proteobacteria* ($R = 0.389$, $P = .008$), *Gammaproteobacteria* ($R = 0.346$, $P = .019$), *Enterobacteriales* ($R = 0.350$, $P = .017$), *Enterobacteriaceae* ($R = 0.350$, $P = .017$), and *Escherichia/Shigella* (Fig 7B). Bacterial diversity (measured as the Shannon Diversity Index at the phylum level) was also positively correlated with neutrophils ($R = 0.293$, $P = .048$).

DISCUSSION

A principal finding of our study was that a high relative abundance of *Actinobacteria*, particularly of *Bifidobacterium*, was positively associated with several measures of both oral and parenteral vaccine responses, including T-cell proliferative responses to OPV, PPD, and TT; the PPD-DTH skin-test response; and vaccine-specific IgG responses. At the genus level, *Actinomyces*, *Corynebacterium*, *Rothia*, and *Bifidobacterium* all showed positive associations with at least 1 of these responses. *B longum* was the most abundant species in infant stool and *B longum* subspecies *infantis* was its

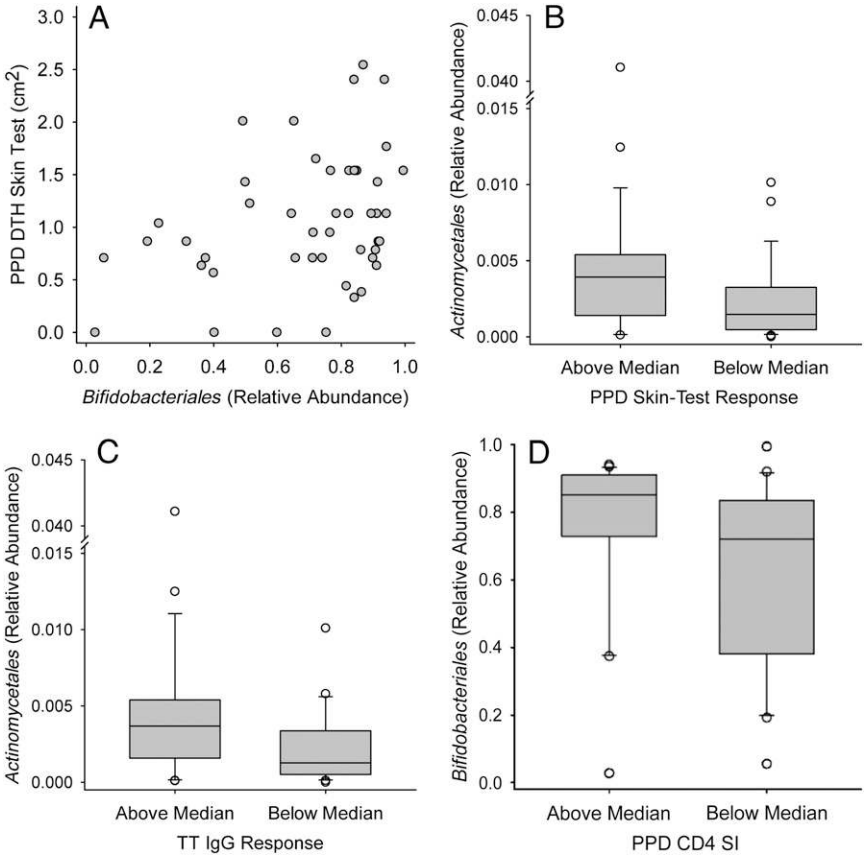

FIGURE 5

Positive association of vaccine responses with stool *Bifidobacteriales*, *Actinomycetales*, and *Coriobacteriales* (from the class *Actinobacteria*) at 15 weeks of age. The relative abundance of stool microbiota at the order level in the class *Actinobacteria* was determined by sequence analysis of the 16S rRNA gene at 15 weeks of age. A and B, The response to BCG vaccination was determined by using the DTH skin-test response to PPD antigen. C, The response to TT vaccination was determined by using the antibody in lymphocyte supernatant assay to measure TT-specific IgG levels (TT IgG). D, The CD4 T-cell SI in response to PPD antigen. A, The scatterplot association was determined by Spearman correlation (*R*): $R = 0.333$, $P = .025$. Box plots (B, C, and D) show the median; 10th, 25th, 75th, and 90th percentiles; and individual outliers. *P* values (Wilcoxon rank-sum test) for comparisons between groups were as follows: B, .018; C, .041; and D, .034.

predominant subspecies and was positively associated with CD4 SI responses to OPV, PPD, and TT, whereas the subspecies *longum* was positively associated with the CD8 SI response to OPV. It is well established that gut microbiota can affect mucosal immune function, as discussed below, but the present observation of an equally strong association of stool microbiota with response to parenteral vaccination is, we believe, novel.

The importance of intestinal microbiota for the development of the mammalian immune system has been well documented in animal models.[12,25] The absence of flora in germ-free mice affects both local[12,25–27] and systemic[12,25] lymphoid compartments, whereas colonization with specific gut bacteria such as segmented filamentous bacteria,[28] *Clostridium*,[29] and bacteria that produce short-chain fatty acids[30] (which include *Bifidobacterium*) can affect development of CD4 T-cell subsets. In humans, the hygiene hypothesis suggests that quantitative or qualitative "deficiencies" in microbial exposure in "hygienic" environments increase subsequent risk of atopic diseases including asthma,[31] whereas the administration of probiotic bacteria is associated with a decreased risk of atopic eczema.[32]

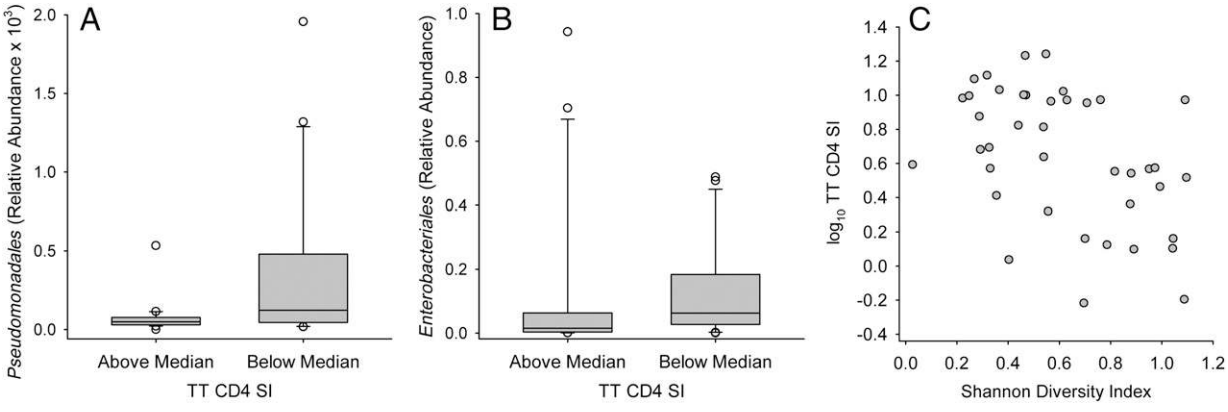

FIGURE 6

Negative association of stool *Pseudomonadales* (A), *Enterobacteriales* (B), and Shannon Diversity Index (measured at the phylum level) (C) with CD4 T-cell SI response to TT vaccine at 15 weeks of age. The relative abundance of stool microbiota was determined by sequence analysis of the 16S rRNA gene. Box plots show the median; 10th, 25th, 75th, and 90th percentiles; and individual outliers. *P* values (Wilcoxon rank-sum test) for comparisons between groups were as follows: A, 0.022; B, 0.047. C, The Spearman correlation coefficient was $R = -0.490$ with $P = .0013$.

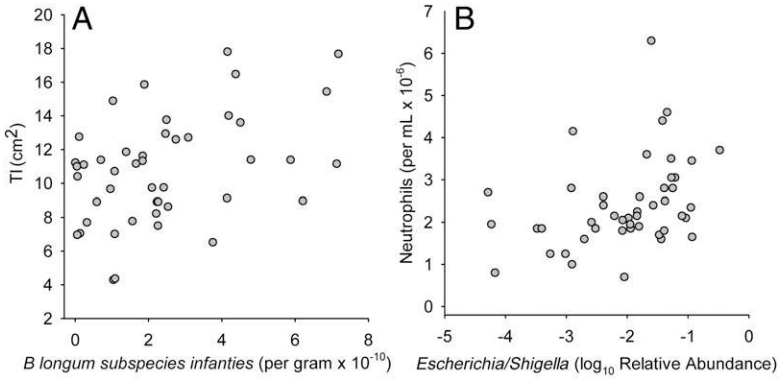

FIGURE 7
Correlation of *Bifidobacterium longum* subspecies *infantis* in stool determined by qPCR with TI (A) and relative abundance of *Escherichia/Shigella* genera in stool determined by sequence analysis of the 16S rRNA gene with blood neutrophil levels (B). Spearman correlation coefficients (*R*) and *P* values were as follows: A, $R = 0.389$, $P = .0083$; B, $R = 0.431$, $P = .0028$.

Perhaps most compelling, administration of *Bifidobacterium* as a probiotic has increased responses to oral vaccines in both animal and human studies,[33] including OPV-specific IgA.[34] Relatively few human studies have examined the relationship of naturally occurring gut bacteria to immune function. Bifidobacteria have been associated with development of IgA- and IgM-secreting plasma cells,[35] memory B cells,[36] and higher salivary IgA.[37] Overall, these data are consistent with the present findings that high *Bifidobacterium* abundance is positively associated with response to immunization.

All of the vaccines used in this study require T-cell help, and the finding that *B longum* subspecies *infantis* was positively associated with both thymus size (as measured by TI) and with a generic measure of peripheral T-cell function (the SI response to SEB) suggests a direct effect of *Bifidobacterium* on the T-cell compartment at sites distant from the intestine. If this is true then 3 mechanisms seem plausible: soluble or cell-associated bacterial metabolites or other molecules could act at a distance on the thymus and other lymphoid tissue via the lymph and blood, they could act locally in the gut to trigger neuroendocrine stimuli that affect distant lymphoid tissue, or cells of the immune system that circulate through systemic and intestinal lymphoid tissue (as do naive T-cells) could be exposed to bacterial products in intestinal lymph nodes and carry this "experience" to systemic sites where they may then respond differently as a result. Mechanistic studies are needed to evaluate these possibilities.

Although bacterial diversity of the gut microbiota is thought to be a benefit in promoting health of the adult host,[38] diversity in the current study in breastfed infants at 15 weeks was associated with higher neutrophilia and a lower response to the TT vaccine and may be a manifestation of dysbiosis. Breastfeeding provides optimal nutrition and immunologic protection through the first 6 months of life[39] and human milk oligosaccharides selectively stimulate growth of *Bifidobacterium*,[40] perhaps acting to limit diversity. Thus, *Bifidobacterium* predominance with low diversity may be optimal for infant health early in infancy, whereas high diversity may be beneficial later in life when the diet is also more diverse.

Dysbiosis of the gut microbiome causes local inflammation.[2] The current study did not examine local inflammation but found that high levels of *Clostridiales*, *Enterobacteriales*, and *Pseudomonadales*, bacterial orders that contain enteric pathogens, were associated with neutrophilia and lower vaccine responses, suggesting that the presence of such organisms in healthy infants may cause systemic inflammation and immune suppression. Consistent with this hypothesis, neutrophil development in the bone marrow is driven by interleukin-17A derived from T-helper 17 cells,[41] which could develop in response to such bacteria. In addition, *Bifidobacterium* can directly protect against enteropathogenic *E coli* infection in mice,[42] data that are consistent with the idea presented here that *Bifidobacterium* predominance early in infancy promotes health by limiting "diversity" resulting from the presence of enteric pathogens.

Extrapolation of these results to the Bangladeshi population is limited by several factors, as seen by reference to countrywide data.[43] First, the study was in an urban population, whereas 79% of Bangladeshi women live in rural areas. Second, 71% of mothers had completed some secondary education, higher than the 35% rate for Bangladesh. Third, study infants were delivered in the hospital; whereas only 15% of births in 2007 were in medical facilities, 31% of births in urban areas were in facilities. The rate of cesarean deliveries was high in this study, but the 2007 national rate was 7.5%, accounting for half of all facility-based deliveries. Families in this study were not affluent because most did not have flush toilets and a quarter did not have drinking water piped into their residences. Data from infants delivered at home and in rural areas would help make this study more representative.

CONCLUSIONS

We report the novel observations that a high abundance of *Actinobacteria*, particularly *Bifidobacterium*, in early infancy is associated with a higher TI, greater vaccine-specific and polyclonal

T-cell proliferation, and a greater PPD-DTH skin-test response. Although this study is observational and not designed to assess causality, the data do suggest that *Bifidobacterium* colonization enhances both oral and systemic vaccine responses by supporting T-cell immunity. Other *Actinobacteria* were positively associated with the TT-specific IgG response. This study raises the possibility that supporting an "optimal" microbial community early in infancy (eg, by supporting breastfeeding) might enhance vaccine responses. Alternatively, a strategy has recently been suggested that could involve using probiotic microbes to enhance vaccine responses.[44]

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Dr Stephensen, working with coauthors, conceptualized and implemented the major hypothesis of the study (evaluating the association of microbiota with immune function), conceptualized and implemented the work to evaluate immune function, conducted statistical analysis, and wrote the first draft of the manuscript; Dr Mills worked to conceptualize, design, and implement the major hypothesis of the study (evaluating the association of microbiota with immune function); supervised acquisition of microbiota data; and participated in analysis and interpretation of the overall data set; Dr Underwood worked to conceptualize, design, and implement the major hypothesis of the study (evaluating the association of microbiota with immune function) and participated in analysis and interpretation of the overall data set; Drs Ahmad, Raqib, and Qadri and Mr Rashid contributed substantially to acquisition of immunologic data and participated in the analysis and interpretation of the overall data set; Mr Huda carried out laboratory studies to analyze microbiota and to characterize immune function, conducted bioinformatic analysis of microbiota data, conducted statistical analysis, and collaborated in writing the first draft of the manuscript; Mr Lewis and Dr Kalanetra carried out laboratory and bioinformatic analysis of stool microbiota data and contributed to the first draft of the manuscript; all authors participated in drafting the manuscript and/or revising it critically for important intellectual content, had final approval authority for the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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