Bifidobacterium Abundance in Early Infancy and Vaccine Response at 2 Years of Age

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abstract

BACKGROUND: The intestinal microbiome in early infancy affects immunologic development and thus may affect vaccine memory, though few prospective studies have examined such associations. We examined the association of *Bifidobacterium* levels in early infancy with memory responses to early vaccination measured at 2 years of age.

METHODS: In this prospective observational study, we examined the association of *Bifidobacterium* abundance in the stool of healthy infants at 6 to 15 weeks of age, near the time of vaccination, with T-cell and antibody responses measured at 6 weeks, 15 weeks, and 2 years of age. Infants were vaccinated with *Bacillus* Calmette-Guérin (BCG) (at birth), oral polio virus (at birth and at 6, 10, and 14 weeks), tetanus toxoid (TT) (at 6, 10, and 14 weeks), and hepatitis B virus (at 6, 10, and 14 weeks). Fecal *Bifidobacterium* was measured at 6, 11, and 15 weeks. *Bifidobacterium* species and subspecies were measured at 6 weeks.

RESULTS: Mean *Bifidobacterium* abundance in early infancy was positively associated with the CD4 T-cell responses to BCG, TT, and hepatitis B virus at 15 weeks, with CD4 responses to BCG and TT at 2 years, and with plasma TT-specific immunoglobulin G and stool polio-specific immunoglobulin A at 2 years. Similar associations were seen for the predominant subspecies, *Bifidobacterium longum* subspecies *infantis*.

CONCLUSIONS: *Bifidobacterium* abundance in early infancy may increase protective efficacy of vaccines by enhancing immunologic memory. This hypothesis could be tested in clinical trials of interventions to optimize *Bifidobacterium* abundance in appropriate populations.

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Dr Stephensen was principal investigator of the parent study, helped develop the study protocol, and mentored Dr Huda to perform laboratory analysis of the immunological assays, statistical analysis and manuscript writing; Dr Mills was a coinvestigator of this study and co-mentored Dr Huda to perform microbiota assay and bioinformatics analysis; Drs Underwood and Raqib were coinvestigators of this study and contributed to the development of the study protocol; Dr Ahmad was a coinvestigator of the parent study and directed research activities at the clinical site and in the laboratory in Bangladesh, contributed to the development of the study protocol, oversaw the overall study operations including providing feedback to the ethical committee, Data and Safety Monitoring Board, and report writing, and mentored Dr Huda to perform laboratory analysis;
(Continued)

WHAT'S KNOWN ON THIS SUBJECT: The composition of the gut microbiome affects many aspects of immune function and is known to affect response to vaccination over the short-term.

WHAT THIS STUDY ADDS: We demonstrate that a high abundance of *Bifidobacterium* in early infancy when infants receive several vaccines is associated with better vaccine memory, as indicated by higher responses to *Bacillus* Calmette-Guérin, polio, and tetanus vaccines measured at 2 years of age.

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Vaccines are estimated to prevent 3 million childhood deaths annually.[1] Vaccine memory involves the development of antibody-producing plasma cells and effector T cells, whereas maintenance of memory involves the persistence of memory B and T cells.[2,3] Vaccine-elicited T-cell and antibody responses as well as protective efficacy vary substantially among individuals for reasons that are not completely understood.[4–6] Recent evidence suggests that part of this variability is due to interactions between the intestinal microbiome and the developing infant immune system.[7]

The importance of intestinal microbes for the development of the mammalian immune system is well documented. The absence of bacteria in germ-free mice affects both local[8–11] and systemic lymphoid compartments,[9,10] whereas colonization with specific commensal bacteria[12–14] affects both innate and adaptive immunity.[15] Disruption of normal development of the intestinal microbiome may have adverse immunologic consequences. Quantitative or qualitative "deficiencies" in microbial exposure during infancy increases subsequent risk of atopic diseases including asthma,[16] whereas the early administration of probiotic bacteria is associated with a decreased risk of atopic eczema.[17] Researchers have examined in only a few human studies the relationship of naturally occurring gut bacteria with immune function, although members of the *Bifidobacterium* genus have been associated with higher levels of immunoglobulin A (IgA)-secreting plasma cells,[18] memory B-cells,[19] and salivary IgA,[20] suggesting a beneficial relationship for the infant.

Establishment of the intestinal microbiome begins early in infancy and follows a typical pattern.[21] Facultative anaerobic bacteria such as *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Escherichia* initially colonize the colon, followed by strict anaerobes that come to predominate during the first few months of life. In breastfed infants *Bifidobacterium longum* subspecies *infantis* often dominates the microbiome because it is specifically adapted to both use human milk oligosaccharides as a carbon source and to restrict human milk oligosaccharides availability to other bacteria.[22] *Bacteroides* and clostridia are also important commensal anaerobes in the infant gut.[21] The abundance of *Bifidobacterium* species (relative to total bacteria) can exceed 60% in breastfed infants in countries like Bangladesh where breastfeeding is widespread.[23,24] The abundance of *B longum* subspecies *infantis* is lower in the United States and other Western countries and appears to have decreased over recent decades,[25] concurrent with the rise in autoimmune and allergic diseases.

In the current study, we evaluate the hypothesis that greater exposure to *Bifidobacterium* (and to *B longum* subspecies *infantis* in particular) early in infancy when vaccines are administered will result in better memory responses to these vaccines. We previously reported that a high abundance of *Bifidobacterium*, and of *B longum* subspecies *infantis*, at 15 weeks was associated with higher vaccine responses measured at the same time.[24] In that study, we examined a subset (n = 48) of infants in a birth cohort of 306 infants. In the current study, we measure the vaccine responses of all available infants at 2 years of age to test the hypothesis that *Bifidobacterium* abundance at 6, 11, and 15 weeks of age is predictive of vaccine responses measured at 2 years. We examine responses to 4 vaccines: tetanus toxoid (TT) and hepatitis B virus (HBV) given at 6, 10, and 14 weeks; *Bacillus* Calmette-Guérin (BCG), given within 48 hours of birth; and oral polio virus (OPV), given within 48 hours of birth and at 6, 10, and 14 weeks. We also examine vaccine responses measured at 6 and 15 weeks to increase the sample size of our previous report.[24]

METHODS

Study Design

In this study, we re-recruited all available participants from a randomized controlled trial of vitamin A supplementation at birth (www.clinicaltrials.gov identifier: NCT01583972) and measured additional vaccine responses at 2 years in the same infants (NCT02027610) (Supplemental Table 4). The study was approved by the Research Review Committee and the Ethical Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh. The inclusion-exclusion criteria, study design, laboratory methodology, clinical procedures, and baseline characteristics of the participants have been described.[26]

Vaccine Responses Assay

Vaccine-specific CD4 T-cell stimulation index (SI), antibody in lymphocyte supernatant (ALS) responses and the purified protein derivative delayed-type hypersensitivity (DTH) skin test response were measured as described in our earlier report.[26,27] All of the vaccines are T-cell–dependent in that a robust T-cell response is needed for protection. Thus, we view the SI as a proxy for the magnitude of memory response (ie, the number of memory T cells). Stool extracts were prepared as described[28] to measure polio-specific IgA by using an in-house enzyme-linked immunosorbent assay (ELISA) described in the Supplemental Information. Antibody avidity index (AI) of plasma polio specific immunoglobulin G (IgG) and IgA, HBV-specific IgG, and TT-specific IgG at 2 years was determined by ELISA by using a chaotropic agent as

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described in the Supplemental Information.

Microbiota Assay

Overall stool microbial composition was determined by using 16s V4 sequencing method[24] modified as described in the Supplemental Information. Abundance at the genus level refers to the abundance of *Bifidobacterium* genomes relative to all bacterial genomes. A terminal restriction-fragment length-polymorphism assay was used to identify bifidobacteria at the species level by comparing fragment lengths to published data.[29] Details are provided in the Supplemental Information and previous publications.[24,30]

Statistical Analysis

The associations between bifidobacteria and vaccine-specific immune responses were determined by multiple regression analysis by using the mean *Bifidobacterium* abundance during early infancy (6, 11, and 15 weeks) and at 6 weeks only, and the abundance of *Bifidobacterium* species and subspecies measured at 6 weeks, as independent variables. The analysis was adjusted for possible covariates, including sex, vitamin A or placebo treatment, birth weight (above or below median), and type of delivery (cesarean delivery or vaginal). Breastfeeding status (exclusive or nonexclusive) during early infancy affects *Bifidobacterium* abundance and could independently affect vaccine responses and was thus evaluated as a covariate in our model. It was statistically significant in only 2 cases and was thus not included in the final model. However, examination of stool IgA responses to OPV at 2 years could be affected if breastmilk contained OPV-specific IgA. Thus, we included breastfeeding status (yes or no) in the regression analysis at 2 years. A *P* value of $<.05$ was considered statistically significant for all analyses. Data are presented as means ± SD unless otherwise indicated. Statistical analysis was performed by using R version 3.4.2.[31]

RESULTS

Characteristics of Study Infants

Microbiota data were available from 291 infants at 6, 11, and 15 weeks, and from 249 of these same infants at 2 years. Half of the participants were boys, and half received a high-dose vitamin A supplement within 48 hours of birth as prescribed by the parent study (Table 1). The majority of infants were born by elective cesarean delivery, as is typical for hospital deliveries in Bangladesh.[26] At 6 weeks, all infants were exclusively or predominantly breastfed, and at 2 years, 55% received supplemental breastfeeding. Infants were healthy on the days of sample collection but a small percentage had elevated C-reactive protein (CRP), indicating an ongoing acute phase response (Table 1).

Bifidobacteria Were the Most Abundant Taxa

All infants had detectable *Bifidobacterium* at 6 weeks and the mean abundance for 6, 11, and 15 weeks was 0.637 with a range 0.020 to 0.921 (Supplemental Table 5). Species and subspecies of *Bifidobacterium* were measured at 6 weeks, and *B longum* was by far the most abundant species, at 0.581, followed distantly by *Bifidobacterium breve* and *Bifidobacterium bifidum* at 0.032 and 0.014, respectively. We further analyzed the 2 subspecies of *B longum* and found that *B longum* subspecies *infantis* (abundance, 0.568) was predominant, being ~10-fold more abundant than *B longum* subspecies *longum* (abundance, 0.058), as expected.

Bifidobacteria Abundance Was Positively Associated With the CD4 T-Cell Response to the BCG Vaccine

The BCG vaccine was given once within 48 hours of birth. Although intestinal bacterial communities were not established at that time, we hypothesized that such communities established between 6 and 15 weeks would affect maintenance of the BCG memory response. We tested this hypothesis using multivariate regression analysis to adjust for other factors that could also affect vaccine responses and found that mean *Bifidobacterium* abundance in early infancy (measured at 6, 11, and 15 weeks; Table 2) and *Bifidobacterium* abundance measured at 6 weeks only (the closest time to vaccination; Supplemental Table 6) were both

TABLE 1 Characteristics of the Participants

Characteristics	6 wk (*n* = 280)	2 y[a] (*n* = 249)
Sex, boy	137 (48.9%)	122 (48.8%)
Vitamin A supplement	139 (49.6%)	121 (48.8%)
Mode of delivery, cesarean delivery	170 (60.7%)	156 (62.7%)
Gestational age, wk	39.1 ± 1.62	39.2 ± 1.61
Preterm birth, <37 wk	20 (7.19%)	17 (6.91%)
Birth wt, g	2735 ± 375	2762 ± 392
Low birth wt, <2500 g	77 (27.5%)	63 (25.2%)
Wasting, WHZ <−2	13 (4.66%)	24 (10.2%)
Stunting, HAZ <−2	48 (17.2%)	81 (34.2%)
Breastfeeding status		
Exclusive	196 (70.0%)	0 (0%)
Nonexclusive	84 (30.0%)	133 (55.0%)
No breastfeeding	0 (0%)	109 (45.0%)
CRP ≥5 mg/L[b]	1 (0.390%)	16 (6.58%)

Data presented as mean ± SD or number (frequency). HAZ, height-for-age *z* score; WHZ, weight-for-height *z* score.
[a] Mean age = 27.7 ± 3.23 m, median age (25th, 75th percentile) = 28.0 (24.8, 30.1) m, range = 18.4 m.
[b] CRP value was not available for 24 subjects at 6 wk and 6 subjects at 2 y.

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TABLE 2 The Association Between *Bifidobacterium* Abundance During Early Infancy (Mean of 6, 11, and 15 Weeks of Age) and Vaccine-Specific CD4 T-Cell SI, DTH Skin Test Response, and Antibody Responses

	Regression Model		Average *Bifidobacterium* Abundance	
	R^2	*P*	β (SE)	*P*
BCG				
6 wk				
$CD4^+$ T-cell SI[a,b]	0.0859	.019	.144 (0.145)	.32
15 wk				
$CD4^+$ T-cell SI	0.0590	.021	.668 (0.214)	<.01
Skin test area, cm^2	0.0201	.34	.427 (0.362)	.24
2 y				
$CD4^+$ T-cell SI[a,c]	0.0883	<.01	.451 (0.221)	.043
TT				
15 wk				
$CD4^+$ T-cell SI[c]	0.0514	.037	.371 (0.131)	<.01
ALS IgG, mIU/mL	0.0166	.49	.408 (0.249)	.10
2 y				
$CD4^+$ T-cell SI	0.0453	.17	.465 (0.222)	.038
Plasma IgG, IU/mL	0.0402	.076	.378 (0.161)	.020
Plasma IgG AI	0.0117	.72	.00523 (0.0404)	.90
HBV				
15 wk				
$CD4^+$ T-cell SI[a]	0.0530	.042	1.09 (0.429)	.012
ALS IgG, mIU/mL[c]	0.0471	.19	−.00162 (0.0119)	.89
2 y				
$CD4^+$ T-cell SI	0.0150	.77	.218 (0.202)	.28
Plasma IgG, IU/mL	0.0228	.35	−.398 (0.233)	.088
Plasma IgG AI[d]	0.0285	.22	−.00723 (0.0599)	.90
Polio				
15 wk				
ALS polio IgG, mIU/mL[c]	0.0334	.11	$-4.03e^{-7}$ ($2.89e^{-7}$)	.16
2 y				
Plasma polio1 IgG, IU/mL	0.0138	.64	−.0117 (0.168)	.95
Plasma polio2 IgG, IU/mL	0.00802	.85	−.0163 (0.153)	.92
Plasma polio3 IgG, IU/mL	0.0156	.57	.155 (0.154)	.32
Plasma polio1 IgA, IU/mL	0.0211	.39	.144 (0.197)	.47
Plasma polio2 IgA, IU/mL	0.0150	.60	.111 (0.183)	.55
Plasma polio3 IgA, IU/mL[d]	0.0349	.12	.307 (0.212)	.15
Stool polio1 IgA, IU/g protein	0.0352	.21	.842 (0.326)	.011
Stool polio2 IgA, IU/g protein	0.0303	.31	.752 (0.311)	.016
Stool polio3 IgA, IU/g protein	0.0394	.16	.974 (0.356)	<.01
Plasma polio IgG AI	0.0317	.17	.123 (0.0595)	.039
Plasma polio IgA AI	0.00162	.9955	.0167 (0.0522)	.75
SEB				
6 wk				
$CD4^+$ T-cell SI[a,b]	0.112	<.01	.253 (0.163)	.12
15 wk				
$CD4^+$ T-cell SI[c]	0.0629	.037	.523 (0.230)	.024
2 y				
$CD4^+$ T-cell SI[d]	0.0646	.046	.397 (0.231)	.088

Association between infant's *Bifibacterium* abundance and 6 wk vaccine response was analyzed by using genus *Bifibacterium* abundance at 6 wk of age. Association between infant's *Bifibacterium* and 15 wk and 2 y analysis were done on mean genus *Bifibacterium* abundance at 6 wk, 11 wk, and 15 wk of age. The regression models were adjusted with sex, treatment group, birth weight, and type of delivery. Stool antibody models were additionally adjusted for breastfeed status at 2 y of age.

[a] Vaccine response was significantly different between boys and girls.

[b] Vaccine response was significantly different between treatment groups.

[c] Vaccine response was significantly different between normal and cesarean delivery.

[d] Vaccine response was significantly different between below and above birth weight median.

positively associated with the CD4 memory T-cell responses (measured as the SI) at 15 weeks and 2 years but not at 6 weeks (Fig 1). For infants with high mean *Bifidobacterium* abundance (90th percentile of study population, abundance = 0.814), the estimated SI at 15 weeks was 85% higher than for infants with low *Bifidobacterium* abundance (10th percentile abundance = 0.396), whereas the difference at 2 years was 64% (Table 3). No significant associations were seen between *Bifidobacterium* abundance and the DTH skin-test response for BCG (Fig 1).

In addition to *Bifidobacterium* abundance at the genus level, *Bifidobacterium* species and subspecies were measured at 6 weeks, and we examined these associations as well, primarily to determine if the predominant species (*B longum*) and subspecies (*B longum* subspecies *infantis*) were also associated with these vaccine responses. In brief, we found that both *B longum* (Supplemental Table 7) and *B longum* subspecies *infantis* (Supplemental Table 8) were significantly associated with the SI response at 15 weeks but not 2 years (Fig 1). We also examined associations for the less abundant *Bifidobacterium* subspecies (*B longum* subspecies *longum* Supplemental Table 9) and 2 minor *Bifidobacterium* species (*B breve* and *B bifidum*; Supplemental Tables 10 and 11, respectively), and no significant associations were seen (Fig 1).

Because bifidobacteria might affect T-cell proliferation in general (which could affect development of vaccine memory responses), we examined the association of bifidobacteria with the SI for *Staphylococcus* enterotoxin B (SEB), a polyclonal stimulator of T-cell proliferation. As was seen for the BCG SI results, *Bifidobacterium*, *B longum*, and *B longum* subspecies *infantis* abundance were all

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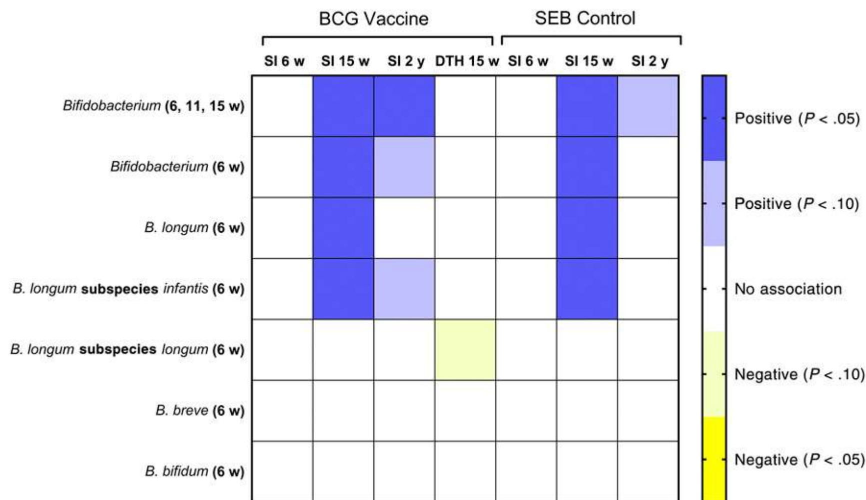

FIGURE 1
Heat map showing associations (and statistical significance) between early life bifidobacteria abundance and BCG vaccine responses measured at 6 weeks, 15 weeks and 2 years of age determined with multiple regression analysis as described in Methods. BCG vaccine responses include the CD4 T-cell SI and the DTH skin test response. Associations with the SI for the positive control for CD4 T-cell stimulation (SEB) are also shown. Bifidobacteria abundance measures were as follows: (1) mean abundance of the genus *Bifidobacterium* measured at 6, 11, and 15 weeks and single measures made at 6 weeks for (2) the genus *Bifidobacterium*; (3) the most abundant species, *B longum*; (4) the most abundant of 2 subspecies, *B longum* subspecies *infantis*; (5) the second subspecies, *B longum* subspecies *longum*; and 2 minor species, (6) *B breve* and (7) *B bifidum*.

significantly associated with the SI for SEB at 15 weeks (Fig 1).

Bifidobacteria Abundance Was Positively Associated With Responses to TT and HBV Vaccines

TT and HBV vaccines were administrated at 6, 10, and 14 weeks, within the period when bifidobacteria abundance was assessed, and we thus hypothesized that bifidobacterial abundance would be positively associated with the SI and IgG responses to both vaccines when they were measured at 15 weeks and 2 years. In partial confirmation of this hypothesis, *Bifidobacterium*, *B longum*, and *B longum* subspecies *infantis* abundance were all positively associated with the SI responses to both TT and HBV when they were measured at 15 weeks and with the TT SI response at 2 years, but they were not positively associated with the HBV response at 2 years (Fig 2). At 15 weeks, the predicted SI values for infants with high *Bifidobacterium* abundance were 76% and 45% higher than for those with low abundance for TT and HBV responses, respectively, whereas the corresponding difference at 2 years for TT was 57% (Table 3).

TABLE 3 Predicted Vaccine Response at Low (10th Percentile) and High (90th Percentile) Mean Bifidobacterium Abundance at 6, 11 and 15 Weeks

Vaccine Response	10th Percentile[a]	90th Percentile[a]	Difference, %
BCG SI at 15 wk	8.63	16.0	85
TT SI at 15 wk	7.15	12.60	76
HBV SI 15 wk	3.14	4.55	45
BCG SI at 2 y	10.0	16.4	64
TT SI at 2 y	4.60	7.23	57
TT plasma IgG 2 y, IU/mL	33.2	47.0	42
Stool OPV IgA at 2 y (strain 2, mIU/g protein)	0.0513	0.1060	107

[a] Predicted from regression models described in Methods, holding covariates constant, and by using mean *Bifidobacterium* abundance at the 10th (abundance = 0.396) and 90th (abundance = 0.814) percentiles.

Bifidobacterium abundance at the genus level was positively associated with the IgG responses to the TT vaccine when measured both at 15 weeks and at 2 years, whereas a similar association was seen at 15 weeks for *B longum* abundance (Fig 2). The predicted TT-specific IgG concentration at 2 years was 42% higher in infants with high versus low *Bifidobacterium* abundance (Table 3). *Bifidobacterium* at the genus level was not associated with the HBV IgG response at either 15 weeks or 2 years, although HBV-specific IgG at 15 weeks and plasma IgG maturation index at 2 years were negatively associated with *B breve* and *B longum* subspecies *longum*, respectively (Fig 2). All infants had protective IgG titres for both TT (≥0.1 IU/mL) and HBV (≥10 mIU/mL) at 2 years.

The generally positive associations of *Bifidobacterium*, *B longum*, and *B longum* subspecies *infantis* abundance with TT vaccine responses found in regression analysis can be seen graphically (without adjustment for covariates) in scatterplots of the raw data at 15 weeks (Supplemental Fig 5) and 2 years (Fig 3).

Bifidobacteria Abundance Was Positively Associated With IgG and IgA Responses to OPV Vaccine

OPV vaccine was administered within 48 hours of birth and again at 6, 10, and 14 weeks. We hypothesized that bifidobacterial abundance would be positively associated with OPV vaccine responses, which included the total IgG response at 15 weeks and strain-specific responses (3 strains are found in the OPV vaccine) at 2 years (plasma IgG and IgA, and stool IgA). In partial agreement with our hypothesis, the abundance of *Bifidobacterium* was positively associated with polio-specific stool IgA at 2 years (Fig 4), with the predicted response being 107% higher in infants with high versus low *Bifidobacterium* abundance (Table 3). This association can also be seen

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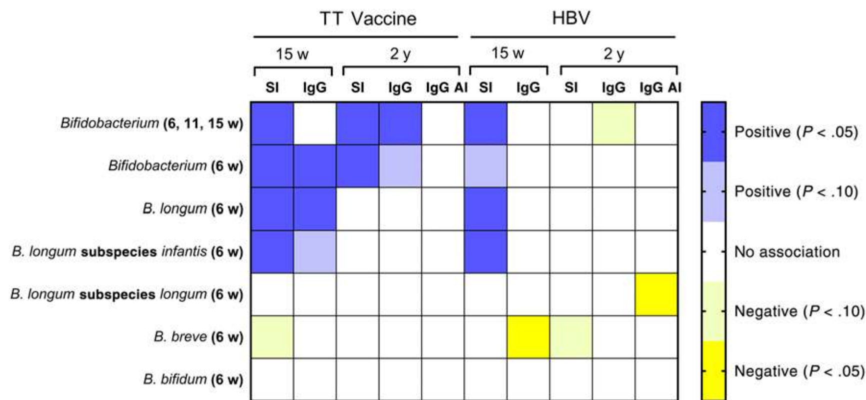

FIGURE 2

Heat map showing associations (and statistical significance) between early life bifidobacteria abundance and TT and HBV responses measured at 15 weeks and 2 years of age determined with multiple regression analysis as described in Methods. Responses include the CD4 T-cell SI, the ALS assay for IgG at 15 weeks, plasma IgG at 2 years, and the IgG AI at 2 years. Bifidobacteria abundance measures were as follows: (1) mean abundance of the genus *Bifidobacterium* measured at 6, 11, and 15 weeks and single measures made at 6 weeks for (2) the genus *Bifidobacterium*; (3) the most abundant species, *B longum*; (4) the most abundant of 2 subspecies, *B longum* subspecies *infantis*; (5) the second subspecies, *B longum* subspecies *longum*; and 2 minor species, (6) *B breve* and (7) *B bifidum*.

graphically with unadjusted data (Fig 3). Plasma IgA at 2 years also revealed some positive bifidobacterial associations (Fig 4), including positive associations of both *Bifidobacterium* and of *B longum* with the poliovirus strain 3 IgA response, whereas a negative association was seen between *B breve* and the strain 1 response (Fig 4). *B bifidum* abundance was positively associated with plasma IgG concentration at 2 years, whereas mean *Bifidobacterium* abundance was positively associated with the IgG AI (Fig 4). Two negative associations were also seen: *B longum* subspecies *longum* with 15 weeks IgG and *B breve* with 2 years plasma polio-specific IgA.

DISCUSSION

Higher bifidobacteria abundance in early infancy is associated with better memory responses to vaccines given at this time, as judged by the magnitude of the vaccine responses measured at 2 years. Mean *Bifidobacterium* abundance measured at 6, 11, and 15 weeks, and *Bifidobacterium* abundance measured at 6 weeks only, performed similarly in predicting later vaccine responses. These findings are novel and support current thinking about how gut microbiota may shape development of the infant immune system.[7,32] For example, higher Th1 responses are associated with bifidobacterial abundance[33] and a microbial state with low *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* in early infancy is associated with atopic CD4 T-cell responses at 2 years and asthma development at 4 years.[34] In addition, vaccinia virus–specific interferon-γ production by CD8 T cells,[35] and human serum albumin–specific and cholera toxin–specific interferon-γ and interleukin-5 production by splenocytes[36] postvaccination are both influenced by gut microbial composition. These studies also suggest a cause-effect relationship between gut microbiota and vaccine responses. Our results suggest a general effect of bifidobacteria on T-cell proliferation or survival, as indicated by the association of bifidobacteria levels with both vaccine-specific and SEB-stimulated T-cell proliferation, whereas the lack of an association with SEB-stimulated proliferation at 2 years suggests an independent association with maintenance of vaccine memory.

Gut bacteria affect development of T cells, particularly Treg and Th17 cells.[12,37] Bifidobacterial effects on T-cells could be direct or might involve effects on dendritic cells which then affect T cells, and these effects may be mediated by production of small-molecule bacterial metabolites including short chain fatty acids.[38–40] Bacterial macromolecules also affect immunity. Both lipopolysaccharide[41,42] and flagellin[43] act as vaccine adjuvants, presumably via toll-like receptor 4 and toll-like receptor 5, respectively, suggesting that commensal bacteria may act as natural vaccine adjuvants.[44] Indirect mechanisms may also be relevant. For example, *Bifidobacterium* protects against enteropathogenic infection[45] and reduces the relative abundance of *Enterobacteriaceae*[46] and thus may improve vaccine responses by reducing the risk of symptomatic infections or subclinical dysbiosis.

Many studies report associations of gut microbiota with vaccine-specific antibody responses.[7] In the current study, we report positive associations of early life bifidobacteria with TT-specific IgG responses both in early infancy and at 2 years, and with polio-specific IgA at 2 years, suggesting a sustained effect on vaccine memory. A study examining rotavirus vaccine response among infants in Ghana[47] found no associations with Actinobacteria (the phylum containing bifidobacteria), but reported that serum IgA was negatively associated with Bacteroidetes and positively associated with *Streptococcus bovis*. The same group performed a similar study in Pakistani infants and found no association with *Bifidobacterium*.[48] The infants in the current study did not receive rotavirus vaccine. A previous study

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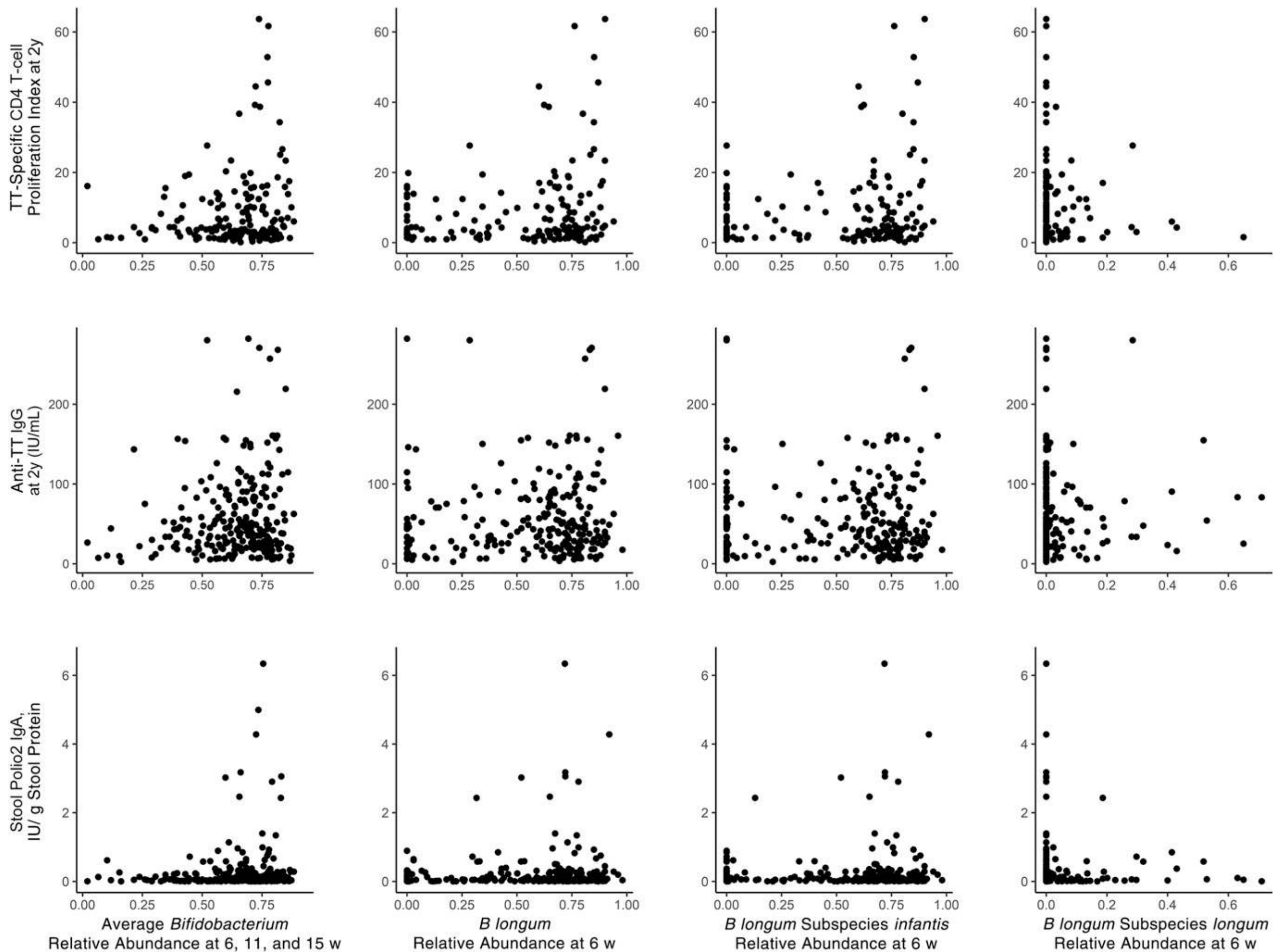

FIGURE 3
Association of stool bifidobacteria in early infancy, at 6 to 15 weeks of age, with vaccine responses at 2 years of age. Top row, TT-specific CD4 T-cell SI (top row); middle row, TT-specific plasma IgG; bottom row, stool polio type 2–specific IgA. The asterisk (*) indicates a single, off-scale value (>7.5).

found that abundance of *B breve* and *B longum*[49] were both positively correlated with polio-specific stool IgA measured shortly after vaccination. In the current study, we also report a nonsignificant (P = .088) positive association between *Bifidobacterium* and HBV-specific IgG at 2 years. A previous human study[50] using *B longum* and *Lactobacillus rhamnosus* supplementation in infants also showed a trend toward an increased HBV-specific IgG response. In another study, the abundance of *Bacteroides ovatus* and *Streptococcus geniculate* in nasal microbiota were positively and negatively associated with IgA responses, respectively, to intranasal influenza vaccination.[51] We also report that the *B breve* was negatively associated with polio-specific plasma IgA, a finding that is consistent with another study[52] revealing that *B breve* supplementation lowers the serum IgA response to cholera vaccination. In our study, we also found a negative association between *B longum* subspecies *longum* and polio-specific ALS IgG at 15 weeks. Such results emphasize the need to identify the mechanisms by which specific bifidobacteria affect immunity.

Strengths of our study include the examination of short- and long-term memory responses, the relatively large sample size, the use of specific assays for bifidobacteria at the species and subspecies level, the use of multiple vaccines and of multiple vaccine end points, and the prospective study design. Our study is limited in that it is observational, thus we cannot infer causality from the associations described here, and participants were all breastfed, limiting the diversity in intestinal microbiota seen in study infants. Additionally, although BCG and the OPV are not used in developed countries, the similarity in associations seen in this study to that seen in a small study of inactivated polio virus,[49] as well as the associations to the more widely administered HBV and TT vaccines, suggest that these results are relevant for many populations of infants around the world.

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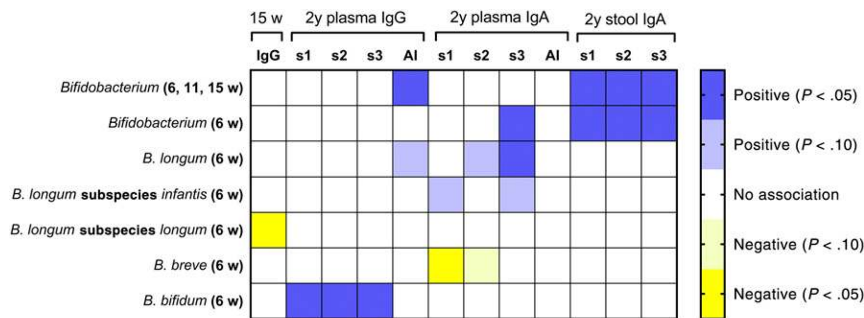

FIGURE 4

Heat map showing associations (and statistical significance) between early life bifidobacteria abundance and OPV vaccine responses measured at 15 weeks and 2 years of age determined with multiple regression analyses as described in Methods. Responses include the CD4 T-cell SI, the ALS assay for IgG at 15 weeks, plasma IgG and IgA at 2 years for the 3 strains of virus included in the vaccine, the IgG AI and IgA AI for all 3 strains at 2 years. Bifidobacteria abundance measures were as follows: (1) mean abundance of the genus *Bifidobacterium* measured at 6, 11, and 15 weeks and single measures made at 6 weeks for (2) the genus *Bifidobacterium*; (3) the most abundant species, *B longum*; (4) the most abundant of 2 subspecies, *B longum* subspecies *infantis*; (5) the second subspecies, *B longum* subspecies *longum*; and 2 minor species, (6) *B breve* and (7) *B bifidum*. s1, strain 1; s2, strain 2; s3, strain 3.

CONCLUSIONS

Colonization with *Bifidobacterium* at the time of vaccination is associated with sustainable systemic and mucosal vaccine-specific memory T-cell and antibody responses. Developing strategies to enhance immunologic memory is a high priority for vaccine research.[53] A recent intervention trial demonstrated that administration of probiotic *B longum* subspecies *infantis* to healthy infants between 7 and 28 days significantly increased the abundance of this organism through at least 60 days,[46] demonstrating prolonged colonization with this organism in breastfed infants. An adequately powered randomized controlled trial of a similar strategy to increase early colonization with *B longum* subspecies *infantis* to enhance responses to early vaccination is indicated by our findings.

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ABBREVIATIONS

AI: avidity index
ALS: antibody in lymphocyte supernatant
BCG: *Bacillus* Calmette-Guérin
CRP: C-reactive protein
DTH: delayed-type hypersensitivity
ELISA: enzyme-linked immunosorbent assay
HBV: hepatitis B virus
IgA: immunoglobulin A
IgG: immunoglobulin G
OPV: oral polio virus
SEB: *Staphylococcus* enterotoxin B
SI: stimulation index
TT: tetanus toxoid

Dr Huda oversaw participant enrollment and follow-up, data collection, and performed laboratory analysis and performed statistical analysis and drafted the manuscript under the mentoring and supervision of Drs Stephensen and Mills. Mr Alam, Ms Khanam, Dr Kalanetra, and Dr Taft all participated in the data collection, laboratory analysis, and editing the manuscript; and all authors reviewed and approved the final manuscript as submitted.

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