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JPEN J Parenter Enteral Nutr 2012 36: 106S

DOI: 10.1177/0148607111430817

The online version of this article can be found at:

http://pen.sagepub.com/content/36/1_suppl/106S

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Bifidobacterium lactis Bb12 Enhances Intestinal Antibody Response in Formula-Fed Infants: A Randomized, Double-Blind, Controlled Trial

Hannah D. Holscher, RD¹; Laura A. Czerkies, MS, RD²; Pamela Cekola, RD²; Richard Litov, PhD³; Marshall Benbow, MD⁴; Sheryl Santema, MD⁵; Dominik D. Alexander, PhD, MSPH⁶; Vanessa Perez, PhD, MS⁶; Shumei Sun, PhD⁷; José M. Saavedra, MD²; and Kelly A. Tappenden, PhD, RD¹

Financial disclosure: This study was sponsored by Nestlé Infant Nutrition, Florham Park, NJ. The publication of the supplement in which this article appears is sponsored by Nestlé Nutrition Institute.

Background: Addition of probiotics to infant formula may positively affect immune function in nonexclusively breastfed infants. This study aimed to investigate the effect of infant starter formula containing the probiotic *Bifidobacterium animalis* subspecies *lactis* (Bb12) on intestinal immunity and inflammation. **Methods:** Six-week-old healthy, full-term infants (n = 172) were enrolled in a prospective, randomized, double-blind, controlled clinical trial with 2 groups studied in parallel to a breastfed comparison group. Formula-fed (FF) infants were randomized to partially hydrolyzed whey formula (CON) or the same formula containing 10⁶ colony-forming units (CFU) Bb12/g (PRO) for 6 weeks. Fecal secretory IgA (sIgA), calprotectin, lactate, and stool pH were assessed at baseline, 2 weeks, and 6 weeks. Anti-poliovirus-specific IgA and anti-rotavirus-specific IgA were assessed at 2 and 6 weeks. **Results:** Among vaginally delivered FF infants, PRO consumption increased (P < .05) fecal sIgA compared to CON. Anti-poliovirus-specific IgA concentration

increased (P < .05) in all infants consuming PRO, whereas anti-rotavirus-specific IgA tended to increase (P = .056) with PRO consumption in cesarean-delivered infants. Anthropometrics and tolerance did not differ significantly between FF infants. **Conclusions:** Infants consuming formula with Bb12 produced feces with detectable presence of Bb12 and augmented sIgA concentration. Furthermore, cesarean-delivered infants consuming Bb12 had heightened immune response, as evidenced by increased anti-rotavirus- and anti-poliovirus-specific IgA following immunization. These results demonstrate that negative immune-related effects of not breastfeeding and cesarean delivery can be mitigated by including Bb12 in infant formula, thereby providing infants a safe, dietary, immune-modulating bacterial introduction. (JPN J Parenter Enteral Nutr. 2012;36:106S-117S)

Keywords: infant formula; pediatrics; probiotics

Clinical Relevancy Statement

Breast milk, the ideal source of nutrition for infants, is also a source of secretory immunoglobulin A (IgA), as well as bacte-

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Received for publication September 1, 2011; accepted for publication November 2, 2011.

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ria, which enhance both passive and active infant immunity. These benefits are not provided by routine-use infant formula. The addition of probiotics to infant formula is reported to increase fecal secretory IgA. This study demonstrates that supplementation of infant formula with *Bifidobacterium animalis* subspecies *lactis* (Bb12) results in detectable fecal levels of Bb12 and increased concentrations of secretory-, anti-rotavirus-specific, and anti-poliovirus-specific IgA. Therefore, including Bb12 in infant formula provides infants a safe, dietary, immune-modulating bacterial experience.

Introduction

Although it is known that breastfeeding is best, infant formulas are often used to supplement breast milk, and mothers of some infants may choose not to breastfeed. Attempts to optimize infant formula have been undertaken to impart some of the benefits of breast milk to the

formula-fed infant. One such strategy is to positively influence the composition and activity of the intestinal microbiota of the formula-fed infant to more closely reflect that of the breastfed infant. An infant starter formula containing the probiotic *Bifidobacterium lactis* is currently being marketed in the United States and is available in many other parts of the world.

Probiotics are live microorganisms that, when administered orally in adequate amounts, survive the digestion process and confer a beneficial effect on the host.¹ Saavedra² proposed probiotics as a concept by which the use of these nonpathogenic agents “constitutes a purposeful attempt to modify the relation with our immediate microbial environment in ways that may benefit our health.” The microbial environment of the intestinal microbiota is an integral part of the immune system. In particular, it has been established that the microbiota of the breastfed infant is dominated by bifidobacteria early in life,³ and this has been thought to contribute to the many health benefits breastfed infants possess. The lower incidence of infection and gastrointestinal disturbances observed in breastfed infants compared to their formula-fed counterparts may be related to differences in their respective microbiota. One such benefit of a balanced intestinal microbiota influenced by probiotics is to provide stimulatory support to the mucosal immune system.

Nonpathogenic bacteria are not considered to be a probiotic until a clinical benefit to the host has been demonstrated. Among those probiotic bacteria that have been better studied is *B lactis*. Safe administration of *B lactis* to pediatric populations has been reported widely in the literature, with no adverse effects on growth noted.^{4,7} Furthermore, *B lactis* consumption is reported to beneficially affect immune function by increasing fecal immunoglobulin A (IgA) levels.^{8,9} Associated outcomes, including reduction in antibiotic use,⁴ reduction in febrile episodes,¹⁰ reduction in number of severe illness days, reduction in otitis media infection,¹¹ and improvements in allergy-related skin conditions, have also been seen.^{12,13} In a small group of toddlers (15–31 months) fed a *B lactis*-containing formula, significantly higher levels of fecal secretory IgA (sIgA) and antipoliavirus IgA were observed after 3 weeks of feeding.⁸ Separately, Mohan et al⁹ demonstrated increased fecal sIgA concentration in a study of premature infants fed a *B lactis*-containing formula for the first 3 weeks of life. Fecal IgA levels of full-term infants exclusively fed a *B lactis*-containing formula have been reported only in one other study to date,¹⁴ but the current trial differs in the timing of probiotic initiation and the composition of the formula vehicle.

sIgA is the predominant immunoglobulin in mucosal surfaces and provides key protection in the innate mucosal immune response to dietary and microbial antigens. Although breast milk contains sIgA, infant formula does not. Significantly higher levels of fecal sIgA in samples from

breastfed infants compared to formula-fed infants have been reported through the first 6 months of life.^{15,16} Probiotics have been shown to increase fecal sIgA levels in a pediatric population after 3 weeks of feeding.⁸ Further investigations to determine ways to increase levels of fecal sIgA in formula-fed infants could lead to improvements in infant formula.

The primary outcome of this trial was to compare the fecal sIgA concentration of 6-week-old healthy infants following 6 weeks of feeding of a partially hydrolyzed whey-based starter formula (control) or the same formula containing *Bifidobacterium animalis* subspecies *lactis* (Bb12). Secondary outcomes, which quantified markers of immunity and inflammation, were fecal sIgA concentration at 8 weeks of age (following 2 weeks of feeding) and fecal abundance of Bb12, anti-rotavirus-specific IgA, anti-poliovirus-specific IgA, calprotectin, lactate, and stool pH after 2 and 6 weeks of formula feeding. We hypothesized that consumption of an infant formula containing Bb12 for 6 weeks would positively affect the intestinal-associated immune system and resident microbiota of formula-fed infants.

Methods

Participants and Study Design

The study was conducted from September 2007 to March 2008 at 5 study sites (Pedia Research in KY/IN; Southwest Children's Research Associates in San Antonio, TX; and Scott & White Memorial Hospital in Temple and Killeen, TX) within the United States. Newborn infants (2–6 weeks of life) were recruited. All participants were required to live within 45 minutes of a study center and have a working freezer in their home. Infants receiving antibiotic therapy in the 30 days prior to enrollment or breastfed infants whose mothers had received antibiotic therapy within the same period were not eligible for the study. Other criteria for exclusion from the study were known or suspected cow milk allergy, congenital illness or malformation that affects infant feeding and/or growth, significant prenatal and/or postnatal disease, immunocompromise, any readmission to the hospital (except for hyperbilirubinemia) prior to enrollment, diarrhea requiring treatment in the 7 days prior to enrollment, or receipt of medication other than multivitamin supplement. Parents of all participants were required to sign a written informed consent prior to enrollment. The studies were approved by the relevant institutional review board (IRB) of each site recruiting participants and the University of Illinois, where all laboratory analyses of samples occurred. The procedures followed were in accordance with the ethical standards of the responsible institutional or regional committee on human experimentation.

Formula-fed (FF) infants were randomized to receive either CON or PRO at 6 weeks of age and consume the

assigned formula exclusively for the following 6 weeks. The control formula was a commercially available, partially hydrolyzed whey infant formula (CON; Nestlé Good Start; Nestlé, Florham Park, NJ), whereas the test formula (PRO) was identical to CON with the exception of the addition of 10^6 colony-forming units (CFU) Bb12/g powder. Both formulas provided 20 kcal/oz with 2.2 g protein/100 kcal from 100% partially hydrolyzed whey. Randomization of FF infants was stratified by delivery (vaginal vs cesarean section). The self-selected breastfed (BF) reference group was exclusively breastfed and followed for 6 weeks starting at 6 weeks of age.

Weight of each infant was taken at baseline (V0) and after 2 (V1) and 6 weeks (V2) of feeding at study visits. Caregivers were instructed to complete daily records for 2 days prior to the visits. Using paper diaries, caregivers of all infants recorded the same data regarding formula intake volumes, stool patterns, and tolerance-related behavior. Caregivers of BF infants documented the length of each feeding or the amount of expressed breast milk given at each feeding, in addition to stool patterns and tolerance-related behavior. All participants were asked to receive immunizations according to the schedule outlined by the Department of Health and Human Services, which included receipt of rotavirus and inactivated poliovirus vaccines at 8 weeks of age. Stool sample for visit 1 was collected prior to vaccinations.

Within 72 hours of each study visit, stool samples were collected at home in 30-mL polypropylene fecal collection tubes (Globe Scientific, Paramus, NJ) within 30 minutes of passing. Caregivers brought the fresh sample in a cooler with ice packs to the site within 30 minutes of collection, or the tube with the sample was placed in a home freezer for 2–72 hours prior to transporting the sample to a study site in the manner outlined above. Upon arrival at the study site, samples were promptly placed at -20°C for a maximum of 2 weeks before shipment on ice to the University of Illinois, where samples were stored at -80°C until analysis.

Fecal Homogenates

Fecal homogenates were prepared by thawing frozen fecal samples on ice and then creating a 10% wt:vol mixture in phosphate-buffered saline (PBS) followed by homogenization with a homogenizer (Model PCU-11; Brinkman Instruments, Westbury, NY) for 1 minute. Aliquots of fecal homogenates for quantification of sIgA, anti-poliovirus-specific IgA, and anti-rotavirus-specific IgA were stored at -80°C until analysis.

Secretory IgA

sIgA was determined from the fecal samples that were collected at baseline, 2 weeks, and 6 weeks of feeding. Analysis of sIgA was performed as described before by

Scholtens and colleagues, with minor modifications.^{14,17} Modifications included a decreased concentration of the secondary antibody, biotin-conjugated mouse anti-human IgA1/IgA2 monoclonal antibody (555884; BD Pharmingen, Franklin Lakes, NJ), to a concentration of 200 ng/mL and use of 1-step Ultra TMB-ELISA (34028; Pierce, Rockford, IL) for 3 minutes prior to addition of 1.8 M H_2SO_4 to stop the enzymatic color development. The plates were read at 450 nm in a plate reader. Fecal sIgA concentrations were determined using serially diluted purified human IgA isolated from colostrum (I-1010; Sigma, St Louis, MO) to generate a standard curve. Results are expressed as change from baseline (mg/sIgA per gram feces).

Anti-Poliovirus- and Anti-Rotavirus-Specific IgA

Measurements of anti-poliovirus-specific IgA and anti-rotavirus-specific IgA from fecal samples collected prior to vaccination (V1) and following vaccination (V2) were conducted in a similar manner as the sIgA protocol with modifications in the original coating of the plate, preparation of the standard curve, sample dilutions, and incubation with 1-step Ultra TMB. Plates were coated with 1:100 dilutions of either IPOL vaccine¹⁸ or oral Rotateq vaccine in a 20-mM NaHCO_3 coating buffer (pH 9.5) for poliovirus-specific or rotavirus-specific IgA, respectively. As standards are not commercially available for either anti-poliovirus- or anti-rotavirus-specific IgA, the respective standard curves were generated by pooling separately all V2 fecal samples from infants receiving the polio vaccine and/or the rotavirus vaccine to create a relative measurement of antibodies present. The total pooled samples used to relatively quantify the amount of anti-poliovirus- or anti-rotavirus-specific IgA were serially diluted and included on each plate. Fecal homogenates were diluted between 10 and 200 times. Incubation time with 1-step Ultra TMB (3,3',5,5'-tetramethylbenzidine) was increased to 30 minutes at room temperature before stopping the enzymatic color reaction with stop solution. Anti-poliovirus-specific and anti-rotavirus-specific IgA concentrations were determined from their respective standard curves and expressed as change in units per gram feces from V1 to V2 as all vaccinations took place after collection of the V1 sample.

Fecal Calprotectin, Lactate, and pH

Fecal calprotectin and lactate were measured using the PhiCal Fecal Calprotectin Immunoassay kit (Genova Diagnostics, Asheville, NC) and Lactate Assay Kit (K607; BioVision, Mountain View, CA), respectively. Fecal pH value was immediately obtained upon thawing the sample by submerging the pH probe (Futura Refillable Probe; Beckman Instrument, Fullerton, CA) into the fecal matter and waiting until the pH meter (UP-5; Denver Instrument, Denver, CO) reading stabilized.

Stool DNA Extraction and Quantitative Polymerase Chain Reaction of Bb12

DNA from stool samples was isolated using the QIamp DNA stool mini kit (Qiagen, Valencia, CA) with minor modifications previously described by Solano-Aguilar and colleagues.¹⁹ Briefly, 1.4 mL of lysis buffer was added to 180–220 mg of feces followed by immediate mixing for 1 minute. Homogenate was then incubated at 95°C for 5 minutes to facilitate increased DNA yield, followed by removal of inhibitors and proteinase K digestion. DNA was then bound to a column, washed, and eluted.

The amount of Bb12 copy numbers in fecal samples was determined using a primer-probe set specific for the *tuf* gene as previously described by Solano-Aguilar and colleagues.¹⁹ Primers and probes were purchased from Applied Biosystems (ABI, Foster City, CA) as follows: *tuf* forward GTG TCG AGC GCG GCA A; reverse CTC GCA CTC ATC CAT CTG CTT; BGB probe ATC AAC ACG AAC GTC GAG A. Commercially available Taqman Gene Expression assays (Applied Biosystems) supplied minor groove binding probes and primers for amplification and analysis of the *tuf* gene of Bb12.

Dr Gloria Solano-Aguilar (The Henry A. Wallace Beltsville Agricultural Research Center, Beltsville, MD) generously donated a genomic *tuf* gene fraction (136.52 nmoles/L; 8.21×10^{10} copies/ μ L) for use to generate the standard curve. The size of the fragment (120 bp) and its molecular mass were quantitatively determined on the DNAChip (Agilent, Waldbronn, Germany) using the Bioanalyzer (Agilent Technologies, Wilmington, DE).

Bb12 *tuf* gene abundance was measured with a Taqman ABI 7900 (Applied Biosystems). Data were analyzed with ABI Sequence Detection System software (version 2.2.1; Applied Biosystems) using standard curve methodology. In this method, the threshold cycles from the Bb12 *tuf* gene were compared with a standard curve generated from a series of dilutions of the *tuf* gene standard. The calculated concentration of the Bb12 copy numbers was normalized to fecal mass (mg). Each sample was assayed in triplicate. Both positive (DNA extracted from the PRO formula) and negative controls were included on each plate.

Statistical Analysis

The statistical power analysis and the number of study participants were based on the primary outcome, level of fecal sIgA, to investigate whether infants consuming infant formula containing Bb12 have a higher level of fecal sIgA compared to infants receiving the control formula. The number of infants was determined by having sufficient statistical power to test the differences in changes in fecal IgA from baseline between the probiotic formula and the control formula, using 2-tailed *t* tests at a level of significance of $P = .05$ (ie, type I error = 0.05).

Fukushima and colleagues⁸ found that the mean fecal sIgA at baseline was 1.49 ± 0.32 mg/g and rose to 4.29 ± 1.09 mg/g after 8 days of feeding with the probiotic formula. We assumed that infants fed with control formula would not experience a change in level of fecal IgA from baseline values to values at 8 days of follow-up. Therefore, we calculated the sample size per group for a statistical power of 0.80 for detecting a difference of 2.8 mg/g or greater. The standard deviation of the fecal IgA was 0.8 mg/g at baseline in the Fukushima et al study and increased to 2.88 mg/g after 8 days of exposure to probiotic-containing formula. To detect a group difference in changes from baseline, the sample size necessary to achieve a power of 0.80 was at least 36 infants per group. As such, 90 infants (45 for each formula group) were recruited to compensate for an anticipated attrition of 20% over the study period. A separate group of exclusively BF infants was included as a reference. In anticipation of a similar attrition in this group, 45 BF infants were recruited in the expectation that 36 of them would complete the study.

Data were analyzed using SAS (version 9.1; SAS Institute, Cary, NC). The generalized linear model included main effects for treatment and delivery and an interaction of treatment and delivery. Treatment means were compared using the Student *t* test. Significance of difference was set at $P < .05$. Change in fecal sIgA from baseline was assessed using per protocol (PP) and intent-to-treat (ITT) approaches. All other analyses were performed only on infants completing the study per protocol. Histograms and q-q plots were used to visually assess normality, and log transformations were utilized as needed. Values reported are means $\pm$ standard error of the mean (SEM).

The secondary outcomes of gastrointestinal tolerance, weight gain, and adverse events were analyzed according to PP and ITT designs. To compare the differences in tolerance data and body weight data between the 2 formula groups and a BF group, a general linear model analysis was performed followed by a Tukey-Kramer procedure to control the overall type I error rate. Mixed-effects models were applied to serial values at baseline (V0), 2 weeks of feeding (V1), and 6 weeks of feeding (V2). The analysis was performed with route of delivery and study center as covariates.

Results

Of 172 recruited infants, 21 FF infants and 6 BF infants did not complete the baseline visit at 6 weeks of age (Figure 1). Infants included in the ITT analysis were 52 infants self-selected to be exclusively breastfed (39 vaginally delivered and 13 by cesarean section) and 93 FF infants randomized to CON (43 infants; 27 vaginally

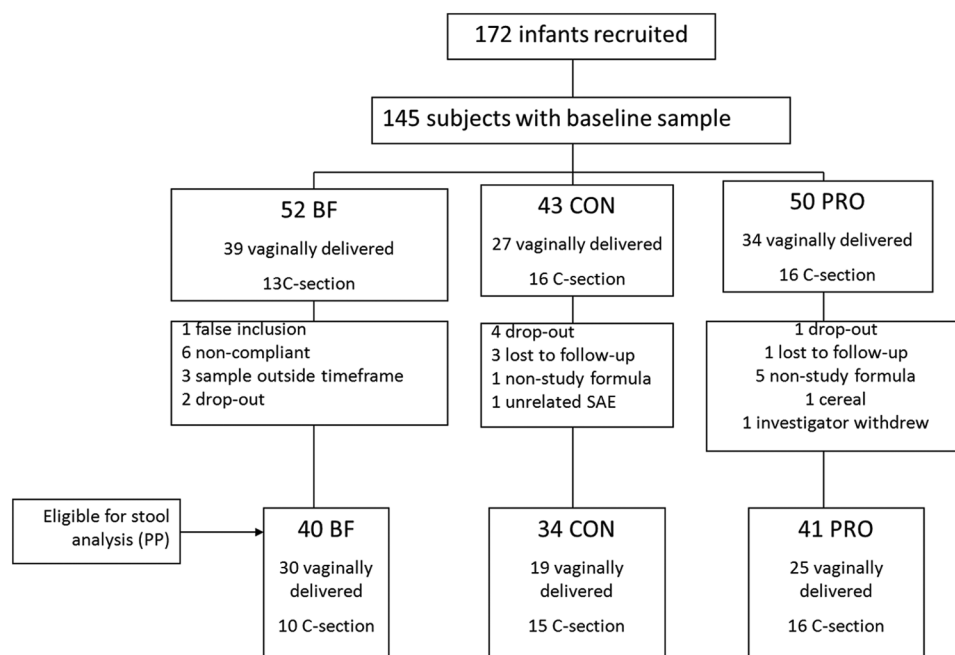


Figure 1. Schematic of participant retention and inclusion in stool per protocol analysis (PP). BF, breastfed; CON, control infant formula group; PRO, probiotic infant formula group; SAE, serious adverse event.

delivered and 16 by cesarean section) or PRO (50 infants; 34 vaginally delivered and 16 by cesarean section). Forty BF and 75 FF infants completed the study per protocol with 41 randomized to PRO (25 vaginally delivered and 16 by cesarean section) and 34 to CON (19 vaginally delivered and 15 by cesarean section). Nine infants in each of the FF groups (18 total; 5 dropped out before study completion, 4 were lost to follow-up, 6 received non-study formula, 1 received cereal, 1 investigator withdrew a participant, 1 study formula-unrelated serious adverse event occurred [hospitalization for respiratory syncytial virus]) and 12 BF infants (6 not exclusively breastfed, 3 incorrect timing of stool collection, 1 false inclusion, 2 dropouts before study completion) were excluded from the PP analysis because of study withdrawal or noncompliance.

Measures of birth weight, length, gestational age, or route of delivery did not differ between feeding groups at baseline. These data as well as infants' prestudy feeding regimen are presented in Table 1. The average formula intake for infants assigned to the PRO was 24 ounces per day (1.0×10^8 colony-forming units [CFU] Bb12) at 8 weeks of age and 25.6 ounces (1.1×10^8 CFU Bb12) per day at 12 weeks of age. The average formula intake for infants assigned to the CON formula was 23 ounces per day at 8 weeks of age and 23.8 ounces per day at 12 weeks of age. There was no difference in intake between PRO and CON ($P = .2829$). BF infants were fed an average of 7 times per day.

Stool samples from 145 infants were analyzed as ITT (primary outcome only), whereas stool samples from 115 infants were included in the PP analysis. Tolerance outcomes and growth are reported for all recruited participants on an ITT basis. Data on fecal samples are reported on ITT and PP infants for fecal sIgA and PP infants only for all other outcomes assessed on stool samples.

Fecal Bb12 abundance was assessed by quantifying *tuf* gene copies and was found to be present in 93% and 88% of PRO fecal samples at V1 and V2, respectively. There was no difference ($P = .12$) in Bb12 *tuf* gene copies in PRO (26.6 ± 15.0), CON (1.5 ± 1.4), or BF (50.1 ± 22.3) samples at baseline, prior to study formula initiation (Figure 2). As expected, Bb12 *tuf* gene copy number abundance was significantly greater ($P = .0095$) in PRO vs CON at V1 (3290 ± 1210 vs 26 ± 11 , respectively) and V2 (5105 ± 1909 vs 39 ± 18 , respectively), thereby validating compliance regarding assigned formula intake within the study population. BF infants were found to have 231.9 ± 46.3 Bb12 *tuf* gene copy numbers at V1 and 203.7 ± 63.3 at V2.

BF infants had significantly higher ($P < .001$) concentrations of fecal sIgA in stool samples as compared to FF infants at all time points (Figure 3). Vaginally delivered PRO infants had increased ($P = .046$; 1.053 ± 1.017 mg/g) fecal sIgA concentration from baseline to 6 weeks of feeding (V0 to V2) compared to CON (-1.809 ± 1.341

Table 1. Baseline Characteristics of the Intention-to-Treat (ITT) Population

	BF		CON		PRO	
	Vaginally Delivered (n = 39)	Delivery by Cesarean Section (n = 13)	Vaginally Delivered (n = 27)	Delivery by Cesarean Section (n = 16)	Vaginally Delivered (n = 34)	Delivery by Cesarean Section (n = 16)
Birth weight, g	3355 ± 79	3552 ± 126	3176 ± 82	3379 ± 78	3277 ± 75	3278 ± 149
Birth length, cm	50.8 ± 0.5	50.4 ± 0.6	50.3 ± 0.6	48.6 ± 0.4	50.3 ± 0.5	49.5 ± 0.5
Gestational age, wk	39.0 ± 0.1	39.0 ± 0.3	38.0 ± 0.2	38.0 ± 0.2	39.0 ± 0.2	39.0 ± 0.2
Prestudy feeding	9 infants received some formula for 1 day	4 infants received some formula for 1.3 ± 0.3 days	7 infants received breast milk for 11.3 ± 4.5 days	6 infants received breast milk for 10.3 ± 5.3 days	5 infants received breast milk for 13.1 ± 4.9 days	3 infants received breast milk for 9.3 ± 4.0 days

Data are expressed as means ± SEM. BF, breastfed; CON, control infant formula group; PRO, probiotic infant formula group.

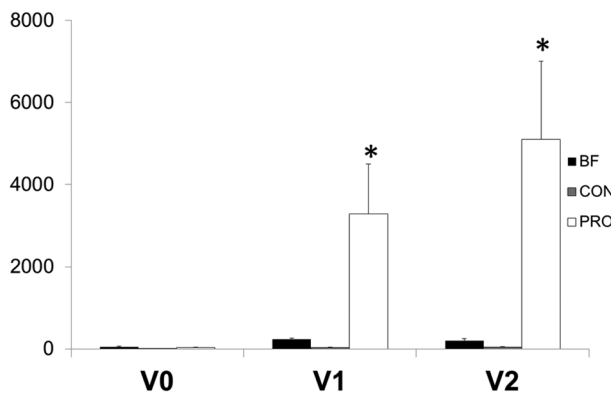


Figure 2. Effect of probiotic supplementation on fecal Bb12 copy numbers per gram feces in all infants. There was no difference at baseline (V0) in copy numbers between groups ($P = .12$). Probiotics increase fecal Bb12 copy numbers in formula-fed infants after 2 weeks and 6 weeks of study formula consumption. PRO infants had significantly greater ($P = .0095$) Bb12 at V1 and V2 as compared to CON at V1 and V2. Data are expressed as means ± SEM. BF, breastfed reference group; CON, control infant formula group; PRO, probiotic infant formula group; V0, baseline sample at 6 weeks of age; V1, 8 weeks of age; V2, 12 weeks of age. * $P < .05$.

mg/g change from baseline) in the ITT groups (Figure 4a). In addition, the change from V0 to V1 also tended ($P = .063$) to be significant in PRO ITT–vaginally delivered infants (1.77 ± 1.52 mg/g change from baseline) as compared to CON (-1.82 ± 1.9 mg/g change from baseline), as shown in Figure 4a. Fecal sIgA concentration was not affected by formula composition in the cesarean-delivered ITT (Figure 4b).

Within PP infants, PP vaginally delivered PRO infants demonstrated a trend toward increased fecal sIgA from

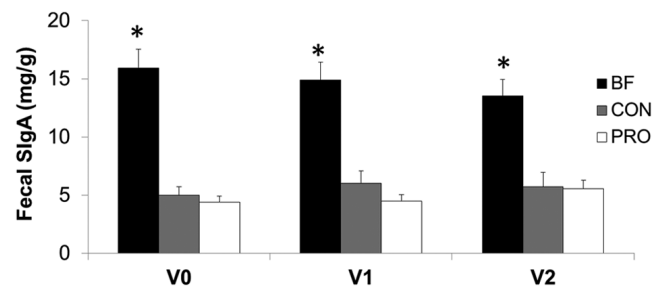


Figure 3. Effect of probiotic supplementation on fecal secretory immunoglobulin A (sIgA) concentration (in milligrams per kilogram feces) of infants at baseline (V0), after 2 weeks (V1), and after 6 weeks (V2). Breastfed infants have higher fecal sIgA ($P < .001$) than formula-fed infants at all time points. Data are expressed as means ± SEM. BF, breastfed; CON, control infant formula group; PRO, probiotic infant formula group. * $P < .05$.

baseline to visit 2 similar to the ITT–vaginally delivered infants ($P = .052$; PRO: 1.80 ± 1.9 mg/g change from baseline; CON: -2.58 ± 1.83 mg/g change from baseline). There was no difference in fecal sIgA in PP-cesarean-delivered infants (data not shown).

In cesarean-delivered infants, PRO (54.9 ± 30.0 U/g change) tended to increase ($P = .056$) anti-rotavirus-specific IgA concentrations from V1 to V2, but no change was observed with CON (11.5 ± 17.0 U/g change) (Figure 5). There was also a trend ($P = .07$) for probiotic supplementation to increase anti-rotavirus-specific IgA concentration from V1 to V2 when all 3 groups were analyzed without stratification by delivery method (PRO: 20.2 ± 20.5 U/g change; CON: 6.0 ± 23.1 U/g change; BF: -59.1 ± 31.6 U/g change).

From V1 to V2, the increase in anti-poliovirus-specific IgA concentration was higher in all PRO ($48.2 \pm$

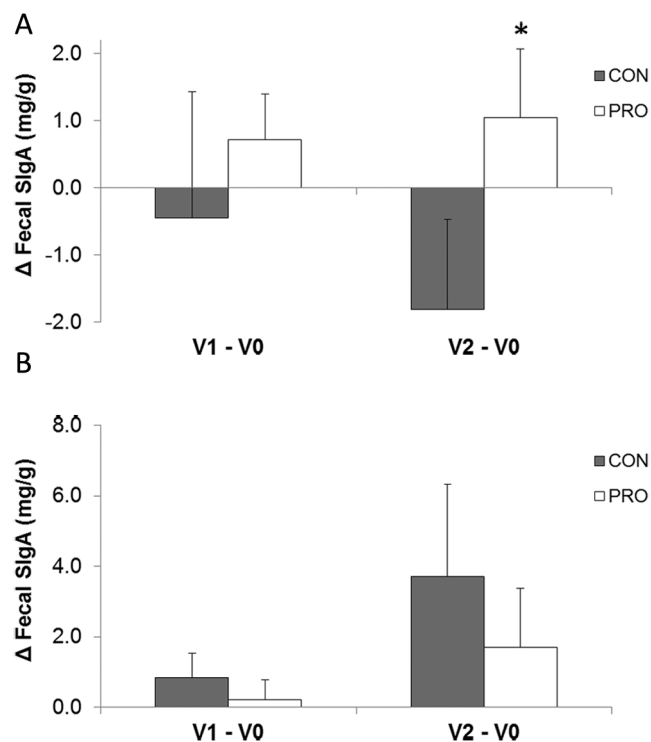


Figure 4. Within the intent-to-treat (ITT) design, probiotics increased fecal secretory immunoglobulin A (sIgA) in vaginally delivered formula-fed infants after 6 weeks of study formula consumption (a). Data are expressed as means $\pm$ SEM. CON, control infant formula group; PRO, probiotic infant formula group. Vaginally delivered PRO infants had a significantly greater ($P = .046$) increase in fecal sIgA concentration from V0 (baseline at 6 weeks of age) to V2 (12 weeks of age) compared to CON. Bb12 supplementation also tended ($P = .063$) to enhance the change of fecal sIgA at V1 (8 weeks of age) in PRO ITT–vaginally delivered infants as compared to CON. There was no treatment effect in ITT–cesarean section infants in change from baseline at V1 ($P = .24$) or V2 ($P = .26$). ($P = .26$) (b). * $P < .05$.

40.2 U/g change) vs CON (-18.0 ± 20.0 U/g change) stool samples ($P = .031$), including those from cesarean-delivered infants ($P = .026$; PRO: 51.6 ± 26.2 U/g change; CON: 19.4 ± 19 U/g change), as shown in Figure 6. A similar trend ($P = .10$) in poliovirus-specific IgA concentration is noted in vaginally delivered infants consuming PRO; however, statistical significance was not achieved because of high variability (PRO: 45.9 ± 66.6 U/g change; CON: -50.7 ± 31.8 U/g change) (Figure 6). Again, there was also a trend ($P = .075$) for probiotic supplementation to increase anti-poliovirus-specific IgA concentration from V1 to V2 when all 3 groups were analyzed without stratification by delivery method (PRO: 48.2 ± 40.2 U/g change; CON: -18.0 ± 20.0 U/g change; BF: -16.5 ± 20.3 U/g change).

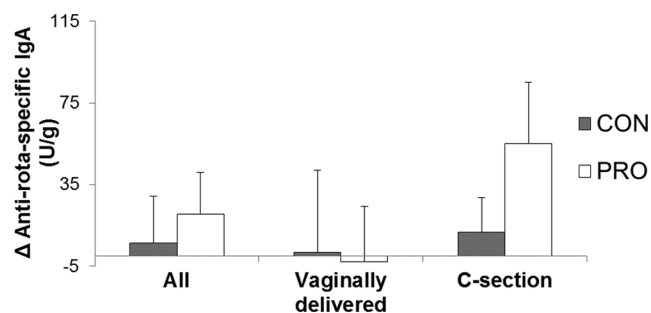


Figure 5. Probiotics increase anti-rotavirus-specific immunoglobulin A (IgA) in infants delivered by cesarean section. Data are expressed as means $\pm$ SEM. CON, control infant formula group; PRO, probiotic infant formula group. PRO infants delivered by cesarean section had greater ($P = .056$) changes in anti-rotavirus-specific IgA concentrations from V1 (8 weeks of age prior to receiving vaccination) to V2 (12 weeks of age after receiving vaccination).

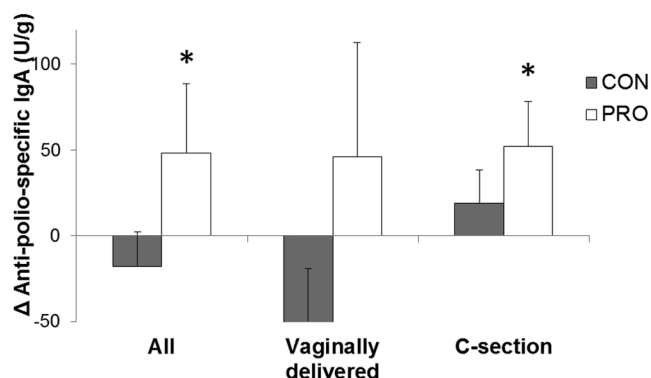


Figure 6. Fecal anti-poliovirus-specific immunoglobulin A (IgA) concentration increased in infants consuming probiotic formula. Data are expressed as means $\pm$ SEM. CON, control infant formula group; PRO, probiotic infant formula group. From V1 (8 weeks of age prior to receiving vaccination) to V2 (12 weeks of age after receiving vaccination), the increase in anti-poliovirus-specific IgA concentration was higher ($P = .031$) for PRO vs CON. When looking at infants based on delivery method, PRO infants delivered by cesarean section had significantly greater ($P = .026$) changes in fecal anti-poliovirus-specific IgA concentration as compared to CON infants delivered by cesarean section. A similar numerical increase in change in anti-poliovirus-specific IgA concentration was noted in vaginally delivered PRO infants as compared to CON infants; however, statistical significance was not achieved.* $P < .05$

Fecal calprotectin concentration increased from V0 to V1 in the all-PRO group ($P = .043$) as compared to CON (Figure 7). This same difference was also seen in infants delivered by cesarean section ($P = .039$) but not by those delivered vaginally ($P = .21$). At V2, there was no

difference ($P = .16$) between PRO (177.9 ± 34.3 $\mu\text{g/g}$ feces) and CON (149.9 ± 15.3 $\mu\text{g/g}$ feces) infants in fecal calprotectin levels regardless of delivery method. The change in fecal calprotectin from V0 to V1 and V2 to V0 did not differ between BF and FF (data not shown). However, BF infants did have significantly higher calprotectin levels than CON and PRO infants at V0 ($P = .0002$; BF: 307.8 ± 34.9 $\mu\text{g/g}$; CON: 195.2 ± 19.4 $\mu\text{g/g}$; PRO: 165.6 ± 19.9 $\mu\text{g/g}$), V1 ($P = .028$; BF: 260.3 ± 36.7 $\mu\text{g/g}$; CON: 153.9 ± 17.2 $\mu\text{g/g}$; PRO: 202.3 ± 29.3), and V2 ($P = .0024$; BF: 319.2 ± 52.3 $\mu\text{g/g}$; CON: 149.9 ± 15.2 $\mu\text{g/g}$; PRO: 177.9 ± 34.3 $\mu\text{g/g}$), as shown in Figure 7.

Fecal pH was not affected by treatment. However, infants in the BF reference group had significantly lower fecal pH at all time points, as shown in Figure 8 (V0: $P = .0155$; PRO: 6.5 ± 0.09 ; CON: 6.5 ± 0.1 ; BF: 6.1 ± 0.1 ; V1: $P = .0135$; PRO: 6.4 ± 0.07 ; CON: 6.4 ± 0.1 ; BF: 6.1 ± 0.1 ; V2: $P = .0255$; PRO: 6.2 ± 0.1 ; CON: 6.3 ± 0.08 ; BF: 5.9 ± 0.1). In addition, there were no differences between formula groups when stratified by delivery method.

Lactate concentration (Figure 9) was found to be significantly higher ($P = .013$) in BF infants as compared to FF infants at V1 (PRO: 4.7 ± 0.9 ; CON: 3.5 ± 0.6 ; BF: 8.4 ± 1.8). When stratified for delivery method, vaginally delivered PRO infants had decreased ($P = .033$) lactate concentrations from V0 to V2 (-0.40 ± 0.1) as compared to CON (0.23 ± 0.2). The opposite trend ($P = .086$) was seen in cesarean-delivered PRO infants, where lactate concentrations increased from V0 to V2 PRO (0.02 ± 0.2) as compared to CON (-0.62 ± 0.9). Lactate concentrations did not differ between formula groups when left unstratified for delivery.

There were no differences in stool frequency, color, or consistency between the 2 formula groups. The BF group had a significantly greater average daily number of bowel movements (3.4 per day at V1 and 2.4 per day at V2) than each of the 2 formula groups (1.3–1.4 per day at V1 and V2). BF infants had yellow stools 85% of the time, and these proportions were significantly higher than in either of the 2 formula groups (18.5% in PRO vs 9% in CON). Approximately 84% of the time, infants assigned to formula had soft stool, which was significantly higher than BF infants. However, BF infants had significantly higher proportions of liquid stool than either formula group ($P < .0001$). There were no differences among the groups in gas, spit-up, or vomiting. Mean weight percentiles generated from the World Health Organization growth charts did not differ among PRE, CON, and BF. BF infant girls had significantly higher weight percentiles than infant girls fed with either formula.

Discussion

B. animalis subspecies *lactis* strain Bb12 was detected within the collected stool samples following ingestion of

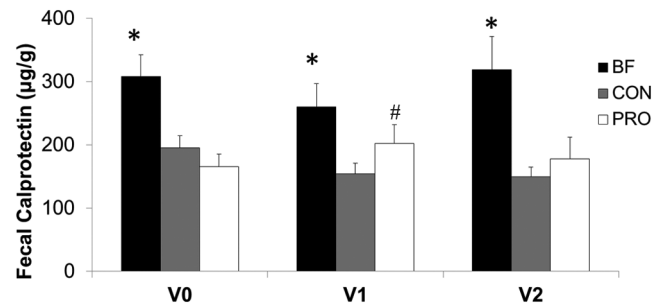


Figure 7. Effect of feeding method on fecal calprotectin concentration in infants at baseline (V0), after 2 weeks (V1), and after 6 weeks (V2). Breastfed (BF) infants had significantly higher calprotectin levels than CON and PRO infants at V0 ($P = .0002$), V1 ($P = .028$), and V2 ($P = .0024$). Fecal calprotectin concentration also increased from V0 to V1 in PRO ($P = .043$) vs CON, denoted with #. Data are expressed as means $\pm$ SEM. CON, control infant formula group; PRO, probiotic infant formula group. * $P < .05$.

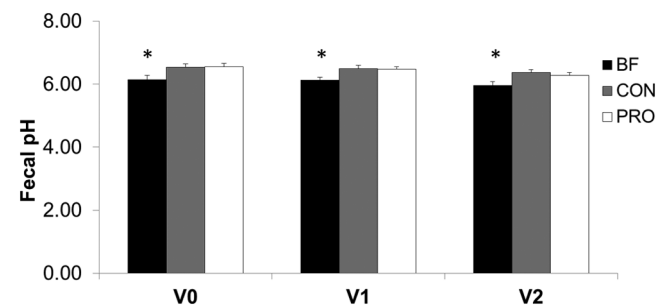


Figure 8. Breastfed infants had significantly lower fecal pH at baseline (V0), after 2 weeks (V1), and after 6 weeks (V2) as compared to PRO and CON. Data are expressed as means $\pm$ SEM. BF, breastfed; CON, control infant formula group; PRO, probiotic infant formula group. * $P < .05$.

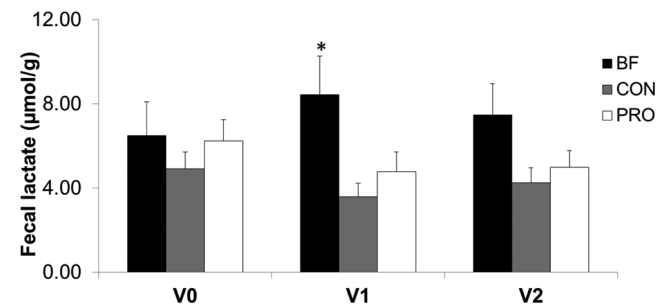


Figure 9. Breastfed infants had significantly higher fecal lactate concentration at after 2 weeks (V1) compared to PRO and CON. Data are expressed as means $\pm$ SEM. BF, breastfed; CON, control infant formula group; PRO, probiotic infant formula group. * $P < .05$.

the probiotic (Bb12)-containing study formula. The use of a primer-probe specific for the *tuf* gene region allowed for highly sensitive and specific quantification of Bb12. The qPCR assay has been shown to have sensitivity to 10 copies of the *tuf* gene and specificity adequate to differentiate between even the most closely related *B animalis* subspecies *animalis* as well as *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium suis*.¹⁹ Our results demonstrated that Bb12 was present in significantly greater levels in PRO infants as compared to CON and BF. Although Bb12 was detected in the majority of fecal samples after 2 weeks (88%) and 6 weeks (93%) of probiotics feeding, it was not detected in 100% of the fecal samples. This likely relates to insufficient *tuf* gene copy numbers present in the tested fecal sample when quantities were limited. In addition, it is possible that unreactive samples were related to noncompliant use of study formula.

sIgA is a predominant mucosal immunoglobulin and plays an integral role in protection from antigens, toxins, and potential pathogens.²⁰ Fecal levels of sIgA are a good measure of levels of sIgA available in the colon and can be assessed noninvasively. Not surprisingly, we observed a significantly higher concentration of total fecal sIgA in BF infants than in FF infants due to the exogenous contribution of sIgA through the mother's breast milk. Within the FF groups, we found that the probiotic formula containing Bb12 significantly increased fecal sIgA from baseline in vaginally delivered PRO infants by ITT analysis after 6 weeks of feeding and had a very strong trend toward increasing sIgA from baseline in vaginally delivered PRO per protocol infants after 6 weeks of feeding. No treatment effect of Bb12 was found on sIgA levels in cesarean-delivered infants or when treatment groups were left unstratified for delivery method. A potential explanation for the treatment effect within vaginally delivered PRO infants and not in cesarean-delivered PRO infants may be related to the composition of the infants' microbiota. Microbiota varies based on delivery method, and it is well established that infants born by vaginal delivery have a higher proportion of bifidobacteria than their cesarean-delivered counterparts.^{3,21-24} In addition, studies have found that bifidobacteria stimulate the production of sIgA.^{8,18,25,26} Thus, the addition of Bb12 to an already bifidobacteria-dominant microbiota in vaginally delivered infants may have enhanced the effects of the presence of the probiotic and thus resulted in larger change from baseline in sIgA during the treatment period. Alternatively, the addition of Bb12 may have contributed to further enhancement of bifidobacteria as was the case in preterm infants supplemented with Bb12 from the first day of birth until 21 days, where a trend was found between bifidobacteria cell counts and fecal sIgA ($r = 0.344$, $P = .08$).²⁷ As these effects were seen only in vaginally delivered infants and not cesarean-delivered infants,

inadequate power may have been an issue within the smaller subsample of infants (14 infants in both PRO and CON in both the ITT and PP groups) stratified for cesarean section delivery. As discussed above, this trial was powered to detect a change in sIgA regardless of delivery method. The effects of Bb12 supplementation on fecal sIgA in infants have been investigated only in a limited number of trials to date. Bb12 supplementation in healthy children and preterm infants has been shown to enhance fecal sIgA.^{8,9} Bakker-Zierikzee and colleagues¹⁴ investigated Bb12 supplementation in healthy full-term infants; however, they did not detect a significant effect of Bb12 supplementation on fecal sIgA levels. Our differing results may be due to inadequate power of the Bakker-Zierikzee and colleagues' study as the post hoc power analysis suggested that a 5-fold increase in each group was needed to reach adequate sensitivity. In addition, statistical analysis on data in the Bakker-Zierikzee et al trial was done only on infants comprising the 10th to 90th percentiles because of high variation between subjects instead of using logarithmic transformations to normalize data, and delivery method was not accounted for in their statistical modeling. In the present study, log transformations were used to normalize data due to high variation, and analysis was stratified by delivery method, thus allowing for detection of the significant increase in fecal sIgA from baseline in PRO infants who were vaginally delivered.

Similar to our findings, *Bifidobacterium longum-infantis* and *B breve* have been shown to increase fecal sIgA and anti-poliovirus-specific IgA in healthy full-term infants after 4 months of feeding.¹⁸ To the best of our knowledge, this is the first trial to investigate the immunomodulating effects of Bb12, or any probiotic, on fecal anti-rotavirus- or anti-poliovirus-specific IgA in healthy full-term infants. Few studies have investigated immune effects related to the interaction of infant delivery method and probiotic feeding. One trial found that probiotic supplementation consisting of 4 bacteria strains (*Lactobacillus rhamnosus* GG, *L rhamnosus* LC705, *B breve* Bb99, and *Propionibacterium freudenreichii* ssp *Shermanii*) provided protection from IgE-associated allergy in caesarean-delivered children until age 5, whereas those born by vaginal delivery were not afforded the same protective effects.²⁸ Our results similarly support enhanced protective effects of probiotics in infants born by caesarean delivery. As our study contained *B lactis* (Bb12) and the other 2 trials each included *Bifidobacteria* spp, it is possible that the microbiotas of the infants in all 3 trials were afforded immunological benefits associated with an enriched *Bifidobacterium* population as an effect of the respective bifidobacteria added as probiotics. As discussed above, it is well established that the dominant bacteria within vaginally delivered infants are bifidobacteria.^{3,21-24} As shown in our trial and the trial by Kuitunen and colleagues,²⁸

further immunological benefit may be gained by a *Bifidobacterium* probiotic to cesarean-delivered infants as it may be contributing to a shift in the commensal bacterial populations to be more similar to the vaginally delivered infant.

At birth, the immune system of infants is skewed toward T helper (Th)–2 responses.²⁹ According to the “hygiene hypothesis,” exposure to bacteria is important in changing the immune response to be more skewed toward Th-1 responses, which in turn has been linked to reducing propensity to allergic diseases.^{30–33} As infants born by cesarean section have been shown to have increased incidences of allergic diseases such as allergic rhinoconjunctivitis and asthma,³⁴ one could speculate that this correlation may also be related to the hygiene hypothesis. By extension, the sterile delivery methods of cesarean section delivery may provide a lesser initial exposure to bacteria and hinder the infant’s progression toward a more skewed Th1 response. In the present study, cesarean-delivered PRO infants saw a significant increase in both anti-poliovirus-specific and anti-rotavirus-specific fecal IgA after 6 weeks of feeding. Thus, it is possible that Bb12 provided immune-modulating bacterial exposure that was more pronounced in the cesarean-delivered infants. In addition, all per protocol PRO infants experienced an increase in anti-poliovirus-specific fecal IgA, suggesting that the addition of Bb12 to infant formula may be helpful in mitigating some of the negative immune-related effects associated with formula feeding.

Calprotectin, a zinc and calcium binding protein that constitutes more than half of the soluble cytosolic protein within human neutrophil granulocytes,³⁵ is a noninvasive marker of gastrointestinal inflammation.^{36,37} Calprotectin has been measured in a number of studies to evaluate inflammation and immune status of the gastrointestinal tracts of infants.^{27,36–44} Existing literature reports conflicting data regarding fecal calprotectin in BF and FF infants. Our findings are in agreement with some published studies in full-term infants monitored during the preweaning period^{38,40} showing calprotectin concentrations were significantly higher in BF infants as compared to FF infants. Few studies have evaluated the effects of probiotic supplementation on calprotectin levels.^{9,45} One study in healthy full-term infants assessed the effects of a probiotic capsule containing 4 bacteria strains (*L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99, and *P. freudenreichii* ssp *Shermanii*) on calprotectin and fecal SIgA levels at 3 and 6 months of age. This trial found a positive correlation between fecal SIgA and calprotectin and reported a correlation between higher fecal SIgA levels and lower IgE-associated atopic diseases at 2 years of age.⁴⁵ Kukkonen and colleagues⁴⁵ concluded from this trial that these findings were indicative of immune maturation with associated reduction in risk for atopic diseases during the first 2 years of life. Furthermore, they pro-

posed that this slight immune activation may be a potential mechanism by which probiotics stimulate immune maturation. Another trial that investigated the effect of probiotic feeding (Bb12) on preterm infant fecal calprotectin levels found the opposite effect in that calprotectin levels were attenuated in the infants consuming Bb12. Our results suggest that the effect of probiotic supplementation varies over time, with an initial increase in calprotectin levels followed by a decrease to levels of non-supplemented infants by 6 weeks of feeding. The results of our trial and that of Kukkonen and colleagues support the hypothesis that Bb12 aids in priming of the gastrointestinal tract of full-term infants to enhance maturation and attenuate the inflammatory responses to bacterial and dietary antigens. Clearly, more research is needed to investigate the effects of probiotic supplementation on calprotectin in infancy.

Fecal pH was lower at each time point in BF vs FF infants; however, there were no differences between FF groups, even when stratified for delivery method. In coordination with the lower pH, fecal lactate concentrations were higher in BF infants as compared to FF infants, but again no effect was seen as a result of the addition of Bb12 to infant formula when infants were not separated by delivery method. When infants within the formula groups were stratified for delivery method, fecal lactate concentrations decreased from baseline to visit 2 (6 weeks of study formula) within the vaginally delivered infants, with the opposite trend occurring within the cesarean-delivered infants. As these small changes in fecal lactate concentration were not mirrored by a significant change in fecal pH, the biological relevance of this is likely minimal. Our results differ from Mohan and colleagues,⁹ where fecal pH was shown to decrease in preterm infants fed Bb12, and the decreased pH was mirrored by an increase in fecal lactate concentration. Trials in full-term infants have not investigated the effects of Bb12 supplementation on fecal lactate levels.

Conclusions

By virtue of containing bioactive substances such as SIgA, microbes, and human milk oligosaccharides, breastfeeding is superior to formula feeding in maintaining high intestinal levels of protection in infants during the first 3 months of life. BF infants’ total fecal SIgA levels decrease over time; however, they remain significantly higher than FF infants at all time points. Infants fed formula supplemented with Bb12 had significantly higher levels of Bb12, and infants born by vaginal delivery had increased concentrations of SIgA. In addition, infants born by cesarean section who received the probiotic formula had enhanced immune response to poliovirus and rotavirus vaccines, as evidenced by an increase in anti-rotavirus-

and anti-poliovirus-specific IgA. These results demonstrate that negative immune-related effects from the absence of breastfeeding and cesarean-section delivery may be mitigated by including *B animalis* subspecies *lactis* Bb12 in infant formula and thereby providing infants a safe, dietary, immune-modulating bacterial experience. Anti-viral-specific mucosal antibodies in this study were evident after only a single dose of vaccines. This enhancement in antibody responses could be of further clinical significance as related to levels of protection achieved from oral viral vaccines in non-BF infants. Further research is needed to investigate the degree and potential for immune-related benefits of probiotics, as well as their relation to infant microbiota and mode of delivery.

Acknowledgments

We thank Nancy Moore for her assistance with site monitoring. In addition, we are grateful to Dr. Gloria Solano-Aguilar for her excellent advice and direction regarding fecal Bb12 quantification.

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