

Effects of *Bifidobacterium* supplementation on intestinal microbiota composition and the immune response in healthy infants

Bing-Bing Wu, Yi Yang, Xiu Xu, Wei-Ping Wang

Shanghai, China

Background: Intestinal microbiotas are thought to be the most important source of maturational stimuli to the development of the immune system. However, few studies have focused on the development of T helper (Th) 1 immune response and antibody response to vaccinations in healthy infants, especially in a large cohort. Through this randomized, double-blind control trial, we investigated the effects of *Bifidobacterium longum* BB536 (BB536) supplementation on intestinal microbiota composition and the immune response in term infants.

Methods: In total, 300 healthy newborns were recruited, randomized and fed formula either supplemented with BB536 or with no supplementation. Stool samples were analyzed at months 2, 4 and 11. The representative cytokine for Th1 [interferon- γ (IFN- γ)] and Th2 [interleukin-4 (IL-4)] secretion cells were measured using enzyme-linked immunospot assay at 4 and 7 months of age. The antibody response to vaccines was measured at months 7 and 11.

Results: A total of 264 infants completed the study. The amount of bifidobacteria and the bifidobacteria/Enterobacteriaceae ratio (B/E) were significantly higher in the BB536 supplementation group at months 2 and 4. The number of IFN- γ secretion cells and the ratio of IFN- γ /IL-4 secretion cells were increased in the BB536 supplementation group at 7 months. Moreover, the higher value of B/E in the early stages seems to be related to the increased Th1 response. No difference was observed between groups in the

antibody response after vaccination.

Conclusions: BB536 has positive effects on establishing a healthy intestinal microbiota early in life, and it also plays an important role in improving the Th1 immune response.

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Key words: intestinal microbiota; probiotics supplementation; term infants; T helper 1/T helper 2 balance; vaccination

Introduction

The gastrointestinal tract is the largest immune organ in the human body.^[1] With the highest number of immune cells and the highest concentrations of bacteria in the body, the gut represents the major site of immune education.^[2,3] It has been reported that the failure to establish a normally functioning gut microbiota early in life is associated with the development of allergic diseases and other immune disorders later in life.^[4-6]

It is known that the immune system of newborns is T helper (Th) 2 biased during pregnancy. After birth, maturation of the immune system is age-dependent, and the development of the Th1 immune response can reset the Th1/Th2 balance. Exposure to environmental microbial components is suggested to play an important role in the maturation process. Probiotics are live microorganisms that, when administered orally in adequate amounts, confer a beneficial effect on the host.^[7] Modulation of the infant gut microbiota with probiotics has been proposed as a potential approach for the treatment and prevention of immune-mediated diseases. Recently, several cohort studies have investigated the immune modulative effects of probiotic supplementation on infants and pregnant women and observed that probiotics supplementation can reduce the risk for atopic diseases.^[8-10] However, the study subjects for the majority of the abovementioned research are children with a

Author Affiliations: Pediatrics Institute, Key Laboratory of Neonatal Diseases, Ministry of Health, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China (Wu BB, Yang Y, Wang WP); Department of Pediatric Healthcare, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China (Xu X)

Corresponding Author: Wei-Ping Wang, MD, PhD, Pediatrics Institute, Key Laboratory of Neonatal Diseases, Ministry of Health, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China (Tel: +86-021-64931883; Fax: +86-021-64931883; Email: wpwang@fudan.edu.cn)

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high risk of allergic diseases, and few studies have been conducted in healthy newborns, especially in a large cohort. In addition, probiotics have been shown to be immunomodulatory and may affect antibody responses following vaccination. To date, only a few studies have evaluated the effects of oral probiotics on the specific immune response of infants following one or several vaccinations in small sample size.^[11,12] There is no report available on the effects of probiotics supplementation on the immune response of Chinese infants following the complete administration of routine vaccinations.

The *Bifidobacterium longum* BB536 (BB536) is a member of the bifidobacteria family and is a component of the intestinal microbiota. It is reported that BB536 has beneficial health effects^[13,14] and has been widely added to commercially available foods for human consumption.^[15]

To demonstrate the effects of BB536 on the development of the immune response in healthy infants, we conducted a double-blind, randomized, placebo-controlled intervention trial to determine if formula supplementation with BB536 can influence the gut microbiota composition, enhance the immune response to vaccination in healthy term infants and provide beneficial effects on the immune balance of the Th1/Th2 response.

Methods

Study design

This randomized, double-blind, placebo-controlled intervention trial study was carried out in the Children's Hospital of Fudan University. The protocol was approved by the ethics committee of the Children's Hospital of Fudan University. Healthy, term infants absent of pre- and post-natal disease were enrolled from day 0 to day 7 after birth if their mother had decided not to breast feed after the 7th day of life. Written consent was signed by a legal representative.

The enrolled newborns were randomized to one of two groups: normal formula (commercially available) or normal formula supplemented with BB536 1×10^7 colony forming units/g. Allocation to formula groups was performed by block-randomization with stratification by gender using a computer program. These groups were utilized from enrollment to 6 months of age. Subsequently, subjects received a commercial standard follow-up formula until the completion of the study at 12 months. The only difference in appearance of the products was the letter printed on the label. Two different letters were used for each formula type, yielding a total of four letters. The identity of the specific product was blind to subjects and investigators.

Stool samples were collected at 2, 4 and 11 months of age to analyze the composition of the gut microbiota. A subgroup of children was chosen for the collection of at least 250 L of fingertip blood samples at 4, 7 and 11 months. Interferon- γ (IFN- γ)/interleukin-4 (IL-4) cytokine-secreting cells were detected at 4 and 7 months using the enzyme-linked immunospot (ELISPOT) assay. Antibody levels were measured at 7 and 11 months with enzyme linked immune sorbent assay (ELISA) kits.

The main objective of the trial was to determine the effects of probiotics BB536 supplementation on immune development and immune response to routine vaccination in healthy infants. The primary outcome was to evaluate the immune development by measuring the representative cytokine for Th1 (IFN- γ) and Th2 (IL-4) secretion cells and the specific antibody serum levels after hepatitis B (HepB), poliomyelitis (Polio), diphtheria, tetanus toxoid and pertussis (DTP)-vaccinations. The secondary objective of the study was to compare the microbiological composition of the stools between the two groups by detecting several main bacterial families.

Stool sample analysis

For determination of the intestinal microbiota, a 1-5 g sample of fresh stool was collected immediately after emission. Serial diluted fecal samples were placed on Eugon tomato medium (Difco, USA)^[16] for a total bifidobacteria count, on MRS plus antibiotics agar (Difco, USA)^[17] for a lactobacilli count and on Drigalski medium (Pasteur, Paris, France) for a Enterobacteriaceae count. The Eugon tomato medium and MRS agar plates were incubated anaerobically at 37°C for 48 hours, and the Drigalski medium plates were incubated aerobically at 37°C for 24 hours. The morphology of each type of colony grown on the Eugon tomato plates and the MRS agar plates were checked by microscopic observation. The Y branched or balloon-shaped bacteria were identified to be bifidobacteria by PCR with the primer sequences F: 5'-GGGTGGTAATGCCGGATG-3', R: 5'-CCACCGTTACACCGGAA-3'.

ELISPOT assay

To evaluate the balance of Th1 and Th2 immune response, IFN- γ (a representative cytokine for Th1) or IL-4 (a representative cytokine for Th2) cytokine-secreting cells were detected using an ELISPOT assay, according to the manufacturer's instructions (U-CyTech biosciences, Netherlands). Peripheral blood mononuclear cells were isolated from finger end blood samples using the Dextran sedimentation method, and the cells were then suspended in RPMI 1640 medium with 10% fetal calf serum at a density of 1×10^6 cells/mL. 100 μ L of cell

suspension and 10 µg/mL phytohemagglutinin (Sigma, USA) were added into each well of a 96-well plate and were incubated for 20 hours at 37°C in a humid 5% CO₂ incubator. The number of spots was counted with a dissection microscope.

Antibody detection

Plasma was obtained after centrifugation and stored at -70°C until analyzed. Antibody response to HepB (XinBo Biological Technology, China), Polio, and DTP vaccines (Hycor Biomedical, USA) were measured with an ELISA kit, according to the manufacturer's instructions.

Statistical analysis

The sample size for the blood test was calculated based on the percentage of infants who responded to the vaccination. According to a study conducted by Pickering et al,^[18] we needed 70 infants in each group to detect a 50% difference in antibody concentration at alpha=0.05 and a power of 80%. Linear mixed effects modeling was used to estimate the effects of probiotics supplementation on the composition of gut microbiota, Th1 and Th2 cytokine-secreting cells and antibody response to the vaccines. Pearson's correlation was used to analyze the relationship between the bifidobacteria/Enterobacteriaceae (B/E) ratios and the numbers of cytokine-secreting cells. A Chi-square test was used to analyze the difference in lactobacilli detectability

Table 1. Demographics and baseline characteristics of the infants at enrollment

Variables	Control (n=129)	BB536 supplementation (n=135)
Female, n (%)	58 (45)	64 (47)
Weight at birth (g)*	3315±483	3320±509
Length at birth (cm)*	49.8±0.43	49.9±0.45

*: Data are presented as mean±standard deviation. BB536: *Bifidobacterium longum* BB536.

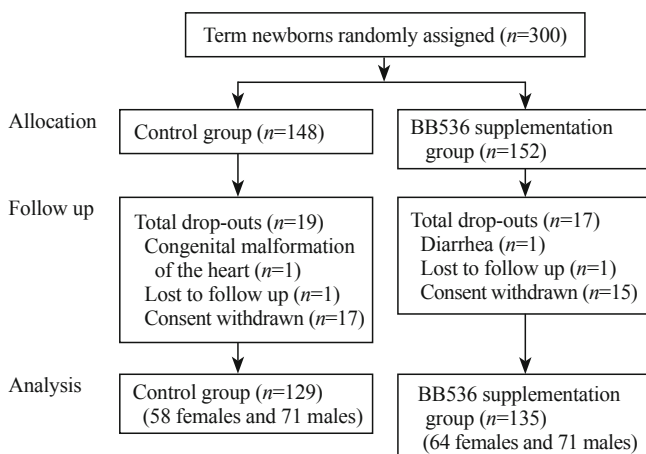


Fig. 1. Study flow diagram. BB536: *Bifidobacterium longum* BB536.

between the two groups. Statistical analyses were performed by the SAS system for windows V8 and SPSS version 16.0. A *P* value <0.05 was considered to be statistically significant.

Table 2. Changes in bifidobacteria and Enterobacteriaceae with age and comparison of the two groups

Variables	Control (n=129)	BB536 supplementation (n=135)	<i>P</i> value
Bifidobacteria			
2 mon	5.93±3.31	7.38±1.69	<0.0001
4 mon	6.79±3.08*	7.59±1.66	0.0096
11 mon	7.28±2.41†	7.27±2.49	0.9843
Enterobacteriaceae			
2 mon	8.86±0.89	8.89±0.75	0.7356
4 mon	8.72±0.73	8.77±0.62	0.6252
11 mon	8.18±0.73‡	8.10±1.15‡	0.3982
B/E ratio			
2 mon	0.68±0.41	0.84±0.23	<0.0001
4 mon	0.79±0.37	0.87±0.21	0.0300
11 mon	0.90±0.32	0.91±0.37	0.7901

Data are presented as log cfu/g wet stool, mean±standard deviation. BB536: *Bifidobacterium longum* BB536; B/E: bifidobacteria/Enterobacteriaceae. *: compared to month 2, *P*<0.01; †: compared to month 2, *P*<0.0001; ‡: compared to month 2 and month 4, *P*<0.0001.

Table 3. Changes in IFN-γ, IL-4 secreting cells (log counts) and IFN-γ/IL-4 ratio in the two groups at 4 months and 7 months of age

Variables	Age	Control	<i>n</i>	BB536 supplementation	<i>n</i>	<i>P</i> value
IFN-γ	4 mon	1.62±0.33	82	1.63±0.36	80	0.797
	7 mon	1.66±0.34	73	1.82±0.34	69	0.007
IL-4	4 mon	1.45±0.41	82	1.46±0.45	80	0.878
	7 mon	1.42±0.40	73	1.51±0.41	69	0.227
IFN-γ/IL-4	4 mon	1.74±1.11	82	1.79±1.01	80	0.771
	7 mon	1.93±0.91	73	2.29±1.09	69	0.044

Data are presented as mean±standard deviation. BB536: *Bifidobacterium longum* BB536; IFN-γ: interferon-γ; IL-4: interleukin-4.



Fig. 2. Peripheral blood mononuclear cells were isolated and suspended in RPMI 1640 with 10% fetal calf serum at a density of 1×10⁶ cells/mL. 100 µL of cell suspension and 10 µg/mL phytohemagglutinin were added into wells coated with anti-IFN-γ or IL-4 antibody and were incubated for 20 h. Spots corresponding to cytokine-secreting cells are indicated by arrow. IFN: interferon; IL: interleukin.

Table 4. Antibody responses to the vaccines DTP, Polio and HepB at 7 months and 11 months of age and comparison of the two groups

Variables	Age	Control	n	BB536 supplementation	n	P value
Diphtheria (IU/mL)	7 mon	2.50±0.33	73	2.45±0.32	69	0.466
	11 mon	2.02±0.40	73	1.99±0.41	69	0.570
Tetanus (IU/mL)	7 mon	2.22±0.26	73	2.22±0.23	69	0.880
	11 mon	2.05±0.48	73	2.04±0.39	69	0.934
Pertussis (U/mL)	7 mon	2.94±0.41	73	3.03±0.48	69	0.209
	11 mon	2.77±0.42	73	2.85±0.43	68	0.279
Polio (U/mL)	7 mon	3.07±0.22	73	3.10±0.22	69	0.423
HepB (mIU/mL)	7 mon	2.48±0.64	70	2.53±0.71	69	0.665
	11 mon	2.44±0.56	68	2.55±0.61	69	0.307

All the data are logarithms transformed before analysis, and presented as mean±standard deviation. DTP: diphtheria, tetanus toxoid and pertussis; Polio: poliomyelitis; HepB: hepatitis B.

Results

Sample size and demographic characteristics

A total of 300 healthy newborns were enrolled from day 0 to day 7 after birth, and 264 infants completed the study. Among the 264 infants, 129 infants (58 females and 71 males) were in the control group, and 135 infants (64 females and 71 males) were in the BB536 supplementation group. There were no statistically significant differences between the groups with respect to gender, birth weight and length (Table 1). The reason for dropouts included one infant with diarrhea whose mother changed the formula, one infant with a congenital heart malformation, two infants who were lost to follow-up and 32 infants who were withdrawn by their parents (Fig. 1).

The effect of BB536 supplementation on the intestinal microbial microbiota

In the control group, total bifidobacteria counts in fecal samples increased with age, while the numbers of Enterobacteriaceae decreased. The number of bifidobacteria at 4 months was significantly more greater than that at 2 months, $P<0.05$. The quantity of Enterobacteriaceae at 11 months of age was significantly lower than that at 2 months and 4 months (Table 2).

In the BB536 supplementation group, the total amount of bifidobacteria and the rate of B/E were significantly higher than those in the control group at 2 and 4 months of age (Table 2). Moreover, the lactobacilli detectability was 81.5%, 79.3% and 74.1% in the BB536 supplementation group at 2, 4 and 11 months of age, respectively, which were slightly higher than that in the control group (72.1%, 73.6% and 69%, respectively), although this difference was not statistically significant. There was no obvious influence of the BB536 supplement on the enumeration of Enterobacteriaceae at 2, 4 and 11 months of age.

The effect of BB536 supplementation on Th1 and Th2 cytokine secretion

We examined the balance of Th1 and Th2 at 4 and 7 months of age by measuring IFN- γ and IL-4 secretion cells and their ratio using an ELISPOT technique (Fig. 2). We found that the number of IFN- γ secretion cells and the ratio of IFN- γ /IL-4 increased significantly at 7 months of age in the BB536 supplementation group compared to those in the control group. However, no significant differences were observed between the two groups for the amount of IL-4 secretion cells (Table 3).

Correlative analysis showed that there was a weak positive correlation between the ratio of B/E at 2 months and the ratio of IFN- γ /IL-4 at 4 months ($R=0.195$, $P=0.013$), as well as between the ratio of B/E at 4 months and the ratio of IFN- γ /IL-4 at 7 months ($R=0.238$, $P=0.004$).

The effect of BB536 supplementation on the immune response to vaccinations

Antibody response to the vaccines of DTP, Polio and HepB at months 7 and 11 were measured using the ELISA technique. However, no statistically significant differences were observed between the two groups (Table 4).

Discussion

The communication between host and bacteria at the gut mucosal interface plays an important role in establishing a competent immune system.^[19] Characterizing the development of the intestinal bacteria composition in healthy newborns may improve our understanding of the interaction between host and microbe. In the current study, we observed that whether BB536 is supplemented or not, total bifidobacteria counts in fecal samples increase with age, while the numbers of Enterobacteriaceae decrease. These results indicate that the probiotics represented by bifidobacteria are steadily colonized in the gut of healthy newborns with age.

It is reported that probiotics supplementation has positive effects on the composition of the indigenous intestinal microbiota. Our results are consistent with the findings of previous studies. In this study, we observed that amounts of bifidobacteria were significantly higher in the BB536 supplementation group at months 2 and 4 than in the control group. Additionally, the ratio of B/E, which may reflect the ability of the intestinal barrier to resist pathogen invasion, was higher in the BB536 group than in the control group at 2 and 4 months. It is known that in healthy breastfed infants, the dominant intestinal microbiota is bifidobacteria,^[20] and this has been thought to contribute to the many

health benefits of breastfeeding. Our findings indicate that BB536 intervention may help artificially fed infants establish a healthy microbial colonization early on that is closer to that of breastfed infants, which may play an important role in building up a well-balanced intestinal barrier during infancy.

The optimal time at which to provide probiotics intervention is an ongoing discussion. According to the results from our study, supplementation of probiotics before 4 months of age will have better effects. After 4 months, the introduction of weaning food and the natural trends of the composition of the intestinal microbiota may weaken the effects of a probiotics intervention.

Because the adaptive immune system is immature and inexperienced during the neonatal period, it cannot generate an appropriate immune response.^[21] Intestinal microbiota are thought to be the most important sources of maturational stimuli to the development of the immune system. In this study, we used IFN- γ and IL-4 as the representative cytokines of Th1 and Th2 cells, respectively, and the rate of IFN- γ /IL-4 was used to investigate the balance of the Th1 and Th2 responses. Our results showed that the IFN- γ secreting cells and the ratio of IFN- γ /IL-4 secreting cells were increased in the BB536 supplementation group compared with the control group at 7 months. We also observed that higher values of B/E were related to an increase in IFN- γ secreting cells and IFN- γ /IL-4 later in life. These results indicate that BB536 has a positive effect on the enhancement of the Th1 response.

Postnatally, the immune system of infants is skewed toward Th2 responses.^[22] The establishment of a balanced Th1/Th2 immune response is a key event of immune development in early life. According to the "hygiene hypothesis",^[23] improvements in public health and hygiene can thus potentially favor a Th2 response, which may lead to atopic sensitization by enhanced immunoglobulin E production. Microbiota exposure is considered to play an important role in changing the immune response to be skewed toward Th1 responses, which in turn has been linked to reducing the propensity to allergic diseases. Our previous study wherein we fed neonatal Sprague-Dawley rats sufficient antibiotics to generate bifidobacteria minimization rats showed that the pre-existing Th2 response in these rats was reinforced by increasing the protein level of IL-4 in plasma. However, after bifidobacteria supplementation, the immune response was shifted to a Th1 type.^[24] Other researchers also obtained positive results of probiotics interventions in modulating the Th1/Th2 balance by measuring the associated cytokines through mRNA^[25] and protein levels.^[26] In this study, we used the ELISPOT technique to measure the Th1 and Th2 cytokine secreting cells directly. Our results from the

live cell expression level analyses further confirm that probiotics supplementation is an effective method to produce a balanced immune response between Th1/Th2 early in life, which may improve anti-infectious immunity and prevent the occurrence of allergic diseases later in life.

Specific antibody response is another important parameter used to evaluate the immune system maturation status in infancy. Accumulating evidence has shown that taking some specific probiotics can enhance antibody responses to vaccines.^[27] In this study, we also evaluated the effects of BB536 supplementation on HepB, Polio and DTP vaccine responses. However, we did not observe much difference in antibody levels between the BB536 supplementation group and the control group. Compared with other studies observing the positive effects of probiotics interventions on vaccine responses, there may be some possible explanations for the inconsistent results. First, different strains of probiotics may have markedly different immunomodulatory functions. Second, probiotics interventions for infants in developing countries may have different antibody responses following vaccination compared to infants from developed countries. Pérez et al^[28] investigated the effect of probiotics supplementation on the antibody response in children of low socio-economic status and did not observe any benefit of probiotics interventions. They concluded that a high natural rate of exposure to infectious agents in their population may account for the absence of an additional stimulation by supplementary probiotics. Youngster et al^[29] did not observe a positive effect of probiotic supplementation on the immune response of healthy infants to live vaccines either. It seems that when there is an adequate immune response, the probiotics do not have a further effect on the quantity of antibodies generated.

In summary, the current study evaluated the effects of BB536 supplementation on the intestinal barrier function, balance of the Th1/Th2 immune response and antibody generation in healthy term infants after vaccination. We observed that BB536 can help to establish a healthy intestinal microbiota in early life and improve Th1 immune response through enhancing the secretion of cytokine IFN- γ . However, no significant effects of BB536 supplementation were observed in terms of enhancing the antibody titer after vaccination with HepB, Polio and DTP.

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Ethical approval: This study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee

of the Children's Hospital of Fudan University. Parents were informed about the study, and written consent was signed by a legal representative.

Competing interest: None declared.

Contributors: Wu BB and Yang Y contributed equally to this work. Wu BB wrote the main body of the article under the supervision of Yang Y. Xu X provided advice on medical aspects. Wang WP is the guarantor.

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