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

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**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.


**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. 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. 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.

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. 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. However, the study subjects for the majority of the abovementioned research are children with a
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. There is no report available on the effects of probiotics supplementation on the immune response of Chinese infants following the completion 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 and has been widely added to commercially available foods for human consumption. 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) for a total bifidobacteria count, on MRS plus antibiotics agar (Difco, USA) for a lactobacilli count and on Drigalski medium (Pasteur, France) for an 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'-GGTGTAATGCGGGATG-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 μL of cell sample of fresh stool was collected immediately after emission. Serial diluted fecal samples were placed on Eugon tomato medium (Difco, USA) for a total bifidobacteria count, on MRS plus antibiotics agar (Difco, USA) for a lactobacilli count and on Drigalski medium (Pasteur, France) for an 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'-GGTGTAATGCGGGATG-3', R: 5'-CCACCGTTACACCGGAA-3'.