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The role of intestinal bifidobacteria on immune system development in young rats

Ping Dong, Yi Yang, Wei-ping Wang *

Children's Hospital, Pediatrics Department of Shanghai Medical College, Fudan University, Shanghai, PR China.

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ABSTRACT

Aim: The effects of intestinal bifidobacteria on the development of immunity in early life were explored.
Methods: Neonatal SD rats born and housed under strict barrier systems were fed from birth with sufficient antibiotics (bifidobacteria minimisation group) or supplemented daily with 1×10^{10} colony-forming units of live *Bifidobacterium longum* (bifidobacteria supplementation group). Relevant indices of immune development were determined at one, three and six weeks old.
Results: Compared to the control group, minimisation of the intestinal bifidobacteria delayed maturation of dendritic cells in Peyer's Patches and the development of T cells in the thymus, increased IL-4 secretion in the plasma, down-regulated IL-12, IL-10 mRNA and the interferon-γ/IL-4 mRNA ratio in intestinal mucosa, decreased interferon-γ mRNA in cultured peripheral blood mononuclear cells (PBMCs), and reduced immunoglobulin-M production in cultured PBMCs. Conversely, supplementation with bifidobacteria promoted dendritic cell maturation in Peyer's Patches, up-regulated IL-12, IL-10, interferon-γ mRNA and the interferon-γ/IL-4 ratio in intestinal mucosa, increased interferon-γ gene expression in cultured PBMCs, and raised immunoglobulin-M secretion in cultured PBMCs.
Conclusions: Intestinal bifidobacteria could promote the maturation of dendritic cells and its expression of IL-12 locally in the gut, influence the development of T cells in the thymus, favour the development of T-helper cell type 1 response by increasing the local and systemic expression of interferon-γ and ensure the intestinal regulatory T cell response by promoting the local expression of IL-10. In addition, they enhance antibody synthesis by PBMCs, thereby affecting the development of both the gut and systemic immunity in early life.

1. Introduction

Comparative studies based on the germ-free animal have provided clear evidence that the body's cross-talk with commensal bacteria is essential for the development and maturation of the immune system in early life [1]. Because the gastrointestinal tract encompasses the largest surface of the human body, all kinds of colonising flora and the gut-associated lymphoid tissue (GALT) constitute the main elements that influence immune system development. Bifidobacteria, known as a kind of probiotic bacteria, represent up to 90% of the total intestinal flora in breast-fed infants [2]. Premature delivery, decreased breast-feeding, abuse of antibiotics and other factors have led to insufficient bifidobacteria colonisation of the gut in early infancy, which may be related to the increased future incidence of inflammatory, autoimmune and atopic diseases [3]. Thus, the impact of bifidobacteria on the development of immunity and the precise mechanisms behind it require further elucidation.

One principal character in immune system development in early life is the establishment of a balanced T-helper (Th) cell response (Th1/Th2). Th1 mainly secretes cytokines, such as interferon (IFN)-γ and IL-2, mediates cellular immunity and thus participates in anti-virus and intracellular bacterial infection responses. Th2 secretes cytokines, such as IL-4 and IL-5, mediates humoral immunity and is involved in the immune responses to bacterial infection, as well as immediate hypersensitivity reactions. An imbalanced Th1/Th2 may contribute, in part, to clinical diseases: e.g. excessive Th2 activity can lead to allergic disease, whereas Th1 predominance is found in inflammatory bowel disease. Adaptive responses of the newborn show a Th2 type and tend toward a Th1/Th2 balance as a result of the postnatal stimulation of initially colonising microbes on Th1 response. Additionally, the development of regulatory T (Treg) cell response is critical in inducing the tolerance to 'self' and in controlling inflammatory diseases, including atopic disorders [4]. A retrospective study indicated that a preventive colonisation of high-risk infants with probiotics after birth decreased the incidence of allergies and repeated infections later in life [5], but its mechanism remains unclear.

Dendritic cells (DCs), the most potent antigen-presenting cells (APCs), possess potent T-cell stimulatory capacities and are important in initiating the immune response [6]. Immature DCs reside in various

Abbreviations: DCs, dendritic cells; IFN, interferon; Ig, immunoglobulin; PPs, Peyer's Patches; PBMCs, peripheral blood mononuclear cells; Th1, T-helper cell type 1; Th2, T-helper cell type 2; Treg, regulatory T cell.

* Corresponding author. Children's Hospital, Pediatrics Department of Shanghai Medical College, Fudan University, 399 Wanyuan Road, Shanghai, 201102, PR China. Tel.: +86 21 64931990; fax: +86 21 65644937.
E-mail address: wpwang@shmu.edu.cn (W. Wang).

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portals of the microorganism entry, such as skin and mucosa, where they guard sites of potential pathogen entry in to the host and capture invading microorganisms, and then migrate to nearby lymphoid organs, with gradual maturation. Mature DCs initiate a primary cell-mediated immune response through the expression of major histocompatibility complex (MHC) and costimulatory molecules. Moreover, DCs are critical in directing the differentiation of naive T cells into Th1, Th2 or Treg cells, thus, controlling the type, amplitude and intensity of the response [7]. The modulation of probiotics on T-cell responses may occur primarily through its action on the differentiation, maturation and functional activities of DCs. Recent in vitro studies have shown that certain probiotic interactions with DCs lead to the polarisation of naive T cells to a Th1 or Treg direction [8,9], however, to date, there are no relevant reports in vivo.

The aim of the present study was to clarify how intestinal bifidobacteria affect the development of both the gut and systemic immunity in early life, especially its modulation on the differentiation, maturation and function of DCs, T-cell development, T-cell responses (Th1, Th2 and Treg) and immunoglobulin synthesis. Thus, the SD rats used in the study were fed from birth with sufficient antibiotics per day (bifidobacteria minimisation group) or administrated a daily dose of *Bifidobacterium longum* (bifidobacteria supplementation group) until one, three and six weeks of age, which are equivalent to the neonatal, infant and adolescent period of childhood, respectively. Various immunological parameters were assessed to study the impact of both bifidobacteria minimisation and supplementation on immune development. It is expected that the research will provide an experimental basis for the proper usage of bifidobacteria to improve the immunity of children in reality.

2. Materials and methods

2.1. Animals

All experimental procedures involving animals were approved by the Ethics Committee of Shanghai Medical Collage of Fudan University and the care of animals was in accordance with institution guidelines. Newborn specific pathogen-free Sprague–Dawley (SD) rats were used. The rat pups were reared in plastic cages in a room kept at 22 ± 3 °C and $55 \pm 5\%$ humidity, on a 12 h light/dark cycle under strict barrier systems with the water, diet and bedding material sterilized and high level air purity (Animal Centre of Public Health, Clinical Centre Affiliated to Fudan University). They were breast fed first and subsequently consume diet and water freely throughout the experimental period. The pups were divided into three groups, bifidobacteria minimisation (BM) group was fed from birth with gentamicin sulphate injection (100 mg/kg) and metronidazole injection (80 mg/kg) per day to minimise the amount of resident bifidobacteria in intestinal tract, while bifidobacteria supplementation (BS) group supplemented from birth with live *B. longum* (BL-11) 1×10^{10} colony-forming units (CFU) per day. The BL-11 (kindly provided by George Food Industries, Shanghai, China) were supplied as freeze-dried powder in sealed packets containing about 1.5×10^{11} CFU/g and stored at a temperature of −20 °C until dissolved in warm water for use. Both antibiotic injections and BL-11 suspension were given to the rats through orogastric gavage, which performed in a clean bench placed in the room. A third group of non-intervened rats was used as control. Ten rats of each group were anaesthetised by intra-peritoneal injection of 7% chloral hydrate (5 ml/kg) at one, three and six weeks old respectively. Blood was collected from the carotid artery into a heparinised tube, and the thymus, spleen and the whole length of the small intestine were removed and placed in sterile Petri dishes containing medium.

2.2. Faecal sample preparation and bifidobacteria quantification

Individual rat faeces were collected before rats were sacrificed and preparation of bacterial DNA were conducted as described by Wang RF [10]. An amount of 0.5 g of faeces was added to 9 ml of sterile phosphate-buffered saline (PBS, 0.05 M, pH 7.4) and mixed well by inverting and surging the tube for 5 to 10 min, then centrifuged at low speed (1440 rpm) for 5 min to collect the upper phases; this was repeated three times. The upper phases were then centrifuged at high speed (9200 rpm) for 3 min to collect the bacterial cells in the pellets. After washing four times with PBS and once with distilled water, the cells in the pellets were resuspended with 50 µl distilled water and 50 µl 1% Triton X-100, boiled at 100 °C for 5 min, and immediately cooled down in ice water. The bifidobacteria in pure cultures with an accurate quantity of 5×10^{10} CFU/ml were directly centrifuged at 9200 rpm for 3 min and then were washed twice with PBS and once with water, suspended, serially diluted, boiled, and cooled in ice water used as standard. The primer set was designed from the 16S rRNA gene sequence available from GenBank: GGGTGGTAATGCCGGATG (P1); CCACCGTTACA CCGGGAA (P2), with the product length 517 base pair. Two microlitres of each sample was directly added to 48 µl of PCR mixture containing 25 µl of Real time PCR Master Mix (TOYOBO, Japan) and 0.2 µM of each primer. The amplifying conditions were: 95 °C for the first 20 min, then 95 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, for 40 cycles. The amount of bifidobacteria in the unknown samples was determined by interpolating the threshold cycle values to the standard curve, and the results were expressed as log10 CFU/g faeces.

2.3. Preparation of single-cell suspensions

The cellular suspensions of thymus and spleen were prepared by chopping the tissues into small pieces with a sterile scissor and then grinding mechanically with a 100 mesh stainless steel sieve. The resulting suspensions were then transferred to a tube and centrifuged at 1500 rpm for 5 min to collect the cell pellets. After washing twice with Hanks, the cells were resuspended and adjusted to a final concentration of 1×10^6 cells/ml in Hanks. To prepare the cellular suspension of Peyer's Patches (PPs), all PPs were carefully excised from the whole length of the small intestine using a curved scissor, chopped and then ground mechanically with a 200 mesh stainless steel sieve. The resulting suspensions were handled in the same way as that of the thymus and spleen.

2.4. Fluorescence staining and flow cytometry analysis

By using direct immunofluorescence, the development of T cells (immature CD3+CD4+CD8+ and mature CD3+CD4+ or CD3+CD8+ T cell) in thymus, the proportion of T-cell subsets (CD3+CD4+ helper T cell, CD3+CD8+ cytotoxic T cell, and CD4+CD25+ regulatory T cell, CD161+ natural killer cell), the number and maturation of DCs (OX62+ cell [11] and fluorescence intensity of CD86) and B cells (CD45RA+ cell [12] and fluorescence intensity of immunoglobulin (Ig)M heavy chain (mIgM)) in peripheral blood, spleen and PPs were determined. For the staining of thymus, spleen and PPs cells, 1×10^5 cells/tube were incubated with antibodies for 20 min, rinsed with PBS, centrifuged at 1000 rpm for 5 min, and then resuspended in PBS. For blood studies, 100 µl whole blood was incubated with antibodies for 20 min. The blood was then lysed with FACS Lysing Solution (BD Biosciences, USA), rinsed with PBS, centrifuged at 1000 rpm for 5 min, and resuspended in PBS. Analysis was done with a FACSCalibur flow cytometer (BD Biosciences). The following mouse monoclonal antibodies were used: FITC anti-CD3 (1 µg/10^6cells), PE anti-CD4 (0.25 µg/10^6cells), APC anti-CD8 (0.5 µg/10^6cells), FITC anti-CD25 (1 µg/10^6cells) and PE anti-CD45RA (1 µg/10^6cells) (all from Biolegend, San Diego, CA), PE anti-OX62 (10 µl/tube), FITC anti-CD86 (10 µl/tube), PE anti-CD161 (10 µl/tube) and FITC anti-mIgM (4 µl/tube) (all from Serotec, UK). Corresponding

2.5. PBMCs cultured in vitro

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised diluted blood by density gradient centrifugation over Ficoll–Hypaque gradients (SABC, Shanghai, China). After being washed twice with D-Hanks, the cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI1640 culture medium (GIBCO, USA) supplemented with 10% heat-inactivated neonatal calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin. The cell suspension was then incubated in a round bottom and ninety-six-well culture plate (Falcon, USA) at 1×10^5 cells/well with the stimulation of phytohaemagglutinin (PHA, 5 μg/ml, Sigma, USA) or Staphylococcus aureus cells (SAC, 0.0056% w/v, Calbiochem, USA). The plates were placed at 37 °C in an environment of 5% CO_2. After being incubated with PHA for 24 h and 48 h, the cells were harvested, mixed well with TRIzol (Invitrogen, USA) and stored at −70 °C until analysis of cytokine expression by RT-PCR. Cell-free supernatants were obtained after the PBMCs were incubated with SAC for 5 d and stored at −70 °C until assayed for antibody production by ELISA.

2.6. RNA isolation and reverse transcription

To investigate the mRNA levels of IL-12 (mainly secreted by DCs), Th1/Th2 (IFN-γ/ IL-4) and regulatory (IL-10, Foxp3) cytokines in intestinal mucosa, the mucosa of the small intestine was scraped and then put in the mortar and ground quickly with liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. For the gene expression of these cytokines in PHA-stimulated PBMCs, total RNA was isolated from the harvested cells, which had been transferred to TRIzol in the same way. The RNA concentration was measured by absorbance at 260 nm in a spectrophotometer (Shimadzu UV-1601, Japan). Two micrograms of total RNA was then reverse transcribed into cDNA with moloney murine leukaemia virus reverse transcriptase (Promega, USA) according to the manufacturer's instructions. After reverse transcription, the 25 μl reaction was diluted to 125 μl so that 2.5 μl of RT product added to a PCR mixture would be equivalent to the reverse transcription of 40 ng total RNA.

2.7. Real-time quantitative PCR

SYBR Green dye and Mastercycler ep realplex4 (Eppendorf, Germany) were used for Real-Time PCR. The sequence of primers, annealing temperature and PCR product size are described in Table 1. Quantitative PCR was carried out on a 25 μl final volume: 12.5 μl of Real time PCR Master Mix (TOYOBO), 0.2 μM of each primer and 2.5 μl of cDNA. The amplifying conditions were: 95 °C for the first 20 min, then 95 °C for 30 s, 54–60 °C (Table 1) for 30 s, and 72 °C for 40 s, for 40 cycles. β-actin was used as quality control. Experiments were performed in duplicate to ensure the reproducibility of the technique. Amplification specificity was checked using a melting curve following the manufacturer's instructions. The relative number of molecules of each transcript was determined by interpolating the threshold cycle values of the unknown samples to each standard curve.

Table 1
Primers, annealing temperatures and product sizes of PCR for β-actin and cytokine gene detection.

Genes	Primers (5′→3′)	Ta (°C)	Product (bp)
β-actin	P1: AAGATCCTGACCGAGCGTGG P2: CAGCACTGTGTTGGCATAGAGG	58	320
IL-12	P1: TCAGGGACATCATCAAACCG P2: ACGCACCTTTCTGGTTACACTC	58	201
IFN-γ	P1: GAAAGACAACCAGGCCATCAG P2: TCATGAATGCATCCTTTTTTGC	54	101
IL-4	P1: CCACGGAGAACGAGCTCATC P2: GAGAACCCCAGACTTGTTCTTCA	60	101
IL-10	P1: GCCAAGCCTTGTCAGAAATGA P2: TCCCAGGGAATTCAAATGCT	56	101
Foxp3	P1: TTCACCTATGCCACCCTC P2: CACTGCTCCCTTCTCACTC	57	198

2.8. Enzyme-linked immunosorbent assay

To determine the basal concentration of cytokines (IL-12, IFN-γ, IL-4 and IL-10) and immunoglobulin (IgG, IgM, IgA and IgE) in plasma, the plasma samples were prepared by having the heparinised blood coagulated naturally at room temperature for 15 min and then centrifuged at 2000 rpm for 10 min to collect the upper phases. The sample quantification was conducted using a double antibody sandwich ELISA with the kits (for cytokines, Jingmei, Beijing, China; for immunoglobulin, ICL, USA) according to the manufacturer's instructions. The level of IgG and IgM in cell-free culture supernatants was measured in the same way to investigate the ability of immunoglobulin secretion by SAC-stimulated PBMCs. The limits of detection of these ELISA kits are as follows: IL-12, 4 pg/ml; IFN-γ, 7 pg/ml; IL-4, 7 pg/ml; IL-10, 6 pg/ml; IgG, IgM and IgA, 5 ng/ml; and IgE, 0.2 ng/ml.

2.9. Statistical analyses

Data are expressed as means and standard deviations (SD). One-way ANOVA was applied to examine group differences, with further multiple comparisons using a Bonferroni test (Stata ver. 7.0, Stata Corp., USA). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. The amount of bifidobacteria in faecal samples

To evaluate the actual results of bifidobacteria minimisation in the BM group, as well as the extra colonisation of it in the BS group, the amount of bifidobacteria in faecal samples was detected by RT-fluorescence quantitative PCR. The results showed that, as compared with the control of the same age, the resident bifidobacteria in the BM group decreased by three orders of magnitude at the end of three weeks and decreased by four orders of magnitude at the end of six weeks, whereas that in the BS group increased by four orders of magnitude at the end of three weeks and maintained this level to the end of six weeks ($n=10$, $p<0.001$ for all, Bonferroni test) (Fig. 1). In addition, bifidobacteria minimisation or supplementation didn't result in any body weight or organ (the thymus, spleen and PPs) weight difference at any age (data not shown), as well as the organ gross observation, except that at the end of six weeks, the intestine of rats in the BM group had excessive gas and mucus (especially the cecum).

3.2. The modulation of bifidobacteria on the differentiation, maturation and function of DCs

To determine the modulation of intestinal bifidobacteria on DCs, the number and maturation of DCs in the peripheral blood, spleen and PPs, the gene expression of IL-12 (mainly secreted by DCs) in intestinal mucosa and cultured PBMCs, as well as the secretion of it in plasma were determined by flow cytometry, RT-PCR and ELISA, respectively. For the level of surface marker CD86 (co-stimulatory molecule) expression increases as DCs mature gradually and thus reflects DC developmental stage [13]. It was found that in PPs and at six weeks, as compared with the control (19.17 (2.32)), the Geo Mean

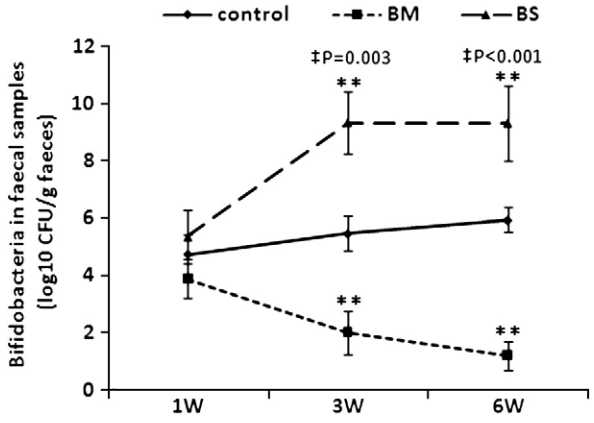

Fig. 1. Amount of bifidobacteria in faecal samples, measured by RT-fluorescence quantitative PCR at the end of one, three and six weeks, showing the effective bifidobacteria minimisation in the BM group and bifidobacteria supplementation in the BS group. ‡p values were obtained using one-way ANOVA to examine group differences, asterisks indicate statistical difference vs. control (**$p<0.01$) through the further multiple comparisons using a Bonferroni test ($n=10$).

significantly (16.89 (1.77)), whereas that in the BS group increased significantly (22.33 (2.09)) ($p=0.013$, One-way ANOVA; $p=0.024$ for the BM, $p=0.015$ for the BS, Bonferroni test) ($n=10$). The results of RT-PCR showed that the BS group had an increased expression of IL-12 mRNA in the intestinal mucosa, both at three weeks and six weeks ($n=10$, $p=0.039$, $p<0.001$ respectively, Bonferroni test); whereas the BM group had a decreased expression of it at six weeks ($n=10$, $p<0.001$, Bonferroni test) (Fig. 2). DC maturation and IL-12 expression outside the gut showed no significant group differences, as well as DC number (the percentage of OX62+ cells) in all areas (data not shown).

3.3. The effect of bifidobacteria on the development of T cells in the thymus

Using flow cytometry, we studied the effect of intestinal bifidobacteria on the development of T cells in the thymus. We found that in the thymus, the immature CD4+CD8+ T cell percentage in the BM group was significantly higher than that in the control group, both at three weeks and six weeks ($n=10$, $p=0.043$, $p=0.022$ respectively, Bonferroni test), but the mature CD4+ or CD8+ T cell percentage was markedly lower than that in the control group at three weeks ($n=10$, $p=0.036$, $p=0.031$ respectively, Bonferroni test). The BS group was not significantly different than the control however. In addition, the proportion of each T-cell subset (CD3+CD4+ helper T cell, CD3+CD8+ cytotoxic T cell, and CD4+CD25+ regulatory T cell, CD161+ natural killer cell) in the peripheral blood, spleen and PPs was also studied by flow cytometry, and none of the T-cell subsets differed significantly among the three groups in all areas (Table 2).

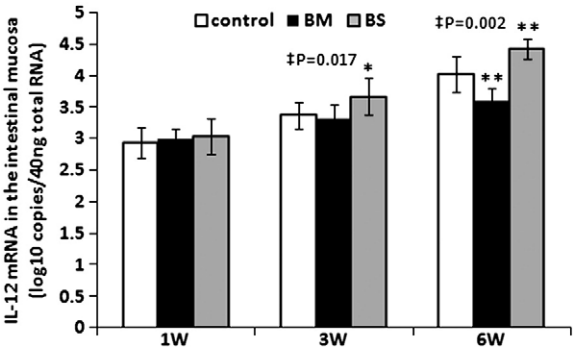

Fig. 2. Effect of intestinal bifidobacteria minimisation (the BM group) or supplementation (the BS group) on the expression of IL-12 mRNA in the intestinal mucosa. ‡p values were obtained using one-way ANOVA to examine group differences, asterisks indicate statistical difference vs. control (*$p<0.05$, **$p<0.01$) through the further

3.4. The impact of bifidobacteria on the T-helper cell response

To determine whether intestinal bifidobacteria affect the balance of Th1 and Th2, the mRNA levels of Th1/Th2 (IFN-γ/ IL-4) cytokines in intestinal mucosa and cultured PBMCs, as well as the secretion of them in plasma were measured with RT-PCR and ELISA respectively. The results showed that, as compared with the control, the BM group had an increased protein level of IL-4 in plasma at six weeks ($n=10$, $p=0.006$, Bonferroni test), but a decreased IFN-γ/IL-4 mRNA ratio in intestinal mucosa both at three weeks and six weeks ($n=10$, $p=0.011$, $p=0.015$ respectively, Bonferroni test), as well as a decreased IFN-γ gene transcript in cultured PBMCs at six weeks ($n=10$, $p=0.034$, Bonferroni test). The BS group, however, had up-regulated IFN-γ mRNA and IFN-γ/IL-4 ratio in intestinal mucosa both at three weeks and six weeks ($n=10$, $p<0.001$ for all, Bonferroni test), as well as increased IFN-γ gene expression in cultured PBMCs at six weeks ($n=10$, $p=0.035$, Bonferroni test) (Fig. 3).

3.5. The modulation of bifidobacteria on the regulatory T cell response

To further evaluate the modulation of intestinal bifidobacteria on the Treg cell response, the number of CD4+CD25+ Treg cells in the peripheral blood, spleen and PPs, the expression of regulatory (IL-10, Foxp3) cytokines in intestinal mucosa and cultured PBMCs, as well as IL-10 secretion in plasma were determined by flow cytometry, RT-PCR and ELISA, respectively. We found that at both three weeks and six weeks, the BM group had a decreased expression of IL-10 mRNA in the intestinal mucosa ($n=10$, $p=0.022$, $p=0.012$ respectively, Bonferroni test), whereas the BS group had an increased expression ($n=10$, $p=0.015$, $p=0.011$ respectively, Bonferroni test), as compared with the control (Fig. 4). Expression of IL-10 outside the gut, Foxp3 gene expression (data not shown) and the percentage of CD4+CD25+ Treg cells (as has been noted) in all areas showed no significant group differences.

3.6. The effect of bifidobacteria on immunoglobulin synthesis

To investigate the effect of intestinal bifidobacteria on immunoglobulin secretion, the number and maturation of B cells in the peripheral blood, spleen and PPs, the basal level of immunoglobulin (IgG, IgM, IgA and IgE) in plasma and the production of IgG and IgM by SAC-stimulated PBMCs were determined by flow cytometry and ELISA, respectively. We found that at six weeks, after PBMCs were cultured with SAC for 5d, the BM group had a decreased production of IgM (299.91 (100.03) ng/ml), whereas the BS group had an increased production of it (749.58 (85.58) ng/ml), as compared with the control (507.16 (89.8) ng/ml) ($p<0.001$, one-way ANOVA; $p=0.012$ for the BM, $p=0.009$ for the BS, Bonferroni test) ($n=10$). The number and maturation of B cells (the percentage of CD45RA+ cells and fluorescence intensity of mIgM) in all areas showed no significant group differences (Table 2); this was also the case for the level of immunoglobulin in plasma (data not shown).

4. Discussion

Early colonising flora of the gut is crucial in the development of the body's immune system. Bifidobacteria, as the predominant bacteria in the gut during the early period of life, could play a major role in this process. In our present study, feeding the neonatal rats daily with sufficient antibiotics and keeping them in a strict barrier environment

Table 2
Effects of intestinal bifidobacteria on T-cell development in thymus, proportion of each lymphocyte subset and B-cell maturation in peripheral blood, spleen and PPs.

	Thymus				Blood			
	Control	[a]BM	[b]BS	[c]*p*	Control	BM	BS	*p*
[d]CD3+CD4+CD8+								
1W	65.13(10.09)	65.31(9.76)	64.98(9.55)	0.998	7.74(1.44)	8.67(2.78)	8.09(2.30)	0.776
3W	49.25(6.13)	55.21(7.20)*	48.81(5.21)	0.039	2.40(0.71)	2.35(1.13)	2.88(0.52)	0.584
6W	30.09(5.06)	44.31(5.98)*	31.21(4.03)	0.012	1.70(0.36)	1.98(0.45)	1.61(0.28)	0.189
[d]CD3+CD4+								
1W	8.21(1.12)	7.89(0.98)	8.05(1.10)	0.896	19.84(2.65)	21.13(1.98)	20.05(3.14)	0.581
3W	11.46(2.30)	8.62(1.51)*	11.95(1.76)	0.043	29.35(5.38)	27.11(6.13)	26.45(4.99)	0.069
6W	12.27(2.17)	11.49(1.25)	12.12(1.34)	0.747	44.55(4.23)	42.99(3.29)	46.31(3.28)	0.055
[d]CD3+CD8+								
1W	4.65(0.24)	4.52(0.19)	4.31(0.12)	0.841	7.66(1.44)	6.31(1.05)	6.06(2.33)	0.772
3W	9.44(0.76)	6.33(0.51)*	9.19(0.58)	0.046	11.73(2.11)	12.47(1.98)	14.55(2.31)	0.084
6W	6.76(0.52)	5.98(0.45)	6.13(0.60)	0.354	22.55(3.35)	24.07(4.84)	24.10(4.29)	0.163
[d]CD4+CD25+								
1W	–	–	–	–	1.47(0.11)	1.22(0.18)	1.28(0.21)	0.065
3W	–	–	–	–	1.97(0.21)	2.16(0.15)	2.03(0.32)	0.282
6W	–	–	–	–	2.30(0.23)	2.65(0.25)	2.57(0.18)	0.059
[d]CD161+								
1W	–	–	–	–	2.08(0.31)	1.79(0.54)	1.76(0.37)	0.069
3W	–	–	–	–	7.66(1.11)	8.25(0.98)	8.31(1.40)	0.233
6W	–	–	–	–	5.29(0.48)	5.57(0.55)	6.02(0.56)	0.340
[d]CD45RA+								
1W	–	–	–	–	17.61(0.93)	15.39(1.12)	15.79(2.04)	0.081
3W	–	–	–	–	21.82(1.39)	21.16(2.27)	20.88(2.41)	0.454
6W	–	–	–	–	18.16(2.21)	19.55(3.43)	16.64(3.07)	0.052
[e]mIgM+								
1W	–	–	–	–	24.94(3.11)	25.21(2.60)	23.39(3.07)	0.399
3W	–	–	–	–	17.96(2.61)	17.77(3.58)	16.49(3.12)	0.652
6W	–	–	–	–	22.47(2.01)	20.35(2.33)	20.68(3.14)	0.113

	Spleen				PPs			
	Control	BM	BS	*p*	Control	BM	BS	*p*
CD3+CD4+CD8+								
1W	4.11(0.31)	4.09(0.45)	3.75(0.39)	0.072	0.99(0.12)	0.98(0.24)	1.23(0.25)	0.255
3W	3.69(0.37)	4.06(0.87)	3.99(0.55)	0.060	1.17(0.14)	1.26(0.05)	1.22(0.18)	0.774
6W	2.02(0.48)	1.19(1.13)	1.61(0.85)	0.225	1.58(0.19)	1.39(0.22)	1.56(0.27)	0.069
CD3+CD4+								
1W	21.94(5.39)	18.19(4.27)	19.82(3.66)	0.060	18.13(1.35)	16.76(2.21)	15.48(1.39)	0.079
3W	22.78(4.55)	24.23(5.17)	25.01(4.44)	0.082	20.38(4.02)	19.61(2.19)	21.11(4.67)	0.661
6W	20.86(4.08)	18.06(5.33)	17.87(5.51)	0.224	21.29(2.87)	19.59(2.95)	22.18(2.06)	0.177
CD3+CD8+								
1W	13.45(1.57)	10.53(2.41)	13.07(3.42)	0.115	5.56(1.94)	4.11(1.19)	6.31(1.71)	0.064
3W	11.31(1.79)	9.36(1.50)	10.55(2.30)	0.644	5.37(1.39)	5.29(1.43)	6.56(2.42)	0.113
6W	10.12(2.41)	7.19(3.68)	9.48(2.19)	0.098	7.43(1.82)	5.78(1.31)	7.76(2.07)	0.472
CD4+CD25+								
1W	1.88(0.14)	1.71(0.21)	1.77(0.13)	0.150	5.36(0.18)	6.03(1.12)	5.93(0.77)	0.054
3W	1.93(0.17)	2.11(0.22)	1.85(0.19)	0.373	6.02(0.19)	5.79(0.34)	6.11(0.25)	0.344
6W	2.39(0.18)	2.43(0.25)	2.16(0.20)	0.551	8.47(1.35)	7.11(0.79)	9.09(1.16)	0.092
CD161+								
1W	7.42(0.95)	5.99(2.11)	5.92(1.72)	0.068	1.99(0.12)	1.64(0.11)	1.73(0.19)	0.221
3W	10.21(0.78)	10.06(1.03)	11.05(0.67)	0.197	2.94(0.41)	3.03(0.58)	3.43(0.44)	0.057
6W	8.45(1.24)	6.39(2.09)	6.88(1.56)	0.058	2.77(0.37)	3.49(0.71)	2.84(0.62)	0.121
CD45RA+								
1W	37.41(6.28)	39.15(7.13)	40.01(6.03)	0.062	53.39(11.45)	49.75(15.42)	51.11(13.39)	0.077
3W	43.51(5.87)	39.11(6.93)	42.55(7.02)	0.055	60.01(16.13)	64.12(15.52)	59.98(11.31)	0.060
6W	30.39(5.04)	30.06(3.98)	33.19(3.71)	0.266	57.62(14.61)	54.19(10.09)	56.05(13.36)	0.112
mIgM+								
1W	22.96(3.05)	19.58(3.66)	23.11(4.05)	0.077	12.12(3.31)	15.13(4.54)	13.93(5.16)	0.227
3W	31.25(2.67)	34.29(3.30)	30.97(4.15)	0.064	19.37(2.60)	22.27(3.79)	20.11(4.12)	0.545
6W	41.03(2.23)	40.22(3.38)	41.18(4.06)	0.549	19.19(4.05)	17.81(3.99)	20.15(5.63)	0.066

Asterisks indicate statistical difference vs. control (*$p<0.05$) through the further multiple comparisons using a Bonferroni test, $n=10$.
[a] : the bifidobacteria minimisation group (BM).
[b] : the bifidobacteria supplementation group (BS).
[c] : *p* values were obtained using one-way ANOVA to examine group differences.
[d] : data were expressed as the percentage of positive cells with respect to total lymphocytes (mean (SD)).
[e] : data were expressed as the fluorescence intensity of mIgM in CD45RA+ cells (mean (SD)).

longum (a bifidobacterial species that is widely used in infant formula and probiotic functional foods) successfully colonised the gut after the daily supplementation of 1×10^{10} CFU from birth. Although none of week (the neonatal period), several aspects of the immunity were affected by both bifidobacteria minimisation and supplementation at the end of three weeks (the infant period), with the most obvious

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