

Infant gut microbiota is protective against cow's milk allergy in mice despite immature ileal T-cell response

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Received 15 July 2011; revised 9 September 2011; accepted 14 September 2011.

DOI: 10.1111/j.1574-6941.2011.01207.x

Editor: Julian Marchesi

Keywords

gut microbiota; cow's milk allergy; gnotobiotic mice; high-throughput pyrosequencing; FoxP3.

Abstract

Faecal microbiota of healthy infant displays a large abundance of *Bifidobacterium* spp. and *Bacteroides* spp. Although some studies have reported an association between these two genera and allergy, these findings remain a subject of debate. Using a gnotobiotic mouse model of cow's milk allergy, we investigated the impact of an infant gut microbiota – mainly composed of *Bifidobacterium* and *Bacteroides* spp. – on immune activation and allergic manifestations. The transplanted microbiota failed to restore an ileal T-cell response similar to the one observed in conventional mice. This may be due to the low bacterial translocation into Peyer's patches in gnotobiotic mice. The allergic response was then monitored in germ-free, gnotobiotic, and conventional mice after repeated oral sensitization with whey proteins and cholera toxin. Colonized mice displayed a lower drop of rectal temperature upon oral challenge with β -lactoglobulin, lower plasma mMCP-1, and lower anti-BLG IgG1 than germ-free mice. The *foxp3* gene was highly expressed in the ileum of both colonized mice that were protected against allergy. This study is the first demonstration that a transplanted healthy infant microbiota mainly composed of *Bifidobacterium* and *Bacteroides* had a protective impact on sensitization and food allergy in mice despite altered T-cell response in the ileum.

Introduction

In early life, intestinal microbiota in healthy infants displays a large abundance of *Bifidobacterium* spp. and *Bacteroides* spp. (Adlerberth & Wold, 2009). This microbiota plays a crucial role in the development of gastrointestinal-associated lymphoid tissue and the modulation of the T-helper Th1/Th2/T-regulatory balance (Sudo *et al.*, 1997; Gaboriau-Routhiau *et al.*, 2009). Intestinal adaptive immune responses can be initiated in Peyer's patches (PP) – the usual site in the gut from where commensal bacteria are sampled – either through translocation or by dendritic cell sensing (Cerf-Bensussan & Gaboriau-Routhiau, 2010). The hygiene hypothesis proposes that disturbances in the gastrointestinal microbiota are linked

to increased prevalence of allergic and autoimmune diseases (Okada *et al.*, 2010). Indeed, changes in the establishment of gut microbiota have been observed in western infants (Campeotto *et al.*, 2007; Adlerberth & Wold, 2009). This is most likely due to improved hygiene and cleanliness in western countries, and excessive use of antibiotics, resulting in reduced bacterial stimulus. Several clinical studies have reported differences in the composition of bacterial communities in the faeces of children with and without allergic diseases. Many of those studies highlighted the involvement of *Bifidobacterium* and *Bacteroides* in the protection against the development of atopy (Stsepetova *et al.*, 2007; Vael *et al.*, 2008; Sjogren *et al.*, 2009a, b), but this observation remains a matter of debate (Adlerberth & Wold, 2009). Moreover, the mechanisms

underlying such protective effects remain elusive. There is increasing evidence that T-regulatory cells derived from the thymus or induced in the periphery including the gut mucosa (Chen *et al.*, 2003; Karim *et al.*, 2004) are key players of the immune regulation (O'Mahony *et al.*, 2008; Lyons *et al.*, 2010; Round & Mazmanian, 2010; Zhang *et al.*, 2010).

Gnotobiotic mouse models, which allow colonization of germ-free animals with a single strain or defined bacterial communities, provide a powerful tool to study the interaction between specific gut microbiota and the development of the immune system (Gaboriau-Routhiau *et al.*, 2009). Using germ-free and conventional mice, we have recently shown that the gut microbiota plays a protective role against allergen sensitization and allergic response in a mouse model of food allergy (Rodriguez *et al.*, 2011). The impact of an infant microbiota on the development of the local immune response in mice and the subsequent effect on food allergies has never been investigated.

To address the role of an infant gut microbiota characterized by a dominance of *Bifidobacterium* and *Bacteroides* on allergy, we chose a well-characterized healthy infant microbiota and transplanted it into germ-free mice at weaning age. Implantation of the infant microbiota into the mice was assessed by high-throughput pyrosequencing prior to investigating its impact on allergic manifestations in a murine model of cow's milk allergy. We also studied the impact of such microbiota on the immune T-cell response in different organs and the translocation and dissemination of commensal bacteria before allergic sensitization.

Materials and methods

Animals and housing conditions

Germ-free (Gf) C3H/HeN mice from Anaxem (INRA, Jouy-en Josas, France) and conventional (Cv) C3H/HeN mice from Charles River Laboratories (CRL, l'Arbresle, France) were purchased at weaning age (21 ± 2 days of life). Gf and gnotobiotic (Gn, ex-germ-free) mice were housed in sterile isolators. Gf status was controlled weekly by standard microbiological methods. Cv mice were also housed in sterile isolators to prevent the impact of any environmental factors on the microbiota. Mice were given autoclaved tap water and a cow's milk protein-free standard pellet chow (R03; SAFE, Augy, France) sterilized by γ -irradiation at 45 kGy *ad libitum*. All procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals. The protocol was approved by the Regional Council of Ethics for animal experimentation (Ile de France-Paris Descartes – P2.

AW.034.07). Experiments were performed in the technical support animal care facilities of the Institut M dicament Toxicologie Chimie Environnement (IMTCE, Paris Descartes University).

Colonization of gnotobiotic mice

A faecal microbiota belonging to a 3-month-old healthy infant was selected for its dominance of *Bifidobacterium* and *Bacteroides* species. Approximately 0.1 g of faeces was transferred into Tryptone–Glucose–Yeast–Hemin liquid medium and incubated at 37 °C for 48 h in an anaerobic cabinet (MACS; AES-Chemunex, Bruz, France; N₂/H₂/CO₂; 80 : 10 : 10) or for 24 h in an aerobic atmosphere. A mix of these two cultures (2 : 1 anaerobic/aerobic culture, v/v) was administered to Gf mice by ingastric infusion at D2 and D3 (Fig. 1). Mice from Cv and Gf groups received sterile water.

Analysis of the microbiota

Culture method

As previously described (Rodriguez *et al.*, 2011), freshly collected faecal samples (day 16, Fig. 1) were resuspended in brain heart infusion broth with 10% (v/v) glycerol and kept frozen (–80 °C) until bacterial counting. Faecal samples were serially diluted and spread onto selective and nonselective agar media with a Spiral System (AES-Chemunex). Agar plates were incubated at 37 °C for 24 h under aerobic conditions or at 37 °C for 48 h in an anaerobic cabinet. This allowed isolation, identification and quantification of aerobic and facultative aerobic bacterial groups, i.e. staphylococci, enterobacteria, enterococci, lactobacilli and anaerobes, including *Bacteroides*, *Clostridium*, *Bifidobacterium* and *Fusobacterium*.

For bacterial translocation and dissemination studies (day 15, Fig. 1), PP, mesenteric lymph nodes (MLN) and a fragment of spleen from 10 Gn and 10 Cv mice were homogenized in brain heart infusion broth and spread onto Trypticase-soja agar medium and Columbia agar base + cysteine (160 mg L^{–1}) + sheep blood (5%) medium. These were, respectively, incubated in aerobic and anaerobic conditions for 48 h.

High-throughput sequencing (HTS) analysis of gut microbiota

DNA was extracted from frozen–thawed samples of infant stool, randomly chosen Gn mouse faeces (at day 15, $n = 3$) and caecal samples of Gn mice with high and low clinical scores of allergy (day 51, $n = 4$). The

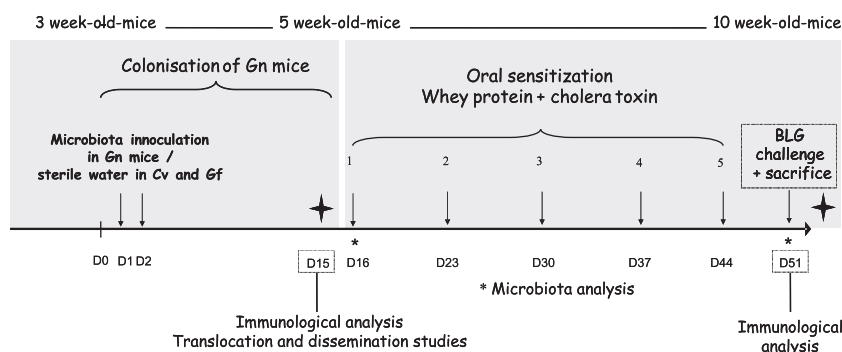


Fig. 1. Experimental procedure. Three-week-old C3H/HeN mice were orally inoculated with the selected infant microbiota cultured in either brain heart infusion (gnotobiotic) or sterile water (germ-free and conventional) at day 0. Establishment of microbiota in gnotobiotic mice occurred over the following 14 days. At day 15, 10 mice from each group were euthanized for immunological and bacterial translocation analysis. From days 16 to 44, 15 mice were orally sensitized with WP and CT once a week (WP-sensitized mice). Control mice were treated with CT alone ($n = 12$ –15 per group). All mice were orally challenged with BLG 1 week after the last sensitization. Faecal samples were collected before the first sensitization, and caecal contents were collected on day 51. Allergic response was assessed on day 51 after the BLG challenge.

influence of the priming sequences on the microbiota profiling by 16S gene pyrosequencing was studied, and two sets of primers were selected, pV1-2-B and pV4. Analysis of the DNA extracts was then performed on these two regions of the 16S gene as previously described (Claus *et al.*, 2011).

Analysis of cytokine production by intestine lamina propria, MLN and PP lymphocytes at day 15

Isolation of small intestine (ileum) and colon lamina propria lymphocytes (LPL)

Ileum and colon fragments were incubated with 5 mM EDTA in PBS to remove epithelial cells. LP leucocytes were extracted from the remaining tissue by a 45-min incubation at 37 °C in 35 $\mu\text{g mL}^{-1}$ Liberase (Roche Diagnostics, France) and 10 U mL^{-1} DNase (Roche Diagnostics), as previously described (Hacini-Rachinel *et al.*, 2009). Leucocytes were enriched by centrifugation over 40% Percoll[®] (GE Healthcare). The resulting cell suspension contained over 90% viable cells.

MLN and PP were removed and gently crushed, filtered through a 70- μm nylon filter (Falcon, VWR, Val de Fontenay, France) and rinsed in RPMI 1640 medium. Extracted cells were cultured for 72 h in 48-well plates (0.5 10^6 cells per well) coated overnight with anti-CD3 and anti-CD28 (5 $\mu\text{g mL}^{-1}$; BD Biosciences, Le Pont-de-Claix, France). IL-10, IL-4, IL-5 and IFN- γ in the supernatants were assayed with the mouse Th1/Th2 4-plex multiplex kit (Meso Scale Discovery, Gaithersburg, MD).

Flow cytometry analysis

Cells were incubated for 15 min at 4 °C with Fc-specific mAb (clone 2.4G2) and then stained using the following mAbs from BD Pharmingen: fluorescein isothiocyanate-conjugated rat antimouse CD45 (clone 30-F11); peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)-conjugated rat antimouse CD4 (clone RM4-5); allophycocyanin-conjugated rat antimouse CD25 (clone PC61) or relevant isotype-matched conjugates. Phycoerythrin-conjugated rat antimouse FoxP3 (clone FJK-16s) staining was performed according to the manufacturer's instructions (eBiosciences, Montrouge, France). Staining was analysed using a FACSCALIBUR (Becton Dickinson, Canada) flow cytometer and FLOWJO software (TreeStar). Data are expressed as percentage of CD4+CD25+ T cells and CD4+CD25+ FoxP3+ T cells gated on CD4-expressing cells.

Allergic response

Oral sensitization and immune challenge

Cv, Gn and Gf mice (27–30 per group) were divided into two subgroups of c. 15 mice each. Oral sensitizations were performed by intragastric infusion. One subgroup received whey proteins (WP, Lacprodan 80[®]; Arla, Lyon, France; 15 mg per mouse) and cholera toxin (CT) as an adjuvant (List Biological, Campbell, California; 10 μg per mouse) in 0.9% NaCl (sensitized group). The other subgroup received CT alone in 0.9% NaCl (control group). The sensitizations were performed five times, at weekly intervals, from day 16 to day 44. One week after the last sensitization, on day 51, all mice received an oral challenge of

60 mg of β -lactoglobulin (BLG; Sigma Aldrich). Clinical scores were recorded as described later, and mice were then euthanized with an intraperitoneal injection of sodium pentobarbital (CEVA santé animale, Libourne, France). Two independent experiments were performed. (Fig. 1)

Evaluation of allergic response

Mice were observed and scored 15–45 min after the BLG challenge by two investigators blinded to the sensitization protocol and the mouse groups, as previously described (Rodriguez *et al.*, 2011). Allergic symptoms were evaluated based on four criteria: drop in rectal temperature, scratching behaviour, loss of mobility and puffiness (including bristled fur, oedema around the nose and eyes, laborious breathing). Rectal temperature was recorded before the challenge and after the clinical evaluation. A drop in temperature was graded as follows: $< 3^\circ\text{C} = 0$, $3\text{--}5^\circ\text{C} = 2$ and $> 5^\circ\text{C} = 4$. Scratching was defined as the number of scratching episodes per 15-min interval and graded as follows: 1–3 episodes = 0, 4–5 episodes = 1 and > 6 episodes = 2. Loss of mobility was graded in terms of duration of absence of any movement, as follows: < 10 min = 0; > 10 min = 1, during the entire trial = 2. Puffiness was graded as none = 0 and puffiness = 2. The clinical score was defined as the sum of the four individual scores and therefore ranged from 0 to 10.

Measurement of plasma mouse mast cell protease-1 (mMCP-1)

On the day of euthanasia, blood was recovered in K₃–EDTA tubes and plasma was stored at -80°C until mMCP-1 measurement by ELISA (Moredun Scientific Ltd., Penicuik, UK) in accordance with the manufacturer's instructions.

Detection of BLG-specific antibodies in plasma by ELISA

Determination of BLG-specific IgE levels was performed by capturing with rat antimouse IgE (Pharmingen, BD Biosciences) antibody and detecting with freshly prepared biotinylated-BLG (Pierce, Rockford, IL) and streptavidin-HRP (Pierce) (Rodriguez *et al.*, 2011). Samples were diluted 20-fold and measured in duplicate, and data were expressed in terms of optical densities (450 nm). Levels of anti-BLG IgG1 were determined using BLG as capture antigen and HRP labelled-Mab goat antimouse IgG1 (Southern Biotech, Birmingham, Alabama) as detection antibody. Titres were expressed as the $\log_{10}$ of the reciprocal of the cut-off dilution. The cut-off dilution was the dilution of samples that gave twice the absorbance of

the negative control. Duplicate wells were run for each sample, and optical densities were read at 450 nm.

Quantification of Th1–Th2–Th17 and T-regulatory gene expression in ileum by reverse transcription quantitative PCR at day 51

Assessment of gene expression assay was performed as previously described (Menard *et al.*, 2008). A total of 2.5 cm of the entire terminal ileum was crushed with Ultra-Turrax instrument for 40 s, and total mRNA was extracted using the TRIzol[®] reagent method (Invitrogen) according to the manufacturer's instructions. mRNA was treated by DNase I (Invitrogen), and first-strand cDNA synthesis was performed using Superscript II and Oligo dT_{12–18} primers (Invitrogen). The cDNA was subjected to quantitative PCR monitored in real time using an ABI Prism 7900HT (Applied Biosystem). The SYBrGreen Quantitect SYBrGreen assay kit and Quantitect Primer Assays (Qiagen, Courtaboeuf, France) were used to quantify IFN- γ , IL-10, IL-4 and transforming growth factor β (TGF- β). Inventoried TaqMan gene expression assays with Taqman universal master mix II (Applied Biosystem) were used to quantify IL-17 and FoxP3. Dosages were performed in duplicate, and gene expression levels were calculated using the ΔC_t method with β -actin (Qiagen) assayed as internal control. Fold increase expression was normalized to expression levels in the Gf-sensitized group.

Statistical analysis

Results were expressed as median and interquartile range, or mean and standard error of the mean. Median data were analysed using the Mann–Whitney test. Differences were considered significant when the *P* value was less than 0.05.

Results

Dominance of *Bifidobacterium* and *Bacteroides* spp. was preserved when human microbiota was transferred to mice

Microbial patterns in the infant stool and in randomly chosen mouse faeces ($n = 3$) were analysed by 16S gene high-throughput pyrosequencing. The number of sequences analysed per sample is shown in Supporting Information, Table S1. Infant and Gn mouse faeces presented similar community members from phylum to genus classifications, with some differences in proportions between group members (Fig. 2). *Bacteroidaceae* were in higher relative abundance than in infant stool, whereas *Bifidobacteriaceae* were in lower relative abundance. The

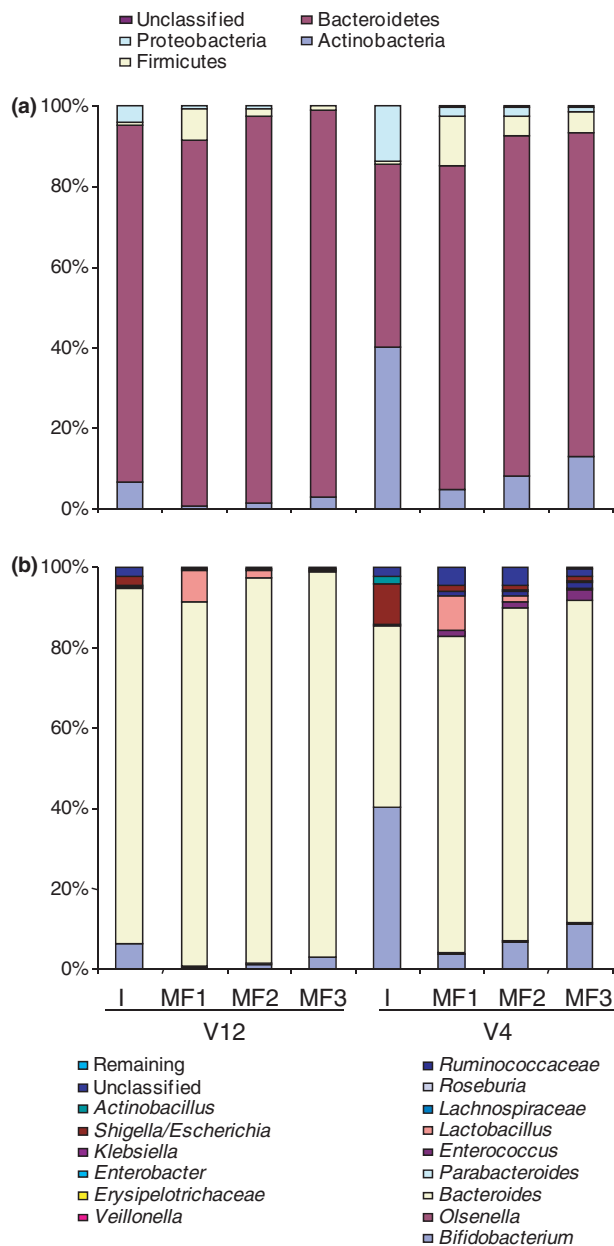


Fig. 2. Microbial patterns of the infant transplanted faecal sample (I) and the gnotobiotic mouse faeces (MF; day 15; $n = 3$) using high-throughput pyrosequencing. Analyses were performed with V1–2 (left panel) and V4 (right panel) 16S rRNA variable gene region, and results are expressed as relative numbers of identified sequences (%) at phylum (a) and genus (b) taxonomic rank.

OTU analysis (> 95% identity with type strains) also suggested minor differences in the community members (Table S1). *Bacteroides* were dominant in both the infant faecal sample and mouse faeces (Fig. 2). However, *Bacteroides stercoris* and *Bacteroides fragilis* were not detected in mouse faeces, whereas the relative abundance

of *Bacteroides uniformis* and *Bacteroides dorei* was different in human and mouse samples (Table S1). *Bifidobacterium* species in mice were qualitatively similar to those detected in infant stool (Fig. 2), with minor differences in relative abundance for some species: *Bifidobacterium pseudocatenulatum* remained stable, but the relative abundance of *Bifidobacterium gallicum* and *Bifidobacterium pullorum* decreased in mice (Table S1). Of note, colonization of mice by the infant microbiota was reproducible because Gn mice chosen at random displayed similar faecal microbial patterns (Fig. 2).

Preservation of the dominance of *Bifidobacterium* and *Bacteroides* in mice was also observed using culture method. Infant stool and mouse faeces showed similar levels of *Bifidobacterium* (10.3 ± 1.2 vs. 9.0 ± 0.0 Log_{10} CFU g^{-1} ; mean $\pm$ SD) and *Bacteroides* (8.9 ± 2.0 vs. 9.1 ± 0.1 Log_{10} CFU g^{-1}) and similar levels of enterococci (7.10 ± 0.28 vs. 8.25 ± 0.35 Log_{10} CFU g^{-1}), enterobacteria (8.5 ± 1.6 vs. 8.8 ± 1.3 Log_{10} CFU g^{-1}) and clostridia (7.7 ± 0.0 vs. 6.3 ± 0.4 Log_{10} CFU g^{-1}). Although lower in infant stool than in mouse faeces (4.9 ± 2.3 vs. 7.5 ± 0.8 Log_{10} CFU g^{-1}), the levels of lactobacilli were not significantly different. The level of staphylococci in infant stool was 5.6 ± 0.4 Log_{10} CFU g^{-1} , but none of these bacteria were isolated from mouse faeces.

A 2-week colonization with the transplanted infant microbiota fails to stimulate the ileal T-cell response

We first investigated the impact of transplanted infant microbiota on the local T-cell response in weaned Gn mice (day 15, Fig. 1). Lymphocytes from MLN, PP, ileum and colon were stimulated with anti-CD3/anti-CD28 prior to quantifying cytokines in the supernatant. LPL isolated from the ileum of Gf, Cv and Gn mice produced different levels of Th1/Th2/T-regulatory type cytokines (Fig. 3). LPL from Gf and Gn mice released significantly lower levels of IFN- γ , IL-4, IL-5 and IL-10 than those of Cv LPL. Flow cytometry analysis of LPL from ileum indicated that Gf and Gn mice displayed similar mean proportions of CD4⁺CD25⁺ T cells (13.5 ± 3.9 vs. $15.3 \pm 0.8\%$) and CD4⁺CD25⁺FoxP3⁺ T cells (8.8 ± 1.8 vs. $9.5 \pm 1.9\%$) among the CD4⁺ T cells (data not shown). Proportions of both populations of cells were lower in the ileum of Cv mice ($8.6 \pm 1.3\%$ for CD4⁺CD25⁺ cells and $4.4 \pm 0.3\%$ for CD4⁺CD25⁺FoxP3⁺ cells). However, the proportion of CD4⁺ T cells was higher in Cv mice (24.7 ± 0.6) than in Gf mice ($16.9 \pm 0.9\%$) or Gn mice ($20.3 \pm 4\%$). LPL isolated from the colon of Cv mice contained higher proportions of CD4⁺CD25⁺FoxP3⁺ cells ($8.2 \pm 0.5\%$) than the LPL of Gf ($6.0 \pm 0.3\%$) and Gn mice ($5.1 \pm 0.1\%$) (data not shown) and tended to

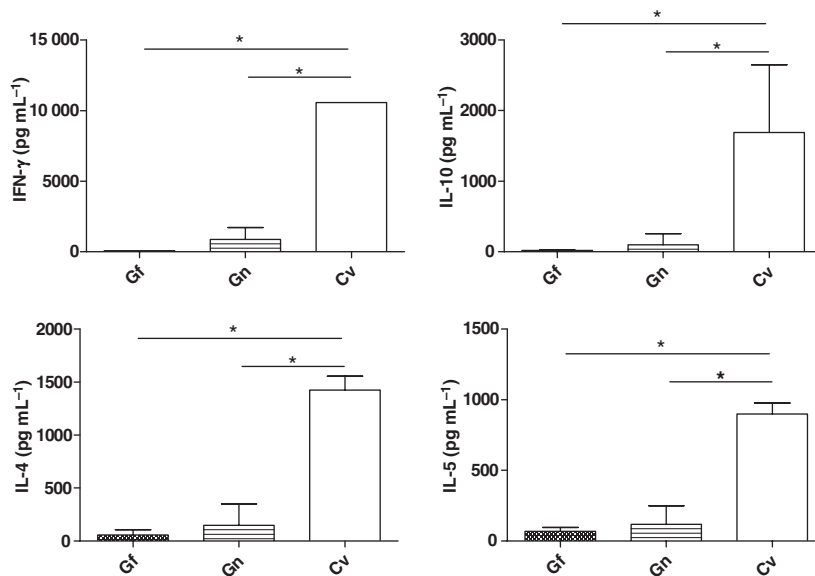


Fig. 3. Immunological analysis at day 15. Cytokine synthesis by ileal LPL from germ-free (Gf), gnotobiotic (Gn) and conventional (Cv) mice. Cytokines were measured by the mouse Th1/Th2 4-plex multiplex kit (Meso Scale Discovery) in culture supernatants of ileal LPL stimulated for 72 h by anti-CD3+CD28. Bars represent mean values of pooled samples ($n = 2-3/\text{pool}$); error bars represent SEM. Using Mann-Whitney test, differences were considered significant when $P < 0.05$ (*).

release higher levels of cytokines *in vitro*. In addition, the proportion of CD4+CD25+FoxP3+ T cells in MLN of Cv mice was higher than in Gf and Gn mice despite nonstatistical differences in cytokine release (Table S2). No statistical differences were observed between the three groups in PP at any level (CD4+ subsets and cytokine release, Table S2). These data show that a 2-week colonization of Gf mice with the infant microbiota therefore fails to fully activate the lymphocyte response in the ileum.

Lower translocation of commensal bacteria into PP in Gn than in Cv mice

Because bacterial translocation may influence the development of the immune system, we used plating to measure the translocation into PP and MLN and the dissemination into the spleen of Cv and Gn mice at day 15. Translocation into PP was observed in 9 of 10 mice in both Cv and Gn groups (data not shown). However, median levels of translocation were significantly higher in Cv ($8.5 \text{ Log}_{10} \text{ CFU g}^{-1}$) than in Gn mice ($3.5 \text{ Log}_{10} \text{ CFU g}^{-1}$) (Fig. 4). There were equal proportions of aerobic (*Enterococcus* and enterobacteria) and anaerobic (anaerobic lactobacilli) genera of translocated bacteria in Cv mice. However, only aerobic genera were translocated into Gn PP. No bacteria belonging to *Bifidobacterium* and *Bacteroides* genera were detected in PP cultures (data not shown). Similar levels of translocation (incidence (70%) and total bacterial counts) were found in the MLN of Cv and Gn mice (Fig. 4). Translocation was limited to aerobic bacteria from *Enterococcus* and enterobacteria genera (data not shown). In the spleen, the

median levels and proportion of dissemination were very low and not significantly different between the two groups. Dissemination in the spleen occurred in 3 of 10 mice for the Gn group and in 5 of 10 mice for the Cv group, although only at very low levels (below $1.3 \text{ Log}_{10} \text{ CFU g}^{-1}$, Fig. 4). Only aerobic bacteria were isolated in spleen cultures (data not shown). These data reveal that Gn mice, which display a weak T-cell function in the ileum, also exhibit a low bacterial translocation rate into PP.

Mice colonized with the infant microbiota were protected against cow's milk allergy

Gf mice have been previously reported to be more susceptible to develop cow's milk allergy than Cv mice (Rodriguez *et al.*, 2011). We therefore aimed to determine whether colonization with the infant microbiota – which led to a weak T-cell response in the ileum similar to that of Gf mice – would protect Gn mice from cow's milk allergy. Gf, Cv and Gn mice were orally sensitized with WP and orally challenged with BLG to trigger allergic reaction. Clinical scores, plasma levels of mMCP-1 and BLG-specific IgG1 were significantly higher in WP-sensitized groups than in control groups regardless of microbial status (Fig. 5). Compared with Gf mice, WP-sensitized Gn and Cv mice displayed a lower drop in rectal temperatures upon oral challenge with BLG (Fig. 5b) as well as lower levels of plasma mMCP-1 (Fig. 5c) and BLG-specific IgG1 (Fig. 5d). Clinical scores of allergy were lower in both WP-sensitized Cv and Gn compared with Gf mice, but reached statistical significance in Gn mice only (Fig. 5a). These data suggest that both Cv and Gn mice are protected against cow's milk allergy.

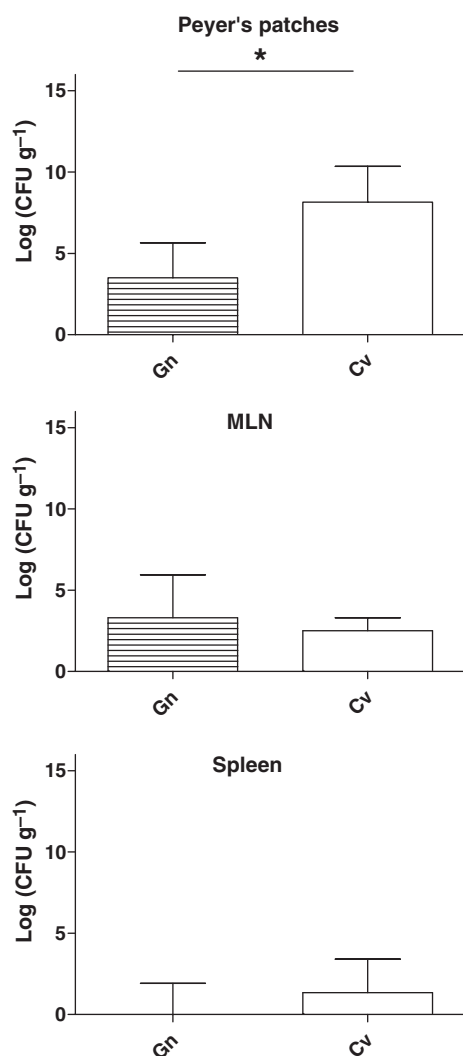


Fig. 4. Comparison at day 15 of the total bacteria translocation levels in Peyer's patches and MLN, and dissemination in spleen, between gnotobiotic (Gn; $n = 10$) and conventional (Cv; $n = 10$) mice, using culture method. Bars represent median levels of isolated bacteria in $\text{Log}_{10} \text{CFU g}^{-1}$ of sample. Error bars represent interquartile range. Using Mann–Whitney test, differences were considered significant when $P < 0.05$ (*).

No significant differences were seen in BLG-specific IgE levels between the three WP-sensitized groups (data not shown).

Differences in caecal microbial communities at the OTU level in Gn mice experiencing low and high allergic responses

Disturbances in the caecal microbiota have been previously reported to be linked to severity of allergic symptoms in Cv mice (Rodriguez *et al.*, 2011). We therefore addressed whether this was also the case in Gn mice. We

investigated the bacterial diversity in caecal contents of sensitized Gn mice displaying high allergic responses (i.e. clinical score = 6 and 8, $n = 2$), as opposed to Gn mice with no clinical symptoms (i.e. clinical score = 0, $n = 2$). HTS analyses of caecal contents revealed differences only at the OTU level, between high and low Gn responders (Table S3), but not at higher classification levels.

Colonization with the infant microbiota induced high ileal *foxp3* gene expression

We showed that Gn mice were protected against cow's milk allergy when challenged with BLG on day 51, despite a weak ileal T-cell response on day 15. We thus investigated whether the protective effect could be linked to a restored T-cell response over time in the ileum. Ileum gene expression levels of *ifn- γ* , *il-4*, *tgf- β* , *il-10*, *il-17* and *foxp3* were assessed in Cv and Gn mice and normalized to Gf gene expression levels at day 51 (Fig. 6). Similar to what was observed at day 15, Gn mice displayed no elicitation of ileal T-cell response at day 51, as shown by no increase in Th1-, Th2- and Th17 cell-related gene expression levels. In contrast, relative gene expressions in the ileum of Cv mice were significantly higher than those of Gf and Gn mice (Fig. 6). Surprisingly, Gn mice showed a *circa* 15-fold relative increase in *foxp3* gene expression vs. Gf mice, but were similar to Cv mice. This increase was observed in control colonized mice when normalized to Gf mice (data not shown) and was therefore independent from the sensitization process.

Discussion

Our study shows that a healthy infant gut microbiota characterized by dominance of *Bifidobacterium* and *Bacteroides* was protective against allergy using a Gn mouse model of cow's milk allergy.

Several studies have reported the successful colonization of germ-free mice with one or several bacterial strains, but only a few of them investigated the adaptation of complex human commensal microbiota to mouse gut conditions (Turnbaugh *et al.*, 2009; Goodman *et al.*, 2011). In our study, infant and Gn mouse faeces presented similar microbial patterns from the phylum to genus classification, while minor differences were observed at the OTU level. This is similar to recent studies where humanized mice were obtained by transferring a frozen–thawed human distal microbiota (Turnbaugh *et al.*, 2009) or a culture-derived faecal microbial community (Goodman *et al.*, 2011) into Gf mice with a remarkable preservation of diversity. However, differences in relative proportions may occur between the microbial pattern of human donors and murine recipients. This is

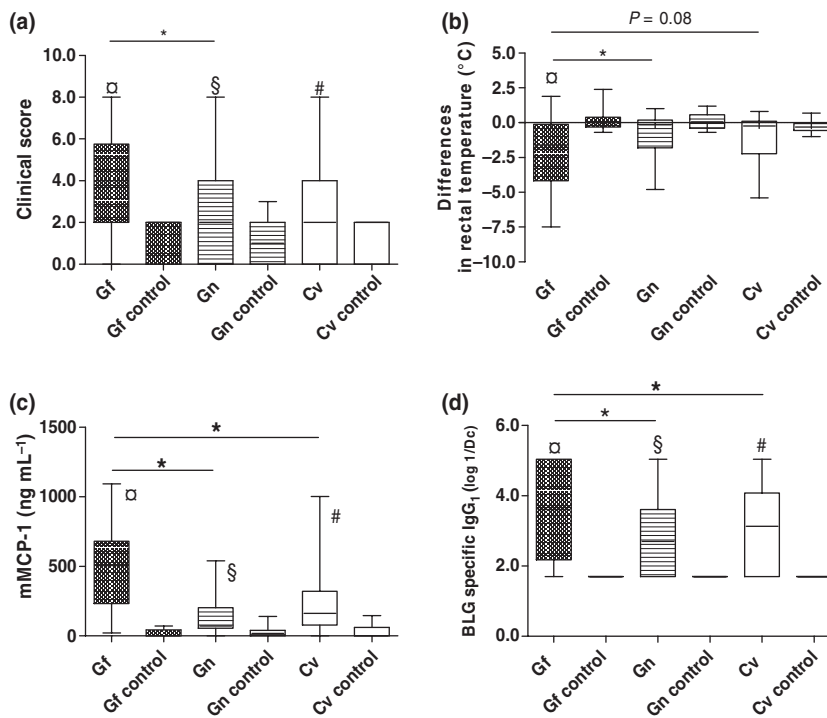


Fig. 5. Allergy response at day 51 after BLG challenge and plasma immunoglobulin in germ-free (Gf), gnotobiotic (Gn) and conventional (Cv) mice using the cow's milk allergy model. (a) Clinical score; (b) differences in rectal temperature (°C); (c) mMCP-1 levels in plasma (ng mL⁻¹); (d) specific BLG IgG₁ (Log1/Dc; Dc = cut-off dilution) levels. Box plots show median value (central horizontal line), the 25th percentile (lower box border) and the 75th percentile (upper box border). The lower and upper horizontal lines refer to the 10th and 90th percentiles, respectively. Using Mann-Whitney test, differences were considered significant when $P < 0.05$: * between sensitized groups; □, § and #: between control and sensitized groups in Gf, Gn and Cv mice, respectively.

likely due to distinct selective pressures imposed by the gut habitat of each host rather than by microbiota transplantation procedure (Rawls *et al.*, 2006). In our study, Actinobacteria and especially bifidobacteria – which are not usual inhabitants of mouse gut (Ley *et al.*, 2005) – may have suffered from murine gut conditions, explaining their lower relative abundance to the benefit of *Bacteroides* in Gn mice. Despite these modifications in relative proportions, the dominance of *Bifidobacterium* and *Bacteroides*, characteristic of the infant microbiota, was conserved in Gn mice after colonization of Gf mice. Moreover, culture analyses showed that the levels of *Bifidobacterium* colonization were similar in the infant stool and mouse faeces.

The present study showed that Gf and Cv mice displayed distinct T-cell responses in the gut. These data are in line with the recent work of Gaboriau-Routhiau *et al.* (2009), who reported higher cytokine release by Cv LPL than Gf LPL following activation with anti-CD3/anti-CD28. Colonization of Gf mice with the infant gut microbiota stimulated the T-cell response in the colon but not in the ileum at 2 weeks post-transplantation. Lack of T-cell activation in the small intestine was also reported in Gn mice after colonization with a human gut microbiota (Gaboriau-Routhiau *et al.*, 2009). Our data at 15 days postcolonization do not exclude a time-dependent effect of gut microbiota on the local immune response (Gaboriau-Routhiau *et al.*, 2009). In our experimental design, the T-cell response in the ileum was still

weak at the end of the sensitization process on day 51. However, we compared the local T-cell responses in Gn mice colonized since the weaning period with those in Cv mice colonized since birth, and this may explain our observations. Indeed, the early colonization has been reported to be of particular importance as colonization of Gf mice with *Bifidobacterium infantis* could restore Th1 responses in neonatal but not adult gnotobiotic mice (Sudo *et al.*, 1997).

Bacterial translocation and dissemination from the gut trigger immune responses locally and in the periphery (Macpherson & Uhr, 2004). The weak translocation in PP of Gn mice could be a key factor underlying the failure to initiate an immune response in the ileum (Macpherson & Uhr, 2004). Indeed, an efficient induction of a gut T-cell response – which includes proliferation of lymphoblasts in PP T-cell areas and T-cell migration into the lamina propria and epithelium (Guy-Grand *et al.*, 1974; Macpherson & Uhr, 2004) – was linked to the capacity of bacteria to adhere and penetrate PP. Pathogens such as *Salmonella* (Salazar-Gonzalez *et al.*, 2006) and commensal bacteria such as segmented filamentous bacteria (Gaboriau-Routhiau *et al.*, 2009) were shown to activate T cells in the gut. In our model, translocated bacteria belonged to genera commonly described in the literature, and no bacteria belonging to the dominant *Bifidobacterium* and *Bacteroides* genera were found in PP of Gn mice. Bifidobacteria have been reported to modulate the immune system (Menard *et al.*, 2008; Lyons *et al.*, 2010; Zhang *et al.*,

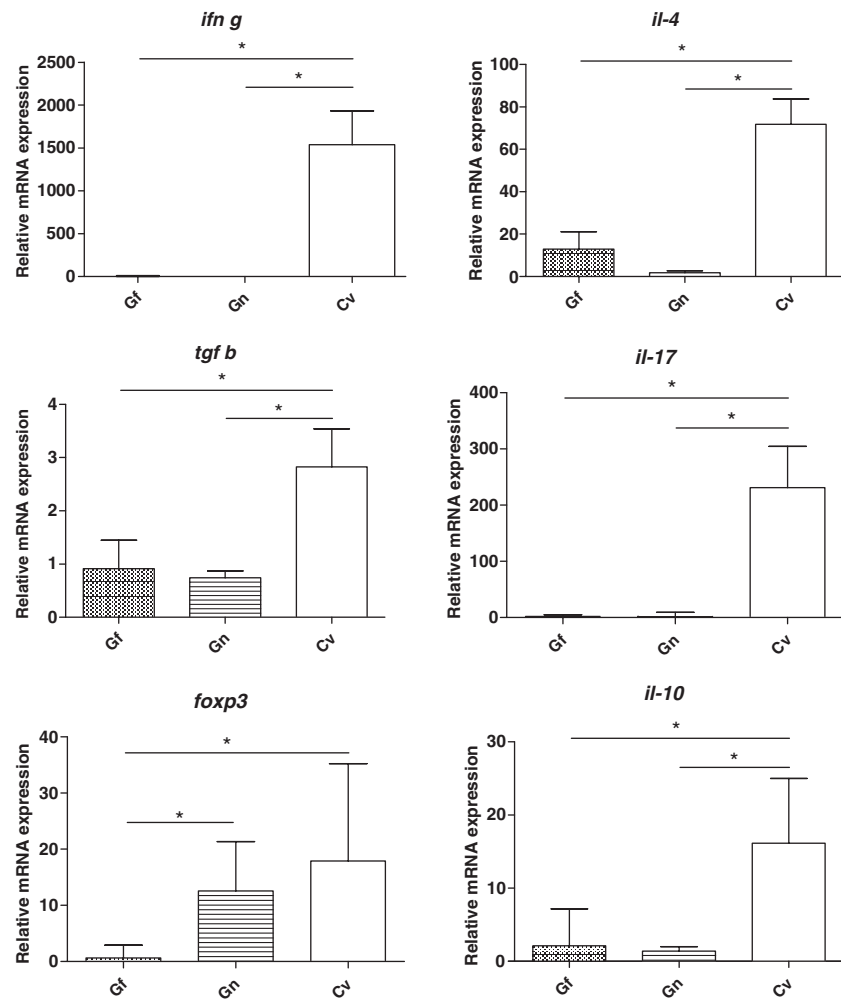


Fig. 6. Ileal gene mRNA expression in WP-sensitized, gnotobiotic (Gn) and conventional (Cv) mice. mRNA expression is relative to germ-free (Gf) mice. Bars represent median values, and error bars represent interquartile range. Using Mann–Whitney test, differences were considered significant when $P < 0.05$ (*).

2010), especially the gut T-cell response, most likely through translocation (Zhang *et al.*, 2010). Differences in PP translocation between Gn and Cv groups could be explained by (i) distinct gut microbial patterns and/or (ii) fewer and less developed PP in Gn mice. Indeed, Cv mouse microbiota did not display bifidobacteria, a genus that decreases bacterial translocation (Romond *et al.*, 2008). In addition, even though ex-germ-free mice were reported to develop normal PP upon bacterial colonization (Cebra, 1999; McCracken & Lorenz, 2001), a 2-week colonization with a simplified microbiota may be insufficient to develop fully matured PP and, in turn, may lead to differences in translocation.

In the present study, Gn mice were protected against cow's milk allergy as indicated by significantly lower clinical scores of allergy vs. Gf mice, as well as low plasma levels of mMCP-1 and BLG-specific IgG1. However, gut colonization with the infant microbiota did not prevent production of BLG-specific IgE. Moreover, disturbances

at the OTU level were associated with the severity of allergic symptoms, in line with the modifications in the microbiota observed in our previous experiment (Rodriguez *et al.*, 2011). Protection against food allergy symptoms without any effects on antigen-specific IgE has already been reported in mice fed with polyphenol-enriched apple extract (Zuercher *et al.*, 2010). Interestingly, the transplanted microbiota induced *foxp3* gene expression in the ileum in an allergen-independent manner. FoxP3 is considered as a master regulatory molecule in regulatory T-cell function (Tang & Bluestone, 2008). The protective effect of regulatory T cells on allergies including airway hyper-responsiveness has been reported in many animal studies (Strickland *et al.*, 2006). In humans, children with food allergy showed significantly lower levels of *foxp3* gene expression in blood cells compared to healthy children (Krogulska *et al.*, 2010).

The exact mechanisms of protection by regulatory T cells are unclear, but the release of suppressor cytokines

and the direct suppression of target cells by cell contact remain the principal purported pathways (Akdis *et al.*, 2005). We observed an induction of *foxp3* expression without an increase in *il10* expression. This suggests the induction of CD4⁺ FoxP3⁺ IL-10[−] T cells, one of the three different CD4⁺ Treg cells subsets, which have been described in the intestinal *lamina propria* (Maynard *et al.*, 2007). Moreover, Gri *et al.* (2008) showed a direct suppressive effect of FoxP3⁺ T-regulatory cells on mast cell degranulation, suggesting a potential role in allergic manifestations. In our model, the *foxp3* gene was highly expressed in the ileum of Cv and Gn mice, which experienced less severe allergic symptoms than Gf mice. The link between *foxp3* gene expression in ileum and protection against food allergy has never been reported and remains to be elucidated. Recently, Tregs have been reported to be generated in MLN prior to homing to the gut where they undergo a local expansion in the context of oral tolerance induction (Hadis *et al.*, 2011). The presence of *Bifidobacterium* and *Bacteroides* in the gut of Gn mice may explain such *foxp3* gene activation. *Bifidobacterium* and/or *Bacteroides* may stimulate antigen-presenting cells and then instruct local naïve CD4⁺ T cells to be converted into FoxP3⁺ Tregs. Indeed, bifidobacteria have been documented to be in close contact with local CD11c⁺ dendritic cells following a transient translocation (Hiramatsu *et al.*, 2011) and to modulate intestinal inflammation by suppressing Th2 response and increasing the number of Tregs in mice with food allergy (Zhang *et al.*, 2010). Treatment of mice with *Bifidobacterium* strains led to the recruitment of FoxP3⁺ T cell in intestinal mucosa and in spleen (O'Mahony *et al.*, 2008; Lyons *et al.*, 2010). *Bacteroides* strains were also shown to strongly promote recruitment of FoxP3⁺ T cells into the colon (Round & Mazmanian, 2010). The specific impact of a microbiota rich in *Bifidobacterium* and *Bacteroides* deserves further investigation using Gf mice associated with different microbial patterns.

In conclusion, the transplanted microbiota characterized by a dominant *Bifidobacterium* and *Bacteroides* population had a protective impact on food allergy in mice, despite a weak ileal T-cell response. The link between *foxp3* expression in the ileum and protection against food allergy deserves further investigation.

Acknowledgements

Bertrand Rodriguez received grant support from NES-TEC. This work was an associated project of the FP7 Marie Curie Actions 'Cross-Talk' ITN project – Grant agreement no21553-2. We thank Chantal Martin from IMTCE for technical support in animal experiments. None of the authors had any conflicts of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Relative abundance (%) of identified sequences at the level of phylum, genus, and operational taxonomic unit (OTU) in infant stool (I) and mouse faeces (MF) for gnotobiotic groups at day 16.

Table S2. Immunological analysis at day 15 of Peyer's patches (PP) and mesenteric lymph nodes (MLN) T cells from germ-free, gnotobiotic, and conventional mice.

Table S3. Relative abundance (%) of identified sequences at phylum, genus, and operational taxonomic unit (OTU) levels in caecal content of low-responding mice (LR; score 0) and high-responding mice (HR1: score 6; HR2: score 8) to β -lactoglobulin challenge at day 51.

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Supplementary Table S1
2

16S rRNA gene variable region	V12				V4			
	LR1	LR2	HR1	HR2	LR1	LR2	HR1	HR2
Phylum								
Actinobacteria	2,3	1,7	1,6	1,3	13,6	12,8	11,2	11,1
Bacteroidetes	95,1	97,3	96,2	96,2	75,6	76,3	80,6	83,8
Firmicutes	1,6	0,6	1,2	1,4	6,8	7,3	5,9	3,3
Proteobacteria	1,0	0,4	1,0	1,1	3,7	3,6	2,4	1,8
Unclassified	0,1	0,0	0,0	0,0	0,3	0,0	0,0	0,0
Genus								
<i>Bifidobacterium</i>	1,8	1,1	1,0	0,9	7,4	6,3	4,3	3,7
<i>Olsenella</i>	0,5	0,5	0,5	0,4	1,6	1,4	1,6	2,2
<i>Bacteroides</i>	95,1	97,3	96,2	96,2	74,6	76,2	80,6	83,4
<i>Enterococcus</i>	0,1	0,0	0,1	0,1	1,5	2,4	0,5	0,7
<i>Lactobacillus</i>	1,0	0,6	0,6	0,8	1,3	1,4	0,8	0,4
<i>Ruminococcaceae</i>	0,4	0,0	0,4	0,2	3,6	3,2	4,3	2,2
<i>Enterobacter</i>	0,1	0,2	0,2	0,1	0,0	0,0	0,0	0,0
<i>Shigella/Escherichia</i>	0,4	0,1	0,4	0,7	2,1	2,0	1,3	1,1
<i>Actinobacillus</i>	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Unclassified	0,5	0,1	0,5	0,3	7,7	6,9	6,4	5,9
Remaining	0,1	0,0	0,1	0,3	0,3	0,2	0,3	0,4
OTU (>95%)								
<i>Bacteroides dorei</i>	51,1	53,6	50,5	43,2	37,7	39,9	44,1	35,8
<i>Bacteroides fragilis</i>	16,9	10,3	6,6	7,1	16,0	9,3	6,6	6,3
<i>Bacteroides uniformis</i>	27,0	32,8	38,6	45,3	21,1	26,8	29,8	40,6
<i>Bifidobacterium gallicum</i>	0,0	0,0	0,0	0,0	4,2	3,9	2,1	1,8
<i>Bifidobacterium longum</i>	0,0	0,0	0,0	0,0	0,7	0,5	0,3	1,8
<i>Bifidobacterium pseudocatenulatum</i>	0,7	0,5	0,4	0,5	0,3	0,0	0,0	0,0
<i>Clostridium orbiscindens</i>	0,0	0,0	0,0	0,0	2,2	1,9	1,9	0,0
<i>Enterococcus canintestini</i>	0,4	0,0	0,3	0,2	3,5	3,2	4,3	2,2
<i>Escherichia vulneris</i>	0,0	0,0	0,0	0,0	1,5	2,7	0,5	0,7
<i>Lactobacillus gasseri</i>	0,0	0,0	0,0	0,0	2,6	2,6	1,9	1,5
<i>Olsenella uli</i>	1,1	0,6	0,6	0,8	1,3	1,4	0,8	0,4
<i>Escherichia coli</i>	0,0	0,0	0,1	0,0	5,7	6,2	6,6	6,6
Remaining	0,8	0,2	0,7	0,9	0,0	0,0	0,0	0,0
Unassigned	2,1	1,9	2,2	2,1	3,1	1,7	1,1	2,2
Number of identified sequences	1661	962	3874	1531	2068	1089	376	271

Supplementary Table S2

	Germ free		Gnotobiotic		Conventional	
	PP	MLN	PP	MLN	PP	MLN
IFN-γ	nd	12450 $\pm$ 341	nd	11925 $\pm$ 697	nd	9941 $\pm$ 671
IL-4	nd	370 $\pm$ 107	nd	440 $\pm$ 78	nd	320 $\pm$ 48
IL-5	nd	469 $\pm$ 78	nd	536 $\pm$ 129	nd	469 $\pm$ 65
IL-10	nd	1293 $\pm$ 302	nd	1574 $\pm$ 567	nd	1356 $\pm$ 303
CD4+CD25+ (%)	10.6 $\pm$ 1.3	6.5 $\pm$ 0.1	9.0 $\pm$ 1.0	6.2 $\pm$ 0.8	7.3 $\pm$ 1.1	8.5 $\pm$ 0.7
CD4+CD25+ Foxp3+ (%)	6.9 $\pm$ 0.0	5.8 $\pm$ 0.4	6.3 $\pm$ 0.2	4.7 $\pm$ 0.5	5.4 $\pm$ 1.1	7.0 $\pm$ 0.7

nd : not detected

Supplementary Table S3

16S rRNA gene variable region	V12				V4			
	I	MF1	MF2	MF3	I	MF1	MF2	MF3
Phylum								
Actinobacteria	6.5	0.7	1.6	2.9	40.2	5.0	8.3	13.0
Bacteroidetes	88.6	90.9	95.9	96.1	45.5	80.4	84.3	80.3
Firmicutes	0.9	7.9	2.0	1.0	0.4	12.4	5.3	5.4
Proteobacteria	4.1	0.5	0.6	0.0	13.9	2.1	2.0	1.1
Unclassified	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.2
Genus								
<i>Bifidobacterium</i>	6.5	0.6	1.4	2.9	40.2	3.9	6.6	11.2
<i>Olsenella</i>	0.0	0.1	0.2	0.0	0.0	0.2	0.5	0.2
<i>Bacteroides</i>	88.2	90.9	95.9	96.1	45.1	78.8	82.8	80.3
<i>Parabacteroides</i>	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Enterococcus</i>	0.0	0.1	0.0	0.1	0.0	1.5	1.5	2.6
<i>Lactobacillus</i>	0.5	7.5	1.8	0.6	0.0	8.6	1.5	0.2
<i>Ruminococcaceae</i>	0.0	0.1	0.2	0.1	0.0	1.0	1.0	1.7
<i>Veillonella</i>	0.2	0.0	0.0	0.0	0.4	0.0	0.0	0.0
<i>Erysipelotrichaceae</i>	0.0	0.2	0.0	0.1	0.0	0.1	0.3	0.4
<i>Enterobacter</i>	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.0
<i>Klebsiella</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Shigella/Escherichia</i>	2.0	0.2	0.4	0.0	10.2	1.3	1.3	0.9
<i>Actinobacillus</i>	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0
Unclassified	2.2	0.1	0.0	0.0	2.3	4.5	4.4	2.2
Remaining	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.2
OTU (>95%)								
<i>Bacteroides dorei</i>	70.3	25.4	29.6	36.3	35.0	21.4	27.2	29.1
<i>Bacteroides fragilis</i>	5.7	13.1	8.3	8.8	1.5	12.7	8.5	6.5
<i>Bacteroides stercoris</i>	9.7	0.0	0.0	0.0	6.8	0.0	0.0	0.0
<i>Bacteroides uniformis</i>	1.5	52.0	57.5	50.9	1.6	44.6	47.0	44.7
<i>Bifidobacterium gallicum</i>	0.0	0.0	0.0	0.0	32.7	2.2	3.0	6.7
<i>Bifidobacterium longum</i>	0.0	0.0	0.0	0.0	0.8	0.9	1.0	1.9
<i>Bifidobacterium pseudocatenulatum</i>	4.3	0.2	0.4	0.8	0.4	0.1	0.2	0.2
<i>Bifidobacterium pullorum</i>	0.0	0.0	0.0	0.0	6.4	0.7	2.3	2.6
<i>Clostridium orbiscindens</i>	0.0	0.1	0.2	0.1	0.0	1.0	1.0	1.7
<i>Enterococcus canintestini</i>	0.0	0.0	0.0	0.0	0.0	1.6	1.5	2.6
<i>Escherichia vulneris</i>	0.0	0.0	0.0	0.0	11.3	1.7	1.6	0.9
<i>Lactobacillus gasseri</i>	0.5	7.5	1.6	0.3	0.0	9.4	1.8	0.4
<i>Olsenella uli</i>	0.0	0.0	0.0	0.0	0.0	0.7	1.5	1.1
<i>Escherichia coli</i>	3.2	0.3	0.4	0.0	0.0	0.0	0.0	0.0
Remaining	4.8	1.3	2.0	2.8	3.8	2.1	2.3	1.5
Unassigned	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0
Number of identified sequences	587	1349	510	716	266	2724	2932	463