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Gnotobiotic Mouse Immune Response Induced by *Bifidobacterium* sp. Strains Isolated from Infants▽

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***Bifidobacterium*, which is a dominant genus in infants' fecal flora and can be used as a probiotic, has shown beneficial effects in various pathologies, including allergic diseases, but its role in immunity has so far been little known. Numerous studies have shown the crucial role of the initial intestinal colonization in the development of the intestinal immune system, and bifidobacteria could play a major role in this process. For a better understanding of the effect of *Bifidobacterium* on the immune system, we aimed at determining the impact of *Bifidobacterium* on the T-helper 1 (T_H1)/T_H2 balance by using gnotobiotic mice. Germfree mice were inoculated with *Bifidobacterium longum* NCC2705, whose genome is sequenced, and with nine *Bifidobacterium* strains isolated from infants' fecal flora. Five days after inoculation, mice were killed. Transforming growth factor β1 (TGF-β1), interleukin-4 (IL-4), IL-10, and gamma interferon (IFN-γ) gene expressions in the ileum and IFN-γ, tumor necrosis factor alpha (TNF-α), IL-10, IL-4, and IL-5 secretions by splenocytes cultivated for 48 h with concanavalin A were quantified. Two *Bifidobacterium* species had no effect (*B. adolescentis*) or little effect (*B. breve*) on the immune system. *Bifidobacterium bifidum*, *Bifidobacterium dentium*, and one *B. longum* strain induced T_H1 and T_H2 cytokines at the systemic and intestinal levels. One *B. longum* strain induced a T_H2 orientation with high levels of IL-4 and IL-10, both secreted by splenocytes, and of TGF-β gene expression in the ileum. The other two strains induced T_H1 orientations with high levels of IFN-γ and TNF-α splenocyte secretions. *Bifidobacterium*'s capacity to stimulate immunity is species specific, but its influence on the orientation of the immune system is strain specific.**

Bifidobacteria are gram-positive, anaerobic bacteria that represent up to 90% of the total gut microflora in breast-fed babies (7) and up to 15% in adults (22). The presence of such high levels of bifidobacteria in the human intestine is suggested to contribute to human health, leading to the use of bifidobacteria as probiotics. Indeed, beneficial effects of bifidobacteria are shown for various diseases, e.g., diarrhea associated with rotavirus or antibiotics and some inflammatory intestinal diseases (27). Though some studies suggest a role for bifidobacteria in prevention of allergic diseases, data are scarce and controversial (2, 5). Moreover, the role of bifidobacteria in immunity has so far been little known.

For a few years, the incidences of allergic diseases have been increasing in developed countries. A disturbance in the balance of T-helper 1 (T_H1)/T_H2 lymphocyte responses to exogenous antigens toward a T_H2 phenotype is considered a major event in the onset of allergic diseases. Though the mechanisms of these disturbances are still controversial, several studies suggest a prominent role for microorganisms from the environment and the normal commensal flora of the gastrointestinal tract in these disregulations or in the prevention of allergic sensitization (15, 28). In fact, intestinal colonization, in which the earliest contact with microbes occurs, has a marked impact on the maturation of the immune system (IS), which is underdeveloped at birth (9). The natural sequential colonization process of the digestive tract is complex and was shown to crucially influence immune response establishment during the neonatal period, especially T_H1/T_H2 balance (8, 34, 35). Several clinical studies showed a relationship between allergic diseases and the gut microbiota, pointing out quantitative and qualitative differences in bifidobacterial colonization (4, 12, 26, 37). These studies showed that allergic patients had lower counts of *Bifidobacterium* than healthy control subjects. Furthermore, differences in species were observed. *Bifidobacterium adolescentis* and *Bifidobacterium longum* were isolated from allergic infants as the predominant bifidobacteria, whereas the predominant ones isolated from age-matched healthy infants were *Bifidobacterium infantis*, *Bifidobacterium bifidum*, and *Bifidobacterium breve* (26). Although these data suggested a link between bifidobacterial species and atopy or tolerance, no clear relation of causes and effects has been demonstrated yet. An in vivo study of newborn mice showed that *B. infantis* establishment was important for restoration of the usual oral tolerance process and especially for immunoglobulin E suppression (34). The impact of bifidobacteria on immunity has been specified with in vitro studies showing the roles of individual bifidobacterial species (11, 21, 29, 39). Nonetheless, no in vivo study has confirmed such observations. Thus, it is essential, at a time when dramatic increases in the prevalences of allergic diseases are a major concern in western countries, to understand the connections between allergy and intestinal flora and more particularly between allergy and bi-

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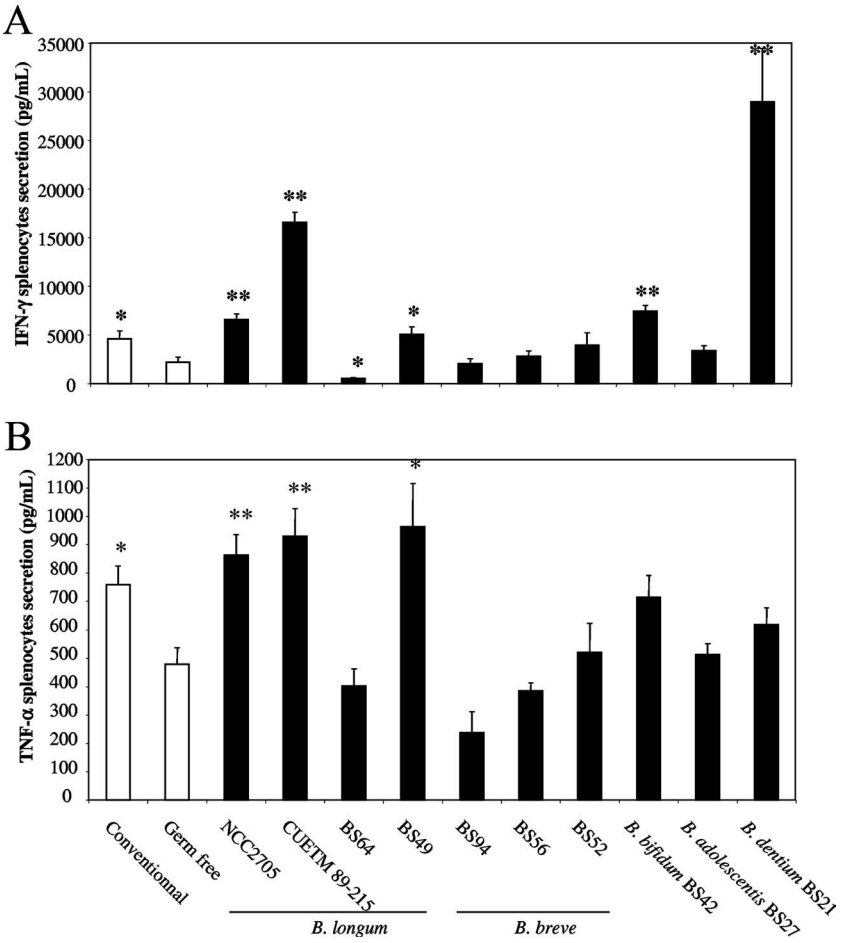

FIG. 1. Levels of pro-T_H1 cytokine secretion by splenocytes stimulated for 48 h with ConA (3 μg/μl). (A) IFN-γ; (B) TNF-α. Data obtained by ELISA are presented as means plus SEMs. *P* was <0.05 (*) and <0.01 (**) for comparison with GF mice. Reference groups are represented by open bars.

fidobacteria, which may be used as a probiotic for preventing these diseases (3, 5).

In this study, using gnotobiotic mice and quantification of pro- and anti-inflammatory-cytokine production and/or gene expression, we aimed to assess the roles of bifidobacterial strains belonging to different species and isolated from the fecal flora of infants on T_H1/T_H2 balance.

MATERIALS AND METHODS

Bacterial strains. Ten *Bifidobacterium* strains were studied. Nine strains were isolated from the dominant fecal flora of 3- to 24-month-old healthy infants, including three *B. longum* strains (BS64, BS49, and CUETM 89-215), three *B. breve* strains (BS94, BS52, BS56), one *B. bifidum* strain (BS42), one *B. dentium* strain (BS21), and one *B. adolescentis* strain (BS27). The tenth strain was *B. longum* NCC2705, whose genome is entirely sequenced (31). Bifidobacterial genera were identified according to their morphological characteristics, by the presence of fructose-6-phospho-phosphoketolase, and by PCR according to Kok et al. (18). Species identification was realized using a multiplex PCR targeting the 16S-23S rRNA gene intergenic spacer according to Mullié et al. (23). All strains were grown on Wilkins Chalgren agar base containing D-glucose (10 g/liter), L-cysteine (0.5 g/liter), and Tween 80 (0.5%, vol/vol) (6) and in trypticase-glucose-yeast extract-hemin broth. They were incubated at 37°C in an anaerobic chamber (dw Scientific, AES laboratoires, Bruzz, France).

Experimental animals. Six- to 7-week-old female germfree (GF) C_3H/HeN and conventional C_3H/HeN mice were purchased from the INRA (Jouy-en-Josas, France). GF and conventional mice were maintained in sterile isolators (JCE Biotechnology, Hauterive, France) and fed ad libitum with a commercial rodent diet sterilized by gamma irradiation (40 megarads). The animal experimentation was conducted in accordance with the rules of our institution and with Council of Europe Guidelines, with license for experimental studies on living animals and animal facility agreement no. A750602 (Direction of Veterinary Services, Prefecture de Police de Paris, France).

Twelve groups of 6 to 12 mice were studied. GF and conventional mice as well as mice which had been mono-associated with one of the 10 *Bifidobacterium* strains were included.

Colonization of GF mice. Bifidobacterial mono-associated mice were obtained by gastric intubation of GF mice with a 48-h culture of one of the bifidobacterial strains comprising 10^6 to 10^8 CFU/ml. This range of doses did not influence the colonization level. Both GF and conventional mice received the same volume (300 μl) of sterile water. Feces were collected 48 h after force feeding to check the sterility in GF mice and the bacterial establishment in monobiotic mice. Cecal contents were collected after sacrifice in order to measure the bifidobacterial levels in monobiotic mice, to determine the fecal microbiota compositions in conventional mice, and to check the sterility in GF mice. Dilutions of feces and cecal contents in a prereduced peptone liquid medium were performed in anaerobiosis and spread on Wilkins Chalgren agar base for monobiotic mice and on various media allowing the isolation and the quantification of the main bacterial genera for conventional mice, as previously described (6).

Lymphocyte culture. Six days after their inoculation, mice were sacrificed with an intraperitoneal injection of pentobarbital sodium (CEVA santé animale, Libourne, France). Spleens were gently crushed, filtered through a 70-μm nylon filter (Falcon, VWR, Val de Fontenay, France), and rinsed in RPMI 1640

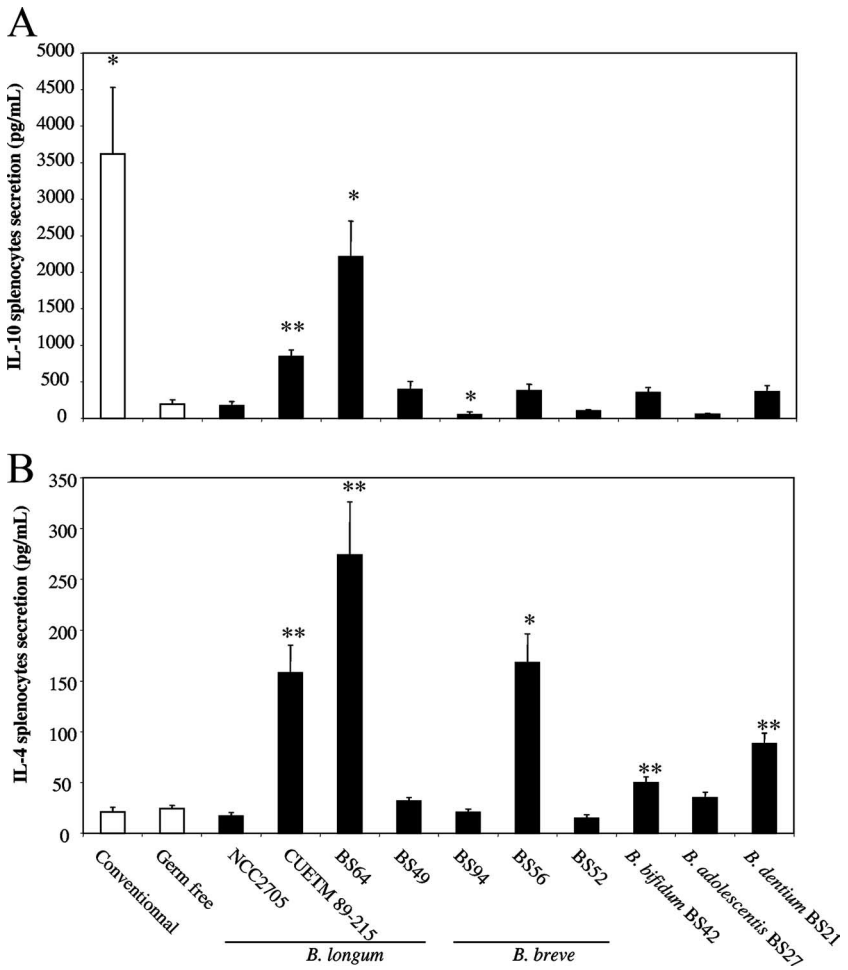

FIG. 2. Levels of pro-T_H2 cytokine secretion by splenocytes stimulated for 48 h with ConA (3 μg/μl). (A) IL-10; (B) IL-4. Data obtained by ELISA are presented as means plus SEMs. *P* was <0.05 (*) and <0.01 (**) for comparison with GF mice. Reference groups are represented by open bars.

(Gibco, Fisher-Bioblock, Illkirch, France). Spleen cells (SC) were then purified for 5 minutes in ice with Tris-buffered NH_4Cl according to Nicaise et al. (25). SC were rinsed and resuspended in 1 ml of RPMI 1640 containing 25 mM of HEPES buffer, 1% of L-glutamine, 1% of penicillin, streptomycin, 1% of fungizon, and 10% of fetal calf serum. The number of viable cells was determined by the trypan blue dye (0.25%) exclusion method. SC were adjusted to 2.10^6 cells/well and cultured in 24-well plates with and without 3 μg/μl of concanavalin A (ConA) (Sigma, France) at 37°C in a 5% CO_2-95% air atmosphere. Supernatants were collected after 48 h of culture by centrifugation at 1,800 rpm, 20°C, for 5 minutes and conserved at −20°C.

Cytokine level measurement. Levels of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10), IL-4, and IL-5 in culture supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) kits (eBiosciences, Montrouge, France) according to the manufacturer's instructions. Detection limits for ELISAs were as follows: IL-4 and IL-5, 4 pg/ml; TNF-α, 8 pg/ml; and IFN-γ and IL-10, 15 pg/ml. Duplicate wells were run for each sample. Results are presented as mean values plus standard errors of the means (SEMs) for each group.

Cytokine expression from the terminal ileum. A total of 2.5 cm of the entire terminal ileum, including the Peyer patches, was crushed with an Ultra-Turrax J25 instrument (Fisher-Bioblock) for 40 seconds, and total mRNA was extracted using the TRIzol reagent method (Invitrogen, Illkirch, France) according to the manufacturer's instructions. mRNA was treated by DNase I (Invitrogen) and converted into cDNA by reverse transcription using the Superscript II and Oligo $dT_{12\text{-}18}$ primers (Invitrogen). The cDNA obtained was subjected to real-time PCR using Smart Cycler (Cepheid, Sunnyvale, Canada). The Quantitect Probe PCR master mix and Quantitect gene expression assay (Qiagen, Courtaboeuf, France) kits were used to quantify the transforming growth factor β1 (TGF-β1)- and IL-4 gene expressions directly on mouse ileum. IFN-γ- and IL-10 gene expressions were quantified by using a Smart kit for Sybr green 1 (Eurogentec, Angers, France) and 900 nM specific primers (IFN-γ forward [5′-AGCAACAG CAAGGCGAAAA-3′] and reverse [5′-CTGGACCTGTGGGTTGTTGA-3′] and IL-10 forward [5′-TTTGAATTCCCTGGGTGAGAA-3′] and reverse [5′-A CAGGGGAGAAATCGATGACA-3′]) (38). Dosages were performed in duplicate. As the efficacy of amplification for each gene was confirmed to be similar to that of the β-actin-encoding gene, which was used as a reference (Qiagen), data analysis was performed with the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (20). For each group and each cytokine, gene expression level is determined by comparison with that in the GF mouse group.

Statistical analysis. The Kruskal-Wallis test was used to determine the significance of the differences between the groups, and the Mann-Whitney U test was performed for pairwise comparison. *P* values of less than 0.05 were considered to be statistically significant. Data were analyzed using SPSS (version 12.0).

RESULTS

Bacterial colonization level. GF mice were sterile before inoculation. After inoculation, bifidobacteria were established at a high level, with a mean of 9.5 ± 0.8 $\log_{10}$ CFU/g of cecal content, ranging from 8.0 ± 0.8 $\log_{10}$ CFU/g of cecal content for *B. adolescentis* to 10.3 ± 0.3 $\log_{10}$ CFU/g of cecal content for *B. breve* BS56. No exogenous bifidobacteria were found.

TABLE 1. Orientation of the IS in GF mice mono-associated with different *Bifidobacterium* strains

Bifidobacterium strain	Bacterial colonization level[a]	Splenocyte secretion (peripheral IS)[b]					Gene expression in final ileum (intestinal IS)[b]			Orientation of T_H1/T_H2 balance according to our in vivo study
		IL-10	IL-4	IL-5	TNF-α	IFN-γ	IFN-γ	TGF-β	IL-4	
B. adolescentis BS27	8.0 ± 0.8	0[c]	0	0	0	0	0	0	0	Noneffector
B. breve BS94	10.3 ± 0.3	/4[d]	0	/2	/2	0	0	×2[d]	/5[d]	Suppressor (peripheral IS)/T_H1/T_H2 (intestinal IS)
B. breve BS52	9.9 ± 0.9	0	0	0	0	0	0	×5.5[d]	/12[d]	Non inducer (peripheral IS)/T_H1/T_H2 (intestinal IS)
B. breve BS56	9.1 ± 0.1	0	×7[d]	0	0	0	0	×2.5[d]	0	Low T_H2 inducer
B. longum BS64	9.6 ± 0.2	×11.5[d]	×11[e]	0	0	/4[d]	0	×3.5[e]	0	T_H2 (peripheral IS)/suppressor (intestinal IS)
B. longum NCC2705	9.8 ± 0.1	0	0	0	×2[e]	×3[e]	0	×4[e]	0	T_H1 (peripheral IS)/suppressor (intestinal IS)
B. longum BS49	9.9 ± 0.1	0	0	0	×2[d]	×2[d]	/7[d]	0	0	T_H1 (peripheral IS)/T_H2 (intestinal IS)
B. longum CUETM 89–215	10.0 ± 0.1	×4.5[e]	×6.5[e]	×1.5	×2[e]	×7.5[e]	/5	×3[e]	0	T_H1/T_H2
B. bifidum BS42	9.1 ± 0.2	0	×2[e]	0	×1.5	×3.5[e]	0	×2	/8[d]	T_H1/T_H2
B. dentium BS21	8.7 ± 0.3	0	×3.5[e]	0	0	×13[e]	0	×3[e]	0	T_H1/T_H2

[a] Expressed as $\log_{10}$ numbers of UFC/g cecal content.
[b] Differences are expressed in comparison with the level for GF mice. ×*n*, upregulation by a factor of *n*; /*n*, downregulation by a factor of *n*.
[c] No difference.
[d] $P < 0.05$.
[e] $P < 0.01$.

The microbiota of conventional mice consisted of 8.2 $\log_{10}$ CFU total aerobes/g of cecal content and 9.4 $\log_{10}$ CFU total anaerobes/g of cecal content, with numerous lactobacilli (8.7 $\log_{10}$ CFU/g of cecal content) and no bifidobacteria.

Cytokine production from splenocytes in vitro. Cytokine secretions by SC are shown in Fig. 1 and 2, and data are compiled in Table 1. Spontaneous secretions of IFN-γ, TNF-α, IL-4, IL-5, and IL-10 were observed in the unstimulated splenocytes' supernatants, but with rates below the detection/quantification threshold. Only the results obtained with SC stimulated with 3 μg/μl of ConA are considered.

T_H1 cytokines. According to their capacities to influence IFN-γ secretion, strains can be significantly separated into three groups in comparison with GF mice, i.e., inducers (three *B. longum* strains out of four, *B. bifidum*, and *B. dentium*), suppressors (the fourth *B. longum* strain), and strains with no effect (the three *B. breve* and *B. adolescentis* strains) (Fig. 1A). Various effects were observed for the *B. longum* strains: *B. longum* CUETM 89-215 was a higher inducer than *B. longum* NCC2705 and BS49, and *B. longum* BS64 was shown to be a suppressor.

Three strains of *B. longum* significantly induced TNF-α secretion, and the other strains, regardless of species, had no effect in comparison with the level for GF mice (Fig. 1B).

T_H2 cytokines. According to their capacities to influence IL-4 secretion, two groups of strains can be observed: two *B. longum* strains, one *B. breve* strain, *B. bifidum*, and *B. dentium* significantly induced this secretion, and the other strains had no effect in comparison with the level for GF mice (Fig. 2B).

No strains had a significant effect on IL-5 secretion.

Regulatory cytokine: IL-10. Strains can be significantly separated into three groups, i.e., inducers (two *B. longum* strains), a suppressor (BS94), and strains with no effect on IL-10 secretion (Fig. 2A).

Cytokine gene expression in the terminal ileum. Cytokine gene expressions in the terminal ileum are shown in Fig. 3, and data are compiled in Table 1. IL-10 gene expression was below the threshold of detection. By contrast, TGF-β1, IL-4, and IFN-γ gene expressions were detected in conventional, GF, and mono-associated mice.

TGF-β1 gene expression. All the strains but three (the *B. bifidum* strain, the *B. adolescentis* strain, and *B. longum* BS49) induced TGF-β1 gene expression compared with the level for GF mice (Fig. 3A).

IFN-γ gene expression. Only the establishment of a conventional flora induced a significant increase in IFN-γ gene expression compared with the level for GF mice (Fig. 3B). This expression was significantly inhibited only in mice mono-associated with *B. longum* BS49 ($P < 0.05$) and CUETM 89-215 ($P = 0.067$) compared with the level for GF mice. By contrast, the two other *B. longum* strains (NCC2705 and BS64) did not exert such an effect.

IL-4 gene expression. Two *B. breve* strains (BS94 and BS52) had suppressor effects on IL-4 gene expression, and the other strains, regardless of species, had no effect compared with the level for GF mice (Fig. 3C). Except for one mouse, *B. bifidum* significantly inhibited IL-4 gene expression compared with the level for GF mice.

DISCUSSION

This is the first time that the impacts of various *Bifidobacterium* strains belonging to several species on immunity have been compared in an in vivo model, i.e., gnotobiotic mice. Our study has shown that *Bifidobacterium*'s capacity to stimulate immunity is species specific but its influence on the orientation of the IS operates in a strain-specific manner.

In our study, we chose to include four strains of *B. longum*

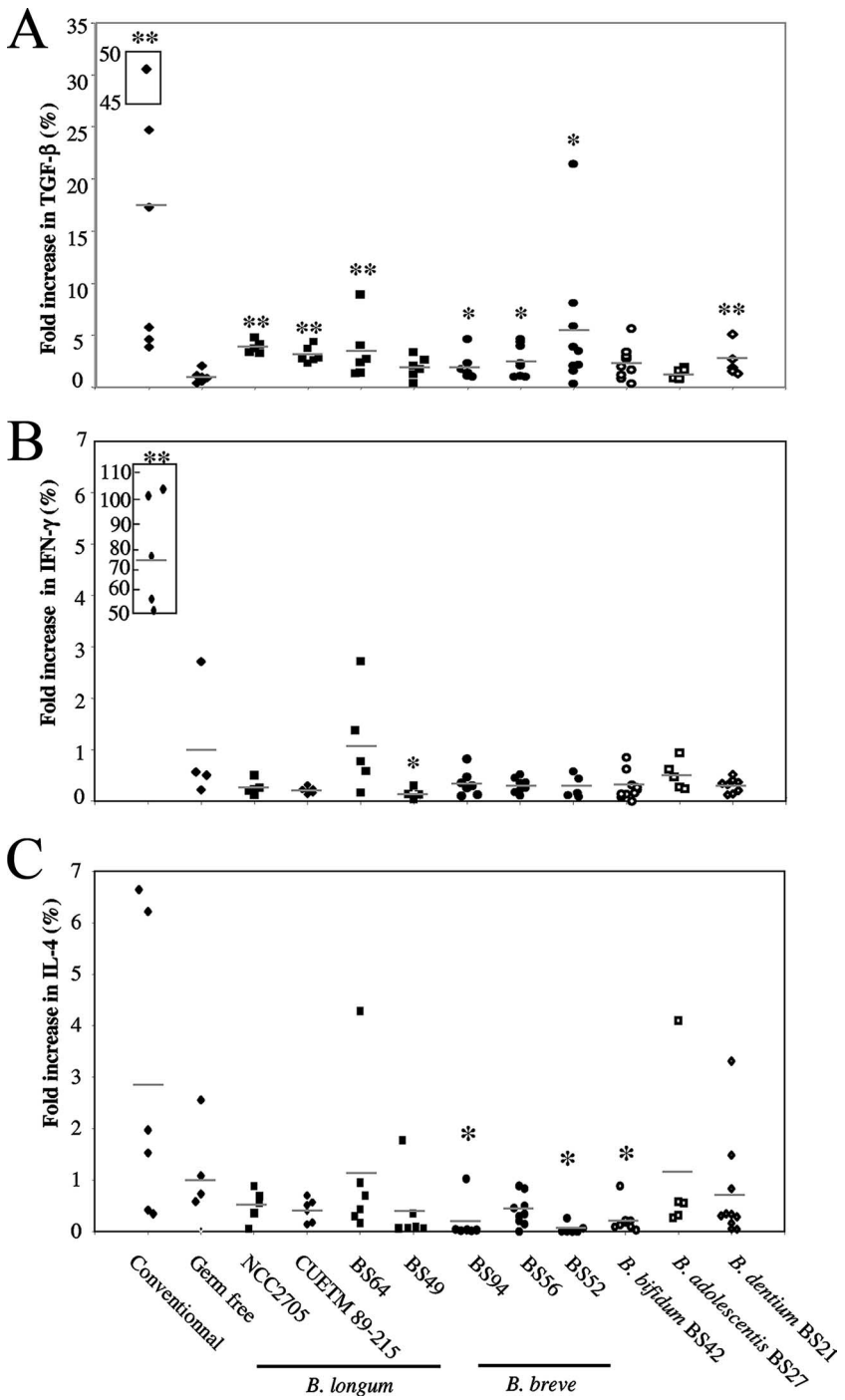

FIG. 3. Increases (*n*-fold) in cytokine gene expression in the terminal ileum. (A) TGF-β1; (B) IFN-γ; (C) IL-4. Results are expressed as individual data points, and bars represent means. The *y* axis represents the amount of the target gene relative to the level for GF mice, normalized with β-actin. *P* values were <0.05 (*) and <0.01 (**) for comparison with GF mice.

and three strains of *B. breve* among the 10 *Bifidobacterium* strains tested. Indeed, in a previous prospective study, these species were isolated as the predominant bifidobacteria in infants (personal data). In GF mice, *Bifidobacterium* colonization occurred at a high level, as high as that in the infants' fecal flora from which the strains were isolated. To investigate the influence of bifidobacteria on T_H1/T_H2 orientation, IL-4 and IL-5 were studied as pro-T_H2 cytokines, IFN-γ and TNF-α as pro-T_H1 cytokines, and IL-10 and TGF-β as regulatory cytokines (28). The last group consists of cytokines that can inhibit T-cell proliferation and differentiation but in some cases can be associated with a T_H2 profile. Indeed, IL-10 and TGF-β can be produced by T_H2 lymphocytes as well as regulatory T cells (16, 28).

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