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Effect of Viability and Integrity of *Bifidobacterium* on Suppression of Allergy in Mice

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Abstract The effects of the cell viability and integrity of *Bifidobacterium* on suppression of allergy were investigated. C3H/HeJ mice were sensitized on weeks 3, 4, 6, and 8 with ovalbumin and choleratoxin to induce an allergic reaction. Mice fed 0.2% of live, disrupted, or heat-killed *Bifidobacterium bifidum* BGN4 in the pellets of their diet for 8 weeks starting 2 weeks before initial sensitization differentially suppressed the allergy response in terms of levels of IgE and IgG1 in their sera, and symptoms on their tails. Viable *Bifidobacterium* was more effective than disrupted or heat-killed cells in suppressing the allergy. Growth inhibition, which occurred in the sham group at week 4, did not occur in the treated groups. These results show that *Bifidobacterium* has a suppressive effect on the allergic response of mice, and that the viability and integrity of the *Bifidobacterium* is required for effective suppression in our experimental model.

Key words: Food allergy, IgE, *Bifidobacterium*

The beneficial effects of bifidobacteria on health have been widely studied. Yoshioka *et al.* [31] showed that bifidobacteria predominated in the feces of breast-fed infants, but, *Escherichia coli*, *Streptococcus faecalis*, and *Bacteroides* sp. were more frequent in the feces of bottle-fed infants. Many beneficial effects of bifidobacteria have been reported such as anticancer activity [5], lowering of plasma cholesterol [6], prevention of constipation [22], and stimulation of the immune system [29]. Bifidobacteria are also thought to protect against intestinal disorders. Hotta *et al.* [9] investigated the effects of administration of *Bifidobacterium* preparations on infant diarrhea, and Hattori *et al.* [8] reported that the administration of *Bifidobacterium* for 1 month to children with atopic dermatitis resulted in significant amelioration of their allergic symptoms, although they did not find any correlation between changes in fecal microflora and levels of allergic symptoms.

There have been only a few studies of the anti-allergic effects of lactic acid bacteria. Matsuzaki *et al.* [21] showed that oral administration of *Lactobacillus casei* Shirota inhibited IgE production. Moreover, administration of *L. casei* induced the formation of Th1 cell-associated cytokines, such as IFN-γ and IL-2, by spleen cells, but suppressed the production of Th2-associated cytokines such as IL-4, IL-5, IL-6, and IL-10. In addition, Shida *et al.* [30] showed that *L. casei* inhibited antigen-induced IgE by stimulating secretion of IL-12 by macrophages. The mechanism by which probiotics prevent allergic response is still unknown. One possible basis of the decrease of IgE-mediated allergic responses could be related to IL-12. Identification of the effective components or structural requirements of the probiotics should be of value in exploiting their allergy preventing potential. Several components of the Gram-positive bacterial cell wall, e.g., capsular polysaccharides, peptidoglycans, and lipoteichoic acids, stimulate the production of IFN-γ and TNF-α in murine cells, and of IFN-γ in human peripheral blood lymphocytes [4, 7, 15, 16, 26, 28].

In the present study, we characterized the immuno-regulatory effects of peroral administration of live, heat-killed, and disrupted cells of *B. bifidum* BGN4 in a mouse model of food allergy. Our aim was to identify an effective way of using *Bifidobacterium* to prevent the occurrence of food allergies.

Materials and Methods

Mice

Three-week-old female C3H/HeJ mice weighing 11–13 g were purchased from Japan SLC (Hamamatsu, Japan) and maintained on ovalbumin (OVA)-free chow. Sensitization

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of the mice was started at 5 weeks of age, and six mice were included in each group. The mice were kept in plastic cages, allowed free access to water, and maintained on a 12:12 h light:dark cycle in an environmentally-controlled animal chamber. Temperature and humidity were controlled at 23±1°C and 55±10%, respectively. The animal experimentation guidelines of Seoul National University were followed.

Microorganisms

B. bifidum BGN4 was isolated from the feces of Korean subjects, as described in a previous study [25]. *Bifidobacterium* and *Lactobacillus* were cultured anaerobically in Lactobacilli-MRS broth (Difco, Detroit, MI, U.S.A.) containing 0.05% L-cysteine (Sigma, St. Louis, MO, U.S.A.) at 37°C for 24 h. To prepare the mouse diets, live bacterial cells were collected by centrifugation (Hanil, Seoul, Korea) at 4,000 ×*g* for 40 min at 4°C, and washed twice with sterile phosphate-buffered saline. To prepare heat-killed and disrupted cells, respectively, the bacteria were exposed to 95°C for 30 min, or disrupted in a cell disrupter (Stansted Fluid Power, Essex, U.K.) for 10 min. The bacterial preparations were then lyophilized (Ilshin, Seoul, Korea) and used as dietary components.

Intragastric Antigen Sensitization and Treatment

Mice were starved for 2 h preceding oral sensitization. Sensitization was performed by intragastric (ig) administration of OVA (50 μg) with 10 μg of cholera toxin (CT) on days 0, 1, 2, 7, and 21, using a blunt stainless steel feeding needle. OVA (Sigma, St. Louis, MO, U.S.A.) served as the antigen. CT and concanavalin A (Con A) were purchased from Sigma (St. Louis, MO, U.S.A.). Five groups of mice were used in this study (Fig. 1). The mice in groups 2 to 5 were gavaged with 0.2 ml phosphate buffer saline solution (PBS, pH 7.2) containing OVA and CT, whereas the mice in the naive group (group 1) were gavaged with PBS without OVA and CT. Mice in groups 2 to 5 were subjected to the same OVA sensitization, and the mice in groups 3 to 5 were administered bacterial powder, whereas those in group 2 acted as sham controls and received OVA and CT but no bacteria. The bacteria-treated mice were fed 0.2% of lyophilized *B. bifidum* BGN4 (BGN4) in the form of diet pellets of live cells, disrupted cells, or heat-killed cells. They were fed the experimental bacterial powders for 7 weeks, starting 2 weeks before the initial sensitization until they were killed. To assess serum antibody responses, tail vein blood was collected weekly after the initial sensitization. Sera were stored at −80°C.

Measurement of Serum OVA-Specific IgE, IgG1, IgG2a, and Total IgE from Sera

Sera were collected weekly from tail veins and stored at −80°C. Levels of OVA-specific IgE, IgG1, or IgG2a were measured by ELISA. Briefly, Nunc-Immuno-Maxisorp plates (Nunc,

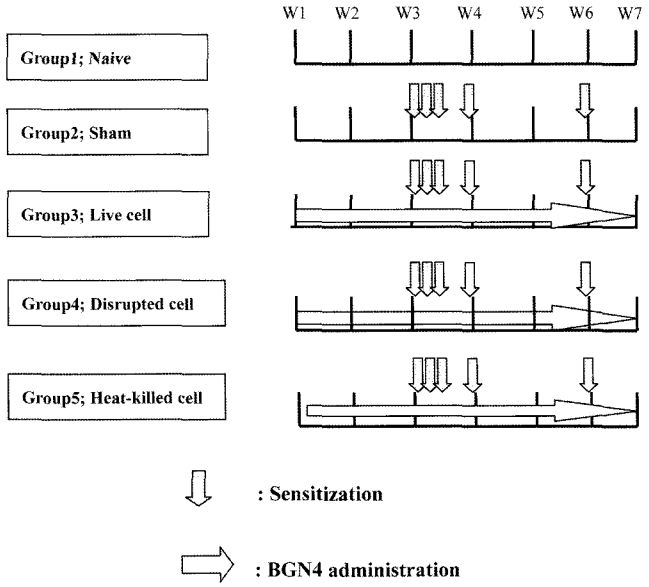

Fig. 1. Experimental protocol for intragastric ovalbumin sensitization and bacterial administration.
Mice were sensitized on weeks 3, 4, and 6 with ovalbumin and cholera toxin. Mice in group 3, group 4, and group 5 were fed 0.2% lyophilized live cells, disrupted cells, or heat-killed cells of *Bifidobacterium bifidum* BGN4, respectively, in the pellets of their diet for 8 weeks, starting 2 weeks before initial sensitization until sacrifice. The naive mice in group 1 were administered PBS buffer instead of ovalbumin and cholera toxin, and the mice in group 2 (sham) were administered corn starch instead of bacterial powder. Each group consisted of 6 mice.

Roskilde, Denmark) were coated with 5 μg/ml of OVA in coating buffer, pH 9.6 (Sigma, St. Louis, MO, U.S.A.), overnight at 4°C. The plates were blocked and washed. Samples were added to the plates and incubated overnight at 4°C. The plates were washed again, and biotinylated rat anti-mouse IgE, IgG1, or IgG2a monoclonal antibodies (2 μg/ml) were added and incubated for 1 h at room temperature to detect OVA-specific IgE, IgG1, and IgG2a, respectively. After incubation with horseradish peroxidase (HRP), (Pharmingen, San Diego, CA, U.S.A.), the reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Fluka, Neu-Ulm, Switzerland) for 30 min at room temperature. The color reactions were stopped with 6 N H_2SO_4 and read at 450 nm. Levels of IgE, IgG1, or IgG2a were calculated using reference curves generated with standards of total mouse IgE, IgG1, and IgG2a, respectively. Total IgE levels in sera were also determined by ELISAs. The plates were coated with 2 μg/ml of rat monoclonal anti-mouse IgE. Serial dilutions of sera were added, followed by 100 μl of a biotinylated rat monoclonal anti-mouse IgE. The subsequent steps were as described above. All of the antibodies used in this study were purchased from Pharmingen.

Measurement of OVA-Specific and Total Fecal IgA

Extracts of fecal pellets were prepared as described by Marinaro *et al.* [20]. In brief, 100 mg of pellet was mixed

with 1 ml of PBS containing 0.1% NaN_3 and incubated at 4°C for 2 h. The fecal pellets were mixed vigorously in PBS for 10 min. After centrifugation (4,000 ×g, 20 min), the supernatants were collected and stored at −70°C. To assay OVA-specific IgA, plates were coated with 5 μg/ml of OVA in coating buffer. After washing and blocking, 100 μl of fecal extracts was added to individual wells and incubated overnight at 4°C. The plates were then washed, and biotinylated rat anti-mouse IgA monoclonal antibody (2 μg/ml) was added and incubated for 1 h at room temperature. After washing, streptavidin-peroxidase (Sigma) was added for 1 h at room temperature. The reactions were developed with TMB for 30 min at room temperature, stopped with 6 N H_2SO_4 and read at 450 nm. Equivalent levels of IgA were calculated using a reference curve generated with mouse total IgA as standard. To measure total IgA, plates were coated with rat anti-mouse IgA capture antibodies (2 μg/ml) in coating buffer, blocked and washed as described above, and fecal extracts (1:50 dilutions) were added to the plates and incubated overnight at 4°C. The plates were washed and 100 μl of biotinylated rat anti-mouse IgA was added to each well. The subsequent steps were as described above. IgA levels were calculated from a reference curve generated with mouse total IgA.

Assessment of Hypersensitivity Reactions

Allergic symptoms were evaluated after sacrifice using the scoring system: 0, no symptoms; 1, puffiness of the tail; 2, 1-2 scabs on the tail; 3, 3-4 scabs on the tail; 4, 5-6 scabs on the tail; 5, more than 7 scabs on tail. Symptoms were scored blind manner, and the scores were evaluated by ten individuals unaware of sample identities.

Statistical Analysis

All data are presented as the mean±standard error of mean (SEM), as indicated by the bars. Data were analyzed using SAS (Release 8.01, NC, U.S.A.). Differences between immunoglobulin and cytokine levels were analyzed by ANOVA followed by Duncan's multiple range tests for multiple comparisons. P values <0.05 were considered significant.

RESULTS

Effect of BGN4 on IgE Production

To monitor possible effects of the BGN4 fractions, sera were obtained from each group. The OVA-specific IgE in the sera of each group are presented in Fig. 2. Levels of OVA-specific IgE and total IgE in the sham group were significantly higher than those in the naive and BGN4-treated groups at week 6.

Symptoms on Tails

Mice sensitized with OVA and CT showed some injuries on their tails. After the administration of OVA and CT, the sham mice started to scratch their tails, and caused some injury including bleeding (Fig. 3). No injuries and scratches were observed in the group treated with live cells, whose symptom scores did not differ from those of the naive group. The symptom scores on the tails of the group treated with disrupted cells were lower than in the sham group but higher than in the other groups, and significantly higher than in the naive group. The symptom scores in the group treated with heat-killed cells were

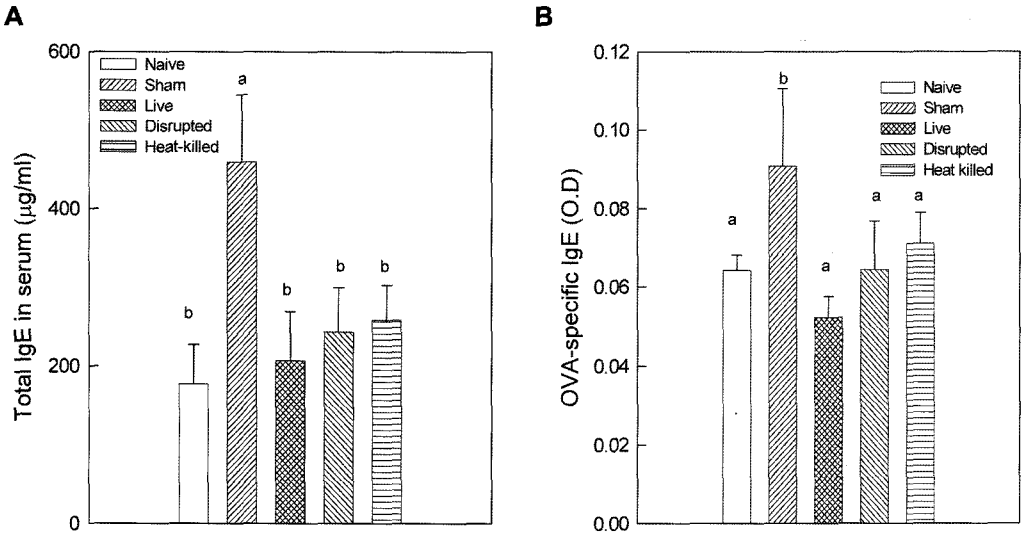

Fig. 2. Effect of administration of bacteria on the levels of total IgE and ovalbumin-specific IgE in sera from ovalbumin-sensitized mice.

IgE levels were determined by ELISAs. Total IgE and ovalbumin-specific IgE levels were determined on week 6. Data are means±SEM of 6 mice per group. Different letters indicate significant differences in Duncan's multiple range tests (p<0.05).

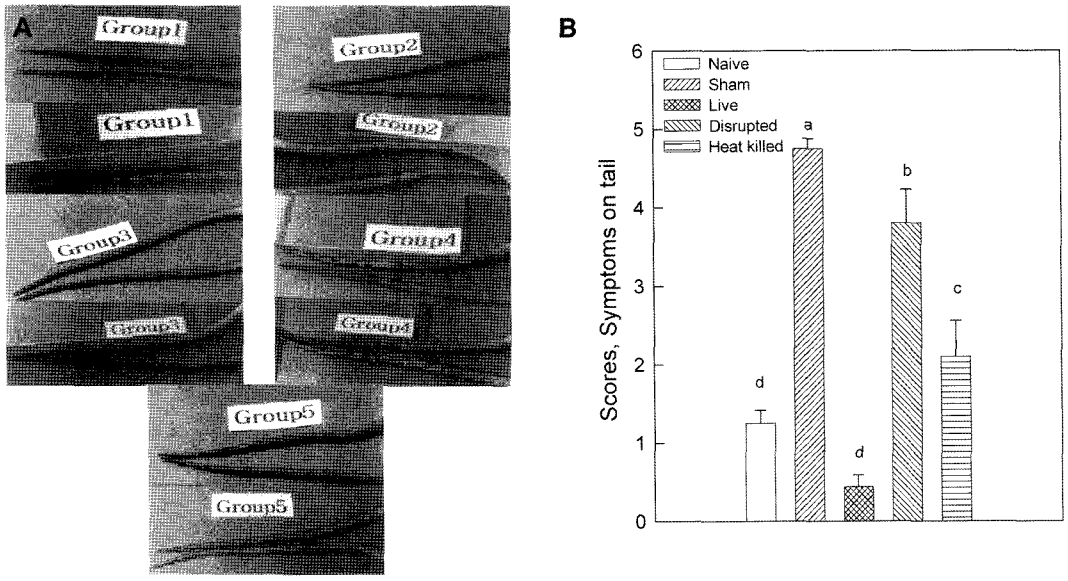

Fig. 3. Severity of allergic symptoms on the tails of ovalbumin-sensitized mice treated with various components of *Bifidobacterium bifidum* BGN4.

The immunized mice showed marked tail bruising and scabs. **A.** The tails of OVA-sensitized mice treated with bacteria. Group 1, naive; Group 2, sham; Group 3, treated with live BGN4; Group 4, treated with disrupted BGN4; Group 5, treated with heat-killed BGN4. **B.** Severity of allergic symptoms on the tails of OVA-sensitized mice. Data are means±SEM. Different letters indicate significant differences in Duncan's multiple range tests ($p<0.05$).

lower than in the sham group or the group treated with disrupted cells (Naive, 1.3±0.2; Sham, 4.8±0.1; Live, 0.4± 0.2; Disrupted, 3.8±0.4; Heat-killed, 2.1±0.5, respectively).

OVA-Specific Mucosal IgA

We evaluated OVA-specific mucosal IgA and total IgA levels in extracts of fecal samples collected on week 7 (Fig. 4). OVA-specific IgA in the BGN4-treated groups was significantly different from that in the sham or naive groups. The levels of OVA-specific IgA in the live cell, disrupted cell, and heat-killed cell groups were markedly lower than those in the sham group. There were no differences in total IgA levels between the five experimental groups (OVA-specific IgA; Naive, 291.8±29.9 g/ml; Sham, 210.6± 34.5 g/ml; Live, 56.2±4.8 g/ml; Disrupted, 89.2±9.2 g/ml; Heat-killed, 60.8±12.9 g/ml, respectively).

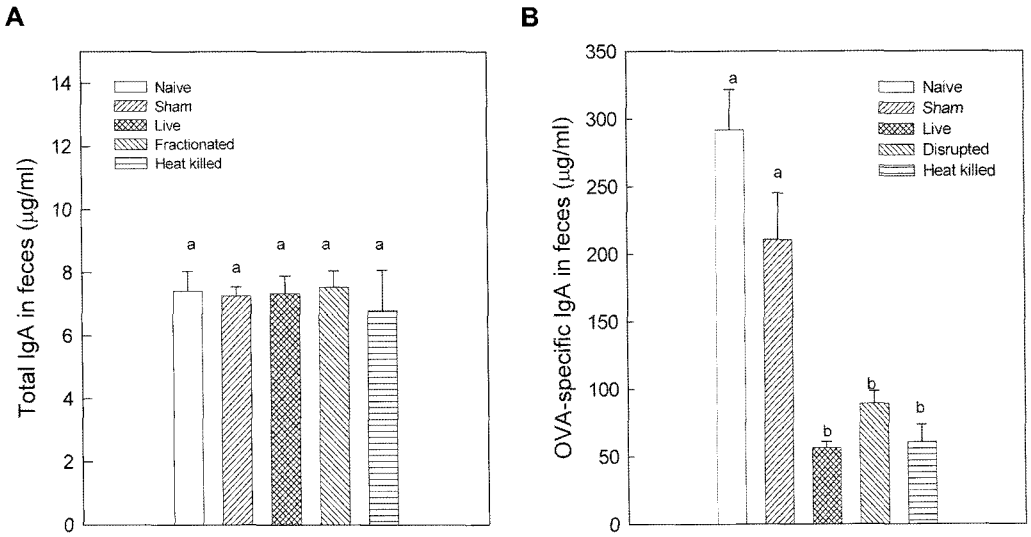

Fig. 4. Effect of *Bifidobacterium* on the production of total IgA (**A**) and ovalbumin-specific IgA (**B**) in fecal samples from ovalbumin-sensitized mice and bifidobacteria-treated mice.

Fresh fecal pellets from each group were collected on week 7. Fecal extracts were prepared and ovalbumin-specific IgA levels detected by ELISA. Data are means±SEM of 6 mice per group. Different letters indicate significant differences in Duncan's multiple range tests ($p<0.05$).

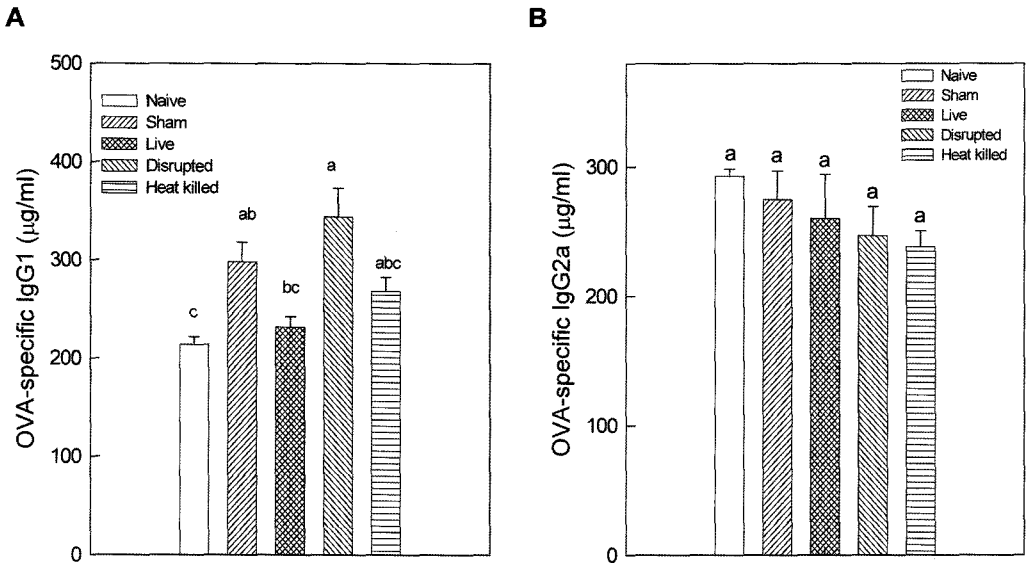

Fig. 5. Effect of *Bifidobacterium* on the production of ovalbumin-specific IgG1 (**A**) and ovalbumin-specific IgG2a (**B**) in sera from ovalbumin-sensitized and bifidobacteria-treated mice.
Levels of antibody were detected by ELISAs. Data are means±SEM of 6 mice per group. Different letters indicate significant differences in Duncan's multiple range tests ($p<0.05$).

IgG1 and IgG2a Levels in Sera

The levels of OVA-specific IgG1 in the sera of the sham group on week 6 were significantly higher than those of the naive group. The levels of OVA-specific IgG1 in the live cell or heat-killed cell groups were as low as those in the naive group. The levels of OVA-specific IgG1 in the disrupted cell group were higher than those in the naive group. On the other hand, the levels of OVA-specific IgG2a in the sham group did not differ from those of the naive group. There were again no significant differences between the five experimental groups (Fig. 5).

Effect of BGN4 Administration on Body Weight

Mean body weights did not differ between the five experimental groups up to week 3 (Table 1). However, the body weights of the sham group were lower than those of the naive group on week 4, whereas no growth inhibition was observed in the *Bifidobacterium* treated groups over the experimental period.

DISCUSSION

In the present study allergic responses were suppressed in mice administered with *Bifidobacterium*. The suppressive effect of live *Bifidobacterium* was higher than that of heat-killed or disrupted cells. The suppressive effect may be due to specific components of the administered *Bifidobacterium*.

It has been shown in several studies that the composition of the microflora differs between healthy and allergic infants. *Bifidobacterium* content was low in allergic infants, and colonization with *Staphylococcus aureus* and enterobacteria was high [1]. In other research, atopic infants had more clostridia and fewer bifidobacteria than healthy infants [11]. These studies suggested that the microflora affect immune responses in allergic disease.

The gastrointestinal tract is a highly complex ecosystem composed of host cells, nutrients, and microflora. This ecosystem plays an important role in nutrition, physiology, and regulation of the immune system. It is known that bacteria in the microflora communicate with each other by

Table 1. Body weight of ovalbumin-sensitized mice and bifidobacteria-treated mice.

Groups	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Naive	15.1±0.2^{a}	18.6±0.2^{a}	20.2±0.3^{a}	20.2±0.3^{ab}	22.1±0.4^{a}	22.7±0.4^{a}
Sham	14.8±0.3^{a}	18.3±0.3^{a}	17.4±0.3^{c}	19.6±0.3^{b}	20.6±0.9^{b}	20.8±0.4^{b}
Live	14.7±0.2^{a}	17.8±0.3^{a}	18.4±0.3^{b}	20.1±0.4^{ab}	20.5±0.4^{b}	21.0±0.4^{b}
Disrupted	16.9±0.3^{a}	19.0±0.2^{a}	20.6±0.4^{b}	21.5±0.3^{b}	21.2±0.5^{b}	21.9±0.1^{b}
Heat-killed	15.3±0.3^{a}	18.6±0.4^{a}	20.1±0.3^{a}	21.3±0.4^{a}	21.8±1.6^{ab}	22.6±0.6^{a}

Values are means±SEM of 6 mice per group.
Different superscripts indicate significant differences as a result of Duncan's multiple range tests ($p<0.05$).

means of signals or antibiotic molecules, and this contributes to maintaining a beneficial ecosystem in the gastrointestinal tract. Microflora are influenced by dietary composition, age, probiotics, and drugs [2]. In order to affect the immune system, a microorganism must activate the lymphoid cells of the gastrointestinal lymphoid tissue [19]. Probiotics have been orally administered with the aim of affecting the microflora. However, bacteria may be damaged by stomach acids, bile salts, or pancreatic enzymes during their passage through the stomach and small intestine. In consequence, some of bacteria will reach the colon in the form of fragments or dead cells, which might still have immunomodulatory effects. Indeed, it is well known that immunomodulatory effects on microflora are related to interactions between the host immune system and bacterial components such as the lipopolysaccharide of Gram-negative bacteria, or lipoteichoic acid of Gram-positive bacteria. Namba *et al.* [23] showed that lysozyme enhanced the adjuvant effect of bacterial cell walls, and they suggested that bacteria are usually broken up enzymatically in the human intestinal tract, and that some components of the cells might be converted in the process into a more immunologically active form. These ideas have been supported by a report that after being fragmented by ozone, bacteria still had an immunomodulatory effect [10]. Therefore, we anticipated that disrupted cells might be effective in suppressing the allergy response. However, in the present study, disrupted cells had a weaker suppressive effect than live or heat-killed cells. The reduced production of OVA-specific IgE and IgA may have resulted from tight control of the Th2 cells that recognize OVA-antigen. The relatively greater reduction in IgG1 than in IgG2 suggests that the live bacteria shifted the balance between Th1 and Th2 toward Th1-type immunity. Although the active components of bacterial cells on allergy are not fully understood, mechanical disruption may damage some of the effective components suggesting that their suppressive effect on OVA-induced allergy is related to their integrity.

In conclusion, the present study shows that viable and intact *Bifidobacterium* are more effective than disrupted or heat-killed cells in modulating the allergic response in mice, after oral administration.

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