

2,4-Dinitrofluorobenzene-Induced Contact Hypersensitivity Response in NC/Nga Mice Fed Fructo-Oligosaccharide

Reiko FUJIWARA¹, Naho SASAJIMA¹, Naoki TAKEMURA¹, Keisuke OZAWA¹, Yuki NAGASAKA¹, Takuma OKUBO¹, Yuraporn SAHASAKUL², Jun WATANABE^{3,†} and Kei SONOYAMA^{4,*}

¹Graduate School of Life Science, ²Graduate School of Agriculture, ³Creative Research Initiative Sousei, and

⁴Research Faculty of Agriculture, Hokkaido University, Kita-9-jyo, Nishi-9-tyome, Kita-ku, Sapporo, Hokkaido 060–8589, Japan

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Summary Strategies to manipulate gut microbiota in infancy have been considered to prevent the development of allergic diseases later in life. We previously demonstrated that maternal dietary supplementation with fructo-oligosaccharide (FOS) during pregnancy and lactation modulated the composition of gut microbiota and diminished the severity of spontaneously developing atopic dermatitis-like skin lesions in the offspring of NC/Nga mice. The present study tested whether dietary FOS affects contact hypersensitivity (CHS), another model for allergic skin disease, in NC/Nga mice. In experiment 1, 5-wk-old female NC/Nga mice were fed diets either with or without FOS supplementation for 3 wk and then received 2,4-dinitrofluorobenzene (DNFB) on the ear auricle 5 times at 7-d intervals. FOS supplementation reduced CHS response as demonstrated by ear swelling. Quantitative RT-PCR analysis showed that mRNA levels for interleukin (IL)-10, IL-12p40, and IL-17 in the lesional ear skin were significantly lower in mice fed FOS. In experiment 2, female NC/Nga mice were fed diets either with or without FOS during pregnancy and lactation. After weaning, offspring were fed the diets supplemented with or without FOS. Three weeks after weaning, offspring received DNFB on the ear auricle 4 times at 7-d intervals. Although FOS supplementation after weaning reduced ear swelling, maternal FOS consumption was ineffective in offspring. The present data suggest that dietary FOS reduces CHS while maternal FOS consumption is ineffective in offspring of DNFB-treated NC/Nga mice.

Key Words fructo-oligosaccharide, 2,4-dinitrofluorobenzene, contact hypersensitivity, gut microbiota, NC/Nga mice

Because gut microbiota early in life profoundly influences later immune responses (1–4), strategies to manipulate the microbiota in infancy have been considered in preventing the onset of allergic diseases. In fact, clinical trials showed that maternal administration of *Lactobacillus rhamnosus* GG (i.e., probiotics) during pregnancy and lactation was beneficial in preventing the development of atopic dermatitis (AD) in at-risk children during the first 4 y of life (5, 6). Likewise, animal experiments showed that dietary supplementation with heat-killed *L. rhamnosus* GG in NC/Nga mice during pregnancy and lactation suppressed the spontaneous development of AD-like skin lesions in offspring (7). In addition, administration of *L. rhamnosus* GG in female BALB/c mice during pregnancy and lactation suppressed the ovalbumin-induced allergic airway inflammation in their offspring (8). Furthermore, administration of *Lactobacillus johnsonii* NCC533 in NC/Nga mice around the weaning period (i.e., 20 to 22 d of age) prevented the development of allergic skin lesions induced

by topical application of mite antigen from 6 wk of age (9, 10).

Because indigestible oligosaccharides (i.e., prebiotics) modulate the composition of gut microbiota, administration of indigestible oligosaccharides during infancy could be also expected to be effective in preventing the development of allergic diseases. Indeed, supplementation of a mixture of long-chain fructo-oligosaccharide (FOS) and short-chain galacto-oligosaccharide (GOS) during the first 6 mo of life reportedly reduced the incidence of AD in formula-fed high-risk infants (11, 12). For animal models, we demonstrated that maternal dietary supplementation with short-chain FOS during pregnancy and lactation modulated the composition of gut microbiota and diminished the severity of spontaneously developing AD-like skin lesions in the offspring of NC/Nga mice (13, 14), suggesting that modulation of gut microbiota in infancy by maternal consumption of prebiotic oligosaccharides is beneficial in preventing allergic diseases. In the NC/Nga mouse model of spontaneously developing AD-like skin lesions, environmental allergens such as mite antigens are thought to contribute to the development of skin lesions (15), and the skin lesions are preceded by immunoglobulin E elevation in sera (16). In contrast, contact hypersensitivity (CHS)

*To whom correspondence should be addressed.

E-mail: ksnym@chem.agr.hokudai.ac.jp

[†]Present address: National Agriculture and Food Research Organization, Tsukuba 305–8642, Japan

induced by topical application with haptens such as 2,4-dinitrofluorobenzene (DNFB) is a T-cell-mediated, antigen-specific type of skin inflammation (17, 18). Thus, CHS is a typical delayed-type hypersensitivity. In order to further confirm the idea that prebiotic manipulation of gut microbiota in infancy is effective in preventing the onset of allergic diseases, the present study tested whether maternal consumption of short-chain FOS affects DNFB-induced CHS in the offspring of NC/Nga mice.

MATERIALS AND METHODS

Animals and diets. The following study protocol was pre-approved by the Hokkaido University Animal Use Committee (approval no. 16), and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

NC/Nga mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled ($23 \pm 2^\circ\text{C}$) room with a dark period from 2000 to 0800 h. Mice were allowed ad libitum access to food and water, and were fed either a synthetic diet prepared according to AIN-93G guidelines (19) or the same diet supplemented with FOS (Meiologo P, donated by Meiji Food Materia, Tokyo, Japan). These diets were referred to as FOS(−) and FOS(+), respectively. The FOS(+) was prepared by adding (50 g/kg diet) FOS to the FOS(−) at the expense of α -cornstarch. According to the manufacturer, FOS is composed of D-glucose and D-fructose (1.3%), sucrose (2.5%), 1-kestose (37.3%), nystose (49.1%) and fructo-oligosaccharides (9.8%).

Experimental design. In experiment 1, female mice (5 wk old) were allocated to two groups of six mice and fed either FOS(−) or FOS(+). Three weeks after starting the test diets, CHS was induced as described below. At 38 d after the first application of DNFB, mice were anesthetized by inhalation of diethyl ether. Whole blood was drawn from the carotid artery, and the right ear auricle of each mouse was excised, snap-frozen in liquid nitrogen, and stored at -80°C for myeloperoxidase (MPO) activity measurement and for RNA isolation. Cecal contents were collected and stored at -80°C for analyses of gut microbial composition.

In experiment 2, mice were divided into two groups: FOS(−) and FOS(+) (two 9-wk-old male and six 8-wk-old female mice in each group). Three females were mated to one male in one cage. Pregnant mice were housed individually and then monitored daily until delivery. The day of birth was referred to as day 0 of neonatal life. Offspring were separated from dams on day 21 and then fed either FOS(−) or FOS(+). FOS(−)-fed offspring whose dam was fed FOS(−) were referred to as FOS(−)(−) ($n=8$). Thus, offspring were divided into four groups: FOS(−)(−), FOS(−)(+), FOS(+)(−), and FOS(+)(+) ($n=8, 9, 8$, and 6, respectively). Three weeks after weaning, CHS was induced as described below. The experimental design is summarized in Fig. 3. On day 73 of age, mice were anesthetized by inhalation of diethyl ether. Whole blood was drawn from the

carotid artery, and the right ear auricle of each mouse was excised, snap-frozen in liquid nitrogen, and stored at -80°C for RNA isolation.

Induction of contact hypersensitivity. CHS to DNFB was induced according to Nagai et al. (20). In brief, 25 μL of 0.15% (v/v) DNFB (Tokyo Kasei, Tokyo, Japan) in acetone/olive oil (4:1, v/v), or the vehicle alone, were applied to each side of right and left ear auricles of mice, respectively, 5 (experiment 1) or 4 (experiment 2) times at 7-d intervals. Ear thickness was measured daily with a digital engineer's micrometer (Mitsutoyo, Kawasaki, Japan) from day 0 to day 3 after each application. Ear thickness measurements were performed by an investigator who was blinded to the treatments of mice. DNFB-specific ear swelling was calculated according to the following equation:

Net swelling

$$= (\text{right ear thickness} - \text{left ear thickness})_{\text{at each time point}} - (\text{right ear thickness} - \text{left ear thickness})_{\text{at 0 h.}}$$

Profile analysis of cecal microbiota by PCR-denaturing gradient gel electrophoresis (DGGE). DNA was isolated from cecal contents using the Isoplant DNA isolation kit (Nippongene, Tokyo) following the manufacturer's instructions. DNA samples served as a template to amplify the 16S rRNA gene fragments using universal primers U968-GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGA GAA CGC GAA GAA CCT TAC) and L1401 (CGG TGT GTA CAA GAC CC), as reported previously (21). PCR was performed in a 25- μL reaction mixture containing 500 nM each of U968-GC and L1401, 1 $\times$ Green Go Taq Flexi buffer (Promega, Madison, WI), 1 mM MgCl_2 , 0.2 mM dNTP, 1.25 U of Go Taq Hot Start polymerase (Promega), and 5 ng/ μL of DNA template. The reaction conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 20 s, and 68°C for 40 s, and a final extension at 68°C for 7 min. The amplicons were separated by DGGE, and a dendrogram of the DGGE-band profile was constructed as previously described (13).

MPO activity. Tissue samples were homogenized in a 50 mM phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethyl ammonium bromide (Sigma, St. Louis, MO). The homogenates were then subjected to three freeze/thaw cycles. After the centrifugation at $13,000 \times g$ for 30 min at 4°C , the supernatants were subjected to MPO activity measurement as described by Bánvölgyi et al. (22). In brief, the samples were added to a 50 mM phosphate buffer (pH 6.0) supplemented with hydrogen peroxide and TMB, and optical density readings were then taken for 15 min at 620 nm. The reaction rate ($\Delta \text{OD}/\text{time}$) was derived from an initial slope of the curve. A calibration curve was then produced, with the rate of reaction plotted against the standard samples of the human MPO (Sigma).

mRNA expression analysis. Total RNA was isolated from the ear auricle as previously described (14). In experiment 2, an equal amount of total RNA isolated from individual mice was pooled in each group, while total RNA samples were analyzed individually in experiment 1. After treatment with DNase to remove any

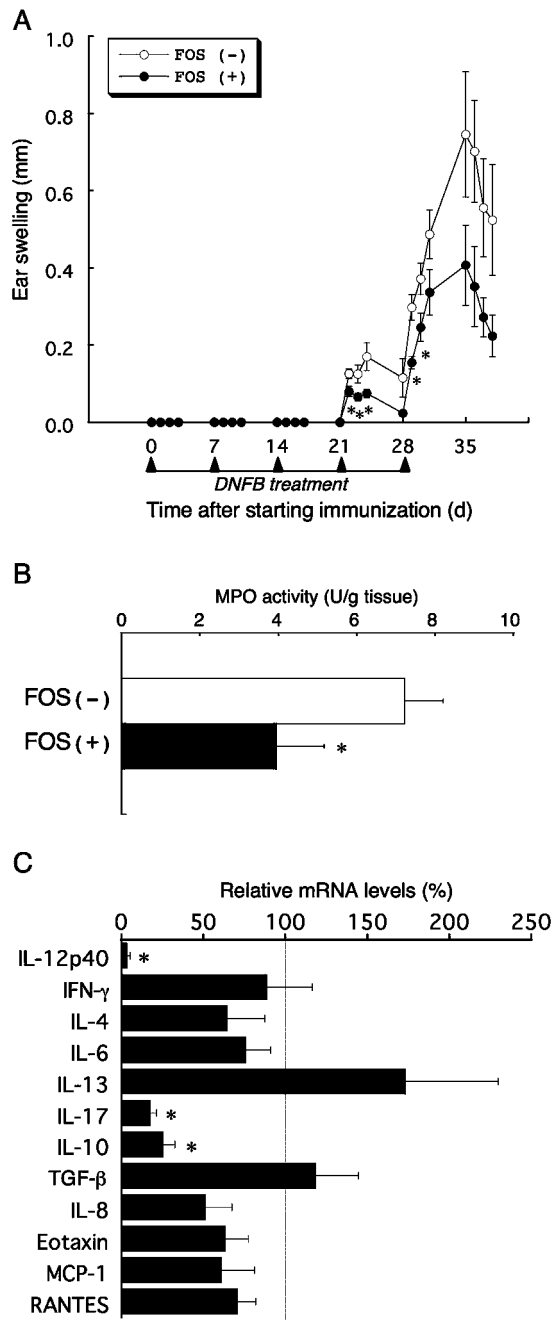


Fig. 1. Contact hypersensitivity response in adult NC/Nga mice in experiment 1. Chart A shows temporal changes in ear swelling after starting 2,4-dinitrofluorobenzene application. Chart B shows the MPO activity in the ear auricles. Chart C shows the relative mRNA levels for cytokines and chemokines in the inflamed ear tissue, and values of mice fed FOS(+) are shown relative to the levels in mice fed FOS(-), which are set to 100. Results are presented as mean $\pm$ SE of 6 mice in each dietary group. Mean values with asterisk were significantly different ($p<0.05$) as estimated by one-way ANOVA.

genomic DNA, the total RNA samples were subjected to reverse transcription, followed by quantitative RT-PCR as previously described (14). Primer sequences for interleukin (IL)-4, IL-6, IL-10, IL-12p40, IL-13, IL-17, interferon (IFN)- γ , transforming growth factor (TGF)- β 1, monocyte chemotactic protein (MCP)-1, and glycer-

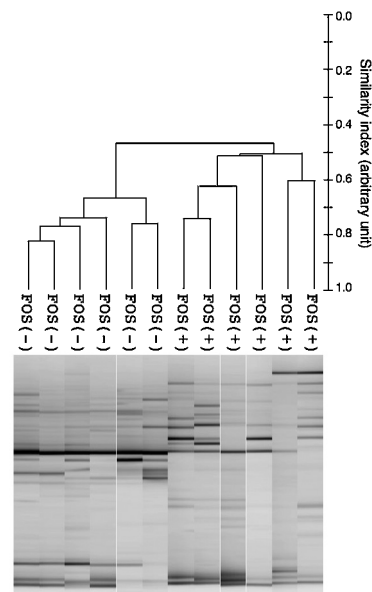


Fig. 2. PCR-DGGE analysis of cecal microbiota based on 16S rRNA gene sequences in experiment 1. DGGE gel image and the dendrogram of DGGE band profiles are shown. Each lane in gels and each line in dendrograms represents an individual mouse.

aldehyde-3-phosphate dehydrogenase are in accordance with Giulietti et al. (23). Primer sequences for IL-8, eotaxin, and RANTES are in accordance with Heishi et al. (24).

Statistical analysis. Results are presented as mean $\pm$ SE. One-way ANOVA or Tukey-Kramer's test following two-way ANOVA was used to analyze the differences among the means of experimental groups. StatView for Macintosh (version 5.0, SAS Institute Inc., NC, USA) was used for the analysis. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

We previously observed that dietary FOS reduced DNFB-induced CHS in BALB/c mice (25). In the present study, experiment 1 tested whether NC/Nga mice also showed the reduction of DNFB-induced CHS by dietary FOS. Ear swelling began to be detected 1 d after the fourth application of DNFB and was further increased by the fifth application (Fig. 1A). One-way ANOVA showed that mice fed FOS(+) had significantly lower ear swelling as compared to mice fed FOS(-) on days 22, 23, 24, 29, and 30 after the first application of DNFB. Additionally, neutrophil infiltration in the inflamed ear auricles was indirectly quantified by MPO activity in the tissue homogenates in experiment 1. In the vehicle-treated left ear auricles, no detectable levels of MPO activity were observed (data not shown). MPO activity in the right ear auricles was significantly lower in mice fed FOS(+) than in mice fed FOS(-) (Fig. 1B). The data suggest that dietary FOS reduced local inflammatory responses of DNFB-induced CHS in NC/Nga mice.

In addition, experiment 2 was performed to test whether maternal consumption of FOS affects CHS in

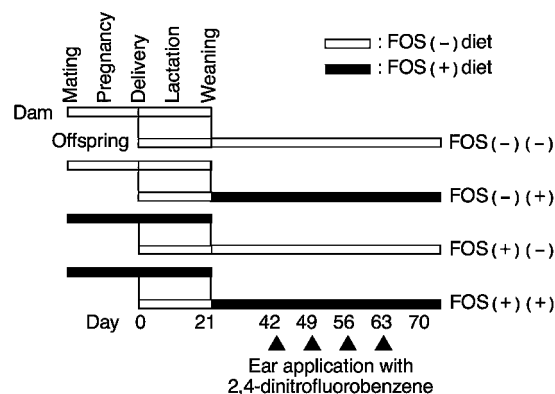


Fig. 3. Schematic presentation of the experimental design in experiment 2. Female NC/Nga mice were fed a diet supplemented with (FOS(+)) or without (FOS(-)) fructo-oligosaccharide during pregnancy and lactation. After weaning on day 21 of age, offspring were fed either FOS(+) or FOS(-) for 11 wk. FOS(-)-fed offspring whose dam was fed FOS(-) were referred to as FOS(-)(-). Thus, offspring were divided into four groups: FOS(-)(-), FOS(-)(+), FOS(+)(-), and FOS(+)(+). 2,4-Dinitrofluorobenzene was applied to ear auricle 4 times at 7-d intervals.

the offspring of NC/Nga mice. Ear swelling began to be detected 1 d after the second application of DNFB and was increased by the repeated application (Fig. 4A). Two-way ANOVA showed that, from 2 to 10 d after the fourth application of DNFB, offspring FOS feeding, but not maternal FOS feeding, affected ear swelling. Thus, ear swelling was significantly lower in the FOS(-)(+) and FOS(+)(+) groups than in the FOS(-)(-) and FOS(+)(-) groups on days 3, 7, 8, 9, and 10 after the fourth application of DNFB. These data clearly indicate that post-weaning FOS consumption, but not maternal FOS consumption, reduced DNFB-induced CHS in NC/Nga mice. Thus, the present findings are in contrast to our previous observations that the severity of spontaneously developing AD-like skin lesions in the offspring of NC/Nga mice was reduced by maternal FOS consumption, and to a lesser extent, by post-weaning FOS consumption (14). Although further investigations are needed to solve this discrepancy, it is assumed that maternal FOS consumption and, perhaps modulation of gut microbiota in infancy by maternal FOS consumption, might have different impacts on the development of symptoms in different types of allergic reaction in the offspring.

The reduction of ear swelling by FOS consumption was accompanied by altered expression of cytokine genes in the lesional ear tissue. In experiment 1, the mRNA levels of cytokines and chemokines in the ear auricle of mice fed FOS(+) are shown relative to the levels in mice fed FOS(-), which are set to 100 (Fig. 1C). Mice fed FOS(+) showed significantly lower mRNA levels of IL-12p40, IL-17, and IL-10 as compared to mice fed FOS(-). In experiment 2, the mRNA levels of IL-12, IL-17, and IL-8 tended to be lower in the FOS(-)(+) and FOS(+)(+) groups than in the FOS(-)(-) and FOS(+)(-) groups (Fig. 4B). Tomimori et al. reported

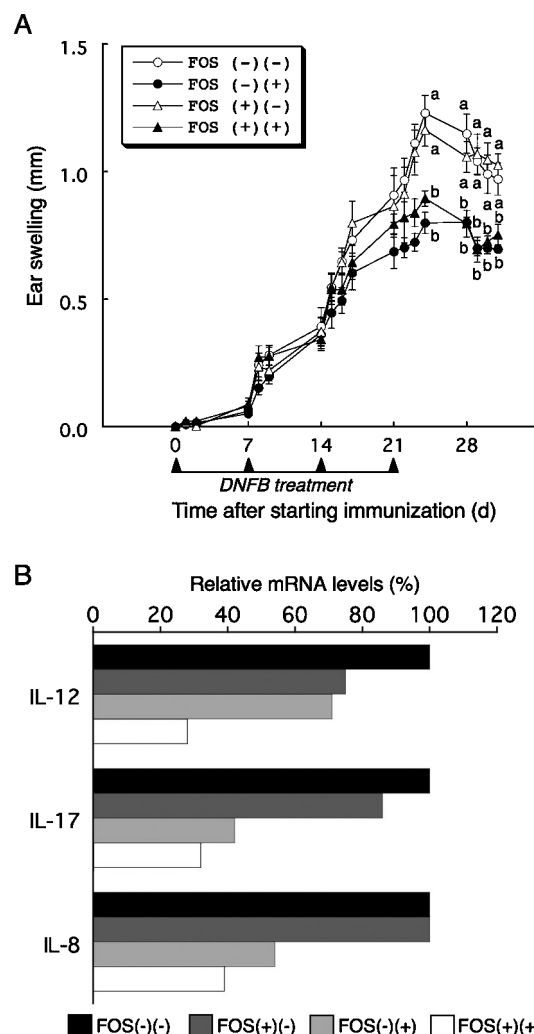


Fig. 4. Contact hypersensitivity response in NC/Nga mouse offspring in experiment 2. Chart A shows temporal changes in ear swelling after starting 2,4-dinitrofluorobenzene application. Results are presented as mean $\pm$ SE, $n=8$, 9, 8, and 6 for FOS(-)(-), FOS(-)(+), FOS(+)(-), and FOS(+)(+), respectively. Mean values with unlike letters at each time point were significantly different ($p<0.05$) as estimated by Tukey-Kramer's test following two-way ANOVA. Chart B shows the relative mRNA levels for cytokines and chemokines in the inflamed ear tissue. Values of mice fed FOS(-)(+), FOS(+)(-), and FOS(+)(+) are shown relative to the levels in mice fed FOS(-)(-), which are set to 100. Results for pooled samples in each group are shown.

that IL-12 protein concentrations increased in the skin lesions of DNFB-treated NC/Nga mice and that administration of anti-IL-12 antibody ameliorated the established dermatitis in NC/Nga mice (26). In addition, Nakae et al. described IL-17-producing T cells, i.e., Th17 cells, as being important for the development of CHS in mice (27), and He et al. showed that IL-17-producing CD8⁺ cells are a central player in effector functions at the elicitation of CHS responses in mice (28). Proliferation and IL-17 production of Th17 cells are driven by IL-23 produced by the innate immune system (29), and IL-12 and IL-23 share the IL-12p40 subunit

(30). Furthermore, IL-10 was reportedly overexpressed in the lesional skin of NC/Nga mice treated with mite antigen (10), although this cytokine is well known to have immune regulatory functions. Therefore, the reduced expression of IL-12p40, IL-17, and IL-10 genes in the ear tissue suggest that dietary FOS suppresses the increased production of IL-12, IL-23-driven IL-17, and IL-10 and that this action might be responsible for the reduction of skin lesions in DNFB-treated NC/Nga mice. In contrast, there was no significant difference in Th1 cytokine (i.e., IFN- γ), Th2 cytokines (i.e., IL-4, IL-6, and IL-13), regulatory cytokine (i.e., TGF- β 1), or chemokines (i.e., IL-8, eotaxin, MCP-1, and RANTES) between the FOS(-) and FOS(+) groups in experiment 1.

Ear swelling response began to be detected earlier and was greater in experiment 2 than in experiment 1. In experiment 1, mice were raised with a commercial rodent diet composed of natural materials in the breeding facility and, after 5 wk of age, they were fed the purified diet in our laboratory. In contrast, pregnant and lactating mice and offspring were fed the purified diet in experiment 2. Therefore, one possible explanation is that maternal diet and/or diet in infancy might influence the CHS response after growing. If that is the case, consumption of some natural compounds in the commercial rodent diet during infancy might influence the development of immune function and then reduce the CHS response later in life.

In our previous study, the DNFB-induced CHS was negatively correlated with the number of bifidobacteria in the gut of BALB/c mice fed FOS(-) and FOS(+) (25). Because sequence analysis revealed that *Bifidobacterium pseudolongum* was the most predominant bifidobacterium in the gut of FOS-supplemented BALB/c mice, we postulated that the reduction in the CHS response by FOS consumption was attributable to the proliferation of *B. pseudolongum* in the gut. Indeed, we showed that oral administration of *B. pseudolongum*, isolated from FOS-supplemented mice, partially reduced the development of DNFB-induced CHS in BALB/c mice (31). In experiment 1 of the present study, analysis of the 16S rRNA gene in the cecal contents showed that PCR-DGGE band profiles are divided into two large clusters: one comprising mice fed FOS(-), and the other comprising mice fed FOS(+) (Fig. 2). The results are consistent with our previous studies (13, 14, 25) and suggest that dietary FOS influenced the composition of gut microbiota. However, both conventional culture analysis and 16S rRNA gene-based analysis showed no detectable levels of bifidobacteria in the gut of NC/Nga mice (data not shown). Therefore, it is unlikely that the proliferation of bifidobacteria in the gut is responsible for the reduction of DNFB-induced CHS in NC/Nga mice.

Previous epidemiological studies showed that differences in the composition of gut microbiota are associated with the development of AD. According to Björkstén et al., babies who developed allergy were less often colonized with enterococci during the first month of life and with bifidobacteria during the first year of life

when compared to healthy infants (32). In addition, allergic infants had higher counts of clostridia at 3 mo, and the prevalence of colonization with *Staphylococcus aureus* was also higher at 6 mo, whereas the counts of *Bacteroides* were lower at 12 mo (32). Kalliomaki et al. reported that atopic children had more clostridia at 3 wk of age (33). Furthermore, *Escherichia coli* and *Clostridium difficile* were reportedly associated with infants who developed AD (34, 35). In our present study, preliminary analysis of 16S rRNA gene clone libraries generated from cecal DNA samples in NC/Nga mice showed that the phyla *Firmicutes* and *Proteobacteria* were the most abundant taxonomic groups in both FOS(-) and FOS(+) groups and that mice fed FOS(+) had more *Firmicutes* and less *Proteobacteria* as compared to mice fed FOS(-) (data not shown). In that experiment, however, only a small number of sequences were analyzed (39 and 34 sequences in mice fed FOS(-) and FOS(+), respectively). Therefore, more precise analysis is needed to elucidate the bacterial species responsible for the reduction of DNFB-induced CHS in NC/Nga mice.

In conclusion, the present data suggest that dietary FOS reduces CHS while maternal FOS consumption is ineffective in offspring of DNFB-treated NC/Nga mice.

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