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Maternal consumption of fructo-oligosaccharide diminishes the severity of skin inflammation in offspring of NC/Nga mice

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Strategies to manipulate the gut microbiota in infancy have been considered to prevent the development of allergic diseases later in life. We aimed to elucidate the effects of maternal dietary supplementation with a prebiotic oligosaccharide on gut microbiota and spontaneously developing atopic dermatitis-like skin lesions in the offspring of NC/Nga mice. Female NC/Nga mice were fed diets either with or without fructo-oligosaccharide supplementation during pregnancy and lactation. After weaning, offspring were fed the diets supplemented with or without fructo-oligosaccharide for 11 weeks in an air-uncontrolled conventional room. Changes in gut microbiota were assessed by denaturing gradient gel electrophoresis of the PCR-amplified 16S rRNA gene. Skin lesions were evaluated by a clinical score and scratching behaviour. Serum antibody levels were measured by ELISA, and expression levels of cytokines and chemokines in lesional tissue were evaluated by quantitative RT-PCR. Maternal supplementation with fructo-oligosaccharide modulated the gut microbiota in sucklings. Although maternal supplementation with fructo-oligosaccharide suppressed the increase in clinical skin severity score and scratching behaviour in offspring, dietary fructo-oligosaccharide after weaning was less effective. The diminution of skin lesions was accompanied by lower serum concentrations of total IgG1 and lower expression levels of TNF-α in the lesional tissue. These data suggest that maternal consumption of fructo-oligosaccharide diminishes the severity of atopic dermatitis-like skin lesions in the offspring of NC/Nga mice.

Fructo-oligosaccharide: Prebiotics: Atopic dermatitis: Gut microbiota: NC/Nga mice

The gut microbiota play an important role in maintaining host health by preventing the colonisation of pathogens, degrading dietary compounds and maintaining normal mucosal immunity. Particularly, the composition of the gut microbiota early in life profoundly influences later immune responses[1–4]. Therefore, strategies to manipulate the microbiota in infancy have been considered in preventing the onset of allergic diseases. This idea is supported by epidemiological data demonstrating that differences in the composition of gut microbiota in infancy precede the development of atopic dermatitis (AD)[5–8]. In addition, clinical trials showed that maternal administration of *Lactobacillus rhamnosus* GG (i.e. probiotics) during pregnancy and lactation was beneficial in preventing the development of AD in at-risk children during the first 4 years of life[9,10]. However, *L. rhamnosus* GG administration did not benefit adolescents suffering from birch pollen allergy[11]. These findings suggest that the time point for exposure to probiotics is important in preventing the development of allergic diseases.

Indigestible oligosaccharides are regarded as prebiotics that affect the host by selectively stimulating the growth and/or activity of beneficial bacteria such as bifidobacteria and lactobacilli in the intestinal tract, and thus improving host health[12–15]. Indeed, a mixture of long-chain fructo-oligosaccharide (FOS) and short-chain galacto-oligosaccharide reportedly reduced the incidence of AD in formula-fed high-risk infants[16,17]. Our animal studies demonstrated that dietary raffinose and α-linked galacto-oligosaccharide reduced allergic airway inflammation in ovalbumin (OVA)-sensitised Brown Norway rats[18,19]. We also showed that dietary short-chain FOS reduced 2,4-dinitrofluorobenzene-induced contact hypersensitivity in BALB/c mice[20]. In addition, Fujitani *et al.* showed that dietary FOS reduced the infiltration of inflammatory cells and oedema formation in duodenal mucosa using an OVA-induced food allergy model in NC/jic mice[21]. Furthermore, Vos *et al.* reported that consumption of a mixture of long-chain FOS and short-chain galacto-oligosaccharide suppressed allergic airway inflammation in an OVA-induced allergic asthma model of BALB/c mice[22]. These findings suggest that administration of indigestible oligosaccharides is effective in the prevention of allergic diseases through modulation of gut microbiota. Given that

Abbreviations: AD, atopic dermatitis; DGGE, denaturing gradient gel electrophoresis; FOS, fructo-oligosaccharide; FOS (−), diet without fructo-oligosaccharide supplementation; FOS (+), diet with fructo-oligosaccharide supplementation; OVA, ovalbumin.

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the time point for administration of probiotics, namely the early phase of life, is important in preventing allergic diseases, modulation of gut microbiota by prebiotics in infancy may produce desirable effects later in life.

We recently demonstrated that supplementation with FOS in female BALB/c mice during pregnancy and lactation altered the composition of gut microbiota in their sucklings[23]. This finding provides a model that enables the examination of whether the modulation of gut microbiota by prebiotics in infancy influences the onset of allergic diseases later in life. Matsuda and colleagues have demonstrated that NC/Nga mice provide an excellent animal model for human AD[24]. When NC/Nga mice are kept in air-uncontrolled conventional surroundings, they develop skin lesions. In contrast, when kept in a specific pathogen-free room, they exhibit no clinical signs. Therefore, environmental allergens such as mite antigens are thought to contribute to the development of skin lesions[25]. Clinical signs begin with scratching behaviour and, starting at the age of 8 weeks, IgE elevation, followed by the onset of eczematous conditions along with the infiltration of various inflammatory cells in the skin lesions[24]. Elevated expression of Th2 cytokines and chemokines is observed in lesional skin areas[24]. The present study examined whether the administration of FOS in female NC/Nga mice during pregnancy and lactation influences the development of dermatitis in their offspring.

Materials and methods

Animals and diets

The following study was approved by the Hokkaido University Animal Use Committee, and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Four male (aged 9 weeks) and twelve female (aged 8 weeks) NC/Nga mice were purchased from Japan SLC (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled (23 ± 2°C) room with a dark period from 20.00 to 08.00 hours. The room was not equipped with air purifiers such as a HEPA (high efficiency particulate air) filter. Mice were allowed *ad libitum* access to food and water, and were fed either a synthetic diet prepared according to AIN-93G guidelines (Table 1) [26] or the same diet supplemented with FOS (Meioligo P, donated by Meiji Food Materia Co., 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-maize starch. 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 fructosylnystose (9·8 %).

Experimental design

Mice were divided into two groups: FOS (−) and FOS (+) (two male and six 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. We used female offspring in the following experiment, because male mice occasionally develop fighting-related skin lesions. Offspring were then fed either FOS (−) or FOS (+) for 11 weeks. FOS (−)-fed offspring whose dam was fed FOS (−) were referred to as FOS (−)(−) (*n* 5). Thus, offspring were divided into four groups: FOS (−)(−), FOS (−)(+), FOS (+)(−) and FOS (+)(+) (five, six, six and six animals per group, respectively). The experimental design is summarised in Fig. 1. In order to induce stable dermatitis, two or three offspring in each group were housed during the experimental period in one cage together with two or three 13-week-old female NC/Nga mice with severe skin lesions, according to Takano *et al.* [27]. Fresh faeces were collected on days 14, 35 and 98 after birth for analysis of intestinal microbiota as described below. At age 98 d, mice were anaesthetised by inhalation of diethyl ether. Whole blood was drawn from the carotid artery, and serum samples were stored at −80°C for antibody measurements as described below. The right ear auricle of each mouse was excised, snap-frozen in liquid N_2, and stored at −80°C for isolation and analysis of RNA as described below.

Table 1. Composition of test diets

Ingredient (g/kg)	FOS (−)	FOS (+)
α-Maize starch*	529·5	479·5
Casein†	200·0	200·0
Sucrose‡	100·0	100·0
Soyabean oil§	70·0	70·0
Cellulose‖	50·0	50·0
Mineral mix (AIN-93G-MIX)¶	35·0	35·0
Vitamin mix (AIN-93G-VX)¶	10·0	10·0
L-Cystine§	3·0	3·0
Choline bitartrate§	2·5	2·5
FOS**	0·0	50·0

FOS (−), diet without fructo-oligosaccharide supplementation; FOS (+), diet with fructo-oligosaccharide supplementation; FOS, fructo-oligosaccharide.
* Amylalpha CL (Chuo-Shokuryou, Aichi, Japan).
† ALACID (New Zealand Dairy Board, Wellington, New Zealand).
‡ Gifted from Nippon Beet Sugar Mfg. Co. (Obihiro, Japan).
§ Wako Pure Chemical Industries, Ltd (Osaka, Japan).
‖ Cellulose powder type D (Advantec Toyo Kaisa Ltd, Tokyo, Japan).
¶ The mineral mixture and vitamin mixture (Nihon Nosan Kogyo Co., Yokohama, Japan) are identical to AIN-93G-MX and AIN-93G-VX as reported by Reeves *et al.* [26], respectively.
** FOS (Meioligo P) was donated by Meiji Food Materia Co. (Tokyo, Japan). 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 fructosylnystose (9·8 %).

Evaluation of severity of atopic dermatitis-like skin lesions

The severity of AD-like skin lesions in mice was scored weekly for the presence of (1) flare haemorrhage, (2) oedema, (3) excoriation and erosion, and (4) incrustation and xerosis[28]. For each of these signs, a score was assigned as follows: 0, no sign; 1, mild; 2, moderate; or 3, severe. The sum of the individual scores was taken as the dermatitis score. In addition, the frequency of scratching behaviour, such as scratching of the nose, ears and dorsal skin with the hind paws, was measured during a 10 min period in each mouse at age 91 d. A series of scratching behaviours generally observed for about 1 s was counted as one incident of scratching according to Kuraishi *et al.* [29]. Observers who were blinded to the experimental groups performed these observations.

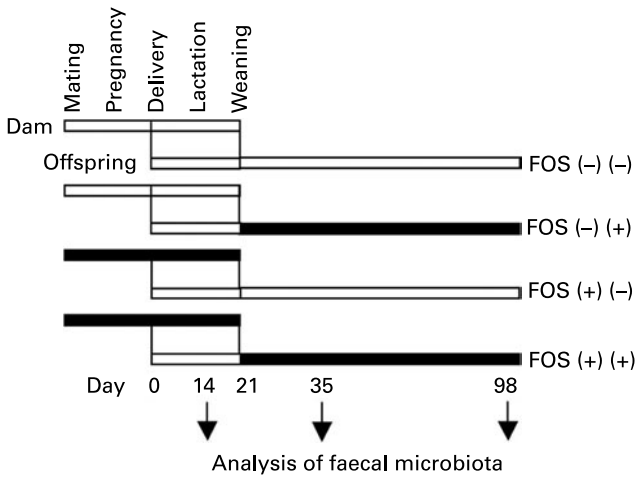

Fig. 1. Schematic presentation of the experimental design. Female NC/Nga mice were fed a diet supplemented with fructo-oligosaccharide (FOS (+); ■) or without fructo-oligosaccharide (FOS (−); □) during pregnancy and lactation. After weaning at age 21 d, offspring were fed either FOS (+) or FOS (−) for 11 weeks. 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 (+)(+). Faecal samples at ages 14, 35 and 98 d were subjected to PCR–denaturing gradient gel electrophoresis analysis to assess changes in gut microbiota.

Antibody ELISA

Ninety-six-well microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with rat anti-mouse IgE (100-fold dilutions, clone ME-01-DE; Yamasa, Choshi, Japan) diluted with carbonate buffer (pH 9·6). After blocking with PBS containing 1 % bovine serum albumin and 0·02 % Tween-20 (PBS-BT) for 1 h at 37°C, serial dilutions of serum with PBS-BT were added and incubated for 2 h at 37°C. Thereafter, biotinylated anti-mouse IgE (1000-fold dilutions, clone ME-02-B; Yamasa) diluted with PBS-BT was added and incubated for 2 h at room temperature. Horseradish peroxide-conjugated streptavidin (1000-fold dilutions; GE Healthcare Life Sciences, Tokyo, Japan) diluted with PBS-BT was added and incubated for 15 min at room temperature. Between each step, wells were washed five times with PBS containing 0·02 % Tween-20. Plates were developed at room temperature after the addition of commercially available substrate reagent (TMB One Component HRP Microwell Substrate; BioFX Laboratories, MD, USA). Finally, 1 M-H_2SO_4 was added, and absorbance at 450 nm was measured using a microplate reader (Genios plus; Tecan, Salzburg, Austria). For IgG1, anti-mouse IgG1 (200-fold dilutions, clone 15H6; Southern Biotech, Birmingham, AL, USA) and horseradish peroxide-conjugated rabbit anti-mouse IgG1 (80 000-fold dilutions; Zymed Laboratories, San Francisco, CA, USA) were used as capture and detection antibodies, respectively. For IgG2a, anti-mouse IgG2a (200-fold dilutions, clone HOPC-1; Southern Biotech) and horseradish peroxide-conjugated rabbit anti-mouse IgG1 (5000-fold dilutions; Zymed Laboratories) were used as capture and detection antibodies, respectively. Correlation coefficient values (r^2) of standard curves for IgE, IgG1 and IgG2a were 0·99, 0·91 and 0·91, respectively. Detection limits for IgE, IgG1 and IgG2a were 0·78 μg/ml, 0·39 mg/ml and 3·9 mg/ml, respectively. The average absorbance in substrate blank wells, to which three times the standard deviation was added, provided the detection limit. Inter-assay and/or intra-assay CV were less than 10 %.

Quantitative RT-PCR for cytokine gene expression in mouse tissue

Because the external ear is one of the constantly lesioned tissues, we used it for assessment of cytokines and chemokines. Total RNA was isolated from the ear auricle as previously described[23]. In order to remove any genomic DNA, RQ1 RNase-free DNase (Promega, Madison, WI, USA) was added to the total RNA sample and then incubated at 37°C for 45 min. After phenol–chloroform extraction, total RNA was precipitated with ethanol and then re-suspended in 8 μl double-distilled water. Approximately 10 ng total RNA was annealed with Oligo $(dT)_{12-18}$ primer (Invitrogen, Carlsbad, CA, USA) at 70°C for 10 min, and first-strand cDNA was then synthesised using Moloney murine leukemia virus RT (M-MLV RT; Invitrogen). In detail, a 20 μl solution composed of 1 × first-strand buffer, 0·5 mM each deoxynucleoside triphosphate (dNTP), 10 mM-dithiothreitol, 40 U of RNase OUT and 200 U of M-MLV RT was incubated for 50 min at 42°C, followed by RNA digestion with DNase-free RNase H (Invitrogen).

Real-time PCR was performed using a Thermal Cycler Dice TP800 (Takara, Ohtsu, Japan). Primer sequences were as follows: TNF-α (forward, 5′ CAT CTT CTC AAA ATT CGA GTG ACA A; reverse, 5′ TGG GAG TAG ACA AGG TAC AAC CC); IL-6 (forward, 5′ GAG GAT ACC ACT CCC AAC AGA CC; reverse, 5′ AAG TGC ATC ATC GTT GTT CAT ACA); IL-8 (forward, 5′ ATG GCT GGG ATT CAC CTC AA; reverse, 5′ AAG CCT CGC GAC CAT TCT T); thymus and activation-regulated chemokine (TARC) (forward, 5′ GAG CTG GTA TAA GAC CTC AGT GGA G; reverse, 5′ TGG CCT TCT TCA CAT GTT TGT C); glyceraldehyde-3-phosphate dehydrogenase (forward, 5′ TCA CCA CCA TGG AGA AGG C; reverse, 5′ GCT AAG CAG TTG GTG CA)[30,31]. Amplification was carried out in a 25 μl reaction volume containing 12·5 μl 1 × SYBR Premix Ex Taq (Takara), 200 nM of each primer and 1 μl of template cDNA. The reaction condition was: 95°C for 10 s, followed by forty cycles at 95°C for 5 s and 60°C for 30 s, with dissociation curve at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Relative gene expression levels for each sample were normalised to the levels for glyceraldehyde-3-phosphate dehydrogenase.

Profile analysis of faecal microbiota by PCR–denaturing gradient gel electrophoresis

DNA was extracted from fresh faeces using a faecal DNA isolation kit (MO Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA samples were used as a template to amplify the fragments of 16S rRNA gene with universal primers, U968-GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (CGG TGT GTA CAA GAC CC)[32], and denaturing gradient gel electrophoresis (DGGE) analysis of the amplicon was carried out as previously described[23]. Quantity One software (version 4.6.0; Bio-Rad, Hercules, CA, USA) was used for band identification

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and normalisation of band patterns from DGGE gels. The Dice similarity coefficient was used for computing sample similarity based on band position and intensity, and the unweighted pair group method using the arithmetic average (UPGMA) algorithm was used to construct a dendrogram of DGGE band profiles as previously described[23].

Statistical analysis

Results are presented as mean values with their standard errors. The Tukey–Kramer test (for frequency of scratching behaviour, antibodies and cytokines) or the Mann–Whitney *U* test (for clinical skin severity score) following two-way ANOVA was used to analyse the differences among the means of the experimental groups. StatView for Macintosh (version 5.0; SAS Institute, Inc., Cary, NC, USA) was used for the analysis. Differences were considered significant at $P<0{\cdot}05$.

Results

Effect of maternal supplementation with fructo-oligosaccharide on gut microbiota in offspring

As shown in Fig. 1, female NC/Nga mice were fed either a diet supplemented with or without FOS during pregnancy and lactation. PCR–DGGE analysis of the 16S rRNA gene in the faeces of lactating mice showed that dietary FOS influenced the composition of the gut microbiota (data not shown), which is consistent with our previous study[23]. In suckling pups at age 14 d, PCR–DGGE band profiles are divided into two large clusters: one comprising pups whose dams were fed FOS (−), and the other comprising pups whose dams were fed FOS (+) (Fig. 2(A) and (B)). At age 21 d, all female offspring were separated from dams and then fed either FOS (−) or FOS (+). At 2 weeks after weaning, no distinct cluster is shown in PCR–DGGE band profiles (Fig. 2(C) and (D)), suggesting the cessation of influence of maternal supplementation with FOS. Thereafter, offspring at age 98 d showed a new cluster pattern with two large clusters comprised of offspring fed FOS (−) and FOS (+) (Fig. 2(E) and (F)).

Effect of maternal supplementation with fructo-oligosaccharide on atopic dermatitis-like skin lesions in offspring

In order to induce stable dermatitis, weaned offspring were housed together with 13-week-old female NC/Nga mice with severe skin lesions under air-uncontrolled conventional conditions[27]. Skin lesions were first observed at age 49 d, and the clinical skin severity score continued to increase up to the end of the experiment, i.e. age 98 d, in all the mice (Fig. 3(A)). Two-way ANOVA showed that maternal feeding with FOS significantly affected the clinical skin severity score in offspring at ages 70, 77, 84, 91 and 98 d. In contrast, consumption of FOS in offspring showed no significant influence. Thus, the clinical skin severity score was significantly lower in the FOS (+)(−) and FOS (+)(+) groups than in the FOS (−)(−) group from age 70 d to age 98 d. The score in the FOS (−)(+) group did not significantly differ from that in the FOS (−)(−) group and from that in the FOS (+)(−) and FOS (+)(+) groups throughout the experimental period.

AD-like skin lesions in NC/Nga mice are accompanied by a higher frequency of scratching behaviour, such as scratching of the nose, ears and dorsal skin with the hind paws[24]. Fig. 3(B) shows the frequency of scratching behaviour in offspring at age 91 d. Two-way ANOVA showed that maternal feeding with FOS significantly affected the frequency of scratching. However, dietary FOS in offspring after weaning showed no significant influence. The frequency of scratching behaviour was significantly lower in the FOS (+)(−) and FOS (+)(+) groups than in the FOS (−)(−) groups. The value in the FOS (+)(−) group was intermediate. Thus, data on the frequency of scratching behaviour were consistent with the clinical skin severity score in offspring.

Effect of maternal supplementation with fructo-oligosaccharide on serum antibodies and tissue cytokines and chemokines in offspring

AD-like skin lesions in NC/Nga mice are accompanied by higher concentrations of serum IgE[24]. In the present study, although there was no significant difference in the serum concentration of total IgE in offspring aged 98 d among the groups (Fig. 4(A)), the concentration of total IgG1, i.e. Th2 response in mice[33], was significantly lower in the FOS (+)(−) and FOS (+)(+) groups than in the FOS (−)(−) and FOS (−)(+) groups (Fig. 4(B)). Two-way ANOVA showed that the concentration of total IgG1 was significantly affected by maternal, but not post-weaning, supplementation with FOS. No consistent change was observed in the concentration of total IgG2a, i.e. Th1 response in mice (Fig. 4(C)).

Expression of inflammation-associated cytokines and chemokines in ear auricles was evaluated by quantitative RT-PCR in offspring at age 98 d. Two-way ANOVA showed that the level of TNF-α mRNA was significantly affected by maternal, but not post-weaning, supplementation with FOS (Fig. 5). There was no significant difference in the level of IL-6, IL-8, and thymus and activation-regulated chemokine (TARC) among the groups.

Discussion

To our knowledge, the present study is the first demonstrating that modulation of the gut microbiota in infancy by maternal consumption with indigestible oligosaccharide diminished the severity of allergic skin inflammation. Our recent study showed that supplementation with FOS in female BALB/c mice during pregnancy and lactation altered the composition of the gut microbiota in their sucklings[23]. This finding provided the impetus for the present study examining whether modulation of gut microbiota in infancy affects the development of AD-like skin lesions in NC/Nga mice. In the present study, reduced progress in spontaneous skin lesions in offspring, under air-uncontrolled conventional conditions where the room was not equipped with air purifiers such as a HEPA (high efficiency particulate air) filter, was associated with the consumption of FOS in their dams during pregnancy and lactation. The diminution of skin lesions was accompanied by a lower frequency of scratching behaviour, lower serum concentrations of IgG1 and lower expression levels of

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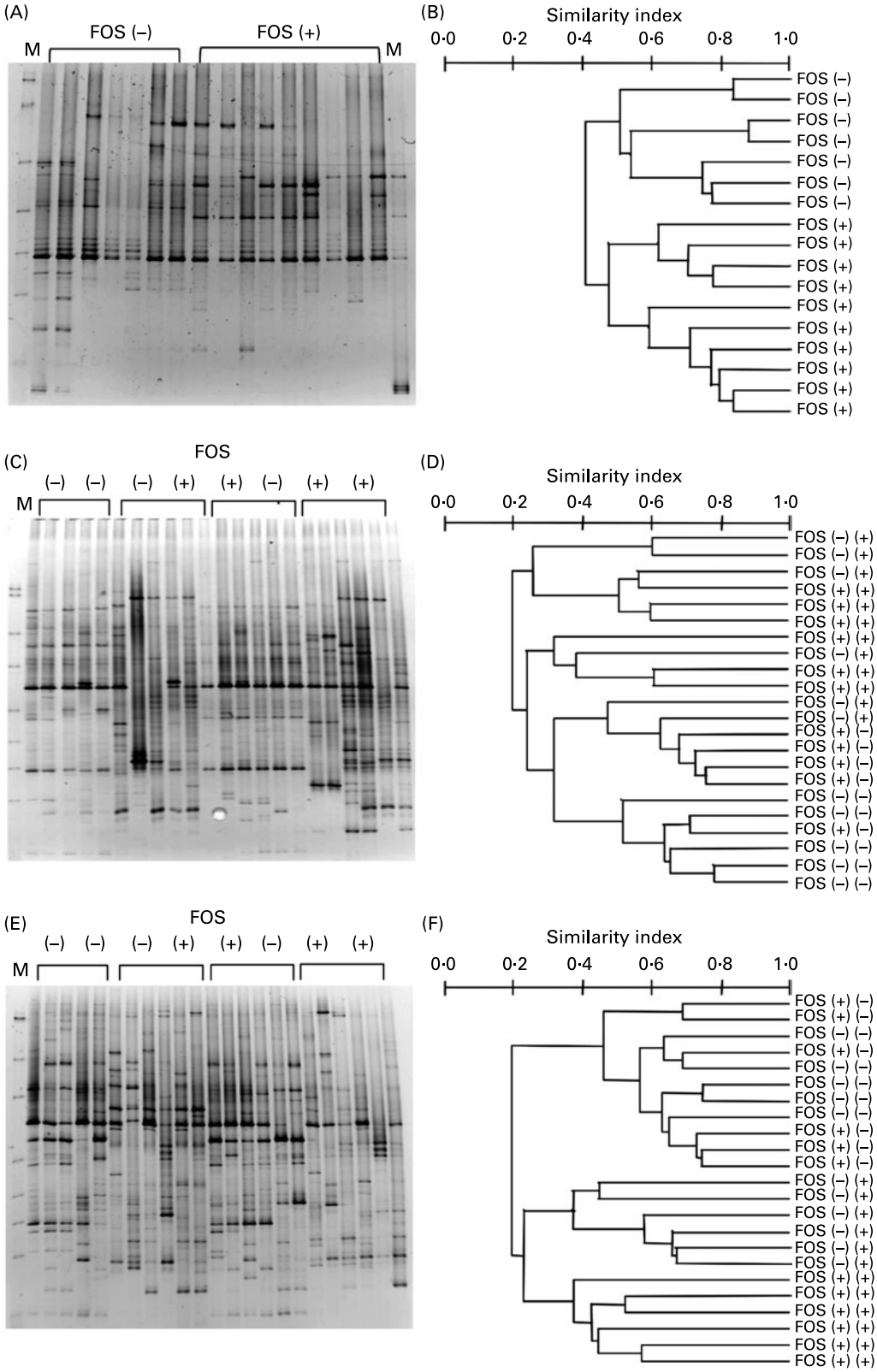

Fig. 2. PCR–denaturing gradient gel electrophoresis (DGGE) analysis of faecal microbiota based on 16S rRNA gene sequences in offspring of NC/Nga mice. DGGE gel images (A, C and E) and respective dendrograms (B, D and F) of DGGE band profiles on days 14 (A and B), 35 (C and D) and 98 (E and F) are shown. Each lane in the gel images and each line in the dendrograms represent individual mice. In (A) and (B), FOS (−) and FOS (+) represent sucklings of dams fed diets without and with fructo-oligosaccharide supplementation, respectively. For explanation of the offspring groups in (C) to (F), see Fig. 1. Distance is measured in arbitrary units. M, size marker.

TNF-α mRNA in the lesional tissue. However, further investigation is required to elucidate the involvement of TNF-α in the diminution of skin lesions, because expression of mRNA is not always equivalent to expression of the corresponding protein. Supplementation with FOS in offspring after weaning was shown to be less effective as compared with maternal supplementation with FOS. PCR–DGGE analysis of 16S rRNA gene profiles in the faeces of offspring suggested that maternal supplementation with FOS modulated the gut microbiota in sucklings, and that the gut microbiota in offspring after weaning were controlled by the diet they were

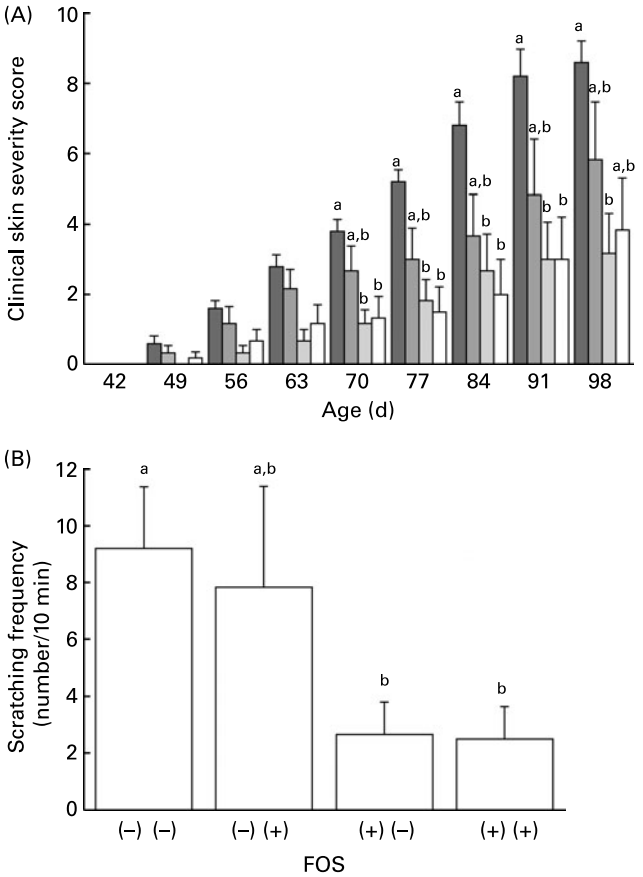

Fig. 3. Severity of spontaneously developing atopic dermatitis-like skin lesions in offspring of NC/Nga mice. (A) Changes in clinical skin severity score in offspring fed a post-weaning diet without fructo-oligosaccharide (FOS) whose dams were not supplemented with FOS (FOS (−)(−); ■), in offspring fed a post-weaning diet supplemented with FOS whose dams were not supplemented with FOS (FOS (−)(+); ■), in offspring fed a post-weaning diet without FOS whose dams were supplemented with FOS (FOS (+)(−); ■) and in offspring fed a post-weaning diet supplemented with FOS whose dams were supplemented with FOS (FOS (+)(+); □). Values are means, with standard errors represented by vertical bars. *P* values at ages 70, 77, 84, 91 and 98 d were $P=0{\cdot}0022$, $P=0{\cdot}0025$, $P=0{\cdot}0074$, $P=0{\cdot}0107$ and $P=0{\cdot}0119$, respectively, for FOS in dams, $P=0{\cdot}4006$, $P=0{\cdot}0860$, $P=0{\cdot}0838$, $P=0{\cdot}1914$ and $P=0{\cdot}4415$, respectively, for FOS in offspring, and $P=0{\cdot}2618$, $P=0{\cdot}1979$, $P=0{\cdot}2017$, $P=0{\cdot}1914$ and $P=0{\cdot}2142$, respectively, for FOS in dams × FOS in offspring (two-way ANOVA). [a,b] Mean values with unlike letters on each day were significantly different ($P<0{\cdot}05$; Mann–Whitney *U* test). (B) Frequency of scratching behaviour for 10 min at age 91 d. Values are means, with standard errors represented by vertical bars. *P* values were $P=0{\cdot}0165$, $P=0{\cdot}7377$ and $P=0{\cdot}7931$ for FOS in dams, FOS in offspring and their interaction, respectively (two-way ANOVA). [a,b] Mean values with unlike letters were significantly different ($P<0{\cdot}05$; Tukey–Kramer test).

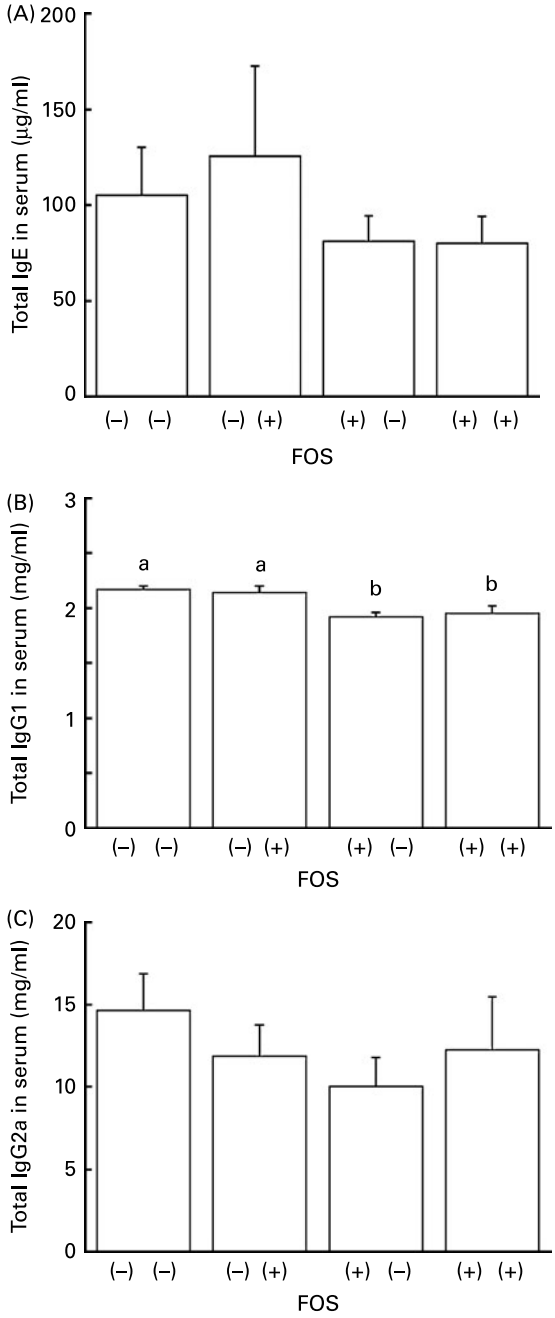

Fig. 4. Serum concentrations of total IgE (A), IgG1 (B) and IgG2a (C) in offspring of NC/Nga mice at age 98 d. FOS, fructo-oligosaccharide; FOS (−)(−), offspring fed a post-weaning diet without FOS, dams not supplemented with FOS; FOS (−)(+), offspring fed a post-weaning diet supplemented with FOS, dams not supplemented with FOS; FOS (+)(−), offspring fed a post-weaning diet without FOS, dams supplemented with FOS; FOS (+)(+), offspring fed a post-weaning diet supplemented with FOS, dams supplemented with FOS. Values are means, with standard errors represented by vertical bars. For total IgE, *P* values were $P=0{\cdot}2020$, $P=0{\cdot}7181$ and $P=0{\cdot}6887$ for FOS in dams, FOS in offspring and their interaction, respectively (two-way ANOVA). Similarly, *P* values for total IgG1 were $P=0{\cdot}0011$, $P=0{\cdot}9248$ and $P=0{\cdot}5928$ for FOS in dams, FOS in offspring and their interaction, respectively. *P* values for IgG2a were $P=0{\cdot}9068$, $P=0{\cdot}3836$ and $P=0{\cdot}3068$ for FOS in dams, FOS in offspring and their interaction, respectively. [a,b] Mean values with unlike letters were significantly different ($P<0{\cdot}05$; Tukey–Kramer test).

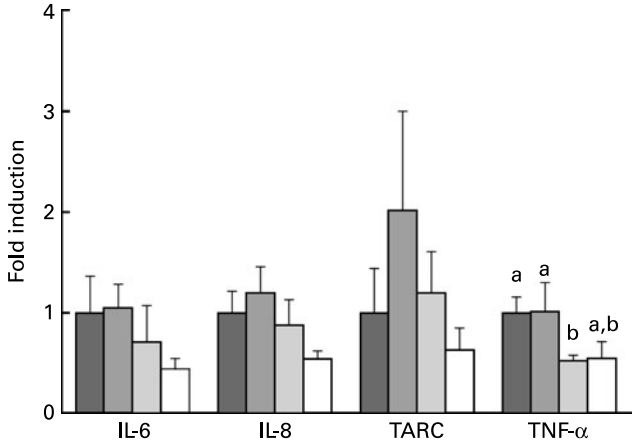

Fig. 5. Ear auricle expression levels of IL-6, IL-8, thymus and activation-regulated chemokine (TARC) and TNF-α evaluated by quantitative RT-PCR in offspring of NC/Nga mice at age 98 d. (■), Offspring fed a post-weaning diet without fructo-oligosaccharide (FOS), dams not supplemented with FOS (FOS (−)(−)); (▨), offspring fed a post-weaning diet supplemented with FOS, dams not supplemented with FOS (FOS (−)(+)); (□), offspring fed a post-weaning diet without FOS, dams supplemented with FOS (FOS (+)(−)); (□), offspring fed a post-weaning diet supplemented with FOS, dams supplemented with FOS (FOS (+)(+)). The levels for FOS (−)(−) are expressed relative to the control values, which are taken as 1. Values are means, with standard errors represented by vertical bars. For IL-6, *P* values were $P=0{\cdot}1251$, $P=0{\cdot}7063$ and $P=0{\cdot}5766$ for FOS in dams, FOS in offspring and their interaction, respectively (two-way ANOVA). Similarly, *P* values for IL-8 were $P=0{\cdot}0882$, $P=0{\cdot}7435$ and $P=0{\cdot}2326$ for FOS in dams, FOS in offspring and their interaction, respectively. *P* values for TARC were $P=0{\cdot}2636$, $P=0{\cdot}6687$ and $P=0{\cdot}1408$ for FOS in dams, FOS in offspring and their interaction, respectively. *P* values for TNF-α were $P=0{\cdot}0275$, $P=0{\cdot}9225$ and $P=0{\cdot}9813$ for FOS in dams, FOS in offspring and their interaction, respectively. [a,b]Mean values with unlike letters were significantly different ($P<0{\cdot}05$; Tukey–Kramer test).

consuming. Therefore, the present study supports the idea that the time point for modulation of gut microbiota is important in preventing the development of allergic diseases.

It remains unclear what kind of gut bacterial species in infant NC/Nga mice was involved in diminishing the severity of AD-like skin lesions later in life. Previous epidemiological studies showed that differences in the composition of gut microbiota in infancy precede the development of AD. According to Bjorksten *et al.* babies who developed allergy were less often colonised with enterococci during the first month of life and with bifidobacteria during the first year of life when compared with healthy infants[5]. In addition, allergic infants had higher counts of clostridia at 3 months, and the prevalence of colonisation with *Staphylococcus aureus* was also higher at 6 months, whereas the counts of *Bacteroides* were lower at 12 months[5]. Kalliomaki *et al.* reported that atopic children had more clostridia at age 3 weeks[6]. Furthermore, *Escherichia coli* and *Clostridium difficile* were reportedly associated with infants who developed AD[7,8]. Therefore, it might be possible that changes in the population of these bacterial species in infant NC/Nga mice are associated with the development of AD-like skin lesions later in life. In our preliminary experiments, quantitative RT-PCR of the 16S rRNA gene showed that bifidobacteria in the faeces of NC/Nga mice were below detection limits (10^6 copies/g faeces; R Fujiwara, J Watanabe and K Sonoyama, unpublished results). Further investigations are thus needed to elucidate the composition of infant gut microbiota beneficial in preventing allergic diseases later in life.

The mechanisms by which the gut microbiota in infancy control the development of allergic diseases later in life remain to be elucidated. Sawada *et al.* described 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[34]. In addition, Inoue *et al.* demonstrated that oral administration of *L. johnsonii* NCC533 in NC/Nga mice around the weaning period (i.e. age 20–22 d) prevented the development of allergic skin lesions induced by topical application of mite antigen from age 6 weeks[35]. Furthermore, Blümer *et al.* reported that administration of *L. rhamnosus* GG in female BALB/c mice during pregnancy and lactation suppressed the OVA-induced allergic airway inflammation in their offspring[36]. These findings, as well as the present study, provide experimental models enabling the investigation of how the gut microbiota in infancy influence the development of the immune system and subsequently control the development of allergic diseases later in life.

Alternatively, the possibility that mediators, transferred from the mother to its offspring, are involved in the suppression of allergic diseases cannot be excluded. Indeed, Verhasselt *et al.* showed that breast milk-mediated transfer of OVA from mouse dams to their sucklings resulted in the induction of oral immune tolerance, which led to the prevention of allergic airway inflammation in OVA-immunised offspring[37]. In addition, Polte *et al.* reported that the tolerance induced with oral OVA in pregnant BALB/c mice was protective against allergic airway inflammation in OVA-immunised offspring through breast milk-mediated mechanisms[38]. Furthermore, Blümer *et al.* demonstrated that prenatal *L. rhamnosus* GG administration in BALB/c mice increased TNF-α gene expression in placenta, which was associated with a reduction in OVA-induced airway inflammation in offspring[36]. In this context, modulation of the gut microbiota in pregnant and lactating NC/Nga mice by dietary FOS might influence the transfer of mediators from dams to offspring, which in turn results in the diminution of AD-like skin lesions later in life.

Because FOS is an indigestible oligosaccharide, dietary supplementation with FOS could lead to reduction of metabolisable energy intake. However, the present study did not measure body weight and food intake in dams and their offspring. Additionally, consumption of indigestible oligosaccharides such as FOS increases the production of SCFA, predominant fermentation products of bacteria, in the distal gut[39]. Therefore, we could not rule out the possibility that the diet-related difference in metabolisable energy intake and/or SCFA production contributed to the difference in the severity of skin lesions in the offspring.

In conclusion, our present study suggests that maternal consumption of FOS diminishes the severity of AD-like skin lesions in the offspring of NC/Nga mice. We therefore propose that the present findings support the idea that the modulation of gut microbiota by prebiotic oligosaccharides early in life may be beneficial in preventing dermatitis, although further investigation is required to reveal the underlying mechanisms involved. The present study provides an experimental model enabling the investigation of how gut microbiota in infancy influence immune system development and subsequently control the development of allergic diseases later in life.

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R. F. and N. T. were involved in the animal experiments, ELISA and RT-PCR. J. W. performed the molecular biological analyses of gut microbiota. K. S. was involved in designing the study and writing the manuscript.

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