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Consumption of fructo-oligosaccharide reduces 2,4-dinitrofluorobenzene-induced contact hypersensitivity in mice

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Strategies to manipulate the intestinal microbiota have been considered to promote immune health. The aim of the present study was to examine whether fructo-oligosaccharide, a typical prebiotic, could suppress antigen-specific skin inflammation by favourably changing the population of intestinal microbiota. Female BALB/c mice were fed a synthetic diet with or without fructo-oligosaccharide supplementation for 3 weeks and were then epicutaneously immunised with 2,4-dinitrofluorobenzene. Afterwards, mice continued to receive their respective diets. At 5 d after immunisation, the mice were ear challenged with the hapten. Ear swelling after the challenge was significantly reduced in the mice fed the diet supplemented with fructo-oligosaccharide than in mice fed the control diet. To characterise the change in the intestinal microbiota, DNA samples isolated from fresh faeces were subjected to PCR–denaturing gradient gel electrophoresis and real-time PCR based on 16S rDNA gene sequences. Dietary fructo-oligosaccharide altered the composition of intestinal microbiota. The numbers of bifidobacteria, but not lactobacilli, were significantly higher in mice fed the fructo-oligosaccharide-supplemented diet than in mice fed the control diet. Ear swelling was negatively correlated with the numbers of bifidobacteria in the faeces. Sequence analysis revealed that *Bifidobacterium pseudolongum* was the most predominant bifidobacteria in the intestine of mice fed the fructo-oligosaccharide-supplemented diet. These results suggest that consumption of fructo-oligosaccharide reduces contact hypersensitivity, which is associated with proliferation of *B. pseudolongum* in the intestinal tract of mice.

Prebiotics: Intestinal microbiota: Contact hypersensitivity: Mice

Allergic contact dermatitis is one of the most prevalent human skin diseases, causing moderate to severe inflammatory damage. This pathological condition arises after contact hypersensitivity (CHS)[1]. CHS is a T cell-mediated, antigen-specific type of skin inflammation that is induced by topical skin contact with haptens in a previously sensitised host[2,3]. When the host is sensitised by application of the hapten, skin dendritic cells capture the hapten and migrate to draining lymph nodes, where they prime specific T cells. These cells differentiate into CHS effector cells, which recirculate through the blood. The second contact with the same hapten leads to a skin inflammatory response that peaks 24–48 h after challenge. Avoidance of causal allergens is one of the emphasised therapeutic suggestions for CHS; however, avoidance is not practical in the majority of cases.

2,4-Dinitrofluorobenzene (DNFB)[4], picryl chloride[5] and oxazolone[6] have been used as typical haptens, which induce CHS in animals. DNFB-induced CHS in mice is the most widely used animal model for allergic contact dermatitis. In this model, mice are sensitised by application of DNFB onto shaved skin and are ear challenged by application of DNFB. Maximal ear swelling is observed 24–48 h after challenge.

Some food components, such as DHA[7], glycyrrhizin[8] and porphyran[9] reportedly suppressed DNFB-induced CHS in mice. Chapat *et al.* [4] reported that oral administration of probiotic *Lactobacillus casei* reduced the DNFB-induced CHS response in mice.

Approximately 400 different microbial species, mostly bacteria, inhabit the intestinal tract of mammals, where the bacterial density reaches more than 10^{11} cells/g contents[10]. Commensal bacteria in the intestinal tract play a major role in the maturation of the immune system, maintenance of homeostasis of the gut-associated immune system[11–13] and induction of oral tolerance[14–16]. Therefore, strategies to manipulate the microbiota have been considered to promote immune health. Some bacteria, such as the *Lactobacillus* and *Bifidobacterium* species, which have been described as 'living micro-organisms exerting health benefits', illustrate the concept of probiotics[17]. These bacteria prevent and treat rotavirus infections and post-antibiotic diarrhoea[18,19], allergic diseases[20–22] and recurrence of inflammatory bowel disease[23,24].

Prebiotics are indigestible oligosaccharides that possess positive health outcomes through promoting colonisation of the gastrointestinal tract with beneficial bacteria[25]. In addition, some

Abbreviations: BSA, bovine serum albumin; CHS, contact hypersensitivity; DGGE, denaturing gradient gel electrophoresis; DNFB, 2,4-dinitrofluorobenzene; FOS, fructo-oligosaccharides.

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indigestible oligosaccharides reportedly ameliorate allergic airway eosinophilia at least partly via post-absorptive mechanisms[26,27]. Stepans *et al.* [28] reported that consumption of human milk oligosaccharides is inversely related to subsequent risk of respiratory and enteric diseases in infants. Growth of intestinal bifidobacteria can be stimulated by dietary supplementation with prebiotics[29]. For example, fructo-oligosaccharides (FOS), which are non-digestible polymers of fructose found naturally in artichokes, leeks, asparagus, onions and bananas, stimulate the growth of faecal bifidobacteria in healthy human subjects[30–32] and in mice[33]. Therefore, we postulated that dietary FOS may reduce the CHS response by stimulating the growth of intestinal bifidobacteria.

Materials and methods

Animals and diets

Female BALB/c mice (age 5 weeks) 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. They were allowed free access to food and water. Mice were allocated to two groups of six or seven mice and fed either a synthetic diet prepared according to AIN-93G[34] or the diet supplemented with FOS (Meioligo P; Meiji Foodmateria, Tokyo, Japan). These diets were referred to as FOS(−) and FOS(+), respectively. The FOS(+) diet was prepared by adding 50 g FOS to each kg of the FOS(−) diet by substituting FOS for α-maize starch. Mice were fed the test diets for 3 weeks. Then, fresh faeces were collected, and CHS was induced as described below. At 12 d after DNFB immunisation, mice were anaesthetised by inhalation of diethyl ether, and blood was drawn from the carotid artery. In a separate experiment, female BALB/c mice (age 5 weeks) purchased from Japan Charles River (Yokohama, Japan) were used, and CHS responses and numbers of faecal bifidobacteria were determined.

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

Induction of contact hypersensitivity

CHS to DNFB was induced as previously described[4]. Briefly, mice were immunised on day 0 by epicutaneous application of 0·5 % DNFB (Tokyo Kasei, Tokyo, Japan) in 25 μl acetone–olive oil (4:1, v/v) onto the shaved abdomen. On day 5, mice were challenged with 0·25 % DNFB in 8 μl acetone–olive oil (4:1, v/v) applied onto the right ear pinna, while 8 μl acetone–olive oil (4:1, v/v) without DNFB was applied onto the left ear pinna. Ear thickness was measured with a digital engineer's micrometer (Mitsutoyo, Kawasaki, Japan) before challenge and 24, 48, 72 and 96 h after challenge. The ear thickness measurements were made by an investigator who was blinded to the treatments of the mice. DNFB-specific ear swelling was calculated according to the following equation:

$$\text{net swelling} = (\text{right ear thickness} - \text{left ear thickness})_{24\,\text{h}} - (\text{right ear thickness} - \text{left ear thickness})_{0\,\text{h}}.$$

The same equation was used for CHS measurements at 48, 72 and 96 h after the challenge.

Antibody enzyme-linked immunosorbent assay

Serum levels of IgG1 and IgG2a specific to the hapten were determined by ELISA. All assays were performed in ninety-six-well microtitre plates (Becton Dickinson, Franklin Lakes, NJ, USA). Wells were coated overnight at 4°C with dinitrophenol-bovine serum albumin (BSA; Calbiochem, San Diego, CA, USA) diluted to 30 μg/ml in carbonate buffer (pH 9·6). Plates were blocked with PBS containing 2 % BSA at 37°C for 1 h. Serial dilutions of serum were prepared in PBS containing 0·2 % BSA and 0·02 % Tween-20 (PBS/BSA-Tween) and then incubated for 2 h at room temperature. After the incubation, horseradish peroxidase–conjugated rat anti-mouse IgG1 (clone LO-MG1-2; Zymed, San Francisco, CA, USA) or rat anti-mouse IgG2a (clone LO-MG2a-3; Zymed) in PBS/BSA-Tween was added and incubated at 37°C for 2 h. Between each step, wells were washed five times with PBS containing 0·02 % Tween-20 (PBS-Tween). Plates were developed at room temperature after the addition of *o*-phenylenediamine (0·4 mg/ml) and H_2O_2 (0·016 %) in citrate-phosphate buffer (pH 5·0). Finally, 1 M-H_2SO_4 was added, and absorbance at 490 nm was measured with a microplate reader (model 550; Bio-Rad, Hercules, CA, USA).

Profile analysis of faecal microbiota by polymerase chain reaction–denaturing gradient gel electrophoresis

DNA was extracted from fresh faeces using a Fecal DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Faecal DNA samples were used as a template to amplify the fragments of 16S rDNA with universal primers, F968-GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and R1401 (CGG TGT GTA CAA GAC CC), and the denaturing gradient gel electrophoresis (DGGE) analysis of the amplicon was performed as previously described[35]. Briefly, PCR was performed in a reaction volume of 25 μl that contained 500 nM each of F968-GC and R1401, 1 × PCR buffer, 0·2 mM-dNTP and 1·25 U of *Taq*-HS polymerase (Takara, Otsu, Japan). The reaction conditions were 94°C for 5 min, followed by thirty-five cycles of 94°C for 30 s, 56°C for 20 s, and 68°C for 40 s, and final extension at 68°C for 7 min. The amplicons were analysed by DGGE on the DCode system (Bio-Rad) on an 8 % polyacrylamide gel (dimensions 160 × 180 × 1 mm) containing a 45 % to 65 % gradient of urea-formamide, where 100 % was defined as 7 M-urea and 40 % formamide. Electrophoresis was performed in Tris–acetate–EDTA (40 mM-2-amino-2-hydroxymethyl-1,3-propanediol; 20 mM-acetic acid; 1 mM-EDTA) at 80 V and 60°C for 16 h. The gel was stained for 30 min in Tris–acetate–EDTA containing SYBR green I nucleic acid gel stain (Cambrex, Hopkinton, MA, USA) and photographed under a UV light transilluminator (model 400EX; Aisin Seiki, Kariya, Japan). Quantity One software (version 4.6.0; Bio-Rad) was used for band identification and normalisation of band patterns from DGGE gels. Subsequently, a dendrogram of the DGGE-band profile was constructed using Pearson's curve-based correlation and the

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unweighted pair-group method using the arithmetic average clustering method in Quantity One software as previously described[36].

Quantification of bifidobacteria and lactobacilli in faeces by real-time polymerase chain reaction

Amplification and detection of faecal DNA were performed with Smart Cycler II (Cepheid, Sunnyvale, CA, USA). *Bifidobacterium* genus-specific (forward, TCG CGT C(C/T)G GTG TGA AAG; reverse, CCA CAT CCA GC(A/G) TCC AC)[37] and *Lactobacillus* genus-specific (forward, TGG AAA CAG (A/G)TG CTA ATA CCG; reverse, GTC CAT TGT GGA AGA TTC CC)[38] primer pairs were used. Real-time PCR was performed in a reaction volume of 25 μl, containing 12·5 μl SYBR Premix Ex Taq (Takara), 200 nM each of the forward and reverse primers, and 1 μl faecal DNA samples. The reaction conditions were: 95°C for 30 s, followed by forty-three cycles at 95°C for 5 s, 64°C for 15 s, and 72°C for 15 s for the quantification of bifidobacteria, and 95°C for 30 s, followed by forty-three cycles at 95°C for 5 s, 57°C for 20 s, and 72°C for 15 s for the quantification of lactobacilli.

Bifidobacterium animalis (JCM 1190^{T}) and *L. murinus* (JCM 1717^{T}) were cultured in De Man, Rogosa and Sharpe (MRS) broth (Becton Dickinson, Rockville, MD, USA) and the genomic DNA was extracted by Isoplant II (Wako Pure Chemical Ind., Osaka, Japan) according to the manufacturer's instructions. Fragments of 16S rDNA were amplified by PCR with the *Bifidobacterium* or *Lactobacillus* genus-specific primer pairs listed above. The amplicons were purified by the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Bioscience, Tokyo, Japan) and cloned in pGEM-Easy T vectors (Promega, Madison, WI, USA). Transformation was performed with competent *Escherichia coli* XL-1 Blue cells and plated onto Luria–Bertani agar plates supplemented with ampicillin (25 μg/ml), X-Gal (30 μg/ml) and isopropyl β-D-1-thiogalactopyranoside (20 μg/ml) and incubated overnight at 37°C. White transformants were picked and grown in Luria–Bertani broth. Plasmid DNA was extracted with QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA) and used as standards for real-time PCR.

Analysis of bifidobacterial diversity by polymerase chain reaction–denaturing gradient gel electrophoresis

Amplification of *Bifidobacterium*-genus specific 16S rDNA fragments and the DGGE analysis of the amplicon were performed as previously described[39]. Briefly, PCR was performed in a reaction volume of 25 μl, containing 500 nM primers g-Bifid F (5′ CTC CTG GAA ACG GGT GG) and g-Bifid R-GC (5′ CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CGT GGT TCT TCC CGA TAT CTA CA), 1 × PCR buffer, 0·2 mM-dNTP and 1·25 U of *Taq*-HS polymerase (Takara). The reaction conditions were: 94°C for 5 min, followed by thirty cycles at 94°C for 30 s, 62°C for 20 s, and 72°C for 50 s, and final extension at 72°C for 7 min. The amplicons were analysed by DGGE as described above, except for using an 8 % polyacrylamide gel containing a 45 % to 55 % gradient of urea-formamide. Fragments on the gel were cut out and amplified by PCR with g-Bifid primers. Amplicons were purified by the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Bioscience) and used for sequence analysis.

Sequence analysis

Purified PCR amplicons (40 ng) were used as a template, and the sequence reactions were performed with a DTCS sequence kit (Beckman Coulter Japan, Tokyo, Japan) according to the manufacturer's instructions with g-Bifid F and g-Bifid R-GC as the sequencing primers. Sequences were compared with those in the GenBank database with the BLAST N algorithm[40].

Statistical analysis

Results are presented as mean values with their standard errors. The unpaired or paired *t* test or Tukey–Kramer's test following one-way ANOVA was used to compare mean values. The correlation between the number of faecal bifidobacteria and ear swelling 24 h after challenge was assessed by Pearson's correlation method. Data analysis was performed with StatView for Macintosh (version 5.0; SAS Institute Inc., Cary, CA, USA). *P* values less than 0·05 were considered statistically significant.

Results

Effect of fructo-oligosaccharides on the contact hypersensitivity response

Representative CHS responses of mice fed either FOS(−) or FOS(+) are shown in Fig. 1. The same experiments were repeated several times, and similar CHS responses were observed. Irrespective of the diet, ear swelling after DNFB challenge in non-immunised mice was routinely less than 10 μm (data not shown). As shown in Fig. 1, 48 h after challenge, ear swelling reached a maximum and then decreased gradually in both the mice fed FOS(−) and those fed FOS(+). Ear swelling was significantly less in mice fed FOS(+) than in mice fed FOS(−) 24 h after challenge, and the reductive effect of FOS intake was still observed even 48 h after challenge (Fig. 1). The area under the curve for ear swelling tended to be smaller in FOS(+)-fed mice when compared with FOS(−)-fed mice (13 123 (SEM 1835) μm × 96 h *v.* 16 856 (SEM 695) μm × 96 h).

In mice purchased from Japan Charles River, the levels of ear swelling were the same between FOS(−)- and FOS(+)-fed animals both 24 h (161·4 (SEM 16·4) *v.* 156·8 (SEM 16·8) μm) and 48 h (190·3 (SEM 7·5) *v.* 196·2 (SEM 13·6) μm) after challenge.

Hapten-specific antibody responses

There was no detectable hapten-specific antibody in the non-immunised mice (data not shown). At 12 d after immunisation, both the mice fed FOS(−) and the mice fed FOS(+) produced detectable levels of hapten-specific IgG1 and IgG2a antibodies. Reciprocal log 2 titres of hapten-specific IgG1 were the same between FOS(−)- and FOS(+)-fed animals (11·0 (SEM 0·3) *v.* 11·3 (SEM 0·3)). Hapten-specific IgG2a titres of the FOS(−) and FOS(+) groups were 6·3 (SEM 0·4) and 7·9

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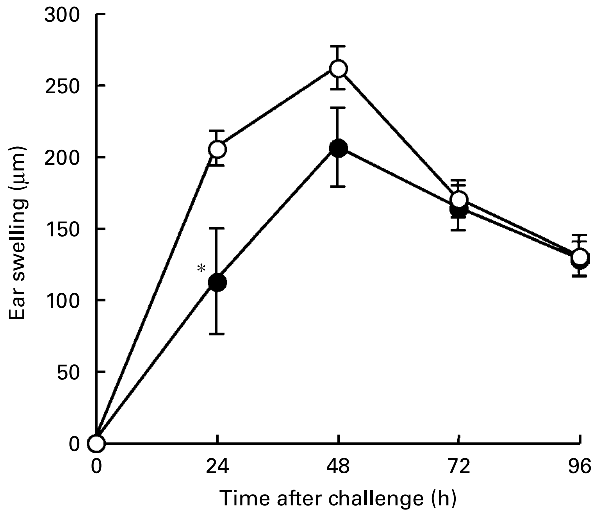


Fig. 1. Time course of ear swelling after 2,4-dinitrofluorobenzene (DNFB) challenge in mice fed no fructo-oligosaccharide (FOS(−); ○) and mice fed fructo-oligosaccharide (FOS(+); ●). Mice were fed the indicated diet for 3 weeks and then immunised epicutaneously with DNFB. Afterwards, the mice continued to receive the indicated diets. Mice were ear challenged with the hapten 5 d after immunisation. Ear thickness was measured before the challenge and 24, 48, 72 and 96 h after the challenge. Values are means for six or seven mice, with their standard errors represented by vertical bars. * Mean value was significantly different from the value for the mice fed FOS(−) ($P<0·05$).

(SEM 0·4), respectively. The serum level of hapten-specific IgG2a in the FOS(+) group tended to be higher than that in the FOS(−) group.

Comparison of bacterial diversity with denaturing gradient gel electrophoresis

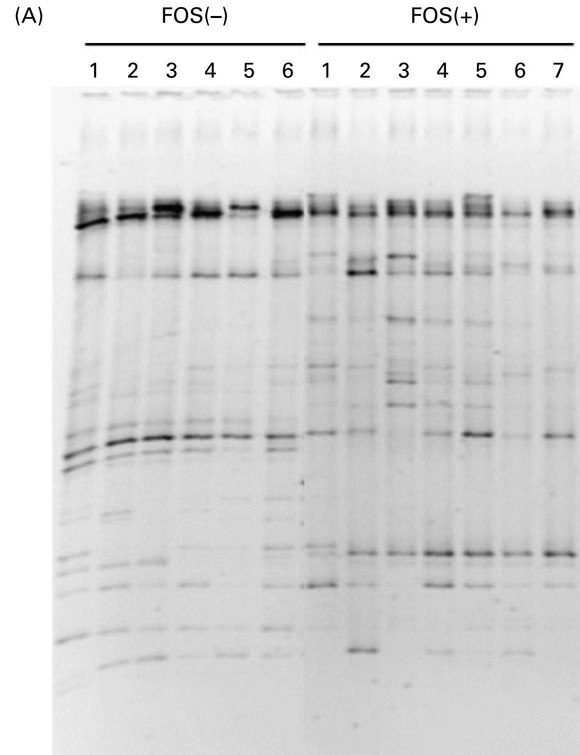
PCR–DGGE analyses for bacterial 16S rDNA extracted from fresh faeces were performed to compare the microbiota in FOS(+)-fed mice with the microbiota in FOS(−)-fed mice. A representative DGGE band profile is shown in Fig. 2 (A). The intensity and position of detected bands were subjected to cluster analysis (Fig. 2 (B)). The dendrogram shows two large clusters of FOS(+) and FOS(−) groups (Fig. 2 (B)).

Quantification of faecal bifidobacteria and lactobacilli

Quantification of faecal bifidobacteria and lactobacilli was performed by real-time PCR with *Bifidobacterium* and *Lactobacillus* genus-specific primers. Detectable levels of bifidobacteria were observed in all faecal DNA samples from FOS(+)-fed mice, although they were observed in low levels in three out of six samples from FOS(−)-fed mice (Fig. 3 (A)). The faecal bifidobacteria level in FOS(+)-fed mice reached $1·6 \times 10^5$ copies/mg faeces and was significantly higher than the level in FOS(−)-fed mice. The level of faecal lactobacilli in FOS(+)-fed mice was not significantly different when compared with that of FOS(−)-fed mice (Fig. 3 (B)).

The numbers of bifidobacteria in the faecal samples were negatively correlated with the ear swelling 24 h after challenge (Fig. 4).

When mice purchased from Japan Charles River were used, neither FOS(−)-fed mice nor FOS(+)-fed mice had



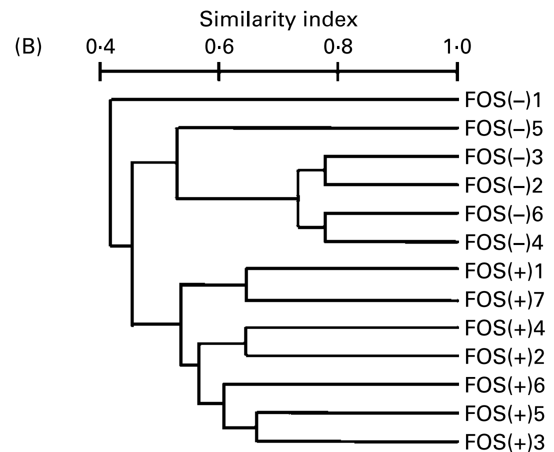


Fig. 2. PCR–denaturing gradient gel electrophoresis (DGGE) analysis of faecal microbiota based on 16S rDNA sequences in mice fed no fructo-oligosaccharide (FOS(−)) and mice fed fructo-oligosaccharide (FOS(+)). (A) SYBR green staining of PCR products separated by DGGE. (B) Similarities among DGGE band profiles of faecal bacteria of mice were calculated based on the position and intensity of bands, and the dendrogram of DGGE band profiles was constructed by the unweighted pair-group method using the arithmetic average clustering method. Distances are measured in arbitrary units.

detectable levels of bifidobacteria in faecal samples even 2 weeks after starting the test diets.

Population profiling of bifidobacteria with denaturing gradient gel electrophoresis

To characterise the population profiling of bifidobacteria in the faeces, PCR–DGGE with *Bifidobacterium* genus-specific primers was performed. DGGE band profiles are shown in Fig. 5. Only one band with high intensity was detected in

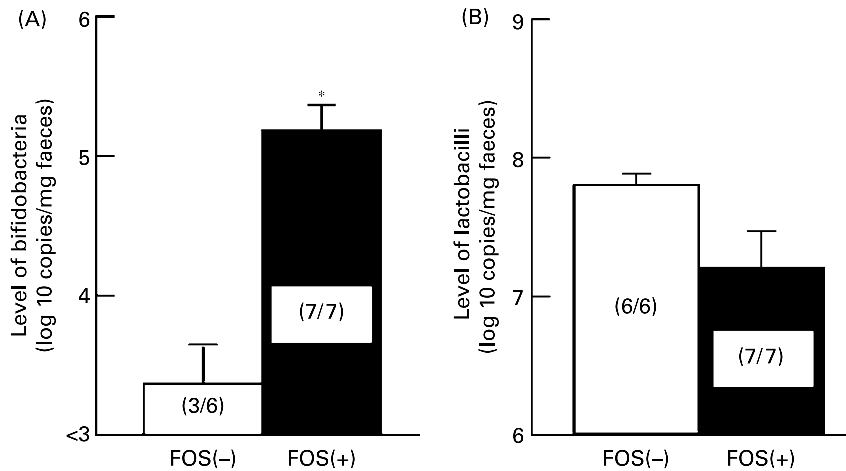


Fig. 3. Bacterial numbers of bifidobacteria (A) and lactobacilli (B) in the faeces of mice fed no fructo-oligosaccharide (FOS(−)) and mice fed fructo-oligosaccharide (FOS(+)). Bacterial numbers in the faeces were quantified by real-time PCR with genus-specific primers. PCR amplicons of genomic DNA from *Bifidobacterium animalis* (JCM 1190^{T}) and *Lactobacillus murinus* (JCM 1717^{T}) were ligated into pGEM-Easy vectors, and the resulting plasmids were used as standards. Values are means for six or seven mice, with their standard errors represented by vertical bars. Fractional values are the frequency of mice possessing detectable levels of bacteria. * Mean value was significantly different from the value for the mice fed FOS(−) ($P<0·05$).

all mice fed FOS(+), while no detectable bands were observed in mice fed FOS(−), with one exception. The bands observed in FOS(+)-fed mice were cut from the gel, and the DNA sequences were analysed. A BLAST search revealed that the sequences showed the highest similarity to *B. pseudolongum* (99·8 %, 486/487).

Discussion

Allergic contact dermatitis, which arises after CHS, is one of the most prevalent human skin diseases[1]. Avoidance of the causal allergen is not practical in the majority of the cases. Some food components[7–9] and probiotic *L. casei*[4] reportedly reduce CHS in mice. We determined whether FOS influences DNFB-induced CHS in mice, the most widely used animal model for allergic contact dermatitis.

DNFB-induced ear swelling 24 h after the challenge was significantly less and the area under the curve for ear swelling tended to be smaller in mice fed FOS(+) when compared with those in mice fed FOS(−) (Fig. 1), indicating that dietary FOS supplementation at a concentration of 5 % retarded the DNFB-induced CHS response in BALB/c mice. DNFB-induced CHS is a widely used model of allergic contact dermatitis and has been used for evaluating the anti-allergic effects of food components[7–9]. However, few reports have addressed CHS-reductive effects of non-digestive oligosaccharides, such as FOS.

It was previously reported that orally administered FOS affects intestinal microbiota, especially the growth of intestinal bifidobacteria[30–33]. Therefore, we hypothesised that the suppressive effect of FOS on CHS was associated with changes in intestinal microbiota. To test this hypothesis, cultivation-independent approaches based on bacterial 16S rDNA gene sequences were performed to study the bacterial composition of fresh faeces. A dendrogram of DGGE band profiles showed two large clusters of FOS(+) and FOS(−) groups (Fig. 2 (B)), clearly indicating that FOS administration changed the composition of intestinal microbiota. Additionally, real-time PCR analysis showed that the faecal bifidobacteria in the FOS(+)-fed mice reached $1·6 \times 10^{5}$ copies/mg faeces, although undetectable levels of bifidobacteria were observed in faeces from half of the FOS(−)-fed mice (Fig. 3 (A)). These results indicate that bifidobacteria, a minor community in the intestinal microbiota, proliferated by FOS administration. It is especially noteworthy that the numbers of faecal bifidobacteria were negatively correlated to the ear swelling 24 h after challenge (Fig. 4). These results suggest that suppression of DNFB-induced CHS by FOS administration is associated with increased numbers of faecal bifidobacteria in mice.

In the present study, mice were fed the synthetic diet to examine the effect of FOS supplementation. FOS supplementation reduced DNFB-induced CHS, which was associated with increased numbers of bifidobacteria. It has been reported that intestinal bifidobacteria proliferated when rats were fed a cereal-based commercial standard chow supplemented with

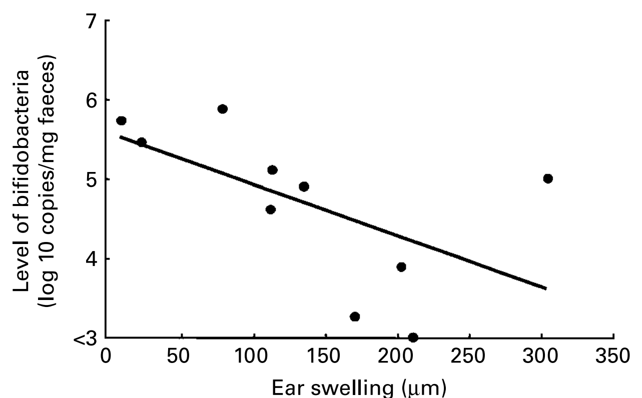


Fig. 4. Correlation between the numbers of bifidobacteria in faeces and ear swelling 24 h after 2,4-dinitrofluorobenzene challenge. The correlation (R −0·585; $P<0·05$) was assessed by Pearson's correlation method.

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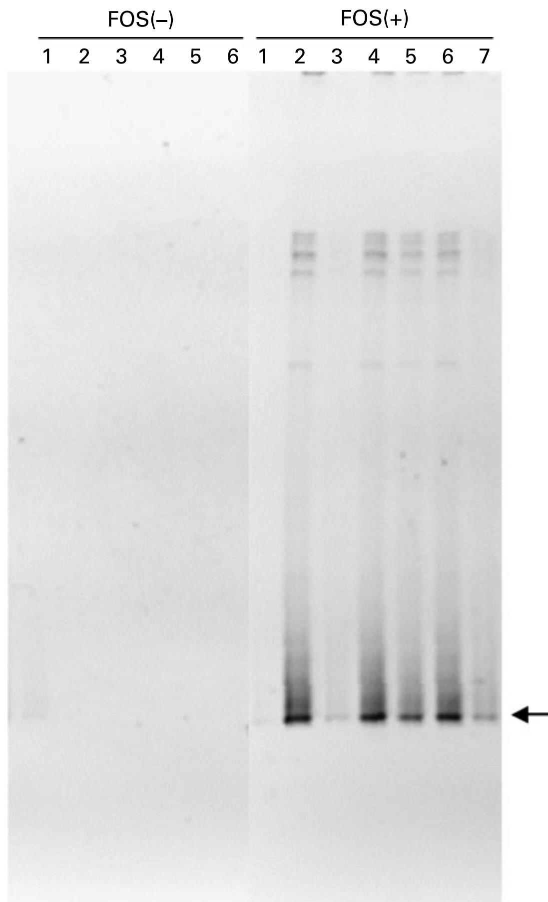

Fig. 5. PCR–denaturing gradient gel electrophoresis (DGGE) analysis of faecal microbiota based on 16S rDNA sequences in mice fed no fructo-oligosaccharide (FOS(−)) and mice fed fructo-oligosaccharide (FOS(+)). PCR amplicons by using *Bifidobacterium* genus-specific g-Bifid primers were separated by DGGE, and the gels were stained with SYBR green. The bands indicated (←) were amplified by g-Bifid primers, and their sequences were analysed.

FOS[41]. Although we have not determined whether FOS supplementation to a cereal-based diet influences the CHS reaction, it would be conceivable that the CHS-reducing effect of FOS is not specific to a synthetic diet.

Chapat *et al.* [4] reported that oral administration of *L. casei* reduced the DNFB-induced CHS response in BALB/c mice. In the present study, however, FOS administration did not induce increased numbers of faecal lactobacilli (Fig. 3 (B)), suggesting that the suppressive effects of FOS on CHS are not associated with intestinal lactobacilli. Nagura *et al.* [42] reported that caecal microbiota of BALB/c mice originating from two different breeders were different and that bifidobacteria were detected only in one breeding colony (from Japan SLC). Indeed, the present results showed that the numbers of bifidobacteria in the faeces of mice purchased from Charles River Japan, Inc., were less than the detection limit, even 2 weeks after FOS(+) administration. In these mice, FOS(+) did not reduce the DNFB-induced CHS response, although BALB/c mice purchased from these breeders are considered to be genetically equivalent. These results also lend support to our hypothesis that increased numbers of bifidobacteria in the intestinal tract are responsible for the suppressive effect of FOS on the CHS response.

We next determined what species of bifidobacteria proliferated in mice fed FOS(+). PCR–DGGE with *Bifidobacterium* genus-specific primers revealed that administration of FOS induced proliferation of *B. pseudolongum* (Fig. 4). *B. pseudolongum* is the most predominant intestinal bifidobacteria in various mammals[43], and oral administration of this bacterial species decreases the frequency of diarrhoea in newborn calves and piglets[44]. However, few reports have addressed the immune-modulating effects of *B. pseudolongum*. In future experiments we will test whether oral administration of *B. pseudolongum* isolated from the faeces of mice fed FOS(+) suppresses the CHS responses.

The cellular and molecular mechanisms involved in the suppressive effect of FOS on the CHS response are unclear. Although it is generally accepted that the DNFB-induced CHS response is independent of $CD4^+$ T-cell help[45,46], the present study showed that epicutaneous immunisation induced the elevation of hapten-specific IgG1 and IgG2a antibodies in sera. Chapat *et al.* [4] demonstrated that oral administration of *L. casei* resulted in diminished levels of hapten-specific serum antibodies induced by skin inflammation. Thus, the probiotic exhibits potent immune-regulatory properties affecting both hapten-specific $CD4^+$ helper T cells and $CD8^+$ cytotoxic T-cell effectors of CHS. In contrast, the serum levels of hapten-specific IgG1 were the same in mice fed FOS(+) and mice fed FOS(−) in the present study. Therefore, increased *B. pseudolongum* by FOS intake may not modulate $CD4^+$ helper T cells. The reason for the upward trend in the serum hapten-specific IgG2a of FOS(+)-fed mice is not clear. However, this change might not be associated with the CHS-reductive effect of FOS, since the CHS response is independent of $CD4^+$ T cells[45,46].

In conclusion, the CHS response induced by skin application of DNFB was reduced by FOS supplementation in the diet. The numbers of intestinal bifidobacteria were increased by FOS, and *B. pseudolongum* was the most predominant bifidobacteria in the mice fed the FOS(+) diet. The severity of CHS was negatively correlated with the numbers of bifidobacteria; this finding strongly suggests that *B. pseudolongum* is responsible for the reductive effect of FOS consumption on the DNFB-induced CHS response.

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