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Role of intestinal *Bifidobacterium pseudolongum* in dietary fructo-oligosaccharide inhibition of 2,4-dinitrofluorobenzene-induced contact hypersensitivity in mice

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Strategies to manipulate the gut microbiota have been explored for preventing allergy development. We previously showed that dietary supplementation with fructo-oligosaccharide (FOS) reduced 2, 4-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity (CHS) in BALB/c mice. Because the CHS response was negatively correlated with the number of faecal bifidobacteria, particularly *Bifidobacterium pseudolongum*, the present study aimed to examine whether oral administration of *B. pseudolongum* affects CHS response. Viable *B. pseudolongum* was successfully isolated from mouse faeces. Female BALB/c mice were fed a synthetic diet with or without FOS supplementation, and *B. pseudolongum* (2×10^7 cells) was administered daily throughout the experimental period. Two weeks after starting the test diets, mice received DNFB on the ear auricle twice at 7-d intervals. Conventional cultivation and molecular biological analyses based on 16S rRNA gene sequences showed that administration of FOS and *B. pseudolongum* resulted in higher excretion of viable bifidobacteria, mainly *B. pseudolongum*. Although dietary FOS reduced the CHS response as demonstrated by ear swelling, *B. pseudolongum* administration resulted in a reduction in the initial phase only of the CHS response. *B. pseudolongum* administration increased hapten-specific IgG1, while dietary FOS decreased IgG2a in sera. Administration of FOS and *B. pseudolongum* decreased interferon- γ production and increased IL-10 production in cervical lymph node cells restimulated with hapten *in vitro*. We conclude that *B. pseudolongum* proliferation in the intestinal tract is partially responsible for the reduction in DNFB-induced CHS response by dietary supplementation with FOS in mice, which may be mediated by the modulation of antigen-induced cytokine production.

Prebiotics: Bifidobacteria: Contact hypersensitivity: Mice

Commensal microbiota in the intestinal tract plays an important role in the normal development of the immune system^(1,2). Therefore, strategies for microbiota manipulation have been explored for the prevention of the onset of immune diseases such as allergy. This idea is supported by epidemiological data demonstrating that differences in the composition of gut microbiota in infancy precede the development of atopic dermatitis⁽³⁾. In addition, clinical trials showed that administration of probiotic bacterial strains, such as *Lactobacillus rhamnosus* GG, was beneficial in both the prevention^(4,5) and treatment⁽⁶⁾ of early allergic diseases. However, some studies reported that *L. rhamnosus* GG and *Lactobacillus acidophilus* did not have a preventative effect in infancy^(7–9). Therefore, there is currently insufficient data to recommend probiotics as part of a standard therapy in the treatment or prevention of allergic conditions⁽¹⁰⁾.

Prebiotics, such as indigestible oligosaccharides, have also been considered to promote immune health by selectively

stimulating the growth and/or activity of beneficial bacteria, such as bifidobacteria and lactobacilli, in the intestinal tract^(11–14). Indeed, a mixture of long-chain fructo-oligosaccharide (FOS) and short-chain galacto-oligosaccharide reportedly reduced the incidence of atopic dermatitis in formula-fed high-risk infants^(15,16). In terms of experimental evidence, we demonstrated that dietary raffinose and α -linked galacto-oligosaccharide reduced allergic airway inflammation in ovalbumin-sensitised Brown Norway rats^(17,18). In addition, Fujitani *et al.*⁽¹⁹⁾ showed that dietary FOS reduced infiltration of inflammatory cells and oedema formation in duodenal mucosa using an ovalbumin-induced food allergy model of NC/jic mice. Furthermore, Vos *et al.*⁽²⁰⁾ reported that consumption of a mixture of long-chain FOS and short-chain galacto-oligosaccharide suppressed allergic airway inflammation in an ovalbumin-induced allergic asthma model of BALB/c mice. These findings suggest that administration of indigestible oligosaccharides is effective in the prevention of allergic diseases.

Abbreviations: BIM, bifidobacterium iodoacetate medium; CHS, contact hypersensitivity; CLN, cervical lymph node; DGGE, denaturing-gradient gel electrophoresis; DNFB, 2,4-dinitrofluorobenzene; FOS, fructo-oligosaccharide; IFN, interferon; RT-qPCR, real-time quantitative PCR.

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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)⁽²¹⁾. 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^(22,23). 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 the challenge. Avoidance of causal allergens is one of the emphasised therapeutic suggestions for CHS; however, avoidance is not practical in the majority of cases.

We previously reported that dietary supplementation with short-chain FOS reduced DNFB-induced CHS response in BALB/c mice⁽²⁴⁾. Molecular biological analyses of gut microbiota in these mice, based on 16S rRNA gene sequences, showed that the CHS response, i.e. ear swelling, was negatively correlated with the numbers of bifidobacteria. Because sequence analysis revealed that *Bifidobacterium pseudolongum* was the most predominant bifidobacterium in the intestine of FOS-supplemented mice, we postulated that a reduction in the CHS response by feeding FOS was associated with the proliferation of *B. pseudolongum* in the intestinal tract. In the present study, therefore, we aimed to examine whether oral administration of *B. pseudolongum*, isolated from FOS-supplemented mice, affects the development of DNFB-induced CHS response in mice.

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 Hokkaido University guidelines for the care and use of laboratory animals.

Female BALB/c mice (5 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled ($23 \pm 2^\circ\text{C}$) 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 eleven mice and fed either a synthetic diet prepared according to AIN-93G guidelines⁽²⁵⁾ 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(+) diet was prepared by adding (50 g/kg diet) FOS to the FOS(–) diet at the expense of α -maize starch. In each dietary group, mice were further divided into two subgroups of five or six mice and intragastrically administered vehicle or 2×10^7 cells of viable *B. pseudolongum* daily as described below. These treatments were referred to as BP(–) and BP(+), respectively. Thus, mice were divided into four groups: FOS(–)BP(–), FOS(–)BP(+), FOS(+)BP(–) and FOS(+)BP(+) (n 5, 6, 5 and 6, respectively). Mice were fed the test diets and administered *B. pseudolongum* throughout the experimental period. *B. pseudolongum* was isolated from the faeces of mice-fed FOS(+) in a separate experiment as described below.

Two weeks after starting the test diets and *B. pseudolongum* administration, fresh faeces were collected, and CHS was induced as described below. At 25 d after the first application of DNFB, mice were anaesthetised by inhalation of diethyl ether and blood was drawn from the carotid artery. Cervical lymph node (CLN) was excised for primary cell culture as described below.

Isolation of *Bifidobacterium pseudolongum*

Fresh faeces were collected from female BALB/c mice-fed FOS(+) for 3 weeks. Faecal samples were homogenised in 450 μl of 50 mM anaerobic phosphate buffer (pH 6.8) that contained 0.05 % (w/v) L-cysteine, 0.05 % (w/v) Tween 80 and 0.1 % (w/v) agar. The samples were diluted, and aliquots were plated on bifidobacteria-selective *Bifidobacterium* iodoacetate medium (BIM) agar⁽²⁶⁾, which consists of MRS agar supplemented with 20 mg/l nalidixic acid, 8.5 mg/l polymyxin B, 50 mg/l kanamycin sulphate, 35 mg/l iodoacetic acid and 25 mg/l 2,3,5-triphenyltetrazolium chloride, and incubated at 37°C for 5 d under anaerobic condition (N_2 – CO_2 – H_2 , 8:1:1, Coy anaerobic chamber, Coy Laboratory Products, Grass Lake, MI, USA). After incubation, a single colony was picked with a platinum loop and streaked onto an agar plate. Following incubation at 37°C for 5 d under anaerobic conditions, a number of single colonies were picked and suspended in BIM broth. After incubation at 37°C for 3 d under anaerobic conditions, the medium was removed by centrifugation (8000g 10 min), and the resultant pellet was resuspended in BIM broth that contained 20 % glycerol medium and stored at -80°C . In addition, aliquots of the pellet were used for isolation of DNA, followed by sequencing analysis as described below. Light microscopic observation following Gram staining was also performed.

Oral administration of *Bifidobacterium pseudolongum*

A frozen stock sample of isolated *B. pseudolongum* was thawed, washed twice with PBS and adjusted to 10^8 cells/ml with PBS before use. Mice were intragastrically administered 200 μl of the bacterial suspension (2×10^7 cells) daily throughout the experimental period. Before administration, viability of bacteria was checked by flow cytometry according to Ben-Amor *et al.*⁽²⁷⁾. In brief, approximately 10^6 cells were suspended in 1 ml anaerobic PBS containing 1 mM dithiothreitol, 0.01 % (w/v) Tween 20, 10^4 particles/ml fluorospheres (Flow-Check fluorospheres, Beckman Coulter, Tokyo Japan), 1 $\mu\text{g/ml}$ propidium iodide (Wako Pure Chemical Industries, Osaka, Japan) and 5 nM SYTO-BC (Molecular Probes, Eugene, OR, USA). After 5 min incubation at room temperature, samples were analysed by flow cytometry (Epics XL, Beckman Coulter). The viability was routinely greater than 80 %.

Species-specific PCR and DNA sequencing for *Bifidobacterium pseudolongum*

B. pseudolongum subsp. *pseudolongum* (JCM1264) was obtained from the Japan Collection of Microorganisms of the Institute of Physical and Chemical Research, and used as a positive control. DNA was isolated from bacterial samples isolated from mouse faeces and *B. pseudolongum* subsp.

pseudolongum (JCM1264) using PrepMan Ultra reagent (Applied Biosystems Japan, Tokyo, Japan), according to the manufacturer's instructions. Bacterial DNA samples were used as a template to amplify the 16S rRNA gene fragments with a primer pair specific to *B. pseudolongum* (forward: IDB41F, CCC TTT TTC CGG GTC CTG T; reverse: IDBC1R, ATC CGA ACT GAG ACC GGT T)⁽²⁸⁾. PCR was performed in a reaction volume of 25 µl that contained 500 nM each of primers, 1 × PCR buffer, 0.2 mM dNTP and 1.25 U *Taq*-HS polymerase (Takara, Otsu, Japan). The reaction conditions were 96°C for 2 min, followed by 35 cycles at 94°C for 30 s, 63°C for 40 s and 72°C for 30 s, and a final extension at 72°C for 5 min. The size of the amplicon (471 bp) was checked by agarose gel electrophoresis. In addition, the nearly complete 16S rRNA gene was sequenced using a BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems Japan) according to the manufacturer's instructions with universal primers (27F, 5' AGA GTT TGA TCM TGG CTC AG; 1492R, 5' TAC GGH TAC CTT GTT ACG ACT T) as the sequencing primers⁽²⁹⁾. The sequence was compared to those in the GenBank database using the BLAST N algorithm.

Induction of contact hypersensitivity

CHS to DNFB was induced according to Nagai *et al.*⁽³⁰⁾. Briefly, 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, twice at 7-d intervals. Ear thickness was measured daily with a digital engineer's micrometer (Mitsutoyo, Kawasaki, Japan) from day 0 to day 10 and on day 14 after the first 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:

$$\begin{aligned} \text{Net swelling} = & (\text{right ear thickness} \\ & - \text{left ear thickness})_{\text{at each time point}} \\ & - (\text{right ear thickness} - \text{left ear thickness})_{\text{at 0 h}}. \end{aligned}$$

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

DNA was isolated 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 16S rRNA gene fragments 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 (GCG TGT GTA CAA GAC CC)⁽³¹⁾. U968-GC primer contains 40-nucleotide GC-rich sequence (GC clamp) at 5' end. Denaturing-gradient gel electrophoresis (DGGE) analysis of the amplicon was performed as previously described⁽²⁴⁾. Quantity One software (version 4.6.0; Bio-Rad, Hercules, CA, USA) 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 unweighted pair-group method using the arithmetic average clustering method in Quantity One software as previously described⁽³²⁾.

Quantification of bifidobacteria in faeces

Populations of bifidobacteria in faecal samples were determined by conventional culture method. Briefly, the fresh samples were diluted tenfold with anaerobic phosphate buffer, and then 50 µl of each dilution were inoculated onto bifidobacteria-selective BIM agar⁽²⁶⁾. Anaerobic incubation was performed at 37°C for 72 h using the AnaeroPack system (Mitsubishi gas, Tokyo, Japan). The number of colonies was counted after incubation and is represented as logarithm of colony-forming units.

Additionally, populations of bifidobacteria were quantified by real-time quantitative PCR (RT-qPCR) as previously described⁽²⁴⁾. Amplification and detection of faecal DNA were performed with the Thermal Cycler Dice Real Time System (Takara). *Bifidobacterium* genus-specific primer pairs (forward: TCG CGT C(C/T)G GTG TGA AAG; reverse: CCA CAT CCA GC(A/G) TCC AC)⁽³³⁾ and *B. pseudolongum* species-specific (IDB41F and IDBC1R as described above)⁽²⁸⁾ primer pairs were used. RT-qPCR 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 of the faecal DNA samples. The reaction conditions were: 95°C for 10 s, followed by forty cycles at 95°C for 5 s, 64°C for 30 s for the quantification of bifidobacteria; and 95°C for 10 s, followed by forty cycles at 95°C for 5 s, 60°C for 30 s for the quantification of *B. pseudolongum*. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was performed after amplification to distinguish the targeted PCR product from the non-targeted PCR product. All samples were analysed in duplicate.

B. pseudolongum subsp. *pseudolongum* (JCM1264) was cultured in BIM broth, and the genomic DNA was extracted by PrepMan Ultra reagent according to the manufacturer's instructions. The 16S rRNA gene fragment was amplified by PCR with the *B. pseudolongum* species-specific primer pair (IDB41F and IDBC1R as described above)⁽²⁸⁾. The amplicons were purified by QIAquick PCR purification kit (Qiagen, 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 the cells were plated onto Luria-Bertani agar plates supplemented with 25 µg/ml ampicillin, 30 µg/ml X-gal and 20 µg/ml IPTG, and incubated overnight at 37°C. White transformants were picked and grown in Luria-Bertani broth. Plasmid DNA was extracted with a QIAprep Spin Miniprep kit (Qiagen) and used as a standard for RT-qPCR.

Antibody ELISA

Serum levels of IgG1 and IgG2a specific to the hapten were determined by ELISA as previously described⁽²⁴⁾. In brief, each well of the ninety-six-well microtitre plates was coated with dinitrophenol-bovine serum albumin (Calbiochem, San Diego, CA, USA). After blocking the wells, serial dilutions of serum were added and then incubated for 2 h at room

temperature. Horseradish peroxidase-conjugated rat anti-mouse IgG1 (clone LO-MG1-2, Zymed Laboratories, South San Francisco, CA, USA) or rat anti-mouse IgG2a (clone LO-MG2a-3, Zymed Laboratories) was added and incubated at 37°C for 2 h. Plates were developed at room temperature after the addition of *o*-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.016 %).

Measurement of in vitro cytokine production in cervical lymph node cells

The excised CLN was gently homogenised with RPMI-1640 medium (GIBCO-BRL, Tokyo, Japan) supplemented with 2 % heat-inactivated fetal calf serum (GIBCO-BRL), 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin, and cells were filtered through nylon mesh (Nippon Rikagaku Kikai, Tokyo, Japan) and centrifuged for 5 min at 300g. To remove erythrocytes, the cell pellet was resuspended in 10 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol-HCl buffer (pH 7.4) supplemented with 0.24 M NH₄Cl and incubated for 5 min at room temperature, followed by washing twice with RPMI-1640 medium supplemented with 2 % fetal calf serum. Thereafter, cells were counted by a Z1 coulter particle counter (Yamato Scientific, Tokyo, Japan), and an equal number of cells isolated from each mouse were pooled in each group. The cells (2×10^5 cells in 0.2 ml) were cultured in round-bottomed ninety-six-well plates (Greiner Bio-One, Tokyo, Japan) in RPMI-1640 medium supplemented with 10 % fetal calf serum and 500 µg/ml dinitrobenzene sulphonic acid sodium salt at 37°C in a 5 % CO₂ atmosphere. Following 3-d-culture, the conditioned medium was collected and subjected to ELISA for cytokines. ELISA was performed in ninety-six-well microtitre plates. Wells were coated overnight at 4°C with primary antibodies (clones XMG1.2 and JES5-16E3 for IFN-γ and IL-10, respectively, eBioscience, San Diego, CA, USA). After blocking with RPMI-1640 medium supplemented with 10 % fetal calf serum at room temperature for 1 h, serial dilutions of conditioned medium, recombinant mouse interferon (IFN)-γ (eBioscience) and recombinant mouse IL-10 (eBioscience) were added and

incubated for 2 h at room temperature. Thereafter, biotin-conjugated secondary antibodies (clones R4-6A2 and JES5-2A5 for IFN-γ and IL-10, respectively, eBioscience) were added and incubated for 1 h at room temperature. Streptavidin-horseradish peroxidase (Zymed Laboratories) was then added and incubated for 30 min at room temperature. Plates were developed at room temperature after the addition of 0.2 M citrate buffer (pH 4.0) containing 0.0123 mg/ml 3,3',5,5'-tetramethylbenzidine and 0.01044 % hydrogen peroxide. Finally, 1 M H₂SO₄ was added, and absorbance was measured at 450 nm with a microplate reader.

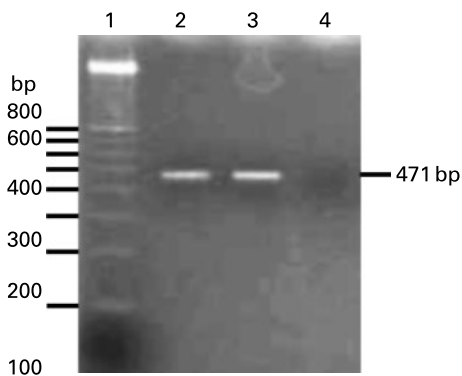


Fig. 1. Representative ethidium bromide staining of PCR products separated on 3 % agarose gel. Lane 1, 100 bp ladder; lane 2, amplicon using DNA from isolated bacterium as template; lane 3, amplicon using DNA from *Bifidobacterium pseudolongum* subsp. *pseudolongum* (JCM 1246) as template; lane 4, negative control without template DNA.

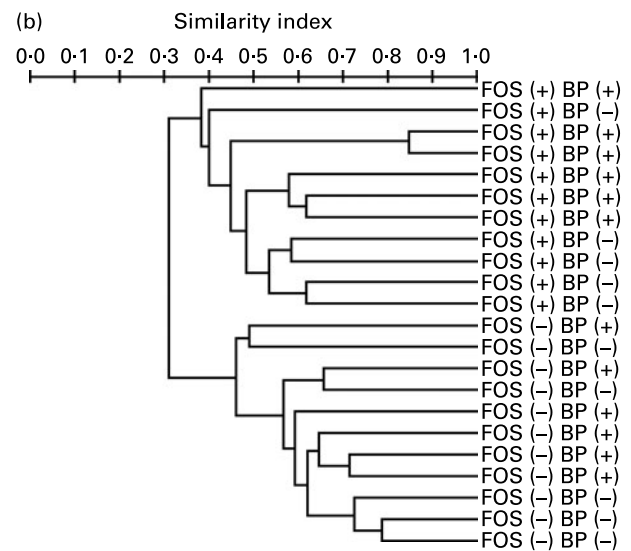
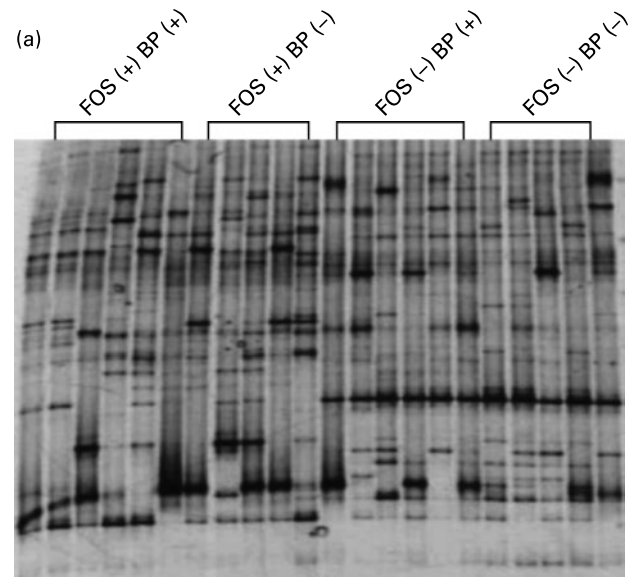
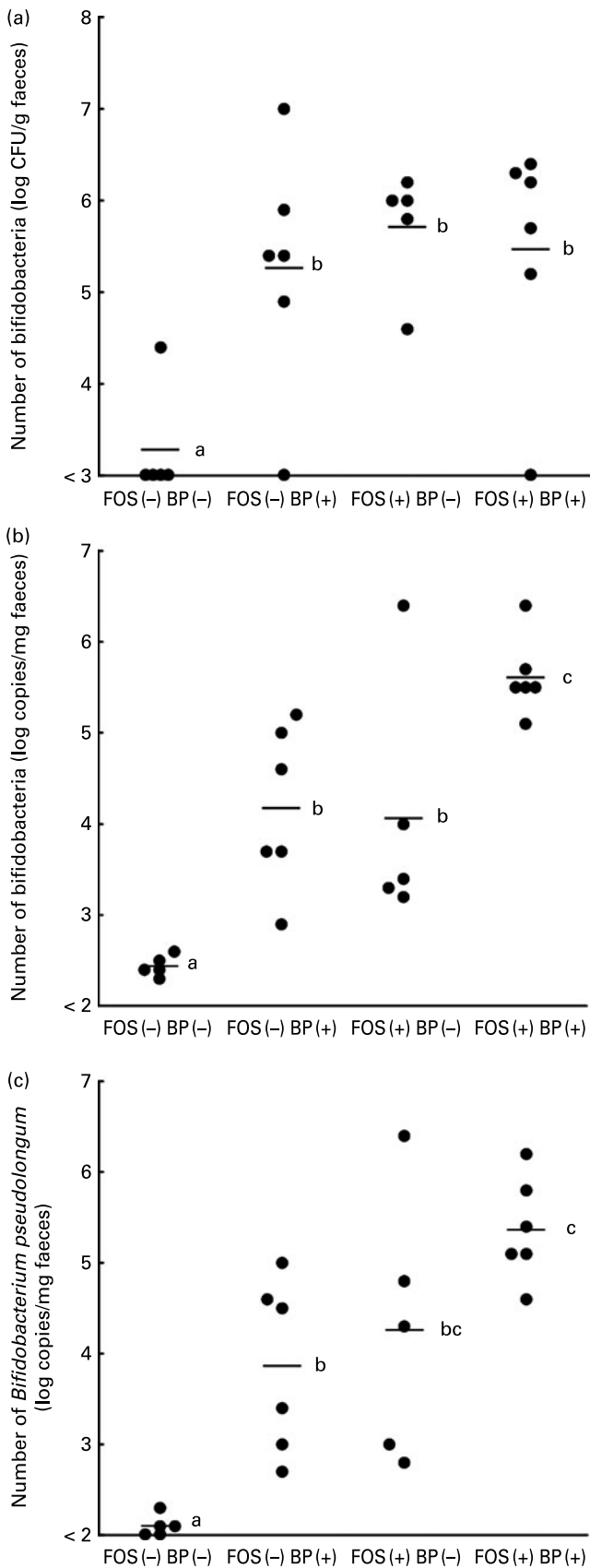


Fig. 2. PCR-denaturing-gradient gel electrophoresis (DGGE) analysis of faecal microbiota based on 16S rRNA gene sequences in BALB/c mice-fed fructo-oligosaccharide (FOS) and *Bifidobacterium pseudolongum* (BP). DGGE gel image stained with SYBR green is shown in chart (a) Similarities among DGGE band profiles of faecal bacteria of mice were calculated based on the position and intensity of bands, and the dendrogram of DGGE band profiles constructed by the unweighted pair-group method using the arithmetic average clustering method is shown in chart (b) Each lane in the gel and each line in the dendrogram represent an individual mouse. Distances are measured in arbitrary units.



Statistical analysis

Results are presented as means with their standard errors. Tukey–Kramer’s test following two-way ANOVA was used to compare mean values. Data analysis was performed with StatView for Macintosh (version 5.0, Statistical Analysis Systems Institute Inc., Spring Valley, CA, USA). *P* values less than 0.05 were considered statistically significant.

Results

Isolation of *Bifidobacterium pseudolongum* from mouse faeces

A Gram-positive obligate anaerobe, in the shape of a club or jelly bean, was isolated from the faeces of mice-fed FOS. DNA isolated from this bacterial sample and *B. pseudolongum* subsp. *pseudolongum* (JCM1264) were subjected to PCR with *B. pseudolongum*-specific primers. Fig. 1 shows the PCR amplicons separated on 3% agarose gel. The size of the amplicons was consistent with the predicted size (471 bp). In addition, the nearly complete 16S rRNA gene sequence (1420 bp) exhibited 99% similarity to *B. pseudolongum* subsp. *pseudolongum* (GenBank accession number, AY174106; Supplementary Fig. 1; the supplementary material for this article can be found at <http://www.journals.cambridge.org/bjn>). These findings indicate that the isolated bacterium is identical to *B. pseudolongum*.

Effect of fructo-oligosaccharide and *Bifidobacterium pseudolongum* on faecal microbiota

The diversity of faecal microbiota in mice was compared by PCR–DGGE analysis based on 16S rRNA gene sequences. A representative DGGE band profile is shown in Fig. 2(a). The intensity and position of detected bands were subjected to cluster analysis. The dendrogram shows two large clusters of FOS(+) and FOS(-) groups (Fig. 2(b)). The results indicate that dietary supplementation with FOS modulated the composition of gut microbiota in mice, being consistent with our previous study⁽²⁴⁾. In contrast, BP(-) and BP(+) groups showed no clear cluster. Thus, it appears that administration of *B. pseudolongum* had no influence on the global composition of gut microbiota in mice.

Effect of fructo-oligosaccharide and *Bifidobacterium pseudolongum* on faecal bifidobacteria

Faecal bifidobacteria were quantified using a conventional cultivation method (Fig. 3(a)). Two-way ANOVA showed that

Fig. 3. Populations of bifidobacteria in the faeces of BALB/c mice-fed fructo-oligosaccharide (FOS) and *Bifidobacterium pseudolongum* (BP). Bifidobacteria (charts (a) and (b)) and *B. pseudolongum* (chart (c)) were quantified by a conventional culture method (chart (a)) and real-time quantitative PCR (charts (b) and (c)). Each ● represents the value for an individual mouse, and horizontal bars represent mean values. In chart (a), *P* values estimated by two-way ANOVA were 0.0281, 0.0281 and 0.0618 for FOS, BP and their interaction, respectively. Similarly, *P* values in chart (b) were 0.0016, 0.0016 and 0.6903 for FOS, BP and their interaction, respectively. *P* values in chart (c) were 0.0002, 0.0017 and 0.4095 for FOS, BP and their interaction, respectively. Mean values with unlike letters were significantly different ($P < 0.05$), as estimated by Tukey–Kramer’s test.

both FOS ($P=0.0281$) and *B. pseudolongum* ($P=0.0281$) increased the faecal excretion of bifidobacteria. The faecal bifidobacterial levels were significantly lower in the FOS(-)BP(-) group than in the other three groups. Only one out of the five mice in the FOS(-)BP(-) group had a detectable level of bifidobacteria in the faeces. There were no significant differences among the other three groups. In addition, RT-qPCR with *Bifidobacterium* genus-specific primers showed that both FOS ($P=0.0016$) and *B. pseudolongum* ($P=0.0016$) increased the faecal excretion of bifidobacteria (Fig. 3(b)). The bifidobacterial levels were significantly lower in the FOS(-)BP(-) group than in the other three groups, and the levels were significantly higher in the FOS(+)BP(+) group than in the FOS(-)BP(+) and FOS(+)BP(-) groups. There was no significant difference between the FOS(-)BP(+) group and the FOS(+)BP(-) group. Furthermore, faecal *B. pseudolongum* levels quantified by RT-qPCR with species-specific primers in each group were roughly comparable with the respective levels of bifidobacteria quantified by RT-qPCR with *Bifidobacterium* genus-specific primers (Fig. 3(c)).

Effect of fructo-oligosaccharide and *Bifidobacterium pseudolongum* on contact hypersensitivity

Ear swelling began to be detected 5 d after the first application of DNFB (Fig. 4). At that time, two-way ANOVA showed that both FOS and *B. pseudolongum* significantly affected ear swelling (Table 1). Thus, ear swelling was significantly higher in the FOS(-)BP(-) group than in the other three groups, and there were no significant differences among the three groups. From 6 d after the first application of DNFB to 3 d after the second application of DNFB, FOS, but not *B. pseudolongum*, affected ear swelling. On days 6 and 7

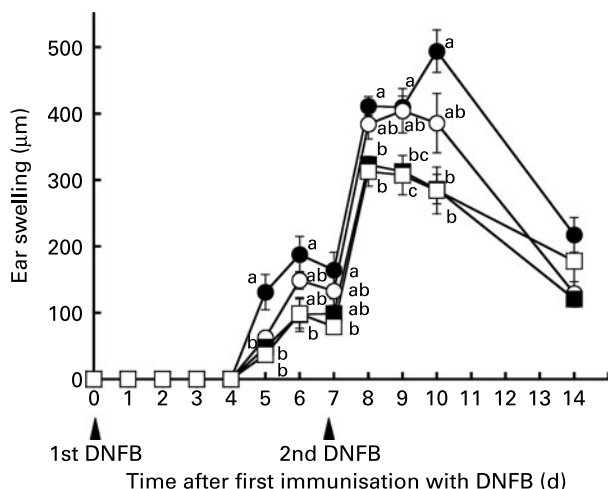


Fig. 4. Temporal changes in ear swelling after starting 2,4-dinitrofluorobenzene (DNFB) application in BALB/c mice-fed fructo-oligosaccharide (FOS) and *Bifidobacterium pseudolongum* (BP). Two weeks after feeding the indicated diet, DNFB was applied to the ear auricle twice at 7-d intervals (▲). Results are presented as means with their standard errors, n 5–6. Mean values with unlike letters at each time point were significantly different ($P < 0.05$) as estimated by Tukey–Kramer's test. Two-way ANOVA was summarised in Table 1. ●, FOS(-)BP(-); ○, FOS(-)BP(+); ■, FOS(+)BP(-); □, FOS(+)BP(+).

after the first application of DNFB, ear swelling was significantly lower in the FOS(+)BP(+) group than in the FOS(-)BP(-) group, and the other two groups were intermediate. On days 1, 2 and 3 after the second application of DNFB, ear swelling was significantly lower in the FOS(+)BP(+) and FOS(+)BP(-) groups than in the FOS(-)BP(+) and FOS(-)BP(-) groups.

Effect of fructo-oligosaccharide and *Bifidobacterium pseudolongum* on serum antibody levels

There was no detectable hapten-specific antibody in the sera of mice without DNFB treatment (data not shown). At 25 d after the first application of DNFB, all the mice produced detectable levels of hapten-specific IgG1 and IgG2a antibodies. Two-way ANOVA showed that *B. pseudolongum* ($P=0.0247$), but not FOS ($P=0.3871$), increased hapten-specific IgG1 levels (Fig. 5(A)). In addition, FOS ($P=0.0002$), but not *B. pseudolongum* ($P=0.7946$), decreased hapten-specific IgG2a levels (Fig. 5(B)). The IgG2a levels were significantly lower in the FOS(+)BP(-) and FOS(+)BP(+) groups than in the FOS(-)BP(-) and FOS(-)BP(+) groups.

Effect of fructo-oligosaccharide and *Bifidobacterium pseudolongum* on in vitro cytokine production in cervical lymph node cells

CLN cells isolated from each mouse were pooled in each group and restimulated *in vitro* with hapten for 3 d (Fig. 6). The concentration of IFN- γ in the conditioned medium tended to be higher in the FOS(-)BP(-) group than in the other three groups. In contrast, the FOS(-)BP(-) group showed an undetectable level of IL-10. The concentration of IL-10 tended to be increased by *B. pseudolongum* and was the highest in the FOS(+)BP(+) group.

Discussion

Our previous study demonstrated that dietary supplementation with FOS reduced the DNFB-induced CHS response in BALB/c mice⁽²⁴⁾. Additionally, analyses of gut microbiota raised the possibility that *B. pseudolongum* proliferation in the gastrointestinal tract was responsible for the inhibitory effect of dietary FOS on the DNFB-induced CHS response. These findings suggest that FOS is a prebiotic oligosaccharide that could prevent allergic contact dermatitis. Because we successfully isolated viable *B. pseudolongum* from the faeces of mice-fed FOS, the present study addressed whether this bacterium is responsible for the inhibitory effect of dietary FOS on DNFB-induced CHS response in mice.

In the present study, conventional culture-dependent analysis showed that dietary supplementation with FOS and intra-gastric administration of *B. pseudolongum* resulted in higher excretion of viable bifidobacteria in the faeces of mice. Likewise, molecular biological culture-independent analysis, i.e. RT-qPCR, showed that FOS and *B. pseudolongum* increased the faecal excretion of bifidobacteria, mainly *B. pseudolongum*. The data suggest that the mouse intestine was exposed to a higher population of viable *B. pseudolongum* by dietary supplementation with FOS and by oral administration of *B. pseudolongum*.

Table 1. Summary of two-way ANOVA *P* values from an analysis of temporal changes in ear swelling, after starting 2,4-dinitrofluorobenzene application, in BALB/c mice-fed fructo-oligosaccharide (FOS), *Bifidobacterium pseudolongum* (BP), and FOS and BP

	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 14
FOS	0.0033	0.0057	0.0087	0.0003	0.0007	0.0002	0.3762
BP	0.0257	0.4196	0.2219	0.3204	0.3204	0.1256	0.5624
FOS×BP	0.0922	0.3917	0.7812	0.6387	0.6383	0.1368	0.0126

FOS(+)/BP(+) mice showed reduced ear swelling in response to topical application of DNFB when compared with vehicle-administered mice-fed FOS(-) diet (i.e. FOS(-)/BP(-)). Although FOS(+)/BP(-) mice also showed a similarly reduced extent of ear swelling as FOS(+)/BP(+) mice, the inhibitory action of *B. pseudolongum* administration without FOS feeding (i.e. FOS(-)/BP(+)) was limited largely to the initial phase of the CHS response (i.e. on day 5 after the first application of DNFB). Because faecal excretion levels of bifidobacteria, mainly *B. pseudolongum*, in FOS(-)/BP(+) group were comparable with those in FOS(+)/BP(-) group as described above, we suppose that *B. pseudolongum* proliferation in the gastrointestinal tract might be partially responsible for the inhibitory effect of dietary FOS on DNFB-induced CHS response in mice. In other words, the proliferation of other bacterial species than bifidobacteria in the intestinal tract of mice-fed FOS might also contribute to the reduction in DNFB-induced CHS response. Indeed, PCR-DGGE analysis of faecal 16S rRNA gene indicated that dietary supplementation with FOS, but not administration of *B. pseudolongum*, influenced the global composition of gut microbiota in mice. Therefore, it is of interest to search for the bacterial species or strains that prevent the CHS response. Chapat *et al.*⁽³⁴⁾ demonstrated that oral administration of *Lactobacillus casei* DN-114 001, a strain used for the preparation of fermented milk, reduced the DNFB-induced CHS response in C57BL/6 mice.

Aberrant regulation between IFN- γ -producing Th1 cells and IL-4-producing Th2 cells is considered important in the development of immune diseases⁽³⁵⁾. For example, CHS responses are coupled with excessive Th1 cells and also IFN- γ -producing CD8⁺ (Tc1) cells^(36,37). In CHS responses, allergen re-exposure in the sensitised tissue results in the production of inflammatory cytokines by epidermal cells, which recruit hapten-specific T cells. Infiltrating T cells releases inflammatory cytokines such as IFN- γ that stimulate keratinocytes to produce chemokines. This in turn results in further recruitment of inflammatory cells, including monocytes and non-antigen-specific T cells. Ultimately, inflammation reaches its maximum, characterised by (epi)dermal infiltrates, oedema and spongiosis⁽³⁸⁾. In order to elucidate whether CHS-reducing action of dietary FOS is mediated by altered response of T cells that produce inflammatory cytokines, the present study examined the IFN- γ production in CLN cells *in vitro*. Dietary FOS reduced the IFN- γ production in CLN cells in response to hapten restimulation. Because oral administration of *B. pseudolongum* also reduced the IFN- γ production, these results suggest that CHS-reducing action of dietary FOS is associated with reduced response of IFN- γ -producing T cells, which might be mediated, at least in part, by increased proliferation of *B. pseudolongum* in the gastrointestinal tract. In addition, serum levels of IgG2a (i.e. the prototypic antibody of Th1-driven B-cell responses in mice) and IgG1 (i.e. the prototypic antibody of Th2-driven B-cell responses in mice)

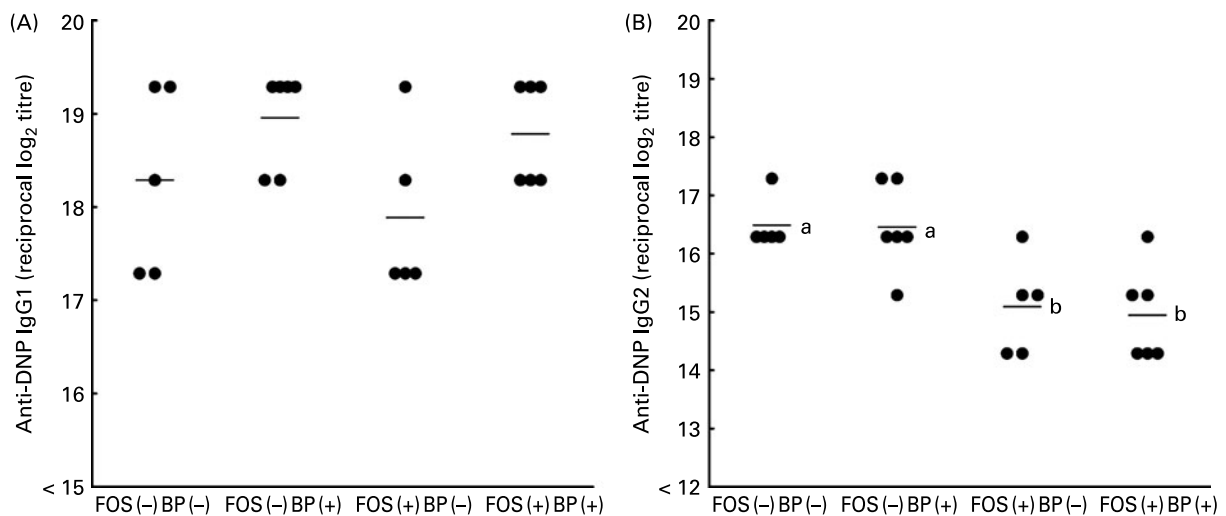


Fig. 5. Serum concentrations of haptenspecific IgG1 (chart A) and IgG2a (chart B) in BALB/c mice-fed fructo-oligosaccharide (FOS) and *Bifidobacterium pseudolongum* (BP). Each ● represents the value for an individual mouse, and horizontal bars represent mean values. In chart (A), *P* values estimated by two-way ANOVA were 0.3871, 0.0247 and 0.7194 for FOS, BP and their interaction, respectively. Similarly, *P* values in chart (B) were 0.0002, 0.7946 and 0.8758 for FOS, BP and their interaction, respectively. ^{a,b}Mean values with unlike letters were significantly different (*P* < 0.05; Tukey-Kramer's test).

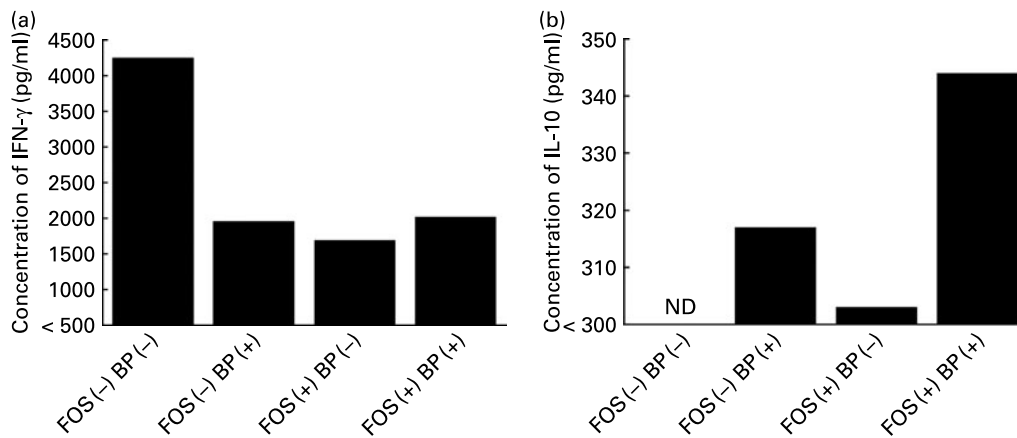


Fig. 6. Cytokine production in cervical lymph node cells isolated from BALB/c mice fed fructo-oligosaccharide (FOS) and *Bifidobacterium pseudolongum* (BP). Cells isolated from individual mice were pooled in each group and restimulated with hapten for 3 d *in vitro*. Concentrations of interferon (IFN)- γ (chart (a)) and IL-10 (chart (b)) in the conditioned medium were measured by ELISA.

were also measured as indicative parameters for Th1/Th2 balance. Dietary supplementation with FOS reduced Th1 response as indicated by reduced IgG2a levels, while administration of *B. pseudolongum* increased Th2 response as indicated by increased IgG1 levels. The data suggest that dietary FOS and administration of *B. pseudolongum* differently influence the Th1/Th2 balance. In other words, other bacterial species than bifidobacteria might contribute to the modulation of cytokine production. Furthermore, the present results are consistent with Chapat *et al.*⁽³⁴⁾ who reported that hapten-specific IgG2a levels in sera and IFN- γ production in splenic CD8⁺T cells *in vitro* were lower in mice-fed *L. casei* DN-114 001. These findings suggest that probiotics and prebiotics exert immunoregulatory actions influencing hapten-specific T cells in DNFB-induced CHS response in mice.

de Waard *et al.*^(39,40) reported that administration of *L. casei* Shirota YIT9029 up-regulated Th1-type immune responses including IgG2b (i.e. the prototypic antibody of Th1-driven B-cell responses in rats) production and delayed-type hypersensitivity reaction in response to infection with *Trichinella spiralis* and *Listeria monocytogenes* in rats and mice. Additionally, Vos *et al.*⁽⁴¹⁾ reported that dietary supplementation with a mixture of galacto-oligosaccharide and FOS promoted Th1-type immune responses in a murine vaccination model. In terms of Th1/Th2 balance, these studies are contradictory to Chapat *et al.*⁽³⁴⁾ and the present results as described above. Thus, probiotics and prebiotics may have both adjuvant properties on immune defence, via up-regulating Th1 responses, and anti-inflammatory actions that prevent CHS responses, via down-regulating Th1 responses. Furthermore, the direction of the immune responses may depend on the bacterial strains relevant to the actions.

However, more recent studies have proposed novel Th cell subsets, i.e. IL-17-producing Th cells (Th17 cells) and regulatory T cells, as key players in the regulation of immune responses and the development of immune diseases⁽⁴²⁾. In particular, Nakae *et al.*⁽⁴³⁾ demonstrated that IL-17-producing T cells rather than IFN- γ -producing Th1 cells are important for the development of CHS in mice. Additionally, He *et al.*⁽⁴⁴⁾ showed that IL-17-producing CD8⁺ (Tc17) cells, but not IFN- γ -producing Tc1 cells, are a central player in

effector functions at the elicitation of CHS responses in mice. In the present study, however, CLN cells produced undetectable levels of IL-17 in response to hapten stimulation *in vitro* (data not shown). In contrast, IL-10 is a cytokine produced by regulatory T cells and reportedly associated with termination of CHS responses by suppressing the response of inflammatory cytokines such as IFN- γ ⁽³⁷⁾. The present study showed a higher production of IL-10 in CLN cells isolated from FOS(-)BP(+) and FOS(+)BP(+) mice, suggesting that CHS-reducing action of dietary FOS is associated with increased production of IL-10, which might be mediated, at least in part, by increased proliferation of *B. pseudolongum* in the gastrointestinal tract. Thus, modulation of antigen-induced production of inflammatory and regulatory cytokines might be involved in the inhibitory effect of gut microbiota on CHS response, although further studies remain to be performed, especially regarding IL-17, a possible key player in CHS responses.

In conclusion, we propose that *B. pseudolongum* proliferation in the gastrointestinal tract is partially responsible for the reduction in DNFB-induced CHS response by dietary supplementation with FOS in mice.

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