

Reduction of allergic airway eosinophilia by dietary raffinose in Brown Norway rats

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Oral administration of raffinose, a naturally occurring indigestible oligosaccharide, has reportedly ameliorated atopic dermatitis in human subjects although the mechanism is unknown. The present study investigated the effect of dietary raffinose on allergen-induced airway eosinophilia in ovalbumin-sensitised Brown Norway rats as an atopic disease model. Brown Norway rats were immunised by subcutaneous injection with ovalbumin on day 0 and fed either a control diet or the diet supplemented with raffinose (50 g/kg diet). The rats were exposed to aerosolised ovalbumin on day 20, and broncho-alveolar lavage fluid was obtained on the next day. The number of eosinophils in the fluid was significantly lower in the rats fed the raffinose diet than in those fed the control diet. Dietary raffinose significantly reduced IL-4 and IL-5 mRNA levels in lung tissue and tended to lower ovalbumin-specific Ig E levels. Suppression of eosinophilia by dietary raffinose was still observed in caecotomised and neomycin-administered rats, suggesting little contribution by the colonic bacteria to the effect of raffinose. Intraperitoneal administration of raffinose also suppressed eosinophilia. Significant concentrations of raffinose were detected in portal venous and abdominal arterial plasma after the intragastric administration of raffinose. Overall, the findings suggest that dietary raffinose ameliorates allergic airway eosinophilia at least partly via post-absorptive mechanisms in Brown Norway rats.

Raffinose: Eosinophilia: Allergy: Ovalbumin: Broncho-alveolar lavage fluid

Raffinose (β -D-fructofuranosyl- O - α -D-galactopyranosyl-(1,6)- α -D-glucopyranoside) is an indigestible oligosaccharide that naturally occurs in many kinds of vegetables and fruits. A series of studies have suggested that raffinose should be considered as a prebiotic agent. Benno *et al.* (1987) have reported that the oral administration of raffinose increases bifidobacteria and decreases bacteroides and clostridia in human faeces. In addition, we have observed that dietary raffinose increases bifidobacteria and lactobacilli in the rat caecum (T Nagura, unpublished results). Furthermore, it has been shown that dietary raffinose decreases faecal putrefactive products such as NH₃, *p*-cresol and indole and improves the defecation frequency in human subjects (Fujisaki *et al.* 1994; Nagura *et al.* 1999). More interestingly, we have observed that the oral administration of raffinose in patients with atopic dermatitis improves symptoms such as eczema and pruritus (Matsuda *et al.* 1998). However, the mechanism by which orally administered raffinose ameliorates atopic dermatitis is unclear.

Clearly, immunological factors contribute to the pathogenesis of atopic dermatitis (Leung & Bieber, 2003). Because there is increasing evidence that prebiotics can

modulate various properties of the immune system (Schley & Field, 2002), an improvement of atopic dermatitis by raffinose could possibly be due to the immunomodulating effects of raffinose. It has been thought that the immunomodulating effects of prebiotics are mainly mediated by lactic acid bacteria, such as lactobacilli and bifidobacteria (Schley & Field, 2002). Actually, the administration of lactic acid bacteria (i.e. probiotics) has been reported to control the clinical symptoms of allergic diseases in human subjects. Administering *Lactobacillus* GG to pregnant and lactating mothers has been reported to reduce the risk of developing atopic eczema in infants (Kalliomäki *et al.* 2001; Rautava *et al.* 2002). In addition, *Lactobacillus* GG and *Bifidobacterium lactis* have reportedly alleviated atopic eczema in children (Majamaa & Isolauri, 1997; Isolauri *et al.* 2000). We therefore hypothesised that the mechanism underlying the improvement of atopic dermatitis by the oral administration of raffinose is associated with the immunomodulating effects of the intestinal microflora.

The Brown Norway (BN) rat model of allergic sensitisation has been extensively characterised and has several inflammatory and immunological features that resemble

Abbreviations: BALF, broncho-alveolar lavage fluid; BN, Brown Norway; DIG, digoxigenin; GAPDH, glyceraldehyde triphosphate dehydrogenase; IFN, interferon; OVA, ovalbumin; PBS-BT, PBS containing bovine serum albumin and Tween-20; Th, T-helper.

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those of asthma, including airway eosinophilia, the development of bronchial hypersensitivity, and the expression of cytokines such as IL-4 and IL-5 in the allergen-exposed sensitised lung (Elwood *et al.* 1991; Haczku *et al.* 1996). Because most patients with atopic dermatitis have eosinophilia in the peripheral blood and chronic skin lesions (Leung & Bieber, 2003), it would be rational to use the BN rat model of allergic airway eosinophilia to investigate the mechanism by which raffinose ameliorates atopic dermatitis. To test our hypothesis mentioned earlier, therefore, we first examined whether dietary raffinose could prevent airway eosinophilia in actively sensitised BN rats. The present study used ovalbumin (OVA) to sensitise the rats since this protein has been very popular as a model allergen. Because atopic diseases are associated with increased serum Ig E, we measured serum levels of OVA-specific Ig E in BN rats. The production of Ig E is regulated by T-helper (Th) cells, which have been classified into Th1 and Th2 subtypes, according to observations in mice (Mosmann *et al.* 1986). Th2 cells synthesise IL-4, which enhances Ig E production by B cells (Coffman *et al.* 1986; Del Prete *et al.* 1988). Conversely, Th1 cells synthesise interferon (IFN)- γ , which inhibits the proliferation of Th2 cells (Sher & Coffman, 1992). The present study also measured serum levels of OVA-specific Ig G1 and Ig G2b as Th1- and Th2-dependent immune responses in rats, respectively (Gracie & Bradley, 1996). Moreover, mRNA levels of IL-4 and IL-5 in the allergen-exposed lung were measured since these cytokines promote eosinophil infiltration (Nakajima *et al.* 1992; Coyle *et al.* 1995). The mRNA levels of IFN- γ , which inhibits eosinophil infiltration (Iwamoto *et al.* 1993), were also determined.

If the effect of dietary raffinose on allergic airway eosinophilia is mediated by the intestinal microflora in BN rats, the effect of raffinose should be attenuated by reducing the intestinal microflora. To test this possibility, we intended to reduce the intestinal microflora by the means of caecectomy and administration of neomycin. Otherwise, a post-absorptive mechanism that is independent of the intestinal microflora may be involved in the effect of dietary raffinose. To test this, finally, the effect of intraperitoneal administration of raffinose on allergic airway eosinophilia was examined.

Materials and methods

Animals and diets

Male BN rats (Charles River, Tokyo, Japan), which were 5 weeks old at the start of the experiment, were housed in individual cages in a temperature-controlled ($23 \pm 2^\circ\text{C}$) room with a dark period from 19.00 to 05.00 hours. They were allowed free access to water and to a purified diet prepared according to the composition of the AIN-93G diet (Reeves *et al.* 1993) before the experiment. This diet was used as the control diet. The raffinose diet was prepared by adding raffinose (50 g/kg diet) to the control diet. Raffinose was used as raffinose pentahydrate (Nippon Beet Sugar Mfg., Obihiro, Japan), which was purified from sugar-beet molasses (Sayama *et al.* 1992), and whose purity determined by HPLC was >995 g/kg DM. The supplementation of

raffinose was based on the significant change in caecal microflora in rats when raffinose was given at 50 g/kg diet (T Nagura, unpublished results). Supplementation of raffinose at this level did not alter the physical characteristics (i.e. texture) of the diet.

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

Experimental design

In experiment 1, after 7 d of consuming the control diet, twelve rats were actively immunised with OVA on day 0 as described later and fed for a further 7 d. Rats were then divided into two groups of six animals; one group was fed the control diet and the other group the raffinose diet. All rats were challenged by exposure to aerosolised OVA on day 20, and broncho-alveolar lavage fluid (BALF) and lung tissue were obtained on the next day as described later.

In experiment 2, we investigated whether the effect of dietary raffinose on allergic airway eosinophilia was affected by reducing the intestinal microflora in BN rats. After 7 d of consuming the control diet, twenty-four rats were actively immunised with OVA on day 0 and divided into two groups (twelve animals per group) of caecotomised and sham-operated rats. The operations were carried out on day 5 as previously described (Sonoyama *et al.* 1995). In brief, under anaesthesia by an intraperitoneal injection of a mixed solution of ketamine hydrochloride (70 mg/kg body weight; Wako Pure Chemical Industries, Osaka, Japan) and xylazine hydrochloride (8 mg/kg body weight; ICN Biomedicals, Aurora, OH, USA), following a laparotomy, the caecum was isolated from the terminal ileum and ascending colon by ligation and then resected. Only the abdominal cavity of the sham-operated rats was opened and exposed for just 10 min, the same length of time as required for the caecectomy. All rats were not allowed food for the first 18 h postoperatively. Thereafter, the caecotomised rats were orally administered with neomycine sulfate (80 mg/kg body weight; Wako Pure Chemical Industries) daily. Rats were fed the control diet for 7 d after immunisation, and the rats in each operation group were then further divided into two groups (six animals per group) of control-diet- and raffinose-diet-fed rats. All rats were challenged by exposure to aerosolised OVA on day 20, and BALF was obtained on the next day. The colonic content of all animals was obtained and subjected to bacteriological analysis.

Experiment 3 was carried out to elucidate whether raffinose is absorbed in the gastrointestinal tract. After 7 d of consuming the control diet, rats were kept without the diet overnight. The animals were then administered 40% (w/v) raffinose solution (4 g/kg body weight) intragastrically. Blood samples were collected from the portal vein and abdominal aorta after a laparotomy under anaesthesia by an intraperitoneal injection of a mixed solution of ketamine hydrochloride (70 mg/kg body weight) and xylazine hydrochloride (8 mg/kg body weight) and then subjected to the determination of plasma concentration of raffinose.

In experiment 4, we investigated the effect of the route of raffinose administration on the allergic airway eosinophilia in BN rats. After 7 d of consuming the control diet, eighteen rats were actively immunised with OVA on day 0 and fed the control diet for 7 d after immunisation. Rats were then divided into three groups of six animals; two groups were fed the control diet and the other group the raffinose diet. Control-diet-fed rats were intraperitoneally injected with either 0.1 ml raffinose solution (0.5%, w/v) or the vehicle (saline) daily. Raffinose-diet-fed rats were intraperitoneally injected with the vehicle. All rats were challenged by exposure to aerosolised OVA on day 20, and BALF was obtained on the next day.

Immunisation and challenge

Each rat was actively immunised by a subcutaneous injection of 1 mg OVA (grade V; Sigma Chemical Co., St Louis, MO, USA) suspended in 0.5 ml Inject Alum (Pierce, Rockford, IL, USA) into the back of the neck. At the same time, 0.2 ml *Bordetella pertussis* vaccine (Wako Pure Chemical Industries) containing 6×10^9 heat-inactivated bacilli in saline was intraperitoneally administered as an adjuvant. On day 20 after immunisation, the rats were exposed to 1% (w/v) OVA solution aerosolised by an ultrasonic nebuliser (NE-U12; Omron, Tokyo, Japan) for 10 min.

Broncho-alveolar lavage and cell count

At 24 h after the OVA challenge, the animals were anaesthetised by an intraperitoneal injection of a mixed solution of ketamine hydrochloride (70 mg/kg body weight) and xylazine hydrochloride (8 mg/kg body weight). Following a laparotomy, the rats were killed by bleeding from the abdominal aorta, and blood samples were collected for antibody measurement. BALF was obtained and subjected to a cell count as previously described (Watanabe *et al.* 2001). In brief, broncho-alveolar lavage was performed with 5×5 ml Hank's balanced salt solution (GIBCO-BRL, Tokyo, Japan), and the cytospin preparations were subjected to staining with Diff-Quick staining solution (International Reagents, Kobe, Japan). In each sample at least 500 cells were identified according to standard structures such as alveolar macrophages, eosinophils, neutrophils, lymphocytes or other cells. After BALF collection, the lung tissues were then subjected to the isolation of RNA and histological analysis.

Isolation and analysis of RNA

Total RNA was isolated from lung tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA samples were treated with DNase RQ1 (Promega, Madison, WI, USA) to remove any genomic DNA. The samples were subjected to semi-quantitative RT-PCR analysis as previously described (Sonoyama *et al.* 2000). Total RNA (5 µg) was annealed with 0.5 µg oligo (dT)₁₂₋₁₈ primer (GIBCO-BRL) at 70°C for 10 min, and first strand cDNA was

then synthesised in 20 µl solution containing 50 mM-tri (hydroxymethyl)-aminomethane-HCl (pH 8.3), 75 mM-KCl, 3 mM-MgCl₂, 10 mM-dithiothreitol, 0.5 mM-deoxyribonucleotide triphosphate, 25 units RNase inhibitor and 200 units Moloney Murine Leukemia Virus RTase (GIBCO-BRL) at 42°C for 50 min, followed by RNA digestion with RNase H (GIBCO-BRL). The first strand cDNA sample (0.5 µl) was added to 50 µl of a PCR reaction mixture containing 0.5 mM-gene-specific primers as described later, 2 mM-MgCl₂, 0.2 mM-deoxyribonucleotide triphosphate and 1.25 units Ex Taq™ polymerase (Takara, Otsu, Japan). Each cycle of PCR included 1 min of denaturation at 94°C, 1 min of primer annealing at different temperatures for each primer pair and 2 min of extension at 72°C. For semi-quantitative PCR, the kinetics of amplification was studied for each combination of primers in preliminary experiments, and PCR was performed at an exponential range. The PCR products, which were separated by 2% agarose gel electrophoresis, were transferred to a nylon membrane (Biodyne Plus; Pall BioSupport, Port Washington, NY, USA), fixed by UV crosslinking, and the blots then hybridised with each inner oligonucleotide probe labelled with digoxigenin (DIG) using a DIG oligonucleotide tailing kit (Roche Diagnostics, Tokyo, Japan). Prehybridisation, hybridisation and detection were all carried out with a DIG luminescence detection kit (Roche Diagnostics). The hybridisation was performed at 42°C overnight, and post-hybridisation washing was performed with 0.1 × Standard Saline Citrate and 0.1% (w/v) SDS at 60°C for 15 min (three times). The bands developed on X-ray film were quantified using NIH *Image* (National Institutes of Health, Rockville Pike, MD, USA). The signal intensity of each band was normalised by comparing with the intensity of glyceraldehyde triphosphate dehydrogenase (GAPDH). IL-4 cDNA (141 bp) was amplified using the primers 5'-GAACCAGGTGACAGAAAAGG-3' (forward) and 5'-ACATCACGTGGGAGTAAAT-3' (reverse) and detected using the inner probe 5'-TTTGCGAAGCACCCCTGGAAGCCCTGCAGATGAGCTCGTTCTCCGTGGTGTTCCT-3'. IL-5 cDNA (163 bp) was amplified using the primers 5'-CCTACTCATAAAATCACCAG-3' (forward) and 5'-TCTCTTCGCACACTTCTCTT-3' (reverse) and detected using the inner probe 5'-TTTCCACAGTGCCCCCTCGGACAGTTTGATTCTTCAGTATGTCTAGCCCT-3'. IFN-γ cDNA (438 bp) was amplified using the primers 5'-TACTGCCAAGGCACACTCATT-3' (forward) and 5'-CTTATTGGCACACTCTCTACC-3' (reverse) and detected using the inner probe 5'-AGTGATCAGGTGCGATTTCGATGACACTTATGTTGTTGCTGATGGCCTGGTTGTC-3'. GAPDH cDNA (702 bp) was amplified using the primers 5'-GCCATCAACGACCCCTTCATT-3' (forward) and 5'-CGCCTGCTTACCACCTTCTT-3' (reverse) and detected using the inner probe 5'-TGATGACCAGCTTCCCATTCTCAGCCTTGACTGTGCCGTTGAACCTTGCCGTGGG-3'.

Antibody measurement

Serum levels of Ig G1, Ig G2b and Ig E specific to OVA were measured by ELISA as previously described (Watanabe *et al.* 2004). All assays were carried out in ninety-six-well

microtitre plates (Becton Dickinson, Franklin Lakes, NJ, USA). For the detection of OVA-specific Ig G1 and Ig G2b, plates were coated overnight at 4°C with 200 µg OVA/ml in 50 mM-carbonate buffer (pH 9.6). Plates were blocked with PBS containing 10 mg bovine serum albumin/ml (fraction V; Serologicals Proteins Inc., Kankakee, IL, USA) at 37°C for 2 h. Test sera diluted with PBS containing 2 mg bovine serum albumin/ml and 200 µg Tween-20/ml (PBS-BT) were then added and incubated at 37°C for 1 h. After the incubation, horseradish peroxidase-conjugated mouse anti-rat Ig G1 (MARG1-2; Zymed, San Francisco, CA, USA) or mouse anti-rat Ig G2b (MARG2b-8; Zymed) in PBS-BT was added and incubated at 37°C for 1 h. Between each step, the wells were washed five times with PBS containing 0.2 mg Tween-20/ml. Plates were developed at room temperature after the addition of *o*-phenylenediamine (400 µg/ml) and H₂O₂ (0.016%) in citrate-phosphate buffer (pH 5.0). Finally, 1 M-H₂SO₄ was added, and the absorbance at 490 nm was measured with a microplate reader (model 550; Bio-Rad, Hercules, CA, USA).

To detect OVA-specific Ig E, plates were coated overnight at 4°C with mouse anti-rat Ig E (MARE-1; Zymed) in 50 mM-carbonate buffer (pH 9.6). After blocking, diluted serum samples were added and incubated overnight at 4°C. After the incubation, DIG-labelled OVA in PBS-BT was added and incubated at 37°C for 1 h. The coupling of OVA with DIG was carried out using a DIG protein labelling kit (Roche Diagnostics) according to the manufacturer's protocol. Horseradish peroxidase-conjugated anti-DIG Fab fragment (Roche Diagnostics) in PBS-BT was added and incubated at 37°C for 2 h. Washing, colour development and measurement were as described for OVA-specific Ig G1 and Ig G2b.

The absorbance units of the diluted test samples were confirmed empirically as tripled concentrations.

Lung histology

After BALF collection, lung tissues were embedded in O.C.T. compound (Miles Scientific, Elkhart, IN, USA), frozen in liquid N₂ and then stored at -80°C for histological analysis. Frozen sections (6 µm) were prepared with a cryostat, thawed onto glass slides and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min. The sections were then stained with haematoxylin and eosin for light microscopy examination or with periodic acid-Schiff for the evaluation of mucus-producing cells.

Bacteriological analysis of colonic content

Bacteriological analysis of the large intestinal content of rats was carried out according to the method of Mitsuoka *et al.* (1965). Briefly, the fresh samples were diluted in 10-fold steps with anaerobic phosphate buffer. A sample (0.05 ml) of each dilution was inoculated onto two non-selective media (glucose-blood-liver and trypticase-soya agars for anaerobic bacteria and aerobic bacteria, respectively) and one selective medium (lactobacillus selection agar for lactobacillus). Anaerobic incubation was carried out at 37°C for 48 h by the steel-wool method, and aerobic

incubation was done at 37°C for 24–48 h. The number of colonies was counted after the incubation.

Determination of raffinose concentrations in plasma

Plasma concentrations of raffinose were determined by HPLC with pulsed amperometric detection. The detector used was a pulsed electrochemical detector-2 (DIONEX, Sunnyvale, CA, USA), the column was a CarboPac PA1 (DIONEX) and the eluate was 150 mM-NaOH.

Statistical analysis

Results are expressed as means and standard errors of the mean. Significance was evaluated by unpaired *t* tests or two-way ANOVA followed by *post hoc* Fisher's protected least significant difference test at *P* < 0.05. The statistical calculations were carried out using StatView 5.0 computer software (SAS Institute, Inc., Cary, NC, USA).

Results

Almost all cells in the BALF of normal animals are alveolar macrophages. Our preliminary experiments showed that more than 95% of total cells in the BALF of both non-immunised OVA-exposed rats and OVA-immunised bovine serum albumin (i.e. unrelated protein)-exposed rats were alveolar macrophages (data not shown). In the OVA-immunised and OVA-exposed rats in the present study, however, approximately 45–60% of total cells in BALF were eosinophils (Figs. 1, 4 and 6), indicating that this treatment induced OVA-specific allergic airway eosinophilia.

In experiment 1, total cell and eosinophil numbers in BALF were significantly lower in the rats fed the raffinose diet than those fed the control diet (Fig. 1 (A)). The number of alveolar macrophages, neutrophils and lymphocytes also tended to be lower in the rats fed the raffinose diet. OVA-specific antibodies were not detected in the plasma of the rats without immunisation (data not shown). At 3 weeks after immunisation, all rats produced detectable levels of OVA-specific Ig G1, Ig G2b and Ig E antibodies. Plasma levels in each antibody subclass were not significantly different between the groups, while Ig E levels tended to be lower in the rats fed the raffinose diet than those fed the control diet (Fig. 1 (B)). In addition, mRNA levels of cytokines in lung tissue were compared between the groups by using RT-PCR and further by Southern hybridisation. Fig. 2 (A) shows the RT-PCR products separated on 2% agarose gel and their corresponding Southern blots. The size of each product was consistent with the predicted size. Under stringent conditions, each individual band was detected by Southern hybridisation of the RT-PCR products with each inner oligonucleotide probe. The findings thus indicated that the RT-PCR products were specific to each mRNA. By semi-quantitative PCR followed by Southern hybridisation using these gene-specific primers and probes, it was shown that IL-4 and IL-5 mRNA levels in the lung tissue of the rats fed the raffinose diet were significantly lower than those of the rats fed the control diet (Fig. 2 (B)). IFN-γ mRNA

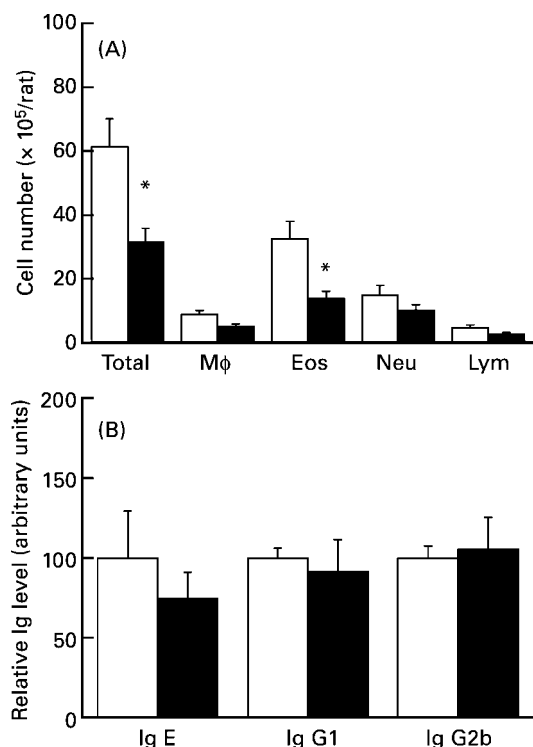


Fig. 1. Effect of dietary raffinose on allergic airway eosinophilia in Brown Norway rats (experiment 1). The rats were immunised by subcutaneous injection with ovalbumin (OVA) on day 0, fed either the AIN-93G diet as a control diet (□) or a diet supplemented with raffinose (50 g/kg diet; ■), challenged by exposure to aerosolised OVA on day 20, and then killed on the next day. (A), Number of total cells (Total) and cell profile in broncho-alveolar lavage fluid; (B), plasma levels of OVA-specific antibodies analysed by ELISA. The antibody levels are expressed relative to the control values, which are taken as 100. Mean values are shown for six rats, with the standard errors of the mean represented by vertical bars. Mφ, Macrophages; Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes. *Mean value was significantly different from that for the control ($P < 0.05$).

levels were, however, the same between the groups. Histology of lung tissue showed that all rats exposed to OVA developed a peribronchovascular inflammation. Similar to the cell numbers in BALF (Fig. 1 (A)), the number of cells of peribronchovascular infiltrates appears to be lower in the rats fed the raffinose diet (Fig. 3 (A)) than those fed the control diet (Fig. 3 (B)). In addition, the number of mucus-producing cells appears to be lower in the rats fed the raffinose diet (Fig. 3 (C)) than those fed the control diet (Fig. 3 (D)). Experiment 1 was repeated four times and similar results were obtained.

Experiment 2 investigated whether the ameliorative action of dietary raffinose on allergic airway eosinophilia was affected by reducing the intestinal microflora in BN rats. Table 1 shows the results of bacteriological analysis of the colonic contents of the sham-operated and the caecectomised and neomycin-administered rats. In the sham-operated rats, the number of anaerobic bacteria in the colon (including caecum) was significantly higher in the rats fed the raffinose diet than in those fed the control diet. According to the gross appearance of the bacterial colony, this change appeared to be due to an increase of

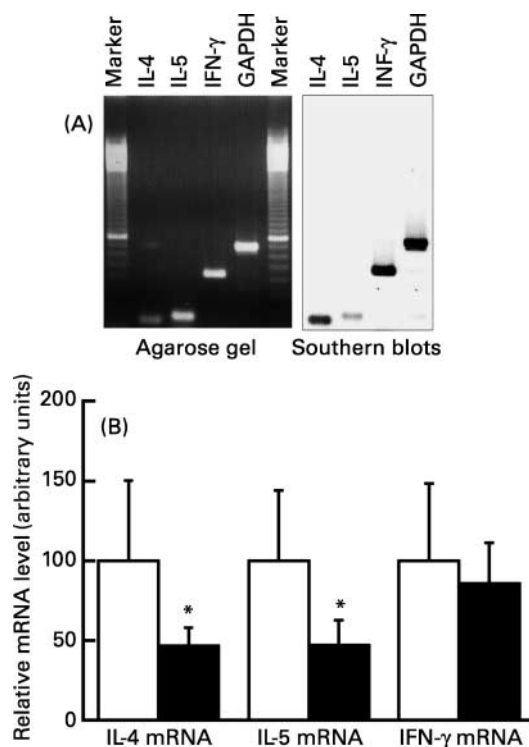


Fig. 2. Effect of dietary raffinose on cytokine mRNA levels in the lungs of Brown Norway rats (experiment 1). Total RNA samples were isolated from lung tissue and analysed by RT-PCR and further by Southern hybridisation using inner oligonucleotide probes. (A), Representative ethidium bromide staining of RT-PCR products separated on 2% agarose gel and their corresponding Southern blots, the marker representing the 100 bp ladder. IFN, interferon; GAPDH, glyceraldehyde triphosphate dehydrogenase. (B), Cytokine mRNA levels in lung tissue determined by semi-quantitative RT-PCR followed by Southern hybridisation. The levels for the raffinose-treated animals (■) are expressed relative to the control (□) values, which are taken as 100. Mean values are shown for six rats, with the standard errors of the mean represented by vertical bars. *Mean value was significantly different from that for the control ($P < 0.05$).

bifidobacteria. The number of aerobic bacteria and lactobacilli was not significantly different between the diet groups in the sham-operated rats. Caecectomy plus neomycin administration significantly reduced the number of anaerobic and aerobic bacteria and lactobacilli in the remaining colon, and there was no significant difference in the number between the diet groups in the caecectomised rats. Fig. 4 shows the total cell number and cell profile in the BALF of rats in experiment 2. In sham-operated animals, the total cell and eosinophil numbers were significantly lower in the rats fed the raffinose diet than those fed the control diet. In the rats fed the control diet, caecectomy tended to reduce the number of total cells and eosinophils. In the rats fed the raffinose diet, the total cell and eosinophil numbers were not significantly different between the sham-operated and caecectomised rats. The number of alveolar macrophages, neutrophils and lymphocytes was not significantly different among the groups. Experiment 2 was repeated three times and similar results were obtained.

To examine whether dietary raffinose is absorbed in the gastrointestinal tract, plasma concentrations of raffinose

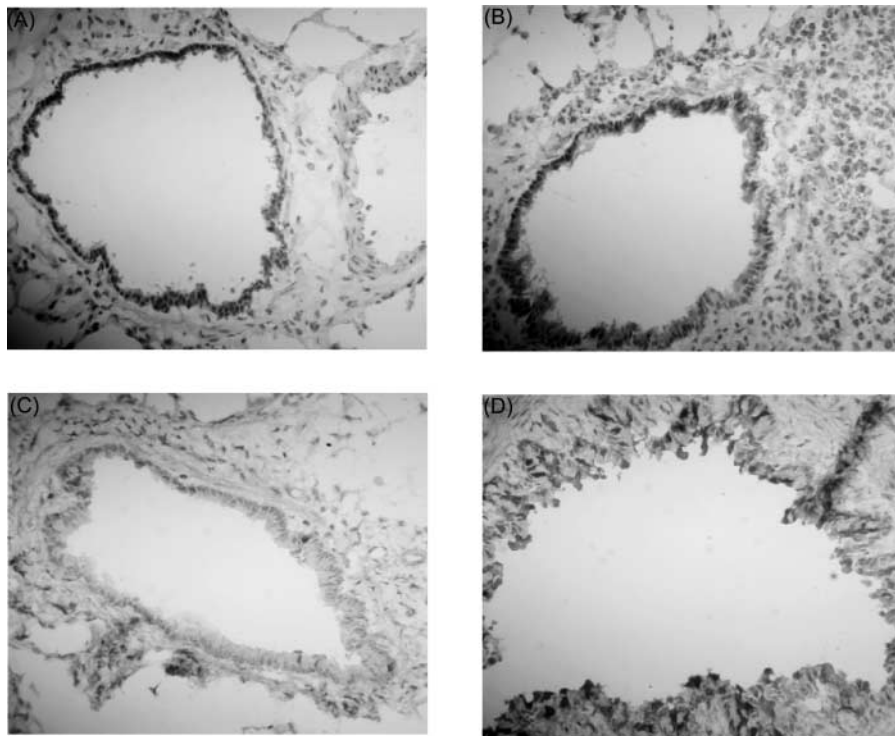


Fig. 3. Effect of dietary raffinose on the histology of lung tissue in Brown Norway rats (experiment 1). Frozen sections ($6\ \mu\text{m}$) of lung were stained with haematoxylin and eosin (A and B) and periodic acid-Schiff (C and D) for the evaluation of inflammatory cell infiltration and mucus-producing cells, respectively. Representative preparations in rats fed the control diet (B and D) and the raffinose diet (A and C) are shown.

were determined after the intragastric administration of raffinose (4 g/kg body weight) in experiment 3. Raffinose was detected at a maximum concentration of approximately $60\ \mu\text{M}$ in portal venous plasma at 60 min after oral administration (Fig. 5). It was first detected within 30 min, reached maximum levels at 60 min and disappeared at 360 min in both portal venous and abdominal arterial plasma. The concentrations were higher in portal venous plasma than in abdominal arterial plasma at 30 and 60 min. These results in experiment 3 were from a single experiment.

Experiment 4 was carried out to clarify whether an intraperitoneal injection of raffinose could also reduce allergic airway eosinophilia. As shown in Fig. 6, an intraperitoneal injection of raffinose significantly lowered the number of eosinophils in the BALF of rats fed the control diet, and the number was similar to that in the rats fed the raffinose diet. Dietary raffinose tended to reduce the number of alveolar macrophages, neutrophils and lymphocytes. Intraperitoneal raffinose also tended to reduce the number of lymphocytes. Experiment 4 was repeated twice and similar results were obtained.

Table 1. Effect of dietary raffinose and caeectomy plus neomycin administration on bacterial counts in rat colon*

(Mean values and standard errors of the mean for six rats)

Diet	Operation	Bacterial count (log no. total content of colon)					
		Anaerobes		Aerobes		Lactobacilli	
		Mean	SEM	Mean	SEM	Mean	SEM
Control	Sham	9.7 ^b	0.2	8.0 ^a	0.0	8.4 ^a	0.6
Raffinose	Sham	10.9 ^a	0.1	8.1 ^a	0.5	9.0 ^a	0.4
Control	Caeectomy	6.1 ^c	0.5	4.7 ^b	1.2	4.0 ^b	0.8
Raffinose	Caeectomy	6.5 ^c	0.5	5.9 ^b	0.4	3.3 ^b	0.8
<i>P</i> value†							
Operation		<0.0001		<0.0001		<0.0001	
Diet		0.0032		0.1145		0.9006	
Operation × diet		0.0909		0.2174		0.1163	

^{a,b,c} Mean values in a column with unlike superscript letters were significantly different ($P < 0.05$).

* The colonic content of rats was subjected to bacteriological analysis, and the number of bacteria was counted as described on p. 250 of proofs.

† Estimated by two-way ANOVA.

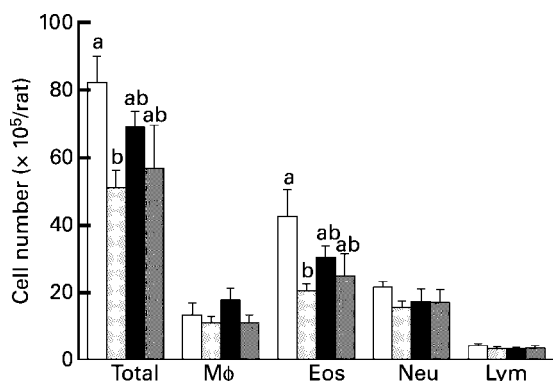


Fig. 4. Effect of caecectomy on the eosinophilia-preventive effect of dietary raffinose in Brown Norway rats (experiment 2). The rats were immunised by subcutaneous injection with ovalbumin (OVA) on day 0, fed either a control diet or a diet supplemented with raffinose, challenged by exposure to aerosolised OVA on day 20, and then killed on the next day. Caecectomies and sham operations were performed on day 5. The number of total cells (Total) and the cell profile in broncho-alveolar lavage fluid are shown. Mean values are shown for six rats, with the standard errors of the mean represented by vertical bars. *P* values for total cells estimated by two-way ANOVA were 0.6878 for operation, 0.0262 for diet and 0.3009 for operation \times diet. *P* values for eosinophils (Eos) estimated by two-way ANOVA were 0.4555 for operation, 0.0208 for diet and 0.1826 for operation \times diet. (□), Sham-operated control rats; (▨), sham-operated raffinose-fed rats; (■), caecectomised control rats; (▩), caecectomised raffinose-fed rats; Mφ, macrophages; Neu, neutrophils; Lym, lymphocytes. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

Discussion

Since the BN rat is a high-Ig E responder strain (Holt & Turner, 1985), the strain has been thought to be suitable for allergy research. In the present study, rats were immunised with OVA via the subcutaneous route with the use of adjuvants, and all rats were successfully sensitised. At this point, one may consider that animals sensitised other than via the enteral route are not suitable to investigate the

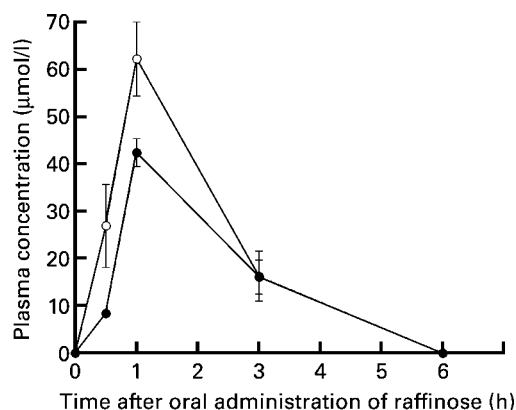


Fig. 5. Changes in time course of raffinose concentrations in portal venous (—○—) and abdominal arterial (—●—) plasma after intragastric administration of raffinose in Brown Norway rats (experiment 3). After keeping the rats without food overnight, the animals were given 40% (w/v) raffinose solution (4 g/kg body weight) by oral administration. Blood samples were then collected after a laparotomy under anaesthesia. Plasma concentrations of raffinose were determined by HPLC. Mean values are shown for three rats, with the standard errors of the mean represented by vertical bars.

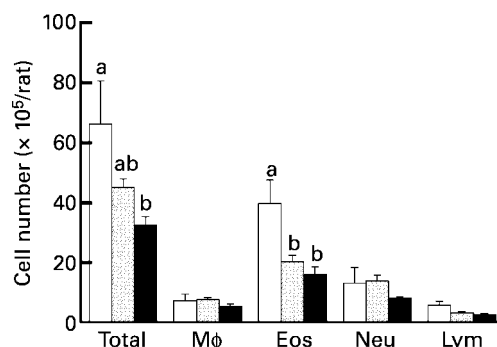


Fig. 6. Effect of intraperitoneal injection of raffinose on allergic airway eosinophilia in Brown Norway rats (experiment 4). The rats were immunised by subcutaneous injection with ovalbumin (OVA) on day 0, fed either a control diet (□) or a diet supplemented with raffinose (▨), challenged by exposure to aerosolised OVA on day 20, and then killed on the next day. One half of the rats fed the control diet were intraperitoneally injected with 0.1 ml raffinose solution (0.5%, w/v) daily (▩), and the other animals belonging to the control and supplemented groups were intraperitoneally injected with the vehicle (saline). The number of total cells (Total) and cell profile in broncho-alveolar lavage fluid are shown. Mean values are shown for six rats, with the standard errors of the mean represented by vertical bars. Mφ, Macrophages; Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

effect of prebiotics on allergic inflammation. However, the intestinal microflora have been known to influence the systemic immune responses beyond gut immunity. It has been demonstrated that the oral administration of *Lactobacillus casei* strain Shirota modified humoral and cellular immune responses to intradermally injected type II collagen (Kato *et al.* 1998). Therefore, we consider that it is not necessarily irrational to use systemically immunised animals to examine whether dietary raffinose modulates allergic inflammation via a prebiotic mechanism.

In the present study, we first demonstrated that dietary raffinose reduced the infiltration of airway eosinophils after exposure to aerosolised OVA in immunised BN rats (Fig. 1 (A)). The histological examination of lung tissue also indicated that dietary raffinose ameliorated peribronchovascular inflammation (Fig. 3). Because atopic diseases are associated with increased serum Ig E, we had expected that dietary raffinose would reduce Ig E and also Ig G1, which is assigned to Th2-dependent immune responses. As shown in Fig. 1 (B), Ig E levels tended to be lower in the rats fed the raffinose diet than in those fed the control diet. We therefore considered that this model could be useful to investigate the mechanisms by which dietary raffinose improved atopic dermatitis in human subjects (Matsuda *et al.* 1998).

One may speculate that oral tolerance is involved in the reduction of allergic airway eosinophilia by dietary raffinose. If that is the case, systemic immune responses against the allergen should be totally suppressed. As shown in Fig. 1 (B), however, plasma levels of antigen-specific antibodies were not affected by dietary raffinose except that Ig E levels tended to be lower in the rats fed the raffinose diet. In addition, the proliferative response to OVA in splenic mononuclear cells *ex vivo* was unchanged between the groups in experiment 1 (data not shown). These findings

do not support the idea that the action of dietary raffinose is associated with an induction of oral tolerance.

It is naturally possible to claim that the improvement of allergic inflammation by dietary raffinose is due to its prebiotic action. If that were the case, the reducing effect of dietary raffinose on allergic airway eosinophilia observed in experiment 1 would be attenuated by the elimination of colonic bacteria. To examine this possibility, BN rats were caecectomised and administered orally with neomycin sulfate in experiment 2. Bacteriological analysis of the colonic content of rats demonstrated that dietary raffinose increased the number of anaerobic bacteria in the sham-operated rats (Table 1). According to the gross appearance of the bacterial colony, the increase in anaerobic bacteria was possibly due to bifidobacteria. The observation is consistent with previous studies on Wistar rats (T Nagura, unpublished results) and human subjects (Benno *et al.* 1987). In addition, caecectomy plus neomycin administration drastically decreased the number of colonic bacteria. Furthermore, dietary raffinose no longer increased the number of colonic bacteria in the caecectomised and neomycin-administered rats. Under these conditions, the eosinophilia-preventive effect of dietary raffinose was still observed in the caecectomised and neomycin-administered rats (Fig. 3). These results suggest that colonic bacteria have little contribution to the reducing action of dietary raffinose on allergic airway eosinophilia. In other words, a reduction of eosinophilic infiltration by dietary raffinose may be associated, rather, with a post-absorptive action of raffinose than a prebiotic action. Because caecectomy tended to decrease the number of eosinophils in the BALF of the rats fed the control diet, however, one may point out that dietary raffinose and caecectomy were both able to eliminate some kinds of colonic bacteria, which may contribute in deteriorating the allergic inflammation. Therefore, we next performed an investigation to elucidate whether dietary raffinose suppressed allergic airway eosinophilia via a post-absorptive mechanism.

Raffinose has been generally known as an indigestible oligosaccharide. In order to consider the post-absorptive action of raffinose, therefore, it should be demonstrated that orally administered raffinose can be absorbed in the gastrointestinal tract. Niewoehner *et al.* (1984a,b, 1990) investigated the kinetics of plasma concentrations after the oral administration (4 g/kg body weight) of glucose, fructose and galactose in 24 h-fasted rats. They showed that peak concentrations of approximately 15, 20 and 2 mM in portal venous plasma were reached at 20, 60 and 10 min for glucose, galactose and fructose, respectively. For comparison with these studies using the major absorbed monosaccharides, we administered a similar dose (4 g/kg body weight) of raffinose in BN rats. A peak raffinose concentration of approximately 60 μ M in portal venous plasma was reached at 60 min after oral administration. Thus, compared with the peak concentrations of the three monosaccharides, this value was exceedingly low. However, the data clearly demonstrated that raffinose was absorbed intact in the gastrointestinal tract. Therefore, even though absorbed raffinose is quite a small proportion of orally administered raffinose, it seems still possible to claim that dietary raffinose acts

via a post-absorptive mechanism. Therefore, we next examined the effect of an intraperitoneal injection of raffinose on allergic airway eosinophilia. Similar to dietary raffinose, an intraperitoneal injection of raffinose significantly suppressed allergic airway eosinophilia (Fig. 6). The findings clearly support the idea that a post-absorptive mechanism is involved in the eosinophilia-preventive effect of dietary raffinose. Although it may be possible that absorbed raffinose influences the immune system and/or inflammation process, the precise mechanisms remain to be elucidated. As shown in Fig. 2, the suppression of airway eosinophilia by dietary raffinose could be associated with a reduced expression of IL-4 and IL-5 mRNA in lung tissue, because these cytokines have been known to promote eosinophil infiltration (Nakajima *et al.* 1992; Coyle *et al.* 1995). Since these cytokines are mainly derived from activated T cells in inflamed lung tissue, it may be possible that absorbed raffinose affects the migration of T cells and/or the expression of such cytokines in T cells. Thus it is necessary to determine whether intraperitoneal raffinose can reduce IL-4 and IL-5 mRNA in lungs exposed to aerosolised OVA in sensitised BN rats.

Recently, Nagura *et al.* (2002) reported that the secretion of IL-12 and IFN- γ from Peyer's patch cells of naive BALB/c mice was significantly increased by dietary raffinose, suggesting suppression of the Th2-type immune response by dietary raffinose. However, the authors observed that adding raffinose to an *in vitro* culture of Peyer's patch cells from BALB/c mice no longer affected the production of IL-12 and IFN- γ (Nagura *et al.* 2002). Thus, it may be unlikely that dietary raffinose influences the production of such cytokines through a post-absorptive mechanism. Further investigations are required to elucidate the post-absorptive mechanism for preventing allergic inflammation by dietary raffinose.

In conclusion, the present findings suggest that dietary raffinose reduces allergic airway eosinophilia in sensitised BN rats at least partly via a post-absorptive mechanism. Therefore, we propose that raffinose is applicable to the prevention and/or treatment of human allergic diseases, even though further investigations are required to understand the mechanisms behind the action of raffinose.

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References

- Benno Y, Endo K, Shiragami N, Sayama K & Mitsuoka T (1987) Effects of raffinose intake on human fecal microflora. *Bifid Microflora* **6**, 59–63.
- Coffman RL, Ohara J, Bond MW, Carty J, Zlotnik A & Paul WE (1986) B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J Immunol* **136**, 4538–4541.
- Coyle AJ, Le Gros G, Bertrand C, Tsuyuki S, Heusser CH, Kopf M & Anderson GP (1995) Interleukin-4 is required for

- the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* **13**, 54–59.
- Del Prete G, Maggi E, Parronchi P, Chretien I, Tiri A, Macchia D, Ricci M, Banchereau J, De Vries J & Romagnani S (1988) IL-4 is an essential factor for the IgE synthesis induced *in vitro* by human T cell clones and their supernatants. *J Immunol* **140**, 4193–4198.
- Elwood W, Lotvall JO, Barnes PJ & Chung KF (1991) Characterization of allergen-induced bronchial hyperresponsiveness and airway inflammation in actively sensitized brown-Norway rats. *J Allergy Clin Immunol* **88**, 951–960.
- Fujisaki H, Nagura T, Kawamoto T & Sayama K (1994) The effects of raffinose administration on the fecal flora, organic acids and putrefactive products in humans (in Japanese). *Bifidus* **8**, 1–5.
- Gracie JA & Bradley JA (1996) Interleukin-12 induces interferon- γ -dependent switching of IgG alloantibody subclass. *Eur J Immunol* **26**, 1217–1221.
- Haczku A, Macary P, Haddad EB, Huang TJ, Kemeny DM, Moqbel R & Chung KF (1996) Expression of Th-2 cytokines interleukin-4 and -5 and of Th-1 cytokine interferon- γ in ovalbumin-exposed sensitized Brown-Norway rats. *Immunology* **88**, 247–251.
- Holt PG & Turner KJ (1985) Persistent IgE-secreting cells which are refractory to T-cell control. *Int Arch Allergy Appl Immunol* **77**, 45–46.
- Isolauri E, Arvola T, Sütas Y, Moilanen E & Salminen S (2000) Probiotics in the management of atopic eczema. *Clin Exp Allergy* **30**, 1604–1610.
- Iwamoto I, Nakajima H, Endo H & Yoshida S (1993) Interferon γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J Exp Med* **177**, 573–576.
- Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P & Isolauri E (2001) Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* **357**, 1076–1079.
- Kato I, Endo-Tanaka K & Yokokura T (1998) Suppressive effects of the oral administration of *Lactobacillus casei* on type II collagen-induced arthritis in DBA/1 mice. *Life Sci* **63**, 635–644.
- Leung DYM & Bieber T (2003) Atopic dermatitis. *Lancet* **361**, 151–160.
- Majamaa H & Isolauri E (1997) Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol* **99**, 179–185.
- Matsuda M, Takeuchi S & Nagura T (1998) Influence of raffinose for atopic dermatitis (in Japanese). *Allergy Practice* **18**, 1092–1095.
- Mitsuoka T, Segi T & Yamamoto S (1965) Eine verbesserte methodik der qualitativen und quantitativen analyse der darmflora von menschen und tieren (Improved methodology for qualitative and quantitative analysis of the intestinal flora of man and animals). *Zbl Bakt Hyg I Abt Orig* **195**, 455–469.
- Mosmann TR, Cherwinski H, Bond MW, Giedlin MA & Coffman RL (1986) Two types of murine helper T cell clone. I Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348–2357.
- Nagura T, Hachimura S, Hashiguchi M, Ueda Y, Kanno T, Kikuchi H, Sayama K & Kaminogawa S (2002) Suppressive effect of dietary raffinose on T-helper 2 cell-mediated immunity. *Br J Nutr* **88**, 421–426.
- Nagura T, Muraguchi H, Uchino K, Aritsuka T & Benno Y (1999) Effects of ingestion of raffinose-rich soup on the fecal flora and daily defecation in humans (in Japanese). *J Intest Microbiol* **13**, 1–7.
- Nakajima H, Iwamoto I, Tomoe S, Matsumura R, Tomioka H, Takatsu K & Yoshida S (1992) CD4⁺ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* **146**, 374–377.
- Niewoehner CB, Gilboe DP & Nuttall FQ (1984a) Metabolic effects of oral glucose in the liver of fasted rats. *Am J Physiol* **246**, E89–E94.
- Niewoehner CB, Gilboe DP, Nuttall GA & Nuttall FQ (1984b) Metabolic effects of oral fructose in the liver of fasted rats. *Am J Physiol* **247**, E505–E512.
- Niewoehner CB, Neil B & Martin T (1990) Hepatic uptake and metabolism of oral galactose in adult fasted rats. *Am J Physiol* **259**, E804–E813.
- Rautava S, Kalliomäki M & Isolauri E (2002) Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. *J Allergy Clin Immunol* **109**, 119–121.
- Reeves PG, Nielsen FH & Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.
- Sayama K, Kamada T, Oikawa S & Masuda T (1992) Production of raffinose: a new byproduct of the beet sugar industry. *Zuckerindustrie* **117**, 893–898.
- Schley PD & Field CJ (2002) The immune-enhancing effects of dietary fibres and prebiotics. *Br J Nutr* **87**, Suppl. 2, S221–S230.
- Sher A & Coffman RL (1992) Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu Rev Immunol* **10**, 385–409.
- Sonoyama K, Kiriya S & Niki R (1995) Expression of apolipoprotein A-I mRNA in liver and intestine of cecectomized rats fed beet fiber. *J Nutr Biochem* **6**, 380–384.
- Sonoyama K, Rutatip S & Kasai T (2000) Gene expression of activin, activin receptors, and follistatin in intestinal epithelial cells. *Am J Physiol* **278**, G89–G97.
- Watanabe J, Fukumoto K, Fukushi E, Sonoyama K & Kawabata J (2004) Isolation of tryptophan as an inhibitor of ovalbumin permeation and analysis of its suppressive effect on oral sensitization. *Biosci Biotechnol Biochem* **68**, 59–65.
- Watanabe J, Tanabe S, Watanabe M, Kasai T & Sonoyama K (2001) Consumption of hypoallergenic flour prevents gluten-induced airway inflammation in Brown Norway rats. *Biosci Biotechnol Biochem* **65**, 1729–1735.