JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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# Degree of Polymerization of Inulin-Type Fructans Differentially Affects Number of Lactic Acid Bacteria, Intestinal Immune Functions, and Immunoglobulin A Secretion in the Rat Cecum

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Supporting Information

**ABSTRACT:** This study examined the role of degree of polymerization (DP) of inulin-fructans in modulating the interaction between lactic acid bacteria and IgA cecal secretion. Rats were fed a control diet or a diet containing one of the fructans with different DP. Consuming fructans increased the cecal IgA concentrations in the order DP4 > DP8 > DP16. Cecal lactobacilli counts were higher in DP4, DP8, and DP16, whereas bifidobacteria were higher in DP8, DP16, and DP23. Cecal IgA concentrations were correlated with cecal lactobacilli counts ($P < 0.01$). DP4, DP8, and DP16, but not DP23, significantly increased IgA-producing plasma cells in the cecal mucosa. IFN-$\gamma$ and IL-10 production in the cecal $CD4^+$ T cells was enhanced solely in DP4. The results show that fructans with lower DP enhance cecal IgA secretion and increase the plasma cells and suggest that the increased lactobacilli may contribute to the stimulation of cecal IgA secretion.

**KEYWORDS:** fructan, immunoglobulin A, lactobacillus, cecum, rats

## INTRODUCTION

The mucosal immune system response in the gut—characterized by secretory IgA and oral tolerance—is different from that of the systemic immune system. IgA production depends on complex mechanisms involving antigen sampling by M cells, processing by underlying antigen-presenting cells, T-cell activation, and class switch to IgA mainly in Peyer's patches and neighboring lamina propria.[1] As the main function of IgA is to agglutinate microorganisms and to prevent the adherence of pathogenic bacteria and viruses to the mucosal surface,[2] increased IgA production induced by a stimulation of commensal bacteria favors the defense against pathogens in the gastrointestinal tract.

Previous studies have shown that administration of lactic acid-producing bacteria (LAB) influences gut-associated lymphoid tissue and promotes intestinal IgA secretion both in humans[3] and in animals.[4,5] In addition, diet supplementation with nondigestible oligosaccharides,[6−8] oligofructose-enriched inulins,[9] or fermentable dietary fibers[10,11] that stimulate LAB proliferation and metabolic capacities has demonstrated increased IgA responses in ileal and cecal contents and feces in animals. These outcomes support the hypothesis that the changes in the number of LAB induced by prebiotic supplementation are a prerequisite for changes in IgA production and secretion into the intestinal lumen.[12]

Inulin-type fructans (INF), typically linked by a $\beta$ (2−1)-fructofuranosyl bond with a glucose moiety at the end of almost every fructose chain, resist small intestinal digestion but are fermented to change the colonic milieu (pH and organic acids), stimulating the proliferation of certain commensal bacteria, including bifidobacteria and lactobacilli.[13,14] However, the fermentation rate and fermentation products of INF may be affected by their degree of polymerization (DP), as any fermentable carbohydrates must be hydrolyzed to monosaccharides prior to fermentation by bacteria.[13,14] This differential fermentation profile may also result in differences in the changes in colonic number of LAB and intestinal immune function, including IgA secretion. At present, however, most of the data related to these changes have been obtained with fructooligosaccharides (a mixture of 1-kestose, nystose, and $1^F$-$\beta$-fructofuranosyl nystose)[6,8] and oligofructose-enriched inulin;[9] relatively little information is available regarding the comparative effects of INF of different DP,[15−17] and analytical data on the relationship between qualitative or quantitative changes in the intestinal microflora and intestinal IgA production are limited.

The present study aimed to better understand the role of DP (fructan-chain length) of INF in modulating the interaction between intestinal microflora and intestinal IgA secretion and to clarify which fermentation patterns of INF favor cecal IgA secretion. Because most INF reach the cecum intact and are extensively fermented and promote LAB proliferation, the immunomodulatory effects of INF are probably exerted mainly in the cecum. Accordingly, we measured cecal IgA concentrations,

**Received:** March 2, 2011
**Revised:** April 21, 2011
**Accepted:** April 21, 2011
**Published:** April 21, 2011

the number of LAB in the cecum, and the proportion of IgA plasma cells and cytokine responses of $CD4^+$ T-lymphocytes in the cecal lamina propria in rats fed four INF with average DPs of 4, 8, 16, or 23.

## MATERIALS AND METHODS

**Materials.** INFs with average DPs of 4, 8, 16, or 23 were prepared as described previously.[15] Briefly, DP4, composed of 44% 1-kestose, 46% nystose, and 10% $1^F$-$\beta$-fructofuranosyl nystose (range 2−4), was purchased from Meiji Seika (Meioligo P, Tokyo, Japan). Raftiline HP (Tienen, Belgium), used as DP23, consisted of a mixture of DP10−60. DP8 (range 3−13) and DP16 (range 5−30), provided by Fuji Nihon Seito Co. (Shizuoka, Japan), were prepared by enzymatic synthesis using a novel bacterial $\beta$-fructosidase enzyme from *Bacillus* sp. 217C-11.[18] The purity of INF used in the present study exceeds 95% (w/w) on a dry matter basis.

**Care of Animals.** The study was approved by the Animal Use Committee of Shizuoka University, and animals were maintained in accordance with the guidelines of Shizuoka University for the care and use of laboratory animals. Male Sprague−Dawley rats (purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were individually housed in wire screen bottom, stainless steel cages in a temperature- (23 ± 2 °C) and lighting- (lights on from 8:00 a.m. to 8:00 p.m.) controlled room. For adaptation, rats were fed a control diet for at least 5 days. This diet[15] was formulated from 250 g/kg casein, 652.25 g/kg cornstarch, and 50 g/kg corn oil. The remainder of the diet consisted of vitamins (10 g/kg), minerals (35 g/kg), and choline bitartrate (2.5 g/kg). The compositions of vitamins and minerals were based on AIN-76.[19] Rats were allocated to groups on the basis of body weight and allowed free access to experimental diets and water. The addition of each fructan was performed at the expense of an equal amount of cornstarch in each diet. Body weight and food intake were recorded every morning before the diet was replenished.

**Experiment 1.** Forty rats weighing 230−260 g (age 7 weeks) were allocated to five groups of eight rats each and were allowed free access to the control diet or diet containing 60 g of DP4, DP8, DP16, or DP23/kg diet for 15 days. After 15 days, rats were killed by decapitation, and the small intestine was excised. Luminal contents were gathered by flushing with 15 mL of ice-cold phosphate-buffered saline (PBS; pH 7.4) containing 0.02 mol of sodium azide/L. After filling up to 20 mL with the same solution, 1 mL of this luminal fluid was centrifuged at 10000*g* for 20 min to obtain a clear supernatant and was used for IgA measurement. The cecum was removed and weighed. Cecal contents were homogenized under nitrogen gas and divided into two portions: one was used for measurements of pH, organic acids, and IgA, and the other was used for measurements of total bacteria and bifidobacteria and lactobacilli counts. The cecal tissue was used for separation of lamina propria mononuclear cells (LMPC) and successive analysis of IgA plasma cells. For IgA measurement, cecal contents (approximately 100 mg wet weight) were homogenized using a Polytron for 1 min in ice-cold PBS (100 mg of cecal contents/mL) containing a protease inhibitor (Complete, Roche Diagnostics, Tokyo, Japan; one tablet/50 mL PBS) and Tween20 (5 g/L). The homogenate was centrifuged at 10000*g* for 20 min at 4 °C, and the supernatant was stored at −80 °C until analysis.

**Experiment 2.** Forty rats weighing 240−250 g (age 7 weeks) were allocated to five groups of eight rats each, and the same dietary treatments used in expt 1 were conducted for 15 days, at which time rats were killed by decapitation and the cecum was excised. The cecal tissue was used for separation of LMPC and successive isolation of $CD4^+$ T cells.

**Cecal pH and Organic Acids.** A portion of homogenate was diluted with the same weight of distilled water, and then cecal pH was measured with a compact pH-meter (model C-1, Horiba, Tokyo, Japan). Cecal organic acids were measured by the internal standard method using a HPLC (LC-6A, Shimadzu, Kyoto, Japan) equipped with a Shim-pack SCR-102H column (8 mm i.d. × 30 cm long, Shimadzu) and an electroconductivity detector (CDD-6A, Shimadzu).[20]

**Immunoglobulin A Analysis.** After an appropriate dilution, luminal and cecal IgA were determined by enzyme-linked immunosorbent assay using Nunc-Immuno plates (MaxiSorb F96; Nunc, Denmark) with a slight modification[21] of the method of Grewal et al.[22]

**Flow Cytometry Analysis of Cecal Bacteria Counts.** Population and viability of bacteria were analyzed by flow cytometry according to the method of Ben-Amor et al.[23] In brief, a portion (≈100 mg) of cecal contents was suspended in 1 mL of anaerobic PBS containing 1 mM dithiothreitol and 0.01% (w/v) Tween20 and homogenized by vortexing for 3 min. After centrifugation at 700*g* for 1 min, the supernatant was carefully recovered and centrifuged at 6000*g* for 3 min. The pellet was washed twice, resuspended in anaerobic PBS, and then serially diluted. Thereafter, the diluted samples were incubated for 15 min at room temperature in anaerobic PBS supplemented with $10^4$ particles/mL fluorospheres (Flow-Check fluorospheres, Beckman Coulter, Tokyo, Japan), 1 mg/mL propidium iodide (PI, Wako Pure Chemical Industries, Osaka, Japan), and 5 nM SYTO-BC (Molecular Probes, Eugene, OR). Samples were analyzed by flow cytometry (Epics XL, Beckman Coulter) (Figure S1 in the Supporting Information).

**Quantification of Bifidobacteria and Lactobacilli in Cecal Contents by Real-Time Polymerase Chain Reaction (PCR).** Amplification and detection of cecal DNA were performed with Light Cycler ST-300 (Roche). *Bifidobacterium* genus-specific (forward, TCG CGT C(C/T)G GTG TGA AAG; reverse, CCA CAT CCA GC(A/G) TCC AC)[24] and *Lactobacillus* genus-specific (forward, TGG AAA CAG (A/G)TG CTA ATA CCG; reverse, GTC CAT TGT GGA AGA TTC CC)[25] primer pairs were used. Real-time PCR was performed in a reaction volume of 20 $\mu$L, containing 10 $\mu$L of SYBR Premix Ex *Taq*II (Takara, Shiga, Japan), 0.8 $\mu$L of 200 nM each of the forward and reverse primers, 6.4 $\mu$L of $H_2O$, and 2 $\mu$L of cecal DNA samples. The reaction conditions were 95 °C for 30 s, followed by 43 cycles at 95 °C for 5 s and 64 °C for 30 s, for the quantification of bifidobacteria and 95 °C for 30 s, followed by 43 cycles at 95 °C for 5 s and 65 °C for 30 s, for the quantification of lactobacilli. *Bifidobacterium animalis* (JCM $1190^T$) and *Lactobacillus murinus* (JCM $1717^T$) were cultured in De Man, Rogosa, and Sharpe broth (Becton Dickinson, Rockville, MD), and the genomic DNA was extracted by Isoplant-II (Wako) 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 GFXe PCR DNA and Gel Band Purification Kit (GE Healthcare Bioscience, Tokyo, Japan) and cloned in pGEMEasy T vectors (Promega, Madison, WI). Transformation was performed with competent *Escherichia coli* XL-1 Blue cells plated onto Luria−Bertani agar plates supplemented with ampicillin (25 mg/mL), X-Gal (30 mg/mL), and isopropyl $\beta$-D-1-thiogalactopyranoside (20 mg/mL) 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, Germantown, MD) and used as standard for real-time PCR.

**Separation of LMPC and Flow Cytometric Analysis of IgA Plasma Cells.** The cecal tissue was cut into small pieces and stirred twice in calcium/magnesium-free Hank's balanced salt solution (HBSS (−), pH 7.2) containing 10 mM dithiothreitol for 15 min. The tissue was then stirred in HBSS(−) containing 1 mM EDTA at 37 °C for 30 min for three cycles. After a washing with RPMI-1640 (GIBCO-Invitrogen, Carlsbad, CA), the tissue was digested in RPMI-1640 containing 10% fetal calf serum (FCS) and 300 U/mL collagenase (Wako) at 37 °C for 30 min for two cycles. The LPMC were separated from undigested tissue by cellular filter (Ikemotorika Ltd., Tokyo, Japan) and subsequently stained with FITC-conjugated anti-rat IgA (MARA-1, AbD Serotec, Oxford, U.K.) and PE-conjugated anti-rat CD45R (HIS24, BD Biosciences, San Diego, CA) for 1 h at 4 °C. Stained cells

**Table 1. Food Intake, Body Weight Gain, and Cecal Variables in Rats Fed the Control Diet or a Diet Containing One of the Inulin-Type Fructans (INF) with Different Degrees of Polymerization (DPs) at 60 g/kg Diet for 15 Days (Experiment 1)[a]**

| | group | | | | |
|---|---|---|---|---|---|
| | control | DP4 | DP8 | DP16 | DP23 |
| food intake, g/15 days | 329 ± 13 b | 298 ± 9 ab | 288 ± 8 a | 288 ± 7 a | 288 ± 8 a |
| body weight gain, g/15 days | 102 ± 9 | 95 ± 5 | 89 ± 5 | 86 ± 5 | 84 ± 7 |
| cecum | | | | | |
| tissue, g | 0.7 ± 0.0 a | 1.4 ± 0.1 b | 1.5 ± 0.1 b | 1.5 ± 0.1 b | 1.5 ± 0.1 b |
| contents, wet g | 2.2 ± 0.2 a | 5.6 ± 0.7 c | 4.5 ± 0.2 bc | 3.5 ± 0.4 ab | 3.9 ± 0.4 bc |
| pH | 7.2 ± 0.1 b | 5.6 ± 0.1 a | 5.7 ± 0.1 a | 5.9 ± 0.2 a | 5.7 ± 0.1 a |
| organic acids, μmol/g wet contents | | | | | |
| acetate | 61.2 ± 5.1 ab | 37.3 ± 9.7 a | 61.9 ± 11.2 ab | 70.6 ± 8.8 ab | 79.6 ± 10.5 b |
| propionate | 17.9 ± 2.6 | 25.1 ± 6.6 | 26.0 ± 5.3 | 23.9 ± 6.2 | 23.3 ± 7.7 |
| *n*-butyrate[b] | 6.9 (3.5−11.2) | 6.1 (2.5−57.6) | 21.4 (15.3−45.8) * | 16.9 (15.2−37.3) * | 14.8 (4.8−32.5) * |
| SCFAs[c] | 86.3 ± 8.2 | 77.4 ± 20.7 | 112.6 ± 19.4 | 114.5 ± 16.2 | 119.5 ± 20.6 |
| succinate | 6.1 ± 2.2 a | 22.6 ± 2.7 b | 14.2 ± 3.1 ab | 12.2 ± 3.4 ab | 12.8 ± 2.6 ab |
| lactate[b] | 0.3 (0.0−21.3) | 35.0 (9.1−82.3) * | 36.3 (2.9−83.2) * | 18.2 (0.4−45.4) | 10.0 (3.2−49.2) |

[a] Data are expressed as the mean ± SE, $n = 8$, unless otherwise noted. Means in a row without a common letter differ ($P < 0.05$) when analyzed by one-way ANOVA, followed by the Tukey−Kramer test. [b] The effects of dietary treatment were examined by Kruskal−Wallis one-way ANOVA, followed by Kolmogorov−Smirnov two-sample tests (*, $P < 0.05$ vs control). Data are expressed as medians (range), $n = 8$. [c] Short-chain fatty acids; the sum of acetate, propionate, and *n*-butyrate.

were analyzed by flow cytometer (EPICS-XL, Beckman Coulter, Inc.), and IgA plasma cells were defined as $IgA^+/CD45R^{low}$ cells[26] (Figure S2 in the Supporting Information).

**Incubation of $CD4^+$ T Cells and Cytokine Analysis.** Cecal LMPC were isolated as described above. LMPC were then layered on a 40−70% Percoll gradient (GE Healthcare Ltd., Buckinghamshire, U.K.) and centrifuged at 760*g* for 20 min to prepare lymphocyte-enriched populations. Enriched $CD4^+$ T cell populations were obtained by negative selection using a magnetic cell-sorting technique.[27] Briefly, the cells were stained with biotinylated antigranulocyte (HIS48, eBioscience, San Diego, CA), anti-NKR/P1A (10/78, BD Biosciences), anti-CD45R (HIS24, eBioscience), anti-RT1B (OX-6, BD Biosciences), and anti-CD8α (OX-8, BD Biosciences) antibodies and subsequently stained with magnetic microbeads conjugated to antibiotin antibody (Miltenyi Biotec, Germany), followed by passage through the magnetized column (LS column, Miltenyi Biotec). $CD4^+$ T-cell purity was checked by flow cytometry (>75%, Figure S3 in the Supporting Information). We purified this population from eight specimens of each dietary treatment, and then two from each treatment were combined before culture. $CD4^+$ T cells (viability of each treatment >95%) were cultured in 96-well plates (Costar 3595, Corning Inc., Corning, NY) at $2 \times 10^5$ cells/well in complete medium (RPMI-1640 containing 10% fetal calf serum, 50 μM 2-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin) with costimulation of plate-bound anti-rat CD3 (10 μg/mL) (G4.18, BD Biosciences) and soluble anti-rat CD28 (1 μg/mL) (JJ319, BD Biosciences) at 37 °C in 5% $CO_2$. After 72 h, cell culture supernatants were collected and used for cytokine analysis. Cytokines (IFN-γ, IL-4, IL-6, and IL-10) were analyzed by commercial ELISA kit (OptEIA, BD Biosciences).

**Statistical Analyses.** Data were analyzed by one-way ANOVA, and significant differences among means were identified by the Tukey−Kramer test. Results were expressed as the mean ± SEM, and a 5% level of probability was considered to be a significant difference in all statements. When variances were not homogeneous by the Bartlett test, data were logarithmically transformed. When variances were not homogeneous even after logarithmic transformation, the data were presented as medians with range and then analyzed by Kruskal−Wallis ANOVA followed by Kolomogorov−Smirnov two-sample tests. The statistical calculations were carried out using Stat View 5.0 computer software (SAS Institute, Cary, NC). Regression analyses were performed using the Stat Cel 2 program (Tokyo Shoseki, Tokyo, Japan). Duplicate assays were performed for each determination.

## RESULTS

In experiment 1, food intake was significantly lower in rats fed the DP8, DP16, and DP23 diets than in those fed the control diet, but there were no differences in body weight gain among the dietary groups (Table 1). The weights of cecal tissue in the fructan diet groups were significantly higher than in the control group. The wet weights of the cecal contents were substantially higher in the fructan diet groups than in the control group and were highest in the DP4 group. Cecal pH declined in all of the fructan diet groups. Cecal acetate concentrations were highest in the DP23 group, intermediate in the control, DP8, and DP16 groups, and lowest in the DP4 group. Cecal butyrate concentrations were higher in the DP8, DP16, and DP23 groups than in the control group. Cecal concentrations of succinate or lactate were higher in the DP4 and DP8 groups, in that order, than in the control group. The amount of IgA in the small intestine did not differ among the groups (Figure 1A), whereas cecal IgA content and concentrations were significantly higher in the DP4, DP8, and DP16 groups (Figure 1B,C).

Bacterial counts/g wet cecal content were the lowest in the DP4 group, and there were significant differences between the control and DP4 groups (Table 2). Total bacterial counts/cecum were significantly greater in rats fed the DP8, DP16, and DP23 diets than in those fed the control diet. The percentage of viable cells was significantly lower in the fructan diet groups than in the control diet group, whereas the opposite was true for dead cells. The percentage of injured cells did not differ among the groups. Lactobacillus counts were higher in the fructan diet groups than in the control group, with no significant differences observed among the fructan diet groups (Figure 2A). Bifidobacteria counts were significantly higher in the DP8, DP16, and DP23 groups than in the control group (Figure 2B). The number of IgA plasma

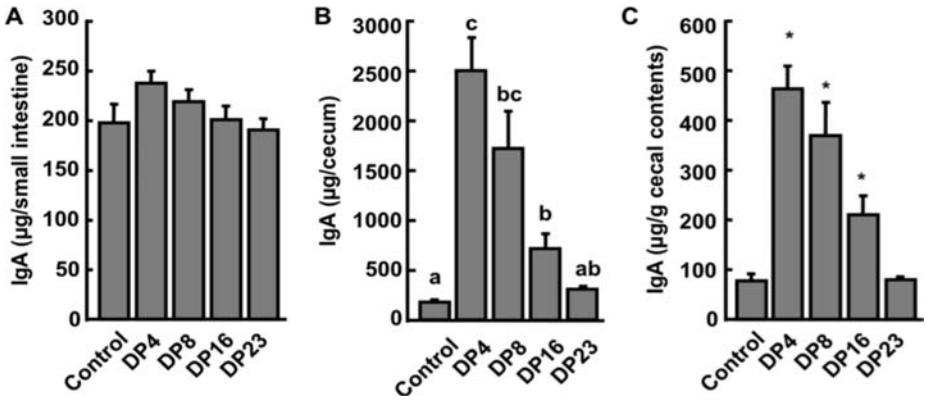


**Figure 1.** Total amounts of immunoglobulin A (IgA) in the small intestinal fluid (A), total cecal contents (B), and cecal IgA concentrations (per g wet cecal contents) (C) in rats fed the control diet or a diet containing one of the inulin-type fructans (INF) with different degrees of polymerization (DPs) at 60 g/kg diet for 15 days (expt 1). Each column and bar represents the mean ± SE ($n$ = 8). Columns not sharing a common letter are significantly different when analyzed by ANOVA followed by the Tukey−Kramer test. Some of the data (B, C) were logarithmically transformed prior to ANOVA. ∗, the effects of dietary treatment were examined by Kruskal−Wallis one-way ANOVA, followed by Kolmogorov−Smirnov two-sample tests ($P$ < 0.05 vs control).

**Table 2. Cecal Bacterial Counts and Viability in Rats Fed the Control Diet or a Diet Containing One of the Inulin-Type Fructans (INF) with Different Degrees of Polymerization (DPs) at 60 g/kg Diet for 15 Days (Experiment 1)[a]**

| | group | | | | |
|---|---|---|---|---|---|
| | control | DP4 | DP8 | DP16 | DP23 |
| bacterial counts[b] | | | | | |
| × $10^{10}$/g wet contents | 5.6 (5.0−7.7) | 2.6 (2.1−4.4) * | 7.8 (2.4−22.0) | 6.1 (5.0−8.0) | 5.2 (3.5−7.4) |
| × $10^{11}$/cecum | 1.4 (1.2−2.0) | 2.1 (1.9−4.1) | 5.1 (1.6−12.1) * | 3.3 (2.0−3.8) * | 2.9 (1.8−4.2) * |
| viable cells, % | 77.5 ± 3.9 b | 65.3 ± 3.2 a | 64.0 ± 5.0 a | 62.5 ± 0.8 a | 69.8 ± 1.8 a |
| injured cells, % | 5.3 ± 1.0 | 6.3 ± 0.6 | 3.8 ± 0.8 | 4.9 ± 1.0 | 1.4 ± 0.5 |
| dead cells, % | 17.2 ± 1.1 a | 28.5 ± 1.4 b | 32.2 ± 5.0 b | 32.7 ± 0.3 b | 28.7 ± 1.6 b |

[a] Data are expressed as the mean ± SE, $n$ = 8, unless otherwise noted. Means in a row without a common letter differ ($P$ < 0.05) when analyzed by one-way ANOVA, followed by the Tukey−Kramer test. [b] The effects of dietary treatment were examined by Kruskal−Wallis one-way ANOVA, followed by Kolomogorov−Smirnov two-sample tests (*, $P$ < 0.05 vs control). Data are expressed as medians (range), $n$ = 8.

cells was lowest in the control and DP23 groups, highest in the DP8 group, and intermediate in the DP4 and DP16 groups, and the differences were all significant (Figure 3). Linear regression analyses showed that there were highly positive correlations among the cecal concentrations of IgA, lactate, and lactobacilli (Figures 4). The correlation between cecal concentration of IgA and lactobacilli plus bifidobacteria was also significant to the same extent ($r$ = 0.97, $P$ < 0.001). Cecal IgA concentrations were not correlated with the cecal concentrations of bifidobacteria alone, total bacteria, or other organic acids.

In experiment 2, lower food intakes were observed repeatedly in the fructan diet groups compared with those in the control group (control, 279; DP4, 244; DP8, 227; DP16, 241; DP23, 225; pooled SE, 7 g), but there were no significant differences in body weight gains (control, 84; DP4, 72; DP8, 62; DP16, 65; DP23, 68; pooled SE, 6 g). After $CD4^+$ T cells, stimulated with anti-CD3 and anti-CD28, were cultured for 72 h, culture supernatants were analyzed for concentrations of IFN-$\gamma$, IL-4, IL-6, and IL-10. Concentrations of IFN-$\gamma$ in rats fed the DP4 diet were greater than in those fed the control, DP16, and DP23 diets. Concentrations of IL-10 in rats fed the DP4 diet were also greater than in those fed the control and DP23 diets. No differences were observed in the concentrations of IL-4 and IL-6 among the dietary groups (Figure 5).

## DISCUSSION

In both experiments, lower body weight gains in rats fed the fructan diets were mainly caused by lower food intakes during the initial 3 days of the experimental periods. We observed soft, unformed feces in the fructan diet groups for the first 3 days, and this might have affected the initial food intakes. Furthermore, Cani et al.[28,29] showed that ingestion of oligofructose or longer chain inulin modified the blood concentrations of gut-derived hormones such as glucagon-like peptide-1 and ghrelin, which might be involved in appetite regulation in rats. Nevertheless, a moderate calorie or protein restriction does not affect secretory IgA level in rodent and human experiments.[30,31]

The present study shows that the cecal concentrations and amounts of IgA were higher in rats fed the DP4, DP8, and DP16 diets (Figure 1B,C) than in those fed the control diet. Little IgA digestion occurs before the large intestine, so that an estimate of IgA in the cecal contents is necessary to account for the sum of both small intestinal and cecal IgA. In the present study, however, the amount of IgA in the small intestinal contents did not differ among the dietary groups (Figure 1A). The results suggest that the increased cecal IgA concentrations in rats fed the DP4, DP8, and DP16 diets reflect greater IgA production and secretion in the cecum, being supported by the results previously reported by Roller et al.[9]

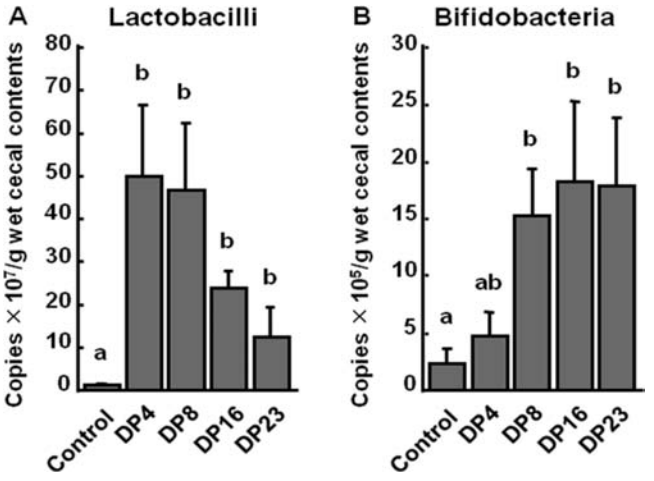


**Figure 2.** Cecal number of lactobacilli (A) and bifidobacteria (B) in rats fed the control diet or a diet containing one of the inulin-type fructans (INF) with different degrees of polymerization (DPs) at 60 g/kg diet for 15 days (expt 1). Data are presented as the mean ± SE ($n$ = 8). Groups not sharing a common letter are significantly different when analyzed by ANOVA followed by the Tukey−Kramer test. The data were logarithmically transformed prior to ANOVA.

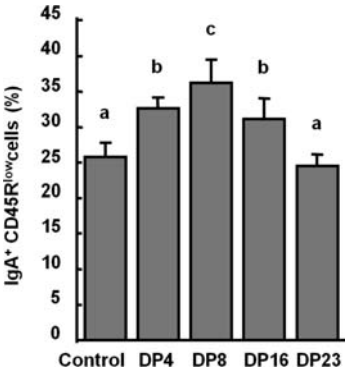


**Figure 3.** Percentage of immunoglobulin A (IgA) plasma cells in rats fed the control diet or a diet containing one of the inulin-type fructans (INF) with different degrees of polymerization (DPs) at 60 g/kg diet for 15 days (expt 1). Data are presented as the mean ± SE ($n$ = 8). Groups not sharing a common letter are significantly different when analyzed by ANOVA followed by the Tukey−Kramer test.

In the present study, cecal IgA concentrations were highly correlated with cecal concentrations of lactate and the cecal number of lactobacilli or lactobacilli plus bifidobacteria. Previous studies have suggested that rapidly fermentable fibers resulting in cecal lactate accumulation increase cecal IgA contents irrespective of the chemical structures of the fermented fibers.[10,11] Because lactate and succinate are absorbed quite poorly in the large bowel compared with short-chain fatty acids,[32] cecal pH is decreased more strongly by these acids, and this acidic condition favors acid-tolerant bacteria such as lactobacilli. Furthermore, lactate is one of the major fermentation products of lactobacilli and bifidobacteria.[33] This might be also the case for INF. In the cecum, however, there are large numbers of endogenous microorganisms, and in addition to LAB, INF can support the growth of these bacteria. Indeed, the number of total bacteria in the cecum was greater in rats fed the DP8, DP16, and DP23 diets than in those fed the control and DP4 diets (Table 2). Recent studies have shown that increases in intestinal bacteria result in the induction of specific mucosal IgA through a pathway independent of T-cell help and subsequent antibody maturation.[1,34] Thus, we cannot entirely exclude the possibility that the increased concentration of cecal IgA was due to an increased level of commensal bacteria other than lactobacilli. However, the cecal IgA concentrations were not correlated with the number of total bacteria in the cecum, including viable, injured, and dead cells. It is therefore plausible to assume that the increased number of cecal lactobacilli may have been involved in the stimulation of IgA production and secretion in the cecum.

The present study showed the tendency that lower DP fructans strongly stimulated the growth of lactobacilli in the cecum, whereas higher DP fructans preferentially support the growth of bifidobacteria. Our results are in accordance with those of previous studies showing that dietary fructans with lower DP increased the proportion of lactobacilli predominantly in healthy normal rats and mice.[6,35] Conversely, other studies showed in normal rats[13] and those associated with a human fecal flora[16] that lower DP fructans were bifidogenic. Furthermore, Vos et al. showed in mice that a combination of medium- and long-chain fructans (Synergy, Orafti) stimulated the growth of both bifidobacteria and lactobacilli more strongly than short-chain fructan or inulin (Raftiline ST, Orafti) alone.[17] These discrepancies may be originated from the differences in the initial number of bifidobacteria and/or lactobacilli among the respective experiments. In our separate experiment, when the initial number of bifidobacteria was relatively high (7.0−7.1 log10 counts/g cecal contents), we also observed a greater extent of stimulation of bifidobacteria in response to the consumption of DP4 (more than 30-fold vs control value). In the case of lactobacilli, what we could only hypothesize is that lactobacilli might have a relatively low potential to metabolize higher DP fructans.

Similar to the gut-associated lymphoid tissue in the small intestine—that is, Peyer's patches—the cecum and the colon contain additional sites of gut-associated lymphoid tissue, such as cecal patches and solitary lymphoid follicles, where the mucosal IgA response is induced.[36−38] We therefore performed phenotype analysis of lamina propria lymphocytes in the cecum using flow cytometry. Ingestion of DP4, DP8, and DP16 diets, but not the DP23 diet, significantly increased the percentage of IgA-producing plasma cells (defined as cells with $IgA^+$ and $CD45R^{low}$) compared with the control, suggesting that isotype switching from IgM to IgA was enhanced in rats fed the DP4, DP8, and DP16 diets. These results are comparable with the analytical data showing that cecal IgA concentrations were greater in rats fed DP4, DP8, and DP16 diets, but not the DP23 diet. Nakamura et al. showed that the proportion of $B220^+IgA^+$ cells in Peyer's patches was significantly higher in mice fed a diet including fructooligosaccharides (virtually the same as DP4 in the present study) than in those fed a control diet.[8] However, to our knowledge, the present study is the first to demonstrate an increased proportion of IgA plasma cells in the cecal mucosa of rats fed INF with certain DPs.

A previous study in mice reported that feeding fructooligosaccharides (DP4) significantly enhanced IFN-$\gamma$ and IL-10 production in $CD4^+$ T cells derived from Peyer's patches when stimulated by a sonicated *Bifidus* component.[4] In addition, feeding oligofructose (fructans with DP2−8)-enriched inulin in rats enhanced the production of IL-10 and IFN-$\gamma$ in Peyer's patch lymphocytes under concanavalin A stimulation.[9] In the present study, we measured cytokine production in $CD4^+$ T cells derived from the cecal lamina propria in response to the cross-linking of

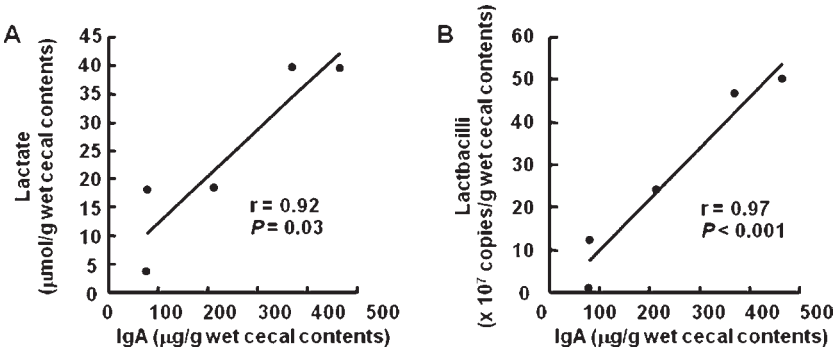


**Figure 4.** Correlations between cecal concentrations (per g wet cecal contents) of immunoglobulin A (IgA) and cecal concentrations of lactate (A) and lactobacilli (B) (expt 1). The regression analyses were performed using the least-squares test.

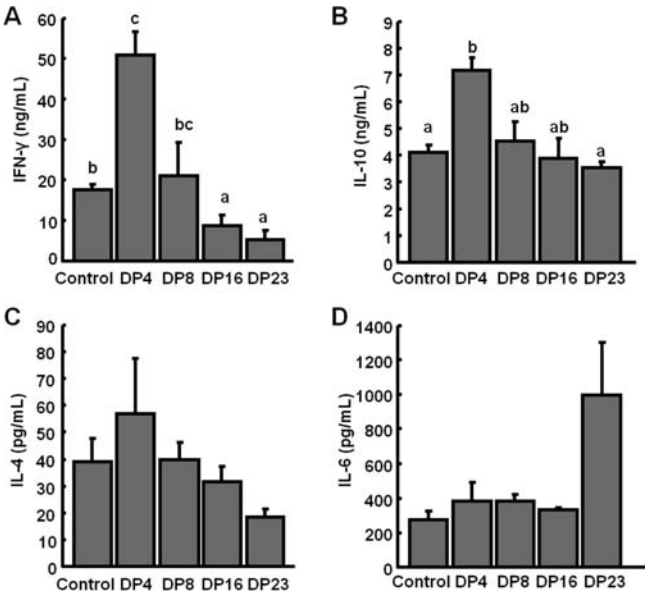


**Figure 5.** IFN-$\gamma$ (A), IL-10 (B), IL-4 (C), and IL-6 (D) concentrations in CD4$^+$ T cells from the cecal lamina propria in rats fed the control diet or a diet containing one of the inulin-type fructans (INF) with different degrees of polymerization (DPs) at 60 g/kg diet for 15 days (expt 2). Cytokine release from CD4$^+$ T cells was measured under CD3/CD28 costimulation. Data are presented as the mean ± SE ($n$ = 4). Groups not sharing a common letter are significantly different when analyzed by ANOVA followed by the Tukey−Kramer test.

CD3 and CD28, which mimics physiological signals in vivo.[39] Given the central role of LAB for the development of intestinal immune function, we hypothesize that all of the DP4, DP8, and DP16 treatments that increased the number of lactobacilli in the cecum enhance the production of cytokines such as IFN-$\gamma$ and IL-10. However, the enhancement of cytokine production was observed only in rats fed the DP4 diet and not in those fed the DP8 and DP16 diets. The reason for this remains unclear, but Roller et al. have shown that oligofructose-enriched inulin, independent of its prebiotic effect, can directly up-regulate IFN-$\gamma$ and IL-10 in Peyer's patch lymphocytes.[9] Therefore, one might expect that INF with a lower DP, such as oligofructose and DP4, may be absorbed intact and that the cells in gut-associated lymphoid tissue are capable of recognizing INF with lower DPs, as has been discussed by Seifert and Watzl.[12]

As stated above, the cecal IgA concentrations were higher in lower DP fructans, in the order DP4 > DP8 > DP16, and were correlated with the cecal number of lactobacillus. However, the number of IgA-plasma cells in the cecal mucosa was higher in lower DP fructans, in the order DP8 > DP4, DP16. Thus, the degree of magnitude of the increased number of IgA-plasma cells was not coincident with the cecal IgA concentrations. We do not have direct evidence to explain this discrepancy. Following secretion, the polymeric form of IgA is transported by its receptor (polymeric immunoglobulin receptor, pIgR) across the epithelium to the mucosal surface. Sollid et al. reported that IFN-$\gamma$ stimulated expression of pIgR for IgA by epithelial cells.[40] Although we did not measure pIgR expression, up-regulation of IFN-$\gamma$ in cecal lamina propria CD4$^+$ T cells may partly explain why the highest cecal IgA concentrations were observed in rats fed the DP4 diet. Furthermore, it is possible that secretory IgA may be digested in the cecum, because some bacterial species, including clostridia, have been shown to possess proteases capable of degrading IgA,[41,42] and these proteases have an optimal pH around neutral range.[41] Therefore, the increased population of cecal lactobacilli and the higher concentrations of cecal lactate and succinate may affect the degradation rate of IgA secreted in the cecum, leading to the higher concentrations of cecal IgA in rats fed the DP4 diet.

The results of the present study show that the ingestion of INF with DP4, DP8, and DP16 enhanced the production and secretion of IgA in the cecum and increased the percentage of IgA-producing plasma cells in the cecal lamina propria and suggest that the increased number of cecal lactobacilli may have been involved in stimulating the production and secretion of IgA in the cecum. The DP of INF has differential effects on the number of LAB, mucosal immune functions, and IgA secretion in the rat cecum, with the most active fractions being DP4 and DP8. Thus, providing these short-chain inulins as ingredients in nutritional formulas could benefit the health of the gastrointestinal tract.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Flow cytometric dot plot images of the cecal lamina propria CD4$^+$ T cells, IgA plasma cells, and cecal bacterial counts. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Funding Sources**
The study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

## ■ ACKNOWLEDGMENT

We thank Dr. Shuhachi Kiriyama for kindly reviewing the manuscript.

## ■ ABBREVIATIONS USED

DP, degree of polymerization; INF, inulin-type fructans; LAB, lactic acid-producing bacteria; LMPC, lamina propria mononuclear cells; pIgR, polymeric immunoglobulin receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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