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Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice

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Immunomodulatory effects of oligosaccharide preparations that resemble chemical and functional aspects of human milk oligosaccharides (HMOS) were studied for the development of new concepts in infant nutrition. A dose range of 1–5% (w/w) dietary pectin-derived acidic oligosaccharides (AOS) was tested in a murine influenza vaccination model. In addition, combinations of AOS and a 9:1 mixture of galacto-oligosaccharides and long-chain fructo-oligosaccharides (GOS/FOS) were tested at a fixed total dietary dose of 2% (w/w). It was found that AOS significantly enhanced vaccine-specific delayed-type hypersensitivity (DTH) responses in a dose-dependent manner. This was accompanied by a reduction in T-helper2 (Th2) cytokine production by splenocytes *in vitro*. Overall, this indicates that the systemic immune response to the vaccine was Th1-skewed by the dietary intervention. Combinations of GOS/FOS and AOS were more effective in enhancing DTH responses than either of the oligosaccharides alone, suggesting interaction effects between these agents. Similar to effects in infants, supplementation of the murine diets with GOS/FOS and combinations of GOS/FOS and AOS for 6-wk enhanced the proportion of fecal bifidobacteria and lactobacilli, but AOS alone did not. In conclusion, these data indicate that GOS/FOS and AOS enhance systemic Th1-dependent immune responses in a murine vaccination model. As Th1-responses are weak in early life in humans, this might suggest that application of these oligosaccharides in infant formulas will be beneficial for the development of the infant's immune system.

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Human milk provides an infant with more than just nutritional value. Among other functions, it also provides immunologic protection through constituents such as antibodies, complement, lysozyme, lactoferrin, and maternal leukocytes (1). Human milk oligosaccharides (HMOS) have been shown to stimulate specific intestinal microbiota (2), to block pathogen adhesion sites in the gut and/or to act as soluble pathogen receptor analogs (3–6). This provides the infant with a non-specific mode of protection against invading pathogens in the gastrointestinal tract. Recently, Eiwegger et al. (7) have shown that HMOS, in particular the acidic fraction, affects the expression of T-cell activation markers as well as cytokine production in cord blood cells *in vitro*. These findings suggest a direct immunomodulatory effect of HMOS and might implicate an additional mechanism of immunologic protection provided by human milk.

For situations in which breast-feeding is not possible, infant nutrition should provide the best alternative care. Human milk contains a mixture of neutral and acidic oligosaccharides (AOS) that

features a complex structural diversity (8). As this diversity of complex structures is not available, a functional analog was developed for infant nutrition. In a first step, a mixture of neutral galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (FOS) in a 9:1 ratio (GOS/FOS) was designed to resemble the molecular size distribution and functional aspects of the neutral fraction of HMOS (9–11). Recently, this concept has been extended by the addition of AOS derived from pectin, to mimic the balance between neutral and acidic HMOS (12). Similar to the described anti-adhesive properties of HMOS (3–5), Kastner et al. (13) have shown inhibitory effects *in vitro* of acidic, galacturonic acid containing oligosaccharides from carrot soup on the adhesion of various pathogens to Hep-2 cells and human intestinal mucosa.

In previous experiments (14), stimulatory effects of GOS/FOS were shown on systemic immune responses in a murine vaccination model. This information, combined with the reported *in vitro* immunomodulatory effects of acidic HMOS (7), has led us to hypothesize that AOS could induce immunomodulatory effects *in vivo*. This hypothesis was tested in a murine vaccination model, using pectin-derived galacturonic acid containing AOS characterized by a degree of methyl esterification of $>40\%$ and an amount of Δ4,5-unsaturated galacturonic acid at the non-reducing terminus of $>35\%$. Additionally, AOS were applied in combination with GOS/FOS, to test for potential interaction of the respective immunomodulatory effects. *In vivo* and *ex vivo* immunologic parameters were analyzed, as well as effects on the intestinal microbiota by measuring the percentage of fecal bifidobacteria and lactobacilli relative to the total bacterial counts.

Materials and methods

Mice

Female, 6-wk-old C57Bl/6JOlaHsd mice were obtained from Harlan (Horst, The Netherlands) and kept under normal conditions, with a 12-h dark and light cycle and access to food and water *ad libitum*. All experiments were approved by an independent animal experiments committee (DEC Consult, Bilthoven, The Netherlands).

Diets

All animals received semi-purified AIN-93G-based diets (Research Diet Services, Wijk bij Duurstede, The Netherlands). All supplemented oligosaccharide products were exchanged for the same amount of total carbohydrates. This approach resulted in a comparable overall carbohydrate composition in different diets, to ensure that the gut flora was minimally influenced by differences between control and test diets in parameters such as gut passage time and fluid retention. The oligosaccharides were mixed into the AIN-93G diet and pressed into pellets. Deviations from the AIN-93G specification are specified in Table 1 for all supplemented diets. AOS are produced from pectin (Südzucker AG, Mannheim, Germany), with a degree of polymerization (dp) of 1–20. It consists of approximately 75% galacturonic acid oligomers, 10% monomers and 15% of moisture and ash. GOS/FOS is a spray-dried powder of GOS (Vivinal GOS, Borculo Domo, Zwolle, The Netherlands; mainly dp 2–6) and FOS with a high degree of polymerization (Raftiline HP, Orafti, Wijchen, The Netherlands; dp > 10) in a 9:1 ratio. Maltodextrin was used as carrier for the spray-drying process. GOS/FOS consists of approximately 51% GOS and FOS oligosaccharides, 19% maltodextrin, 16% lactose, and 14% glucose.

Vaccination protocol and DTH response

The vaccination protocol has been described previously (14), and was shown to induce submaximal immune responses that allow the detection of stimulatory as well as inhibitory immunomodulatory effects. In short, the mice received primary and booster vaccinations,

Table 1. Composition of the diets

Ingredients (g/kg)	Control diets (AIN93G)	AOS dose–response diets			Diets containing 2% oligosaccharide preparations
		1%	2.5%	5%	
Cornstarch	397.5	391.6	382.9	368.2	385.8
Dextrinized cornstarch	132.0	130.1	127.1	122.3	128.1
Sucrose	100.0	98.5	96.3	92.6	97.1
Cellulose	50.0	49.3	48.2	46.3	48.5
Added carbohydrates		10*	25*	50*	50†

*AOS; †GOS/FOS, AOS or a combination of both; see text for details on the added carbohydrates.

consisting of an s.c. injection of a 1:1 mix of vaccine solution (100× diluted Influvac 2002/2003 in PBS, Solvay Pharmaceuticals, Weesp, The Netherlands) and adjuvant (Stimune; Cedidiagnostics, Lelystad, The Netherlands). Sham vaccination groups received injections of a 1:1 mix of PBS and adjuvant. Booster vaccinations were given 21 days after the primary vaccination. Delayed-type hypersensitivity responses (DTH) reactions were induced 9 days after booster vaccination, by s.c. injection of 25 μl Influvac into the ear pinnae of both ears. Ear thickness was measured in duplicate before antigen challenge, as well as 24 h afterwards, with a digital micrometer (Mitutoyo Digimatic 293561; Veenendaal, The Netherlands). The DTH response was calculated by subtracting the basal ear thickness from the value at 24 h after challenge. The experiments ended 10 days after booster vaccination.

Dietary intervention experiments

A dose–response experiment with AOS supplementation was performed (experiment 1), using doses of 1%, 2.5% and 5% (w/w) in the diets ($n = 10$ in all groups). In addition, a combination experiment was carried out by using GOS/FOS, AOS and combinations thereof (experiment 2). All test groups in experiment 2 featured a total supplementation dose of 2% (w/w) of the oligosaccharide preparations; (I) 2% GOS/FOS ($n = 10$), (II) 2% AOS ($n = 10$), (III) 1% GOS/FOS + 1% AOS ($n = 8$) and (IV) 1.8% GOS/FOS + 0.2% AOS ($n = 10$) were tested. Expressed as the net oligosaccharide ratios between the GOS/FOS and AOS oligosaccharides, group III contains GOS/FOS:AOS in a 2:3 mix and group IV contains GOS/FOS:AOS in a 6:1 mix, because of the differences in specific oligosaccharide content of the different oligosaccharide preparations.

All mice were given control AIN-93G diets upon arrival at the animal facility for a minimum of 5 days. The dietary interventions in supplementation groups started 14 days before the primary vaccination and lasted until the last day of the experiment, 31 days after the primary vaccination. In both experiments, a control group was included ($n = 10$) that was vaccinated normally and received the control diet (unsupplemented AIN-93G). Sham vaccination groups ($n = 3$) were also included in both experiments. These groups were not used for statistical calculations or comparisons, but served solely to demonstrate the specificity of vaccine-induced responses.

Splenocyte proliferation assay

Splenocytes were isolated by gently pressing spleen tissue through nylon mesh filters (Falcon cell strainer; Becton Dickinson, Alphen a/d Rijn, The Netherlands). After erythrocyte lysis using an ammonium-chloride buffer, cells were counted and diluted to the appropriate concentrations. Cultures were stimulated with Influvac vaccine at a pre-determined optimal concentration, and proliferation was measured by tritiated thymidine incorporation. In short, 1×10^6 cells were distributed in 96-well plates in triplicate in RMPI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were stimulated with control medium or with 0.1 μg/ml Influvac vaccine solution. After 4 days of culture, 0.4 μCi/well tritiated thymidine was added, and 24 h later cells were harvested (Filtermate, Perkin Elmer) and counted in a scintillation counter (Wallac Microbeta, Perkin Elmer). Data are presented as the difference in c.p.m. between control medium and Influvac-stimulated wells.

Splenocytes that were not used for initiating cell cultures were kept on ice and taken up in RPMI-1640 medium with 25% FCS and 12.5% DMSO at 1×10^7 cells/ml. Cell suspensions were dispensed in cryo-vials (Greiner; Alpen aan de Rijn, The Netherlands), which were placed in cryo-containers (Nalgene; Omnilabo, Breda, The Netherlands) and stored overnight at −80°C. Samples were stored in liquid nitrogen until further analysis.

Ex vivo cytokine production assay

Splenocytes cultures were set up for the measurements of *ex vivo* cytokines production, in parallel with the cultures for proliferation (described above), using the identical procedure. Supernatants were harvested after 5 days of culture and stored at −80°C until further analysis. The cytokines interferon (IFN) gamma, interleukin (IL) 2, IL-4, IL-5 and IL-10 were measured by using a multiplex bead array according to the manufacturer's instructions (x-plex reagents; BioRad, Veenendaal, The Netherlands) on a Bio-Plex workstation (BioRad). Results are shown as the difference in cytokine concentrations between stimulated and unstimulated wells, with the exception of the IL-4 values, as all unstimulated wells showed IL-4 levels that were below the detection limit. The value of the detection limit was used in the calculation of

group statistics in the case of undetectable cytokine concentrations.

Flow cytometric analysis of splenocyte populations

For flow cytometric analysis, splenocyte suspensions were thawed, washed and taken up in cold PBS with 1% FCS. Cells were incubated with fluorescently labelled antibodies for 30 min on ice. After washing, cells were analyzed on an Epics XL flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands). Propidium iodide staining and forward- and side-scatter profile was used to identify the intact living cells. The following monoclonal rat-anti mouse antibodies were used: CD3-FITC (Cat. Nr. IM2768), CD8-PE (Cat. Nr. IM2780), CD19-FITC (Cat. Nr. IM3233), CD25-PE (Cat. Nr. IM 2795), NK1.1-PE (Cat. Nr. IM2904; all from Beckman Coulter) and CD4-PE-CY5 (Cat. Nr. 553654, Becton Dickinson).

Fecal and colon content sample isolation

In experiment 2, fecal samples were collected from the cages from a 12-h period at the end of study and were stored at −20°C. Samples of the colon content were isolated directly after the animals were sacrificed and stored at −20°C.

Quantitative real time PCR of the genus *Bifidobacterium* and *Lactobacillus*

For the relative quantification of the genera *Bifidobacterium* and *Lactobacillus* in relation to the total bacterial load, a duplex 5′ nuclease quantitative real-time PCR (qPCR) assay was used as previously published (15, 16).

Briefly, thawed samples were pooled per experimental group (using a fixed weight of sample per animal) and 10× diluted in saline (w/v). Different primers and probes for the genus *Bifidobacterium* or *Lactobacillus* in combination with primers and probes for the total bacterial load (17) were used in a temperature profile consisting of 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95 and 60°C for 1 min, run on ABI Prism 7700 PCR equipment (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Thereafter, the relative amounts of the genus *Bifidobacterium* or *Lactobacillus* in the samples were calculated with respect to the total bacterial load according to Liu et al. (18), and expressed in percentages. All samples were measured in triplicate.

Statistical methods

Statistical calculations were performed in SPSS version 12.0.1 (SPSS Inc., Chicago, IL, USA) regression analysis and graph plotting was performed in GRAPHPAD PRISM 4.03 software (San Diego, CA, USA). Dose–response relationships in experiment 1 were analyzed by using linear regression, testing the slopes of the regression lines for a deviation from zero. Statistical differences between groups in experiment 2 were analyzed by ANOVA and *post hoc* Bonferroni's test. Differences in microbiologic parameters at different time points were analyzed by using Student's *t*-test, using Bonferroni-adapted p-values to correct for multiple comparisons. p-values < 0.05 were considered to be statistically significant.

Results

Experiment 1

Acidic oligosaccharides supplementation at 1–5% in the diet was well tolerated by the mice; no adverse effects were noted during the experiment. Feed uptake and body weights were slightly higher in AOS supplemented mice compared with control mice (Table 2). Regression analysis showed a significant dose–response for both parameters.

Mice that received AOS supplementation showed significantly enhanced, dose-dependent DTH responses after vaccination in comparison with control mice (Fig. 1a). Background responses after antigen challenge were low in the sham group (4.8 ± 8.2 μm; mean ± s.e.m.) compared with the control group (88.7 ± 7.3 μm), demonstrating that the responses were vaccination-dependent secondary responses. DTH responses were 40.1% higher in the group that received the highest AOS supplementation dose (124.3 ± 5.5 μm) than in control animals.

In addition, *ex vivo* cytokine production and proliferation by vaccine-stimulated splenocyte cultures were affected by dietary intervention with AOS (Fig. 1b–h). No significant effects were observed on the production of Th1-type cytokines; IFN-gamma production showed a decreasing trend and IL-2 production was elevated in the 1% dose-group but not in other dose-groups, showing no clear dose–effect relationship. AOS supplementation decreased the production of Th2-type cytokines significantly in a dose-dependent manner. IL-4 and IL-5 production were significantly reduced; the effect was most pronounced on IL-4 production, ranging from

Table 2. Animal weights and feed intake

Experiment	Group	Animal weight at the end of the experiment		Weekly feed intake per animal	
		Mean	s.e.m.	Mean	s.e.m.
AOS dose–response experiment†	Control	19.67*	0.25	16.99*	0.70
	1%	21.50*	0.43	17.90*	0.48
	2.5%	20.70*	0.56	17.66*	0.72
	5%	22.36*	0.43	18.41*	0.67
Combination experiment‡	Control	19.67	0.36	16.34	0.40
	GOS/FOS	20.05	0.35	16.58	0.42
	AOS	19.89	0.28	17.05	0.49
	1:1	19.43	0.31	16.95	0.41
	1.8:0.2	20.48	0.43	17.81	0.49

Animals were weighed weekly; data are shown from the last measurement. Feed intake was measured weekly per animal cage; the data are shown as the mean intake per animal per week. †Data were analyzed by linear regression analysis; *indicates a dose–response relationship with $p < 0.05$. ‡Data were analyzed by anova; no significant differences were observed.

320.9 ± 43.8 pg/ml in the control group to 105.2 ± 25 g/ml in the highest dose group. The production of IL-10, a cytokine produced by Th2 cells as well as other cell types, was also dose dependently reduced. To further analyze the Th1/Th2 balance, the ratio of IL-4 and IFN-gamma production was calculated per animal and averaged per experimental group. Fig. 1g shows that the IL-4/IFN-gamma ratio decreased dose-dependently with AOS supplementation. In parallel to Th2-type cytokine production, antigen-specific proliferation was dose-dependently decreased as well (Fig. 1h).

Experiment 2

Supplementation with combinations of AOS and GOS/FOS strongly enhanced vaccine-specific DTH responses compared with control-fed animals; 1% GOS/FOS + 1% AOS (group III) showed a response of 201 ± 11 μm, compared with 95 ± 9 μm in control animals. As in experiment 1, background responses were low in the sham group (6.8 ± 3.9 μm). Animals in group III exhibited significantly higher DTH responses than animals that received either GOS/FOS or AOS (groups I and II). In addition, a combination of GOS/FOS with a small amount of AOS in group IV (net GOS/FOS:AOS ratio 6:1) resulted in significantly higher DTH responses than supplementation with only GOS/FOS in group I (Fig. 2a). There were no differences in feed uptake or animal weight in experiment 2 (Table 2).

Ex vivo proliferation responses in the control group from experiment 2 (5025 ± 823 c.p.m.) were similar to those in experiment 1 (5302 ± 430 c.p.m.). IL-4 production in the control group of experiment 2 was low (38 ± 22 pg/ml) and IFN-gamma production was high (847 ± 396 pg/ml) compared with experiment 1. The intra-group variation in cytokine concentrations was high in experiment 2, and proliferation responses or cytokine production did not differ significantly between experimental groups (data not shown).

To investigate whether the difference in DTH responsiveness between both groups was related to changes in systemic lymphocyte populations, splenocytes from control and group III animals from experiment 2 were analyzed by flow cytometry (Fig. 2b). T-cell $CD4^+$ and $CD8^+$ subsets were measured, including analysis of $CD4^+$ $CD25^+$ activated or regulatory T cells. T-cell subsets did not differ significantly between control and group III animals. In addition, CD19 + B-cell and NK1.1 + natural killer cell populations did not differ significantly between the groups.

Freshly collected fecal samples from experiment 2 were analyzed from two timepoints; after 2 wk and after 6 wk of supplementation. The analyses of the relative amount of bifidobacteria and lactobacilli are shown in Fig. 2c,d. Exposure to AOS as a single agent (group II) did not induce changes compared with control animals, whereas GOS/FOS supplementation (group I) resulted in a significantly elevated percentage of bifidobacteria. Combinations of AOS and GOS/FOS resulted in significant relative increases in bifidobacteria as well as lactobacilli in groups III and IV. All supplemented groups, except group II, showed significantly elevated percentages of both bifidobacteria and lactobacilli in the samples taken after 6 wk, compared with the samples taken after 2 wk.

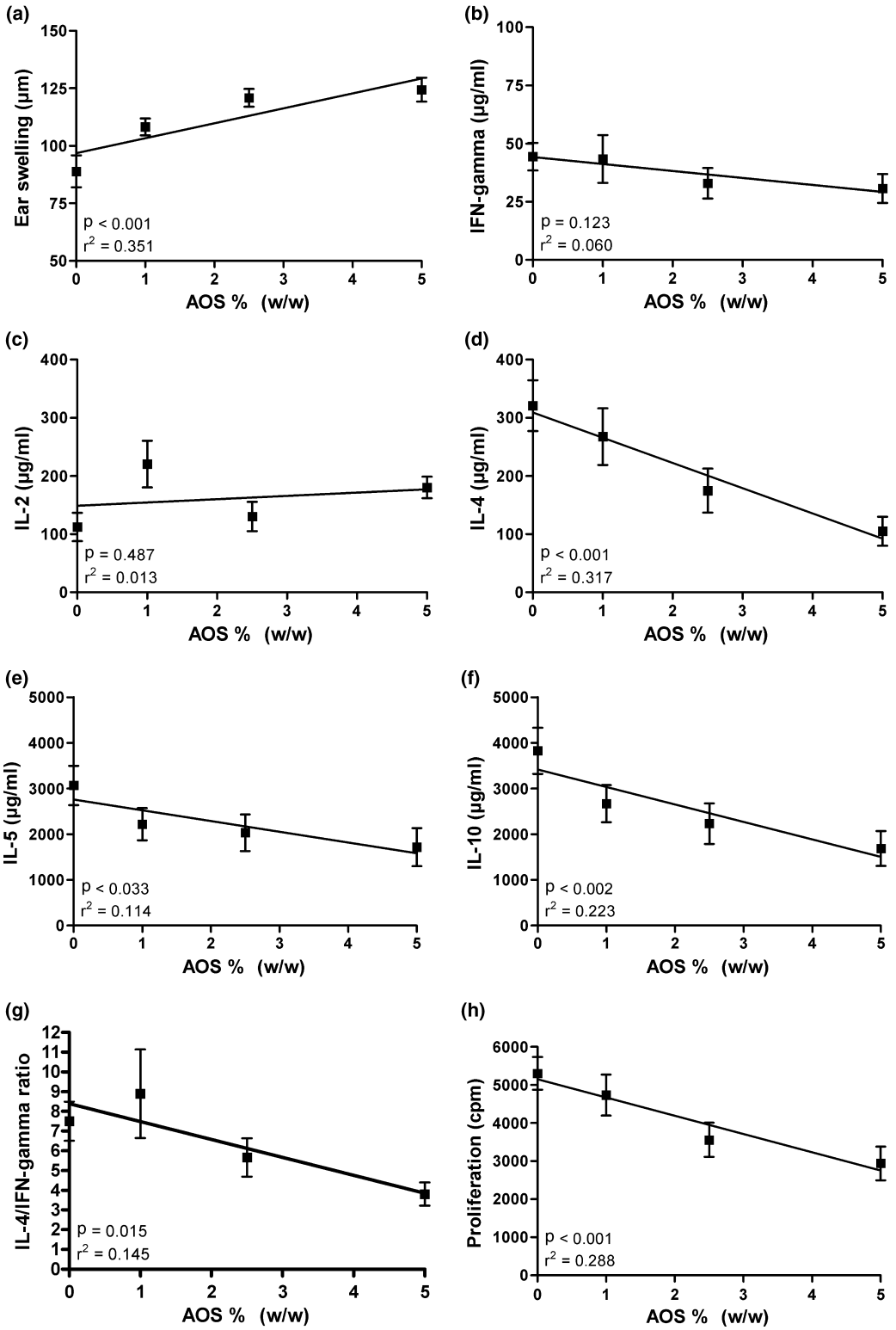


Fig. 1. Dietary acidic oligosaccharides (AOS) affect parameters of immune function *in vivo* and *ex vivo*. Supplementation doses of 1–5% AOS were tested in a murine vaccination model as indicated on the *x*-axes. (a) At the end of the experiment, *in vivo* vaccine-specific delayed-type hypersensitivity responses were analyzed by measuring ear swelling 24 h after antigen injection. (b–g) Splenocytes were isolated and cultured *in vitro*. After 5 days of antigen-specific stimulation, interferon (IFN)-gamma (b), interleukin (IL)-2 (c), IL-4 (d), IL-5 (e) and IL-10 (f) concentrations were measured in culture supernatants, and proliferation was measured by using 3H-thymidine incorporation (g) Data are represented as group means and error bars indicate s.e.m. (h) The IL-4/IFN-gamma ratio in culture supernatants was calculated per animal and averaged per experimental group.

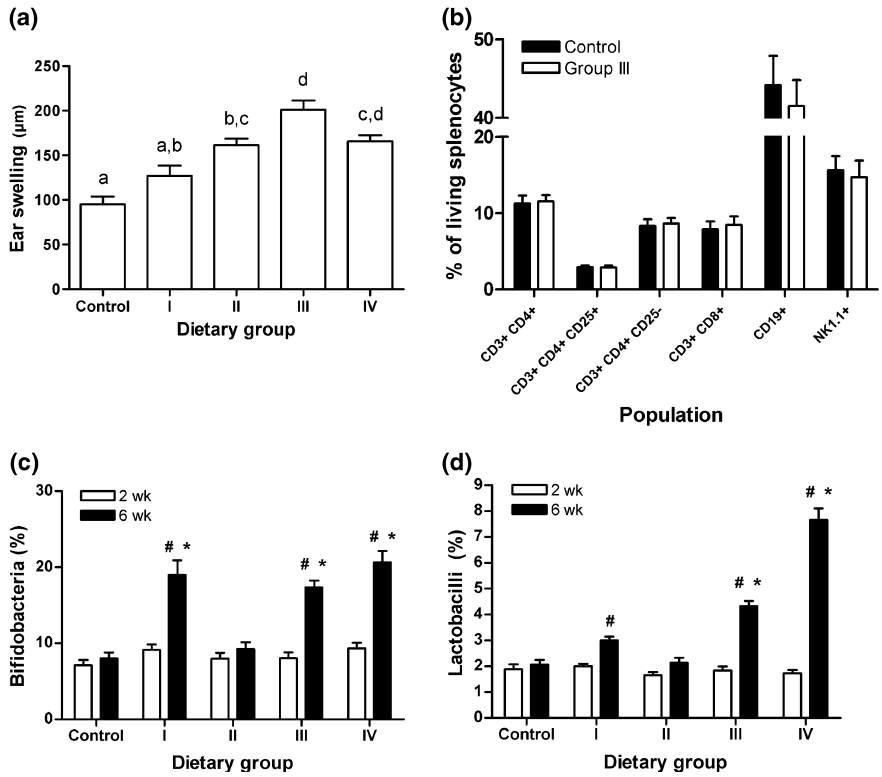


Fig. 2. The effects of combinations dietary of galacto-oligosaccharides and long-chain fructo-oligosaccharides (GOS/FOS), acidic oligosaccharides (AOS) and combinations thereof on DTH responses and the intestinal microflora. Dietary supplementation of (I) 2% (w/w) GOS/FOS, (II) 2% AOS, (III) 1% GOS/FOS + 1% AOS and (IV) 1.8% GOS/FOS + 0.2% AOS (IV) was tested vs. control diet (Control) in a murine vaccination model, as indicated on the *x*-axis or in the legend. (a) At the end of the experiment, *in vivo* DTH responses were analyzed by measuring ear swelling 24 h after antigen injection. Statistical differences between groups are indicated in the graph. Groups that do not share an indicator are different at $p < 0.05$. (b) Splenocyte populations from the control group and group III were analyzed by flow cytometry. The data are presented as the percentage of total living splenocytes. (c, d) bifidobacteria (c) and lactobacilli (d) were measured in fecal samples as a proportion of the total bacterial load. The fecal samples were pooled per group and measured in triplicate. Samples were collected after 2 wk of supplementation (white bars) and after 6 wk of supplementation (black bars). *Indicates $p < 0.05$ compared with the control group. # Indicates $p < 0.05$ between values at two and 6 wk of supplementation within a dietary group. Data in all graphs represent group means and error bars indicate s.e.m.

Discussion

The immune system at birth is not yet fully matured. Th1 responses are weak shortly after birth and tend to increase progressively with age, causing immune responses in early life to be polarized toward Th2 responses in mice and man [reviewed in (19)]. In addition to the well-known role that breast milk plays in providing passive protection via immunoglobulins and other factors, Pabst has hypothesized (20) that breast milk also plays an active role in the development of the infant's immune system. Pabst et al. (21) also provided some evidence of more effective Th1 responses in infants that had received breast milk, compared with infants that had received infant nutrition. In support of this view, there are multiple reports in literature showing lower incidences of infections (22–24) and atopy-related disorders (25–27) in breast-fed children compared with formula-fed children. These immunologic effects could potentially be mediated by a myriad of factors in breast milk, precluding the incorporation of all relevant ingredients in an infant nutrition. A practical approach is to mimic breast milk functionally, by including ingredients that support the development of Th1 responses, for use in situations when breast-feeding is not possible.

In our murine model, AOS induced a dose-dependent increase in DTH response after vaccination, a Th1-dependent *in vivo* parameter. Additionally, *in vitro* production of Th2- but not Th1-type cytokines was decreased dose-dependently. Overall, the *in vivo* and *in vitro* data indicate that dietary intervention with AOS causes a shift in the Th1/Th2 balance in a murine systemic immune response toward Th1. The observed decrease in antigen-specific proliferation might be related to the decreased IL-4 production, which is a factor that can strongly promote T-cell

division (28). AOS containing diets increased feed uptake and animal weight dose-dependently for unknown reasons. Although it cannot be ruled out that this might have played a role in the immunologic effects that were observed, this is not likely, as the second experiment showed significant immunologic effects between groups without differences in animal weight or feed uptake.

The oligosaccharide concept for application in infant nutrition features a combination of GOS/FOS and AOS (12). Because it was shown in previous work that GOS/FOS enhanced vaccine-specific DTH responses (14), combinations of GOS/FOS and AOS were also tested in this study by using a fixed total supplementation dose in all groups. A combination of 1% GOS/FOS and 1% AOS resulted in higher DTH responses than either single preparation at a 2% dose. In addition, a combination of 1.8% GOS/FOS and 0.2% AOS resulted in significantly increased DTH responses compared with 2% GOS/FOS, while 0.2% AOS alone is not expected to affect significantly the DTH response based on an intrapolation of Fig. 1d. Overall, combinations of GOS/FOS and AOS resulted in unexpected higher DTH responses than either of the single preparations, suggesting a synergistic interaction.

In contrast to experiment 1, there were no significant effects on cytokine production or cell proliferation in experiment 2, which might be due to the relatively low levels of AOS in experiment 2. This might indicate differential effects of the oligosaccharide preparations on DTH responses and *ex vivo* cytokine production. On the other hand, it is also possible that effects on cytokine productions were masked by the high intra-group variation in experiment 2.

The mechanism of action of dietary oligosaccharides in causing enhanced DTH responses is not yet clear. Mucosal and systemic Th1-stimulating effects of dietary oligosaccharides were recently published by Mizubuchi et al. (29), using a very high dose (20% w/w) of isomalto-oligosaccharides. Parallel effects of these oligosaccharides were observed on microbiota and immunologic parameters. However, direct effects were also shown on cytokine production in a macrophage-like cell line, indicating that multiple mechanisms might play a causal role in the immunomodulatory effects. Eiwegger et al. (7) described that the acidic fraction of HMOS enhanced the expression of CD25 on $CD4^+$ T cells, an activation marker that is also expressed constitutively on regulatory T cells. This prompted us to conduct a flow cytometric analysis of splenocyte populations in animals from the control group and group III. There were no differences between the groups for any of the analyzed T-cell populations including $CD4^+CD25^+$ cells, or in B- and NK-cell populations, suggesting that the observed immunomodulatory effects were not caused by phenotypic changes in major systemic lymphocyte or NK-cell populations.

In clinical studies, the inclusion of GOS/FOS into infant nutrition resulted in a significant increase in the number of bifidobacteria and lactobacilli in stool samples compared with standard infant nutrition (9, 30, 31). AOS in infant nutrition did not affect these parameters, but a combination of GOS/FOS and AOS increased fecal bifidobacteria and lactobacilli as well (12). In the current study, AOS supplementation did not affect parameters of the fecal microbiota, but GOS/FOS and combinations of GOS/FOS and AOS did. Despite the differences in gut physiology between mice and man, these results indicate that the pre-biotic effects of oligosaccharides in current murine model system are similar to the human situation.

Acidic oligosaccharides supplementation alone enhanced DTH responsiveness, but did not affect the proportions of bifidobacteria and lactobacilli in experiment 2, leaving the possibility open that the immunomodulatory effect of dietary AOS is not causally related to changes in the microbiota. On the other hand, AOS might have caused changes in certain microbiota parameters that were causally related to the immunomodulatory effects but were not measured. Either way, the observed differences in the effects of GOS/FOS and AOS on the levels of bifidobacteria and lactobacilli indicate differences in the working mechanisms of the oligosaccharides, which might explain the observed results in the groups receiving combinations of GOS/FOS and AOS on the DTH responses.

In conclusion, the data presented here show that AOS supplementation induces Th1-skewing effects and stimulatory effects on vaccine-specific DTH responses in a murine model. The data suggest interaction effects between GOS/FOS and AOS with respect to DTH responses, in parallel to differential effects of the oligosaccharide preparations on the microbiota. Optimal Th1-dependent DTH responses were observed by using combinations of GOS/FOS and AOS, indicating that this concept holds promise for application in an infant nutrition, with the aim to mimic breast milk as close as possible in composition and function. Clinical trials will need to be performed to determine the efficacy of this concept in infants, for use in situations when breast-feeding is not possible.

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