

Cow Milk Allergy Symptoms Are Reduced in Mice Fed Dietary Synbiotics during Oral Sensitization with Whey¹

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Abstract

Cow milk allergy is the most common food allergy in children. So far, no effective treatment is available to prevent or cure food allergy. The purpose of this study was to compare effects of dietary supplementation with a prebiotic mixture (Immunofortis), a probiotic strain [*Bifidobacterium breve* M-16V], or a synbiotic diet combining both on the outcome of the allergic response when provided during oral sensitization with whey in mice. Mice were fed diets containing 2% (wt:wt) Immunofortis and/or the *B. breve* M-16V ($n = 6/\text{group}$). The acute allergic skin response was determined by measuring ear swelling. Antigen-induced anaphylaxis was scored. Furthermore, whey-specific serum immunoglobulins and mouse mast cell protease-1 (mMCP-1) were determined. In mice fed the synbiotic mixture, the allergic skin response and the anaphylactic reaction were strongly reduced compared with whey-sensitized mice fed the control diet ($P < 0.01$). Immunofortis or *B. breve* M-16V alone were significantly less effective in reducing the allergic skin response than the synbiotic diet and did not reduce the anaphylactic reaction. The whey-specific IgE and IgG₁ responses were not affected; however, IgG_{2a} was greater in all treated groups than in the control group ($P < 0.05$). Serum mMCP-1 concentrations, reflecting mucosal mast cell degranulation, were lower in mice fed synbiotics compared with those fed the control diet ($P < 0.01$). Dietary supplementation with Immunofortis, *B. breve* M-16V, and particularly the synbiotic mixture, provided during sensitization, reduces the allergic effector response in a murine model of IgE-mediated hypersensitivity that mimics the human route of sensitization. This model shows the potential for dietary intervention with synbiotics in reducing the allergic response to food allergens. J. Nutr. 139: 1398–1403, 2009.

Introduction

The incidence of food allergy is rising in the western world (1). Approximately 2–5% of children in the western world are affected with cow milk allergy (CMA).⁴ Many studies suggest that food allergy in early life is associated with the development of respiratory allergy and even asthma later in life (2–4). So far, no effective preventive strategy or treatment protocol is available for CMA (5). Avoidance of the affecting food in the diet is the single effective solution. Some studies suggest that breastfeeding protects the infant from developing allergic disease. Breast-feeding supports maturation of the immune system of the young infants and protects the child from infections (6,7). In

human milk, there are many components with immunological properties, e.g. nondigestible oligosaccharides, secretory IgA (sIgA), mucins, cytokines, long-chain PUFA, and hormones. In particular, oligosaccharides may support immunocompetence, a prerequisite for adequate capacity to induce oral tolerance, either via direct effects or via stimulation of a beneficial intestinal microbiota (8–10).

Nondigestible prebiotic oligosaccharides are defined as “food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of 1 or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health” (11). Nondigestible carbohydrates, which mimic the oligosaccharides in human milk, are known to have immune regulatory properties (12). In a mouse vaccination model, the vaccine-specific T helper cell (T_H1) response was improved when a prebiotic diet was given starting 1 wk prior to the first vaccination, whereas it failed when the diet was provided after the first vaccination (13). This indicated that the prebiotics are especially effective during the priming phase of an immune response. Therefore, the prebiotic concept was suggested to be a relevant tool for the prevention of immune

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⁴ Abbreviations used: AD, atopic dermatitis; CMA, cow milk allergy; CT, cholera toxin; FOS, long-chain fructo-oligosaccharide; GOS, short-chain galacto-oligosaccharide; i.d., intradermally; IL, interleukin; mMCP-1, mouse mast cell protease-1; sIgA, secretory IgA; T_H, T helper cell.

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disorders such as allergies. In clinical preventive studies in children, early dietary intervention resulted in reduced incidence of allergic manifestations and infections (14), improved microbiota (15), increased fecal sIgA (16), and reduced incidence of atopic dermatitis (AD) (17). Prebiotic oligosaccharides are known to stimulate the growth and/or activity of probiotic bacteria. *Bifidobacteria* together with *Lactobacilli* are well known for their health-promoting functions and selected species are used as probiotics (18–20). Neonates receiving breastfeeding have higher counts in *Bifidobacteria* compared with babies fed standard infant formula. When added to standard milk formula, the prebiotic mixture Immunofortis enhanced *Bifidobacteria* fecal counts in bottle-fed infants to levels similar to those found in breast-fed infants (21). In a double-blind, placebo-controlled trial, a mixture of probiotics containing *Lactobacillus rhamnosus* GG, *Bifidobacterium breve*, and *Propionibacterium freudenreichii* spp. *Shermanii* JS alleviated atopic eczema/dermatitis syndrome in IgE-sensitized infants (22). In a recent review by Savilahti et al. (23), the positive effects of probiotics on allergy prevention and treatment have been evaluated. They concluded the effects are highly variable and that optimal treatment remains unsettled. Because prebiotic oligosaccharides selectively stimulate the growth and/or activity of bacterial species such as *B. breve*, synbiotic concepts are currently introduced combining pro- and prebiotics enabling optimized function of the pro- and prebiotics.

The potential of selective pre-, pro-, and synbiotics to prevent the development of allergic disease has been shown in some clinical trials (17,20,24–26). However, the outcomes of these studies vary and questions remain concerning the mechanisms of action. In the present study, we used a mouse model of orally induced CMA to assess preventive effects of dietary intervention with Immunofortis, a probiotic diet, or a synbiotic diet (27,28).

Methods

Chemicals. Whey proteins were obtained from DMV International. Cholera toxin (CT) was purchased from Quadrant Diagnostics. PBS was from Cambrex Bio Science, and biotin-labeled rat anti-mouse IgE, IgG₁, IgG_{2a}, and IgA were from BD Biosciences. All other chemicals were obtained from Sigma-Aldrich-Chemie.

Diets. Semipurified cow milk protein-free, AIN-93G-based diets were composed and mixed with pro-, pre-, or synbiotics by Research Diet Services (29). The probiotic strain, *B. breve* M-16V (freeze-dried on maltodextrin carrier and conserved at –20°C), was obtained from Morinaga (Morinaga Milk Industry) and 2% (wt:wt) 2×10^9 colony-forming units/g was mixed through the diets and pressed into pellets. Viability of *B. breve* after diet manufacturing was confirmed by plating on MRS agar. The prebiotic diet contained 2% (wt:wt) Immunofortis, a 9:1 (wt:wt) mixture of short-chain galacto-oligosaccharides [GOS; obtained by enzymatic elongation of lactose with galactose by β -galactosidase, with a degree of polymerization of 3–8] and long-chain fructo-oligosaccharides (FOS; derived from chicory inulin with a mean degree of polymerization of >23). Immunofortis is a spray-dried powder of GOS (Vivinal GOS, Borculo Domo) and long-chain FOS (Raftiline HP). Immunofortis consists of ~50% GOS and FOS, 19% is maltodextrin (glucidex 2, Roquette), 16% lactose, 14% glucose, and 1% galactose (13). Immunofortis was exchanged against 2% (wt:wt) of total carbohydrates present in the diet. In total, 4 diets were prepared: control, probiotic, prebiotic, and synbiotic (containing 2% Immunofortis and *B. breve* M-16V). The diets were stored at –20°C prior to use.

Oral sensitization and challenge of mice. Three-week-old specific pathogen-free female C3H/HeOJ mice were purchased from Charles River Laboratories. Animal care and use were performed in accordance

with the guidelines of the Dutch Committee of Animal Experiments. Sham- and whey-sensitized mice were fed a control, *B. breve*, GOS/FOS, or synbiotic diet ($n = 6/\text{group}$). Two weeks before the first sensitization, the dietary intervention was started and continued during the whole study protocol. Mice were sensitized i.g. with 0.5 mL homogenized whey (40 g/L PBS) with CT (20 mg/L PBS) as an adjuvant using a blunt needle. Control mice received CT alone. Mice were boosted weekly for a period of 5 wk as described previously (27). One week after the last sensitization, feces were collected and mice were challenged with whey intradermally (i.d.) in the ear to determine the acute allergic skin response and anaphylaxis scores. After 24 h, mice were challenged i.g. with 1.0 mL homogenized whey (100 g/L PBS) and 30 min later, blood samples were collected in Minicollect Z Serum Sep vials (Greiner bio-one). Collected blood was centrifuged at $16,000 \times g$; 15 min and sera were stored at –70°C. The weight of the mice was monitored weekly.

Acute allergen-specific skin response. The acute allergen-specific skin response was measured after i.d. injection with 20 μL homogenized whey protein (0.5 g/L in PBS) in the ear pinnae. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo) at $t = 0$ and 1 h after challenge. Delta ear swelling (μm) was calculated by subtracting the specific whey-induced ear swelling in sensitized mice after 1 h from the basal ($t = 0$ h) ear thickness. Additionally, the mean Δ ear swelling from the group of sham-sensitized mice fed the corresponding diet was subtracted.

Systemic anaphylaxis. Anaphylaxis was scored after i.d. challenge in the ear. Symptoms of systemic anaphylaxis were scored [adapted from Li et al. (30)]: 0 = no symptoms; 1 = pilar erecti, scratching and rubbing around the nose and head; 2 = pilar erecti, reduced activity; 3 = activity after prodding and lowered body temperature; 4 = no activity after prodding, labored respiration, and lowered body temperature; and 5 = death.

Measurement of specific serum Ig and mouse mast cell protease-1. Concentrations of whey-specific IgE, IgG₁, and IgG_{2a}, were determined in serum using ELISA as described previously (27); specific IgA was measured in feces. In short, microtiter plates (Greiner) were coated with 100 μL whey (20 mg/L), washed, and blocked with 5% bovine serum albumin. Samples were applied in several dilutions; for determinations in feces, 1.0 g feces was dissolved in 10 mL PBS and centrifuged at $16,000 \times g$; 5 min. The supernatant was collected for IgA measurements. Samples were incubated for 2 h at room temperature. Plates were washed and incubated with biotin-labeled rat anti-mouse IgE, IgG₁, IgG_{2a}, or IgA (1 mg/L), washed, and incubated with streptavidin-horseradish peroxidase, washed, and developed with o-phenylenediamine. The reaction was stopped with 4 mol/L H₂SO₄ and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-Rad). Results were expressed as arbitrary units with pooled sera from whey- and alum-intraperitoneally-immunized mice used as a positive reference serum to make the titration curve. Serum concentrations of mouse mast cell protease-1 (mMCP-1) were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific).

Preparation of fecal samples for PCR of *B. breve* M-16V. For PCR analysis, 0.1 g feces was placed in a Lysing Matrix E tube with 1.0 mL stool lysis buffer (Qiagen). These tubes were placed in a mini-BeadBeater (BioSpec Products) for 3 min, heated for 5 min at 95°C, and centrifuged at $14,000 \times g$; 5 min. Then 200 μL Lysozym (10 g/L MiliQ) was added to the supernatant, heated for 30 min at 37°C, and centrifuged at $4000 \times g$; 10 min. DNA was extracted from the collected supernatant using a NucliSENS easyMAG (BioMerieux). PCR was performed as described previously (21) using *B. breve* M-16V-specific primers. Briefly, primers and probes of the *B. breve* were used in a temperature profile consisting of 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60°C for 1 min.

Statistics. All data were analyzed using 1-way ANOVA and post hoc Bonferroni's multiple comparison test except for the anaphylaxis data,

these were analyzed using the Kruskal-Wallis test. Statistical analyses were conducted using GraphPad Prism software (version 4.03). *P*-values < 0.05 were considered significant. Data are represented as means $\pm$ SEM.

Results

Acute allergic skin response. One hour after dermal challenge, the Δ ear swelling in the whey-sensitized mice was $117.6 \pm 8.6 \mu\text{m}$ (remaining ear swelling upon subtraction of ear swelling in sham-sensitized mice fed control diet, see Methods section). The ear swelling of the whey-sensitized mice fed the pro- and prebiotic diet was lower than in those fed the control diet ($P < 0.05$). In mice fed the synbiotic diet, this effect was even more pronounced ($P < 0.001$; Fig. 1). The ear swelling of mice fed the synbiotic diet was reduced ($P < 0.01$) compared with mice fed the pro- and prebiotic diets. The diets themselves did not affect ear swelling in sham-sensitized control mice (data not shown).

Systemic anaphylaxis after i.d. challenge. The mice were i.d. challenged in the ear with whey 1 wk after the last sensitization. In the whey-sensitized group fed the control diet, the anaphylaxis score was enhanced compared with control sham-sensitized mice ($P < 0.01$; Fig. 2). In the synbiotic group, anaphylaxis scores were lower than whey-sensitized mice fed the control diet ($P < 0.01$). Neither the pre- nor probiotic diet alone affected shock scores.

Decreased mMCP-1 levels in serum. To assess the effect of dietary intervention on mucosal mast cell degranulation, mMCP-1 concentrations were determined in the serum. Serum mMCP-1 was lower in mice fed the synbiotic diet than in those fed control diet ($P < 0.01$; Fig. 3) but was unaffected in mice fed the prebiotic or probiotic diet.

Whey-specific Ig levels in serum or feces. Thirty minutes after oral challenge, serum was obtained from all mice. Whey-specific serum IgE, IgG₁, IgG_{2a}, and fecal IgA were analyzed using ELISA. In whey-sensitized mice, specific IgE and IgG₁ increased compared with sham-sensitized mice ($P < 0.01$; Fig. 4A,B). Overall dietary intervention did not affect serum IgE and IgG₁ levels. In contrast, the probiotic, prebiotic, and synbiotic diets enhanced whey-specific IgG_{2a} levels in whey-sensitized mice ($P < 0.01$) compared with sham-sensitized mice (Fig. 4C). The IgG_{2a} levels in whey-sensitized mice fed the control diet

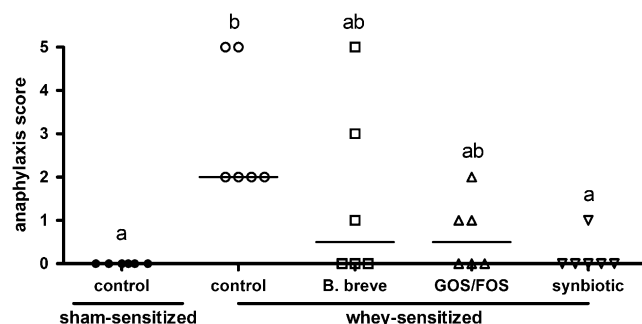


FIGURE 2 Anaphylactic symptom scores of sham-sensitized mice fed a control diet and whey-sensitized mice fed a control, *B. breve*, GOS/FOS, or synbiotic diet. Values are medians, $n = 6$. Medians without a common letter differ, $P < 0.01$.

were not significantly enhanced compared with sham-sensitized mice. Specific IgA levels were increased in feces of whey-sensitized mice fed the control or *B. breve* diet ($P < 0.01$; Fig. 4D) and in mice fed the GOS/FOS or synbiotic diet ($P < 0.001$) compared with sham-sensitized mice fed the control diet.

PCR analysis. PCR analysis on the fecal samples was performed to verify whether the *B. breve* M-16V was present in the feces of the control or pro-, pre-, and synbiotic treated mice. Indeed, the presence of *B. breve* M-16V was confirmed only in the mice fed the pro- and synbiotic diets, whereas feces of control mice and mice fed Immunofortis did not contain *B. breve* M-16V (data not shown).

Discussion

Both pro- and prebiotics modulate immune function and have been shown to reduce the risk of developing allergic disease in human trials (17,31,32). Host factors, bacterial strain differences, dosage, and timing of supplementation may underlie the differences in effectiveness. There is general agreement that, although there are promising effects, more data are needed (23,33). In the present study, dietary intervention with synbiotics combining probiotic strain *B. breve* M-16V with prebiotic GOS/FOS mixture Immunofortis was shown to protect against the development of allergic symptoms in mice orally sensitized with whey. Synbiotics effectively reduced the acute allergic skin response, the anaphylaxis scores, and mast cell-derived mMCP-1 concentrations. Pro- and prebiotics alone were less effective.

The skin response is a robust parameter and was associated with enhanced serum IgE and mast cell-derived mMCP-1 in

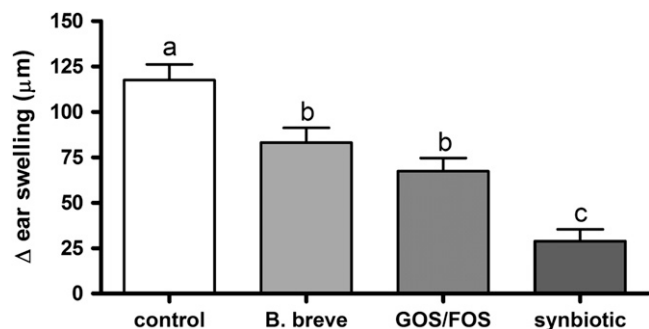


FIGURE 1 Acute allergic skin response of whey-sensitized mice fed a control, *B. breve*, GOS/FOS, or synbiotic diet. The acute ear swelling response was measured 1 h after i.d. challenge with whey. Values are means $\pm$ SEM, $n = 6$. Means without a common letter differ, $P < 0.05$.

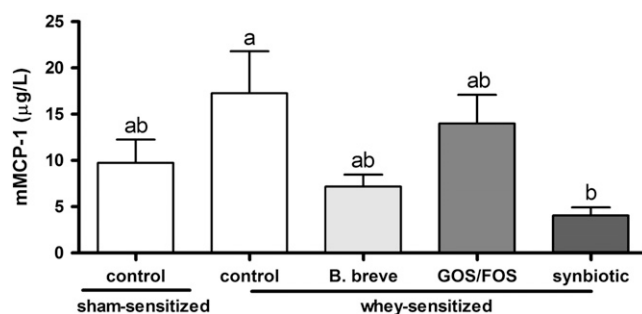


FIGURE 3 Serum mMCP-1 concentrations of sham-sensitized mice fed a control diet and whey-sensitized mice fed a control, *B. breve*, GOS/FOS, or synbiotic diet. Values are means $\pm$ SEM, $n = 6$. Means without a common letter differ, $P < 0.01$.

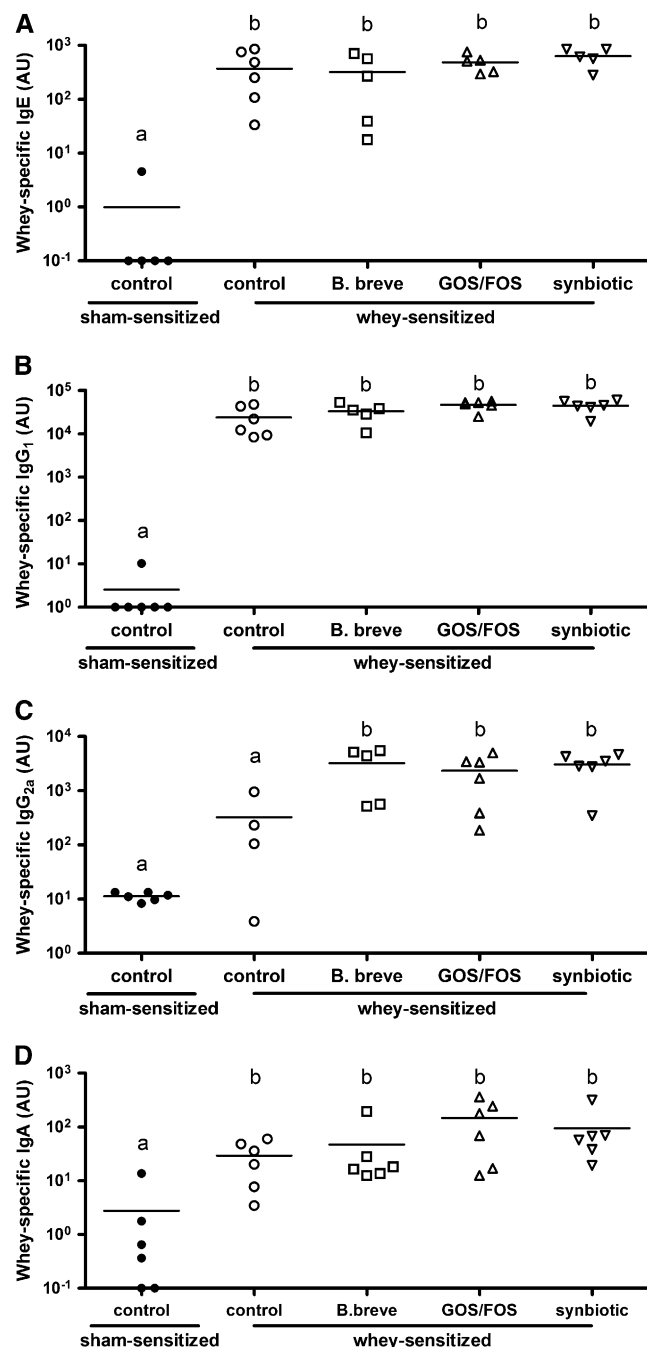


FIGURE 4 Whey-specific Ig of sham-sensitized mice fed a control diet and whey-sensitized mice fed a control, *B. breve*, GOS/FOS, or synbiotic diet. Whey-specific serum IgE (A), serum IgG₁ (B), serum IgG_{2a} (C), and fecal IgA (D) levels were measured. Values are means $\pm$ SEM, $n = 4$ –6. Means without a common letter differ, $P < 0.01$.

previous studies (27). Mice fed pro- and prebiotic diets had a significantly reduced acute allergic skin response upon whey challenge in the ear compared with whey-sensitized mice fed the control diet. The synbiotic diet was even more effective and almost completely prevented occurrence of the acute skin response as well as anaphylactic shock reactions. The clinical effectiveness of the prebiotic mixture used in this study has previously been shown to reduce the incidence of AD in infants at risk. The newborn infants were randomly assigned to standard formula milk or formula milk containing Immunofortis until 6 mo of age, reducing the incidence of AD by >50% (17). Furthermore, infants at high risk for allergy supplemented with

the prebiotic mixture had a slight reduction in total IgE and IgG₁₋₃ and specific IgG₁ (34). IgG₄ tended to increase, which might be suggestive for tolerance induction/outgrowing food allergy. Also probiotics have been shown to reduce AD development in infants. When pregnant mothers with a family history of allergic disease and their babies were supplemented with *Lactobacillus GG*, the cumulative incidence of atopic eczema was still reduced after 7 y by 30% compared with placebo controls (35). Although the incidence of eczema was reduced, IgE levels were not affected. In a similar study, *Lactobacillus reuteri* reduced the incidence of IgE-associated eczema and the occurrence of a positive skin prick test for almost 50%, whereas IgE was not affected (32). As in the human studies, the allergic effector response in the current study using mice orally sensitized with whey was reduced upon dietary intervention with pro-, pre-, and synbiotics, whereas IgE levels remained enhanced. In young infants, development of AD can objectively be scored. Approximately one-third of these children are affected with food allergy. Nevertheless, the occurrence of eczema in AD patients and the acute allergic skin response as measured in mice form reliable measures for skin responses to allergens and, in this respect, this parameter in mice might reflect at least in part some aspects of AD. Indeed, a synbiotic diet has been shown to reduce AD-like skin lesions in mice (24).

Dietary intervention with pro-, pre-, and, in particular, synbiotics reduced the acute allergic skin response and the anaphylaxis scores very effectively, which could not be explained by a reduction in whey-specific T_H2 type IgE or IgG₁ levels. However, increased levels of whey-specific IgG_{2a} and enhanced fecal type IgA levels may reflect improved T_H1 or regulatory T-cell responses in the mice fed a pro-, pre-, or synbiotic diet (16,36,37). In a preclinical murine model, heat-treated *L. rhamnosus GG* reduced the development of A,D probably through the induction of interleukin (IL)-10 (38). These studies and our study are in accordance with at least 2 clinical studies in which probiotic treatment enhanced (fecal) IgA and IL-10 and alleviated allergic symptoms (23,39). Hence, dietary intervention with pre-, pro-, and synbiotics in mice orally sensitized with whey may have supported T_H1 responses and/or regulatory T-cell function.

Besides the acute allergic skin response, anaphylactic scores upon i.d. challenge were dramatically reduced in whey-sensitized mice fed the synbiotic diet. Although in humans the shock usually appears after an oral challenge, at least for food allergens, anaphylaxis in our mouse study occurred after i.d. challenge with whey proteins. Eigenmann et al. (40) studied allergic shock in a murine model of orally induced β -lactoglobulin sensitization. In this study, the anaphylactic shock reaction occurred after oral challenge. Oral inoculation with an avirulent *Salmonella typhimurium* strain prior to allergic sensitization partially prevented anaphylaxis. This is in accordance with the effects of the *B. breve* on anaphylaxis as observed in the current study. So far, to our knowledge, there is no literature available about the preventive effects of pre- and synbiotics on allergen induced anaphylaxis.

The dietary effects on the acute allergic skin response and the anaphylactic shock coincided. Mast cell degranulation most probably underlies both symptom scores. Indeed, mMCP-1 serum concentrations reflecting mucosal mast cell degranulation were reduced, particularly in mice fed the synbiotic diet. Hence, the diets may have altered mast cell function. In a murine model of ovalbumin-induced allergy, oral administration of different bacterial strains prevented mast cell degranulation (41). IgE receptor expression on mast cells can be diminished by

CD4⁺CD25⁺ regulatory T cells upon direct contact or via the secretion of IL-10 and transforming growth factor- β (42). Because whey-specific fecal IgA was enhanced upon dietary intervention, this may be an indication of enhanced regulatory T-cell function. In a clinical study with healthy infants receiving a GOS/FOS diet, fecal sIgA increased and it was suggested to have a regulatory function and a positive effect on mucosal immunity (16). Alternatively, IgG-blocking antibodies may have inhibited IgE-mediated anaphylaxis in mice, as was shown by a study from Strait et al. (43). IgG_{2a} that was enhanced upon dietary intervention may have a function as blocking antibody.

The current study shows very promising effects of the synbiotic concept in prevention of the acute allergic skin response, reducing the incidence and severity of anaphylaxis and reducing mMCP-1 a marker for mast cell degranulation. Little is known about the use of synbiotics to prevent or cure allergic disease. A synbiotic mixture of *Lactobacillus casei* and dextran reduced pollen allergy in humans (24). Kukkonen et al. (25) showed that a synbiotic mixture containing *L. rhamnosus* GG and LC705, *B. breve* Bb99, and *Propionibacterium freudenreichii* ssp *shermanii* combined with GOS supplied to newborn infants was safe, reduced the cumulative incidence of IgE-associated (atopic) diseases, and decreased the occurrence of respiratory infections.

In the present study, a preventive dietary intervention study was performed comparing the effects of pro-, pre-, and synbiotics in mice orally sensitized to whey. In particular, the synbiotic combination of Immunofortis and the probiotic strain *B. breve* M-16V, and to a lesser extent the pro- and prebiotic diets, reduced the acute allergic skin response and anaphylactic scores, which are clinical relevant allergic parameters. Although the exact mechanism of action is not yet known, dietary intervention enhanced whey-specific T_H1 type serum IgG_{2a} and specific IgA in feces while reducing mast cell degranulation. Synbiotics comprise a promising concept that may be more effective in reducing allergic symptoms than single preparations of pre- or probiotics.

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