

Received Date : 26-Aug-2015

Accepted Date : 25-Sep-2015

Article type : Original Article: Experimental Allergy and Immunology

Editor : Hans-Uwe Simon

Maternal exposure to GOS/Inulin mixture prevents food allergies and promotes tolerance in offspring in mice.

Bouchaud Grégory, PhD ^{†,1}	gregory.bouchaud@nantes.inra.fr
Castan Laure, MS ^{†,1,2,3,4}	laure.castan@etu.univ-nantes.fr
Chesné Julie, PhD ^{1,2,3,5}	julie.chesne@univ-nantes.fr
Braza Faouzi, PhD ^{1,2,3,5}	faouzi.braza@etu.univ-nantes.fr
Aubert Philippe, MS ^{3,5,7}	philippe.aubert@univ-nantes.fr
Neunlist Michel, PhD ^{3,5,7}	michel.neunlist@univ-nantes.fr
Magnan Antoine, MD PhD ^{2,3,4,6,7}	Antoine.Magnan@univ-nantes.fr
Bodinier Marie, PhD ¹	marie.bodinier@nantes.inra.fr

¹INRA, UR1268 BIA, rue de la géraudière, BP 71627, F-44316 Nantes, France; ²INSERM, UMR1087, l'institut du thorax, Nantes, F-44000 France; ³CNRS, UMR6291, Nantes, F-44000 France; ⁴Université de Nantes, Nantes, F-44000 France; ⁵INSERM UMR913, Institut des Maladies de l'Appareil Digestif (IMAD), Faculté de Médecine, Nantes, F-44000 France; ⁶CHU de Nantes, l'institut du thorax, Service de Pneumologie, Nantes, F-44000 France; ⁷DHU2020 médecine personnalisée des maladies chroniques, Nantes, F-44000 France;

[†]These authors contributed equally to the work.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12777

This article is protected by copyright. All rights reserved.

Corresponding Author: Dr Marie Bodinier, INRA, UR1268 BIA, rue de la géraudière, BP 71627, F-44316 Nantes, France - marie.bodinier@nantes.inra.fr, phone : +33 240675035

Maternal exposure to GOS/Inulin mixture prevents food allergies and promotes tolerance in offspring in mice.

Grégory Bouchaud^{†,1}, Laure Castan^{†,1,2,3,4}, Julie Chesné^{2,3,4}, Faouzi Braza^{2,3,4}, Philippe Aubert^{3,5,7}, Michel Neunlist^{3,5,7}, Antoine Magnan^{2,3,4,6,7} and Marie Bodinier¹.

¹INRA, UR1268 BIA, rue de la géraudière, BP 71627, F-44316 Nantes, France

²INSERM, UMR1087, l'institut du thorax, Nantes, F-44000 France;

³CNRS, UMR6291, Nantes, F-44000 France;

⁴Université de Nantes, Nantes, F-44000 France

⁵INSERM UMR913, Institut des Maladies de l'Appareil Digestif (IMAD), Faculté de Médecine, Nantes, F-44000 France.

⁶CHU de Nantes, l'institut du thorax, Service de Pneumologie, Nantes, F-44000 France;

⁷DHU2020 médecine personnalisée des maladies chroniques, Nantes, F-44000 France;

[†]These authors contributed equally to the work.

Abstract

Background: Food allergies affect 4 to 8% of children and are constantly on the rise, thus making allergies a timely issue. Most importantly, prevention strategies are non-existent, and current therapeutic strategies have limited efficacy and need to be improved. One alternative to prevent or reduce allergies, particularly during infancy, could consist of modulating maternal immunity and microbiota using non-digestible food ingredients, such as prebiotics. For this purpose, we studied the preventive effects of Balb/c mothers' consuming

prebiotics during pregnancy and breastfeeding on food allergy development in offspring mice.

Methods: After weaning, the offspring from mothers who were exposed to GOS/inulin mixture or fed a control diet were intraperitoneally sensitized to wheat proteins to induce a systemic allergic response and orally exposed to the same allergen. Immunological, physiological and microbial parameters were analyzed.

Results: GOS/Inulin mixture diet modified the microbiota of mothers and their offspring. Offspring from mothers who received GOS/Inulin prebiotics were protected against food allergies and displayed lower clinical scores, specifically of IgE and histamine levels, compared to offspring from mothers fed a control diet. Moreover, GOS/Inulin supplementation for the mother resulted in stronger intestinal permeability in the offspring. Enhancement of the regulatory response to allergic inflammation and changes in the Th2/Th1 balance toward a dampened Th2 response were observed in mice from GOS/Inulin mixture-exposed mothers.

Conclusion: The treatment of pregnant and lactating mice with non-digestible GOS/Inulin prebiotics promotes a long-term protective effect against food allergies in the offspring.

Short title: Maternal prebiotic diet prevent pups food allergy.

Key messages

- Maternal GOS/Inulin diet protects offspring from clinical symptoms of food allergies.
- Maternal GOS/Inulin diet reduces Th2 response in offspring with food allergies.
- Maternal GOS/Inulin consumption modifies offspring's microbiota.

Capsule abstract

A mother's GOS/Inulin-supplemented diet protects the offspring against food allergies through a modulation of immune response.

Key words

gut, immunity, microbiota, symptoms, wheat.

Abbreviations: Alum: aluminum hydroxide; ANOVA: analyze of variance; CTL: control group; FAs: food allergies; F-ELISA: fluorometric enzyme-linked immunosorbent assay; FBS: fetal bovine serum; HBSS: Hank's balanced salt solution; HRP: horseradish peroxidase; IFN: interferon; Ig: immunoglobulin; IL: interleukin; PBS: phosphate buffered saline; RPMI: Roswell Park Memorial Institute; SD: standard deviation; TGF: transforming growth factor; Treg: regulatory T cell; GOS: galacto-oligosaccharides; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BCA: bicinchoninic acid; REA: Research Executive Agency; AU: arbitrary unit; FITC: fluorescein isothiocyanate; H&E: hematoxylin and eosin.

Introduction (word count : 3458)

In developed countries, the prevalence of food allergies (FAs) is estimated to affect up to 8% of children and 2% of adults(1). FAs most often begin within the first two years of life and can be caused by an adverse immunologic reaction involving immunoglobulin E (IgE) to an ingested food antigen. The low-affinity IgE receptor FcγRII (CD23), expressed on enterocytes, has been implicated in the bidirectional transport of allergen-IgE complexes from the lumen to the serosa in the intestine(2). Wheat is one of the 8 foods that most frequently promote allergies in childhood; wheat allergy affects up to 0.5% of the population(3). To date, wheat has been considered a common childhood FA that is usually

outgrown by school age(4). The major wheat allergens are found among the relatively insoluble gluten proteins, consisting of gliadins and glutenins(6, 7). One explanation for the increased prevalence of allergies lies in the well-known "hygiene hypothesis", which postulates that low or no exposure to exogenous antigens in early life may increase the risk of allergic diseases(8, 9). It has been described that an alteration of the microbial flora favors the persistence of Th2 cytokines (IL-4, IL-13, and IL-5), which are predominant at birth, and limits the shift toward a predominant Th1 response and the development of regulatory T cells. Moreover, declining microbial diversity, particularly in the gut, is implicated in the dramatic increase in allergies and other inflammatory and immune diseases(10, 11).

To date, clinical management of wheat allergies has been based on avoidance and the use of rescue medication for reactions. However, 35% of patients remain allergic by the age of 12, suggesting that new treatments are necessary. Over the last several years, new alternatives have been proposed to prevent and treat allergic diseases. Among these, research has focused on the use of probiotics and prebiotics.. Despite more than 19 randomized controlled trials to assess the effects of probiotics in allergy prevention, there are still no definitive benefits or guidelines. There is early promise in using prebiotics to selectively stimulate the growth and activity of bacteria in the colon and improve host health. Prebiotics are non-digestible carbohydrates that stimulate the growth of probiotic bacteria in the intestine. The most common prebiotics are indigestible oligosaccharides, such as inulin and galacto-oligosaccharide (GOS). Both can interact with the gut microflora and play a central role in the maturation of the immune system. They regulate allergic inflammation through an inhibition of the Th2 profile and an up-regulation of the Th1 and Treg responses(12-14). Recent data indicate that prebiotics might also exert a direct action on the immune system, notably by interacting with "galectins" that can exert various immunomodulatory properties

via their binding to some T cell receptors(15). However, none of these mechanisms of action of prebiotics (direct or indirect) on the immune system are well understood. Previous studies in allergic mouse models have described prebiotics (i.e., FOS, GOS) as prophylactic immune-modulators that attenuate allergic responses against house dust mites or pollen(16). Prenatal life and early infancy are crucial periods during which different systems are established, including the immune system, microbiota, gut barrier integrity and oral tolerance(17). Recent work has shown that a maternal diet supplemented with prebiotics (a mix of oligosaccharides) during pregnancy or lactation can affect the development of allergic symptoms in the offspring(18, 19). Nevertheless, the effect of supplementing the diet of adult mice with prebiotics during perinatal period (pregnancy and breastfeeding) on the development of the microbiota and the immune system in the offspring has not been evaluated in the context of allergies and whether this period could be of interest.

We hypothesized that prebiotics might be a promising dietary concept for early-life intervention in the prevention of wheat allergies. Therefore, in this study, we aim to investigate the effect of maternal supplementation with GOS/Inulin mixture during pregnancy and lactation on the development of food allergies in the offspring.

Methods

Animal studies: Balb/c mice from Charles River, France, were used and housed in a ventilated cage system. The protocol was approved by the Ethics Committee on Animal Experimentation of the Pays de la Loire region (CEEA.2011.52; 128; accreditation number: 4478). Mice were fed either a standard diet or a diet supplemented with 4% galacto-oligosaccharides (GOS) and inulin in a 9/1 ratio (Safe, France) during pregnancy and breastfeeding. The pups were fed a normal diet until weaning at 3 weeks of age, and at 6 weeks were sensitized to wheat-deaminated gliadins (DG) by two intraperitoneal injections of

10 µg of DG in 100 µl of PBS and 100 µl of aluminum hydroxide. After eight days, the pups were orally administered (gavage) 20 mg of DG to induce an allergic reaction (**Figure 1**). Body temperature was measured with a rectal probe before and 30 minutes after the oral challenge. The clinical score was evaluated 1 hour after the oral challenge according to the symptomatic scale described previously(20).

Immunoglobulin assays: Blood samples were collected at the end of the protocol and centrifuged at 2000 g for 20 minutes. The quantifications of specific IgE, IgG1, IgG2a, IgA and histamine levels were assayed on serum samples by indirect F-ELISA(20).

Cytokine assays: Mesenteric lymph nodes and Peyer's patches were collected, ground to obtain cell suspension. Pooled cell suspension was then specifically stimulated with DG at 10 µg/ml. After 5 days of growth, supernatants were collected and the concentrations of cytokines were measured by ELISA as described(20).

Flow cytometry: Mesenteric lymph nodes and Peyer's Patches were collected from mice and ground. 1.10^6 cells were transferred to a 96-well round-bottom plate and stimulated for 5 h with 50 ng/ml of phorbol-12-myristate-13-acetate and 1 µg/ml of ionomycin (Sigma-Aldrich) together with brefeldin A (1 mg/mL; BD Bioscience). Cells were stained with surface markers (CD3-FITC, CD4-Alexa 405, CD25-PE-Cy7, BD Biosciences) in the presence of Fc blocker mouse CD16/CD32 antibody (BD Biosciences) and fixed in 4% paraformaldehyde (PFA). For intracellular staining, the cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained with Fox p 3-Pe, IL-4-Pe or IFN-γ-APC (BD Biosciences). Cells were analyzed on a Canto II flow cytometer (BD Biosciences). Data were acquired using Diva 8.0 software and analyzed with FlowJo X (TreeStar).

Western blot: A total of 60 mg of intestine (jejunum) were immersed into 500 µl of lysis buffer for 30 minutes. After sonication, the concentration of the intestinal proteins was assessed using Bi-succinimide acid (BCA). After migration on a 10% SDS-PAGE, the

Accepted Article

proteins were transferred to a nitrocellulose membrane. In an attempt to quantify the proteins, the membrane was incubated with antibodies directed against CD23 and MUC2 (Life Technologies SAS, Saint Aubin, France) and then revealed by chemiluminescence according to the manufacturer's instructions.

Ussing chambers: Pieces of jejunum were extracted from mice, washed with a Krebs solution and mounted in Ussing chambers. The paracellular permeability was assayed using a fluorescein-sulfonic acid gradient over time by fluorimetry. The transcellular permeability was measured by the Horseradish Peroxidase (HRP) activity over time using a spectrofluorometer as previously described(21).

Short-chain fatty acid measurement and 16S DNA analyses from feces: To detect short-chain fatty acids, the content of ceca from mice was collected and centrifuged at 10000 g for 10 minutes. The concentration of short chain fatty acids was calculated from the supernatant by gas-liquid chromatography. The bacteria were quantified by quantitative PCR (qPCR) in feces collected from pups on day 21 after birth (before allergy sensitization) and on day 54 (after allergy sensitization). Briefly, feces were added to 180 μ l of a solution of lysozymes (20 mg/ml), Triton (1.2%) and Tris-HCl/EDTA (1/0,5 M) and incubated at 37°C for 30 minutes. Then, 300 mg of zirconium beads (diameter 0.1 mm, BioSpec products, VWR International, Fontenay-sous-Bois, France) were added with 1 ml of Inhibitex solution from the QIAamp Fast DNA Stool mini kit, and the suspension was disrupted for 3 minutes at 30 Hz in a mini-bead beater (MM301, Retsch GmbH & Co, Haan, Germany). Afterward, the DNA was quantified by Nanovue (GE Healthcare). Following the extraction, qPCR was conducted using the iCycleriQ real-time PCR detection system instrument (Bio-Rad). The analyzed mixture

consisted of a total volume of 15 μ l containing 5 μ l of the extracted DNA, 2.5 μ l of specific primers and 7.5 μ l of QuantiTect SYBR Green PCR Kit (Qiagen).

Statistical analysis: Data were analyzed using GraphPad Prism 6.0. Values were expressed as the mean $\pm$ SEM and compared using one-way ANOVA. In the case of a positive ANOVA, means were compared using the Mann-Whitney U test and the results were corrected by a Bonferroni multiple comparisons test. A p-value of less than 0.05 was considered significant.

Results

Offspring from GOS/Inulin mixture-exposed mothers during pregnancy and lactation are protected from food allergies.

An allergy to DG was induced in offspring from both control diet (CTL allergic) and GOS/Inulin mixture-supplemented mothers (PN allergic) (**Figure 1A**). Non-allergic mice from both groups were used as controls to determine the effect of perinatal supplementation with GOS/Inulin mixture on the development of allergies. Interestingly, feeding mothers the GOS/inulin diet limited the severity of the disease and dampened allergic inflammation in offspring. Indeed, our results showed that the introduction of GOS/Inulin reduced the clinical score (2.1 ± 0.3 AU *versus* 0.8 ± 0.3 AU), body temperature ($2.3 \pm 0.6^{\circ}\text{C}$ *versus* $0.6 \pm 0.2^{\circ}\text{C}$) histamine level (242 ± 41 ng/ml *versus* 76 ± 17) and wheat-specific IgE level (104 ± 16 μ g/ml *versus* 38 ± 7 μ g/ml) compared to the control diet, respectively (**Figure 1B, D and E**). Interestingly, plasma levels of wheat-specific IgG2a and IgA significantly increased in allergic offspring from GOS/Inulin-supplemented mothers compared with the control diet (3.7 ± 0.4 mAU *versus* 11.9 ± 2.5 mAU and 7.8 mAU ± 0.7 *versus* 14.1 mAU ± 1.5 , respectively) (**Figure 1G and H**). Together, these results demonstrate a protective effect of GOS/Inulin

mixture against food allergies in offspring when administered to mothers during gestation and lactation.

Maternal supplementation with GOS/Inulin during gestation and lactation reduces the Th2 response in allergic offspring and increases regulatory responses in allergic offspring.

We examined inflammatory and suppressive cytokines in the guts of offspring from mothers who did and did not receive GOS/Inulin supplements (**Figure 2**). IL-4 and IL-5 production by specifically stimulated cells such as T lymphocytes from mesenteric lymph nodes and Peyer's patches were up-regulated in allergic mice from both control and GOS/Inulin-supplemented diet mothers compared with non-allergic mice. Moreover, GOS/Inulin supplementation during pregnancy and lactation had a profound effect on preventing the secretion of IL-4 and IL-5, as significant decreases were found in pups from GOS/Inulin-supplemented mothers compared to control-diet mothers (43.5 ± 31 pg/ml *versus* 1.7 ± 3 pg/ml and 176.6 ± 42 pg/ml *versus* 42.3 ± 7 pg/ml, respectively) (**Figure 2A and B**). However, we observed no difference for IFN- γ secretion in the guts of allergic offspring from control- or supplemented-diet mothers (**Figure 2C**). Similar results were obtained for the expressions of IL-4 ($3.9 \pm 3.8\%$ *versus* $9.8 \pm 3.9\%$) and IFN- γ in CD4⁺ T cells from lymphoid organs (**Figure 2D and E**). Regarding regulatory T cells, CD4⁺CD25^{high}Foxp3⁺ Treg frequency decreased in allergic pups compared with non-allergic pups from control-diet mothers (**Figure 2F**). However, this decrease was suppressed in offspring from GOS/Inulin-supplemented mothers (**Figure 2F**). The persistence of the Treg response is particularly associated with the production of the cytokine TGF- β , which was found to increase in the allergic offspring of PN mothers compared with other groups (**Figure 2G**). However, no effect on IL-10 production was observed in food allergic mice but IL-10 production was increased in non-allergic mice from GOS/Inulin supplemented mothers compared with control diet mothers confirming the effect of GOS/Inulin in regulatory responses (**Figure 2H**).

GOS/Inulin feeding during the perinatal period protects offspring from increase intestinal permeability from allergies.

To identify whether the changes in the allergic marker and the immune response in the presence of allergies are associated with the modification of the intestinal physiological parameters, jejunum permeability and intestinal protein expression were measured (**Figure 3**). Allergic mice breastfed by mothers supplemented exhibited a 5-fold decrease in FITC-sulfonic acid and a 2.5-fold decrease in the HRP area under the curve compared to pups from control mothers (**Figure 3A**). Interestingly offspring from GOS/Inulin-fed mothers were protected from the increase of paracellular (*left panel*) and transcellular (*right panel*) permeability that occurred in the presence of food allergies (**Figure 3A and B**), suggesting an important role of GOS and inulin in the maintenance of gut barrier integrity. Based on these results, we were interested in examining histological features of mice with allergies. **Figure 3C** highlights less cellular infiltration and villi deterioration in allergic offspring from GOS/Inulinsupplemented mothers compared to offspring from control-diet mothers. In addition to the tissue inflammation, we verified gut protection through the quantification of mucus protein, such as Mucin 2 (MUC2), which is secreted by goblet cells in the epithelial lining into the lumen of the large intestine. Western blot analysis of jejunum lysate revealed that MUC2 was overexpressed by 6-fold in allergic mice from supplemented mother compared with others groups, proving the effect of GOS/Inulin mixture on the protection of gut permeability in the presence of food allergies (**Figure 3E**). Finally, the measurement of CD23 expression in the jejunum of allergic pups from supplemented mothers showed a drastic decrease compared with other groups (**Figure 3D**). To conclude, this shows that perinatal treatment with GOS/Inulin protects the gut barrier from allergic reactions through a modulation of the expression of intestinal proteins.

GOS/Inulin feeding during the perinatal period alters the composition of microbiota in pups.

Accepted Article

To investigate the impact of early exposure to GOS/Inulin mixture on the bacterial composition, we determined the caecum mass content, the bacterial activity by measuring the short-chain fatty acid (SCFA) level and the composition of feces microbiota (**Figure 4**). In non-allergic mice, the caecum content mass did not differ significantly between mice from GOS/Inulin-diet mothers and control-diet mothers (0.168 mg *versus* 0.211 mg), whereas in allergic mice, the mass content decreased compared to non-allergic mice from the PN group (**Figure 4A**). Concerning metabolic activity of microbiota, no difference was found between groups (**Figure 4B**). Quantitative PCR analysis on 3-week-old offspring's feces revealed that GOS/Inulin supplementation lead to a significant increase in bacterial load (**Figure 4C**). Similar results were obtained in the mothers (**data not shown**). At weaning, mice from supplemented mothers displayed higher proportions of the *Lactobacillus* and *Clostridium leptum* phyla and lower proportions of *Clostridium coccooides* compared with the control group (**Figure 4D**). However, changes in microbiota occurring in the presence of food allergies were similar in both groups, with a higher abundance of *Lactobacillus* phylum and a lower abundance of *Clostridium* and *Erysipelothrix* (**Figure 4E**).

Discussion

The early stages of life are of great importance in the development of an organism and specifically in microbiota and immune system maturation. The mother and her diet affect fetal development *in utero* and during breastfeeding. Studies suggest a preventive effect of a prebiotic-supplemented diet in adult mice on contact hypersensitivity(22), cutaneous allergies and asthma(23). Maternal exposure to dietary factors may have immune regulatory effects on the promotion or prevention of skin or respiratory allergic diseases in offspring(18, 19). Here, we show that mothers fed a GOS/Inulin-supplemented diet gave birth to mice protected against an allergy to wheat. Those mice display lower clinical scores, and a preservation of

the intestinal barrier integrity compared to pups from mothers who were fed a normal diet. Moreover, changes in the Th2/Th1 balance toward a dampened Th2 and stronger Treg response were observed in those mice, demonstrating that the mother's diet could affect immune development in the offspring. This study and others have shown that non-digestible oligosaccharides are involved in the induction of beneficial effects through specific molecules or cell populations, such as regulatory T cells, and have a profound effect on the tolerance maintenance(24). These effects coincide with a lower production of Th2-related cytokines, which might change the Th2/Th1 balance toward a dampened Th2 response. Moreover, this is accompanied by an absence of Th2 population increase in the presence of allergies in the PN group, reinforcing the immune regulatory effect of maternal exposure to GOS/Inulin mixture. Previously, it has been shown that the intensity of allergic reactions to food can be attenuated by Treg(25), and Treg induction may underlie the protective effect of certain dietary interventions, including non-digestible oligosaccharides, in food allergy models. While wheat protein sensitization and challenge lead to a decrease in CD4⁺CD25^{high}Foxp3⁺ T cells in Peyer's patches and lymph nodes in controls, treating the mothers with GOS/Inulin mixture prevents the decrease of this population in the presence of allergies. Moreover, treatment with GOS/Inulin mixture leads to an increase of suppressive-related cytokines, such as TGF- β and IL-10. These data suggest that GOS/Inulin may induce suppressive responses in the intestine. However, supplementing the mother's diet had no effect on the Treg cells in non-allergic mice, suggesting that the GOS/Inulin mixture enhance the regulatory response to allergic inflammation rather than inducing a T regulatory population that protects against allergy development. Together, these results show that maternal supplementation with GOS/Inulin can affect *in vivo* immune responses in the adult offspring.

Prebiotics also impact gut physiology, and the mechanisms by which prebiotics improve gut permeability – in the particular context of allergies – are not fully understood. Among the suspected mechanisms, it has been demonstrated that the prebiotic modulation of the gut microbiota increases the villus height and crypt depth and leads to a thicker mucosal layer in the jejunum and the colon(26). These effects are related to the bacterial fermentation of the prebiotics. The resulting products of fermentation are mainly short-chain fatty acids (SCFAs), with butyrate acting as an energetic substrate for the colonocytes and having a trophic effect on mucosa. In addition to the putative role of SCFAs and specific bacterial strains, the exact mechanism underlying the relation between prebiotic-induced changes in gut microbiota and improved gut barrier function has not yet been described. It has been proposed that this higher permeability and allergic symptoms in mice are dependent on a disruption of key intestinal proteins, such as MUC2 and CD23(27). In the vast majority of studies, prebiotics displayed a protective effect in increasing gut barrier integrity by stimulating the secretion of anti-microbial peptides, mucus (acidic mucins, MUC-2, -3 and -4) or the expression of occludins(28). We demonstrated that maternal exposure to particular prebiotics (GOS/Inulin mixture) protects offspring from the alteration of the gut barrier, probably by limiting allergic inflammation characterized by an increase in mucus protein expression and a decrease in CD23 expression.

Prebiotics have repeatedly been shown to selectively stimulate the growth of bifidobacteria and, in some cases, lactobacilli, leading to significant changes in gut microbiota composition(29). Prebiotics can also provide energy for the growth of endogenous favorable bacteria in the gut, thus improving the host microbial balance and increasing specific growth rate resulting in a transient increase in total amount of bacteria as we observed. This could be link to the elevation of the caecum content weight after the oligosaccharide gavage which may result from an increase crypt depth and bacteria density by providing energy source and

normalizing proliferation which in turns allow a higher bacterial count. There is now good evidence that the composition of the gut microbiota influences the development of allergies, and exposure to specific bacteria, including bifidobacteria and lactobacillus strains, can attenuate allergic responses(30). More specifically, studies with germ-free mice have identified key species within the microbiota, such as clostridia, that can strongly influence the development of T regulatory cells in the intestine(31). There is also evidence linking the immunomodulatory function of certain commensal bacteria and components to the induction of Treg cells and their associated cytokines(32). Under *in vitro* conditions, selective bacteria have been shown to induce Treg(30). It therefore seems reasonable to suggest that at least some of the preventive effects of GOS/inulin are derived from the prebiotic nature of these oligosaccharides. However, it is in keeping with existing evidence that the GOS/inulin can directly modulate immune cell function. Further studies will be required to differentiate between the direct immune-modulatory actions of GOS/inulin, which can act via glycan receptors, and the anti-allergic effects mediated through changes in the gut microbiota(33).

In conclusion, although it is clear that prebiotics have some effects on microbiota (modification, stimulation, anti-pathogenic effects), the consequences on the development of allergic symptoms are less clear. Even if no murine model adequately mimics human disease, it should be stressed that in the present study, the mothers were supplemented, and the effects observed in the adult offspring were not an effect of direct exposure to GOS/inulin. Our results demonstrate that maternal supplementation with GOS/Inulin affects the maternal microbiome and immune status, which can be transfer to the offspring through different mechanisms, such as the transfer of molecules. Nevertheless, future direction needs to address whether pregnancy, breastfeeding or both perinatal period constitute the optimal window of supplementation for allergy prevention.

References

1. Chafen JJ, Newberry SJ, Riedl MA, Bravata DM, Maglione M, Suttorp MJ, et al. Diagnosing and managing common food allergies: a systematic review. *JAMA* 2010;**303**(18):1848-1856.
2. Perrier C, Corthesy B. Gut permeability and food allergies. *Clin Exp Allergy* 2011;**41**(1):20-28.
3. Nwaru BI, Hickstein L, Panesar SS, Muraro A, Werfel T, Cardona V, et al. The epidemiology of food allergy in Europe: a systematic review and meta-analysis. *Allergy* 2014;**69**(1):62-75.
4. Kanny G. [Food allergy]. *Rev Prat* 2007;**57**(12):1331-1338.
5. Keet CA, Matsui EC, Dhillon G, Lenehan P, Paterakis M, Wood RA. The natural history of wheat allergy. *Ann Allergy Asthma Immunol* 2009;**102**(5):410-415.
6. Matsuo H, Morita E, Tatham AS, Morimoto K, Horikawa T, Osuna H, et al. Identification of the IgE-binding epitope in omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. *J Biol Chem* 2004;**279**(13):12135-12140.
7. Battais F, Mothes T, Moneret-Vautrin DA, Pineau F, Kanny G, Popineau Y, et al. Identification of IgE-binding epitopes on gliadins for patients with food allergy to wheat. *Allergy* 2005;**60**(6):815-821.
8. Chen J. [The role of food allergy in gastrointestinal disorders in children]. *Zhonghua Er Ke Za Zhi* 2010;**48**(4):241-243.
9. Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989;**299**(6710):1259-1260.
10. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. *Nat Immunol* 2011;**12**(1):5-9.

- Accepted Article
11. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* 2009;**461**(7268):1282-1286.
 12. de Kivit S, Kraneveld AD, Garssen J, Willemsen LE. Glycan recognition at the interface of the intestinal immune system: target for immune modulation via dietary components. *Eur J Pharmacol* 2011;**668 Suppl 1**:S124-132.
 13. Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, et al. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. *Pediatr Allergy Immunol*; **21**(8):1179-1188.
 14. Schley PD, Field CJ. The immune-enhancing effects of dietary fibres and prebiotics. *Br J Nutr* 2002;**87 Suppl 2**:S221-230.
 15. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BC, et al. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. *Allergy* 2012;**67**(3):343-352.
 16. Verheijden KA, Willemsen LE, Braber S, Leusink-Muis T, Delsing DJ, Garssen J, et al. Dietary galacto-oligosaccharides prevent airway eosinophilia and hyperresponsiveness in a murine house dust mite-induced asthma model. *Respir Res* 2015;**16**:17.
 17. Prescott SL. Early-life environmental determinants of allergic diseases and the wider pandemic of inflammatory noncommunicable diseases. *J Allergy Clin Immunol* 2013;**131**(1):23-30.
 18. Hogenkamp A, Knippels LM, Garssen J, van Esch BC. Supplementation of Mice with Specific Nondigestible Oligosaccharides during Pregnancy or Lactation Leads to Diminished Sensitization and Allergy in the Female Offspring. *J Nutr* 2015;**145**(5):996-1002.

19. Hogenkamp A, Thijssen S, van Vlies N, Garssen J. Supplementing pregnant mice with a specific mixture of nondigestible oligosaccharides reduces symptoms of allergic asthma in male offspring. *J Nutr* 2015;**145**(3):640-646.
20. Gourbeyre P, Denery-Papini S, Larre C, Gaudin JC, Brossard C, Bodinier M. Wheat gliadins modified by deamidation are more efficient than native gliadins in inducing a Th2 response in Balb/c mice experimentally sensitized to wheat allergens. *Mol Nutr Food Res*;56(2):336-344.
21. Bouchaud G, Gourbeyre P, Bihouee T, Aubert P, Lair D, Cheminant MA, et al. Consecutive Food and Respiratory Allergies Amplify Systemic and Gut but Not Lung Outcomes in Mice. *J Agric Food Chem*;63(28):6475-6483.
22. Watanabe J, Sasajima N, Aramaki A, Sonoyama K. Consumption of fructo-oligosaccharide reduces 2,4-dinitrofluorobenzene-induced contact hypersensitivity in mice. *Br J Nutr* 2008;**100**(2):339-346.
23. Vos AP, Haarman M, van Ginkel JW, Knol J, Garssen J, Stahl B, et al. Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. *Pediatr Allergy Immunol* 2007;**18**(4):304-312.
24. van't Land B, Schijf M, van Esch BC, van Bergenhenegouwen J, Bastiaans J, Schouten B, et al. Regulatory T-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides. *Vaccine*;28(35):5711-5717.
25. Kanjarawi R, Dercamp C, Etchart N, Adel-Patient K, Nicolas JF, Dubois B, et al. Regulatory T cells control type I food allergy to Beta-lactoglobulin in mice. *Int Arch Allergy Immunol*;156(4):387-396.
26. Chang WH, Li JJ, Zhang S, Zheng AJ, Yuan JL, Cai HY, et al. Effects of glucocorticoid-induced stress on absorption of glycylsarcosine in jejunum of broilers. *Poult Sci*;94(4):700-705.

27. Gourbeyre P, Desbuard N, Gremy G, Tranquet O, Champ M, Denery-Papini S, et al. Perinatal and postweaning exposure to galactooligosaccharides/inulin prebiotics induced biomarkers linked to tolerance mechanism in a mouse model of strong allergic sensitization. *J Agric Food Chem*; **61**(26):6311-6320.
28. Shimotoyodome A, Meguro S, Hase T, Tokimitsu I, Sakata T. Sulfated polysaccharides, but not cellulose, increase colonic mucus in rats with loperamide-induced constipation. *Dig Dis Sci* 2001; **46**(7):1482-1489.
29. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* 2012; **22**(9):1147-1162.
30. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* 2014; **20**(2):159-166.
31. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 2013; **500**(7461):232-236.
32. Lyons A, O'Mahony D, O'Brien F, MacSharry J, Sheil B, Ceddia M, et al. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. *Clin Exp Allergy* 2010; **40**(5):811-819.
33. Julia V, Macia L, Dombrowicz D. The impact of diet on asthma and allergic diseases. *Nat Rev Immunol* 2015; **15**(5):308-322.

Acknowledgments

This study was supported by “Région Pays de la Loire” through a “REAL²” research grant for GB in the context of “CRNH-Ouest”. We thank “Laiterie de Montaigu” for providing GOS and the Bénéo groups for providing inulin. GB was supported by The People Programme

(Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007-2013) under REA grant agreement n°624910. LC was supported by the “ministère de la recherche” and the University of Nantes. We thank Catherine Michel for her support in analysis microbiota and Julie Chabauty-Chartier for her excellent technical support in the care of the mice.

Author contributions

GB carried out the experiments on the mice; LC, JC and FB realized the experiments and analyzed cell populations. PA and LC realized the analyses on the gastrointestinal tract. GB, LC, JC and FB participated in the design of the study, performed the statistical analysis and wrote the manuscript. LC did the histological assessment. MB, MN and AM conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

None of the authors has any conflict of interest with respect to the contents of this article.

Figure legend

Figure 1: Maternal GOS/inulin diet protects offspring against symptoms of wheat protein food allergies. (A) Experimental protocol. Mothers were fed either a control diet (CTL) or a GOS/inulin diet (PN), and, after weaning, their pups were left untouched (unsensitized) or intraperitoneally sensitized and orally challenged with wheat proteins (allergic). The mice were analyzed 1 hour after the challenge for their (B) clinical score and (C) body temperature. Concentrations of wheat-specific (D) IgE, (E) IgG2a and (F) IgA in the plasma of the offspring of mothers fed either a control or a GOS/inulin diet were evaluated. Values are mean $\pm$ SD ($n=8$ animals per group), * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

Figure 2: Maternal exposure to GOS/inulin modifies immune status of offspring related to allergies. The production of IL-4 (A), IL-5 (B), IFN- γ (C) and TGF- β (G) cytokines measured by ELISA in the supernatant from lymphoid cells specifically stimulated with DG. Quantifications of IL-4- and IFN- γ -producing CD4⁺ T cells (D, E) and CD3⁺CD4⁺CD25^{high}Foxp³ Treg Cells (F), TGF- β (G) and IL-10 (H) secretion were measured by flow cytometry after stimulation with PMA/Ionomycin in the presence of Brefeldin A for cytokine secretion. The data represent the mean $\pm$ SEM ($n=8$ animals per group); * $p < 0.05$, * $p < 0.01$, and *** $p < 0.005$.

Figure 3: Maternal exposure to GOS/inulin protects offspring from intestinal barrier degradation in the presence of food allergies. Jejunum segments were isolated 1 hour after the wheat protein challenge, and the intestinal transcellular permeability to sulfonic acid (A, left panel) and paracellular permeability to HRP (B, left panel) were measured in an Ussing chamber over a 150-minute period in allergic pups of mothers from the CTL and PN groups. The AUC for all groups of pups for transcellular (A, right panel) and paracellular (B, right panel) permeability are also shown. Representative jejunum sections H&E stained from gliadin-challenged mice from the CTL and PN groups (C). Expression of intestinal proteins was measured in jejunum lysate in all groups of mice for CD23 (D) and MUC2 (E). Data are the mean $\pm$ SEM ($n = 8$ mice/group); * $p < 0.05$, * $p < 0.01$, and *** $p < 0.005$. Scale bar = 250 μ m.

Figure 4: Maternal diet enriched with GOS/inulin alters the intestinal microbiota in offspring before but not after allergy sensitization. Analysis of caecum content mass (A) and total level of AGCC (B). Before food allergy sensitization, the total amount of bacteria in the feces (C) of pups from mothers fed with the control or GOS/inulin-supplemented diet were measured. Pyrosequencing analysis of the fecal microbiota composition in pups from control diet mother (CTL) and GOS/Inulin mixture-supplemented mother (PN) on day 21

after birth (before food allergy) (D) and on day 54 (after food allergy) (E). Results are representative of data generated ($n=8$ animals per group) and relative abundance of the following phyla is represented: Clostridium Coocoïdes (black), Clostridium Erysipelotrix (striped), Clostridium leptum (dark grey), lactobacillus (dot grey) and Bifidobacterium (light grey). Data are expressed as the mean $\pm$ SEM. * $p < 0.05$, * $p < 0.01$, and *** $p < 0.005$.

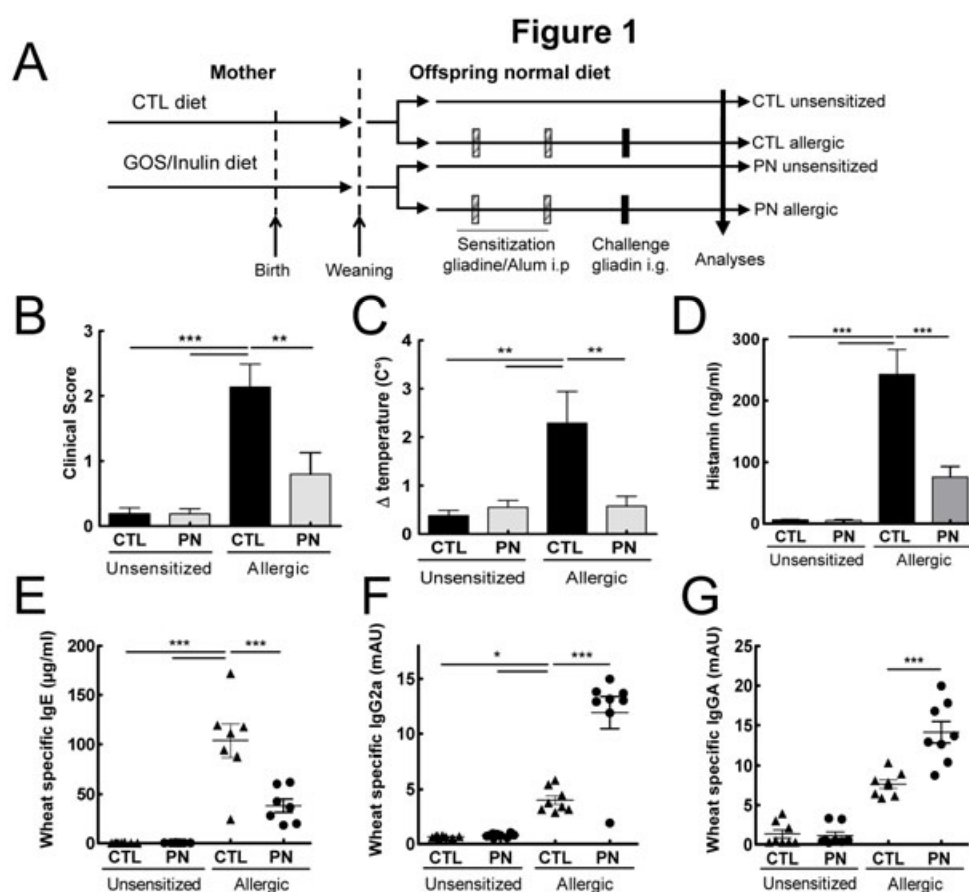


Figure 2

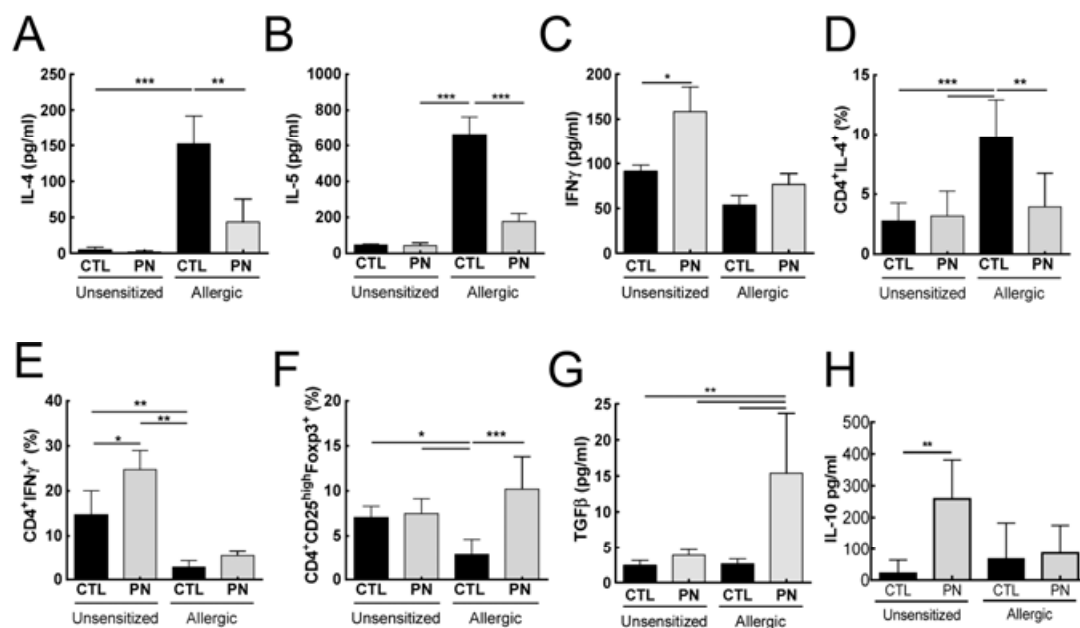


Figure 3

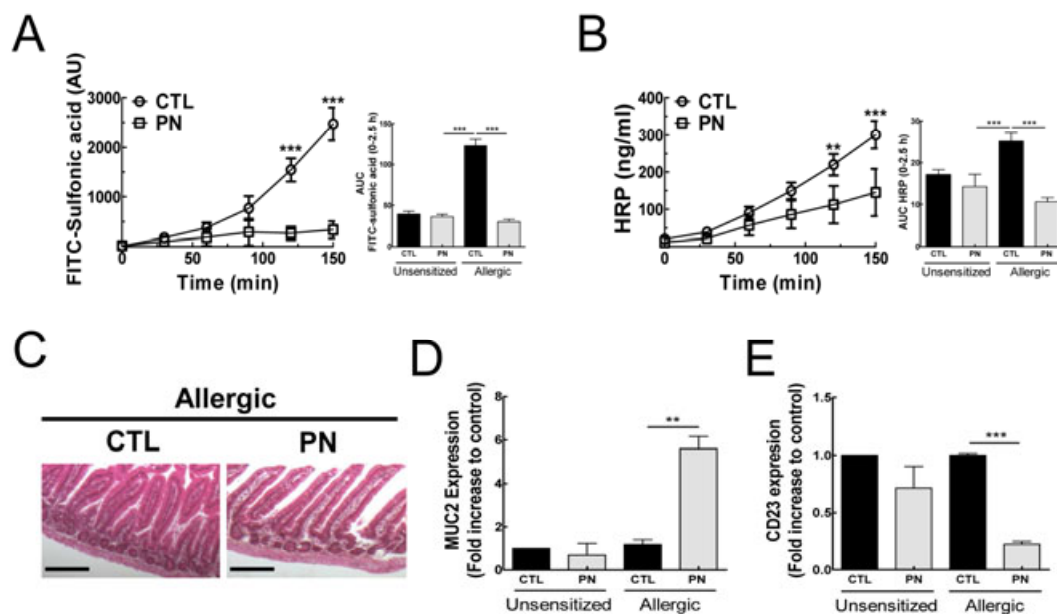


Figure 4

