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Exposure to a Galactooligosaccharides/Inulin Prebiotic Mix at Different Developmental Time Points Differentially Modulates Immune Responses in Mice

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ABSTRACT: Prebiotics constitute emerging tools to alleviate immune pathologies. This study aimed to evaluate the effect of prebiotic exposure during perinatal and postweaning periods on immune and gut regulations. Mice were fed either a galactooligosaccharides/inulin prebiotic mix-enriched diet or a control diet during the perinatal and/or postweaning periods. Biomarkers related to gut barrier function (SCFA, heat shock proteins, zonula occludens protein-1, and mucin-2) and immune mechanisms (IgA, IgE, IgG1, IgG2a, IL-10, TGF-β, IL-4, IL-17A, and IFN-γ) were analyzed. The milk of dams fed the prebiotic diet was more concentrated in both IgA and TGF-β when prebiotics were introduced during both the perinatal and postweaning periods; IL-10, IgA, and IgG2a were increased in pups; and expression of intestinal markers was more pronounced. Postweaning exposure to prebiotics alone induced higher INF-γ and TGF-β levels, whereas IgA levels fell. Combined exposure periods (perinatal/postweaning) to prebiotics increased tolerance-related immunoglobulins in pups and reinforced gut barrier functions.

KEYWORDS: *perinatal, short-chain fatty acids, gut barrier, neonates, maternal milk*

INTRODUCTION

Prenatal life, early infancy, and childhood are crucial periods during which different systems become established: the immune system, the microbiota, gut barrier integrity, and oral tolerance.

Prenatal life constitutes the first period during which the maternal environment can influence the immune system of a fetus in utero. In fact, a prospective birth cohort in rural populations evidenced that maternal exposure to animal sheds and unpasteurized cow's milk had an impact on some immunological biomarkers (decrease of IgE and increase of both IFN-γ and TNF-α) in the cord blood of the neonate.[1] The maternal microbiota can also act on the fetus: recent findings indicate that both the cord blood from healthy neonates[2] and the murine amniotic fluid[3] have some bacteria. Thus, the maternal exposure to various environmental factors is the first step for the development of the immune system and microbiota in children.

During early infancy, the immune system and the microbiota increase in terms of both their complexity and diversity.[4,5] From birth, the infant immune system becomes increasingly more mature. This increased maturity is exerted on both lymphoid organs and cells. For example, the secondary lymphoid organs, such as mesenteric lymph nodes and Peyer's patches, increase in size after birth, and the germinal centers develop.[6] In the case of T cell responses, the immune dominant profile changes from a Th2- to a Th1-biased response.[4] B cell localization is also modified during early infancy, at which point these cells, particularly the IgA-secreting B cells, start to populate numerous mucosal effector sites (i.e., the intestine, urogenital tract, mammary glands, salivary glands, and respiratory tract).[7] The maturation of the immune system is crucial for the mechanism of immune tolerance, which results from the equilibrium between the so-called "Th1/Th2 balance"[8] and regulatory T-cells (Tregs),[9,10] in which the tolerance is maintained via the inhibition of the Th2-biased pathway.[11] Tregs secrete mainly TGF-β, IL-10, and low levels of IL-4 and are involved in the production of IgA.[12] The IgA-secreting B cells are largely responsible for the induction of tolerance mechanisms.[13] Tregs may also contribute to the down-regulation of the novel and discrete Th17 cells that secrete the pro-inflammatory cytokine interleukin (IL)-17.[14] During prenatal life, early infancy is the second most crucial period during which the mother, via her breast milk, can exert a major influence on the maturation of the immune system of her newborn. In fact, a mother's breast milk contains immunological factors, such as IgA, TGF-β, and traces of food antigens,[15,16] that play a key role in the tolerance processes. For example, it has been demonstrated that breast milk mediates the transfer of an antigen that may prevent antigen-specific responses.[17] The mother also plays a key role in the implantation of the microbiota in her infant. Starting at birth, the newborn's intestinal tract will acquire microbiota during the first hours of life through contact with the mother's perineal and vaginal microbiota and with the environment.[18] This bacterial ecosystem is then modified by components in the breast milk, which includes micro-organisms.[19] Microbiota plays a major role in immune response modulation (notably in oral tolerance)[10,20] and in gut barrier maturation, which is enhanced during the first 2 weeks of life.[6] Indeed, some bacteria are known to modulate the gut barrier functions by acting on the tight junctions (zonula occludens proteins (ZOs) and occludins)[21,22] by inducing the

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production of cytoprotective epithelial substances (e.g., heat shock proteins (HSPs) and mucins (MUCs))[23,24] and by secreting short-chain fatty acids (SCFAs).

Early childhood and weaning (4−6 months in humans; 3 weeks in mice) is the third period during which the immune tolerance to food proteins is induced[25,26] and the exposure to microbiota is modulated. These immune and microbial modifications are dependent on the lifestyle and notably the introduction of solid foods, which alter the relative proportions of bacterial phyla in the gut and allow the establishment of food tolerance. These modifications are followed by the establishment of an adult-like microbiota profile with greater stability[27] and a well-established immune system that is able to fight pathogens and tolerate food. Nevertheless, defects in the establishment of these microbiota and the mucosal immune tolerance that are linked to disruptions in the Th1, Th2, and Th17 responses during early life are commonly associated with the later development of allergies, autoimmune diseases, and chronic inflammation of the gut mucosa.[5,6]

Various compounds that are commonly known as "prebiotics" may modify the autochthonous microbiota. Prebiotics have been defined as "nondigestible food ingredients that beneficially affect the host organization by selectively stimulating the growth and/or activity of one or a limited number of bacterial species that are already present in the gut and can thus improve the "host's health".[28] To date, the most widely studied prebiotics are fructans, that is, inulin and fructooligosaccharides (FOS), galactooligosaccharides (GOS), and β-glucans.[29] These food supplements are fermented by the microbiota and generate SCFAs, such as acetate, butyrate, and propionate,[30,31] that can modulate the expression of certain genes.[32] Therefore, a potential strategy for the modification of the autochthonous microbiota is the use of prebiotics, which may in turn have a major impact on sculpting the immune system, first through the mother (via delivery and breast milk) and then through the newborn. This indirect effect has been assessed by studies in which FOS were administered to adult and young mice during lactation and thereafter because an up-regulation of the expression of the IgA and IgA receptors in the intestinal Peyer's patches was found to be linked with an increase in the cecal production of SCFA.[33,34] Recent data indicate that prebiotics might also exert a direct action on the immune system, notably by interacting with some glycan-binding proteins, called "galectins", that can exert various immunomodulatory properties (e.g., induction of Treg cells and inhibition of effector T cells) via their fixation to some T cell receptors.[35] In a recent study, De Kivit et al.[36] observed that galectin-9 is secreted in the serum of both infants and mice following exposure to a GOS/inulin prebiotic mix, which suggests that these proteins may be fixed to galactooligosaccharides. Numerous data are also available on the effect of oligosaccharides (e.g., GOS[37] and fructans[38−40]) and dietary fibers (e.g., cellulose,[38] pectin,[38] arabinoxylans,[41] β-glucans,[42,43] resistant starch,[38,44] and others[45]) on the modulation of the gut barrier, particularly when these are administered after weaning or to adult animals.[38,39,41,43−45] In the large majority of studies, prebiotics display a protective effect in increasing the gut barrier integrity by stimulating the secretion of antimicrobial peptides,[41] mucus (acidic mucins, MUC-2, -3, and -4)[38,39,42,44,45] and/or the expression of occludins[37] and HSP-25.[44] However, another study showed that postnatal exposure to a diet enriched in β-glucans decreased the intestinal integrity of mice by down-regulating ZO-1 and antimicrobial peptides.[43]

Although a large body of evidence indicates that the perinatal period constitutes a crucial "window of opportunity" for the induction of immunological tolerance and the strengthening of the gut barrier, this exposure period to prebiotics has not been widely studied. Therefore, the aim of this study was to evaluate the effect of prebiotic exposure during the perinatal and postweaning periods on the modulation of the immune responses, especially on the Th1-, Th2-, Th17-. and Treg-mediated responses and on the gut barrier functions. We thus chose to feed mice a prebiotic diet during both the perinatal (gestation and lactation) and postweaning periods and to compare the results obtained with those found in another study that was conducted in parallel, in which weaned mice were fed the same prebiotic diet only during the postweaning period. We chose a mixture of two oligosaccharides (GOS and inulin) that were previously used in mouse diets[46] and in some infant milk formulas.[47] These two studies, which were performed in an allergy context, demonstrated an immunomodulatory effect of this prebiotic mix that is associated with a decrease in Th2-related biomarkers. During our study, the effect of prebiotics on the immune responses was investigated in dams and their (6- and 12-week-old) pups. The effect of prebiotic exposure during both the perinatal and postweaning periods on the microbiota metabolism and on several gut markers that are potentially targeted by these metabolites (HSP-25, ZO-1, and MUC-2) was also studied.

MATERIALS AND METHODS

Prebiotics and Diets. The oligosaccharides used in this study were short-chain GOS extracted from Vivinal GOS syrup (Domo, Amersfoort, The Netherlands). The extract from Domo has been extensively characterized by Coulier et al.[48] The Vivinal GOS syrup was spray-dried by La Laiterie de Montaigu (France). The final extract contained 40.5% pure GOS, which had an average degree of polymerization of >3. The GOS were mixed with inulin (long-chain FOS), which has an average degree of polymerization of <23 (Beneo-Orafti, Tienen, Belgium), at a ratio of 9:1. Both oligosaccharides have been biochemically characterized by their respective manufacturers. The prebiotic mix was added to a final concentration of 4% (w/w) to purified diet 210 (SAFE, Augy, France).[46] The preservation of the prebiotic amount in the diet after processing was controlled by HPAEC. The analysis of the oligosaccharides was performed on an ICS3000 HPLC system (Thermo Scientific Dionex) with pulsed amperometric detection. The GOS levels observed in the prebiotic-supplemented diet were very close to those expected: 3.49% compared to the expected 3.6% for the GOS supplementation in the diet. The same finding was observed with the inulin levels: 0.36% compared to the expected 0.4%.

Mice and Housing Conditions. BALB/c mice and their progenitors were obtained from the Centre d'Elevage René Janvier (St. Berthevin, France). Mice were housed in a ventilated cage system (IVC Racks Allentown, PA, USA) under controlled temperature and humidity conditions with a 12 h light/dark cycle. Mice were acclimatized to these conditions for 2 weeks prior to mating. The pups were weaned at 3 weeks of age. The experimental protocol was approved by the Ethics Committee on Animal Experimentation of the Pays de la Loire region (accreditation no. C44502). In the study covering the perinatal and postweaning periods, the progenitors were fed either a semisynthetic control diet (−Prb dams, n = 12; purified diet 210, SAFE, Augy, France) or the same diet supplemented with 4% of the GOS/inulin mixture[49] (+Prb dams, n = 13). The dams received the same diets during the gestation and lactation periods. After weaning, the pups were fed ad libitum the prebiotic-supplemented diet for an additional 9 weeks (−Prb mice, n = 10; +Prb mice, n = 15). In the postweaning study, the progenitors were fed the semisynthetic control diet only. Upon weaning, the pups were fed either the control diet or the prebiotic-supplemented diet for 9 weeks (−Prb mice, n = 18; +Prb mice, n = 18).

Detection of Immune Biomarkers Related to the Activation States of T Cell Populations. During the study, a characterization of

the prebiotic effect on the immune responses was investigated in the dams and their (6- and 12-week-old) pups. A number of different immune biomarkers related to the activation states of the T-cell populations (Th1, Th2, and Treg responses) were assayed (Ig and cytokine production). The involvement of the Th1 response was evaluated through the production of IgG2a and IFN-γ, the Treg response involvement was assessed through the production of IgA, IL-10, and TGF-β, and the Th2 response involvement was investigated through the production of IgG1, IgE, and IL-4.

Blood Sample Collection. Blood samples were collected from the retro-orbital venous plexus. In the study combining the perinatal and postweaning periods, two sets of blood were collected: from T6 mice (6 weeks old) and T12 mice (12 weeks old). The blood samples were incubated for 20 min at 37 °C and then centrifuged for 20 min at 2000*g*. The supernatant was removed to assay the immunoglobulins in the serum.

Extraction of Protein from Feces. Fresh fecal pellets were collected from lactating dams exposed to the different diets for 8 weeks and from the T6 and T12 mice. The extraction was performed as previously described.[50] The pooled fecal pellets (0.1 g) were added to 1 mL of phosphate buffer saline (PBS) containing 1% bovine serum albumin, 50 μg/mL bacitracin, 300 μg/mL benzamidine, 80 μg/mL leupeptin, 20 μg/mL chymostatin, 25 μg/mL pepstatin, and 200 μM phenylmethanesulfonyl fluoride (Sigma-Aldrich Chimie S.A.R.L., Lyon, France) and incubated on a rotary shaker overnight at 4 °C. The tubes were vortexed to disrupt all solid materials and then centrifuged at 16000*g* and 4 °C for 5 min. The supernatant was used to analyze the presence of total IgA in the feces.

Measurement of Fecal and Milk IgA and Milk TGF-β Levels. The dams were anesthetized (0.6 mg of Rompum and 3 mg of Imalgene per mouse) before being milked. The dams were injected intraperitoneally with 0.15 IU oxytocin and then milked by hand following stimulation of the mammary glands. The milk droplets were collected using a Pasteur pipet. The tubes were centrifuged at 20000*g* and 4 °C for 2 min. The supernatants were removed and used for further analyses. The levels of IgA were assayed with a sandwich F-ELISA using a capture goat antibody directed against mouse IgA (Southern Biotechnology Associates, Birmingham, AL, USA) and a detection goat antibody directed against mouse IgA and conjugated to alkaline phosphatase (Southern Biotechnology Associates, ref 1040-04). The capture antibody was diluted 1:30 in carbonate buffer (30 mM Na_2CO_3 and 70 mM $NaHCO_3$, pH 9.6). The detection antibody was diluted 1:3000 in PBS with 0.1% Tween-20 and 0.5% gelatin. The fluorescence intensity (emission at 440 nm and excitation at 360 nm) was measured after incubation with an alkaline phosphatase substrate (4-methylumbeliferin). The levels of TFG-β were assayed using the mouse TGF-β ELISA Ready-Set-Go! Kit (eBioscience, Paris, France) in accordance with the manufacturer's instructions.

Measurement of Total IgE, IgG1, and IgG2a in Serum. The mouse total IgE was measured by F-ELISA as previously described.[51] The total IgG1 and IgG2a levels were quantified using an F-ELISA protocol based on an assay developed by Gourbeyre et al.[52] In our study, the plates were coated with a rat monoclonal IgG antibody directed against mouse IgG1 (Santa Cruz Biotechnology, 14G11, Santa, Cruz, CA, USA), which was diluted 1:50 in carbonate buffer, or a rat monoclonal IgG antibody directed against mouse IgG2a (Santa Cruz Biotechnology), which was diluted 1:20 in carbonate buffer. To determine the total IgG1 and IgG2a, the samples were diluted 1:500 in PBS with 0.1% Tween-20 and 0.5% gelatin. A detection goat antibody directed against mouse IgG1 (diluted 1:1000 in PBS with 0.1% Tween-20 and 0.5% gelatin) or IgG2a (diluted 1:2000 in PBS with 0.1% Tween-20 and 0.5% gelatin) and conjugated to alkaline phosphatase (Southern Biotechnology Associates) was used. The fluorescence intensity (emission at 440 nm and excitation at 360 nm) was measured after incubation with an alkaline phosphatase substrate (4-methylumbeliferin).

Nonspecific Activation of Spleen Cells and Quantification of Cytokines (IL-4, IL-10, IFN-γ ,and TGF-β). Mice were euthanized by vertebral dislocation, and the spleens were collected. Single spleen cell cultures were activated in the presence of either PBS (negative control) or concanavalin A (1 μg/mL), as previously described.[52] The supernatants were then collected and stored at −80 °C until further assay. The levels of IL-4, IL-10, IFN-γ, and TGF-β were assayed using two sets of kits, the mouse IL-4, IL-10, and IFN-γ CytoSet kits (Life Technologies SAS, Saint Aubin, France) and the mouse TGF-β ELISA Ready-Set-Go! Kit (eBioscience), in accordance with the manufacturers' instructions.

Microbial Metabolism: Detection of Short-ChainFatty Acids by Capillary Gas−Liquid Chromatography. The contents of the ceca collected from 12-week-old mice belonging to the combined study (perinatal and postweaning) were collected for SCFA analysis. Approximately 1 g of the cecal content was homogenized in 4 volumes of sterile water. After centrifugation of the thawed samples (10000*g* for 10 min), 0.1 mL of the supernatant was supplemented with 0.9 mL of oxalic acid (0.5 M). The SCFA contents were analyzed by capillary gas−liquid chromatography (SGE BP21 capillary column, 25 m × 0.53 mm; nitrogen as carrier gas, 17 mL/min). The injector and detector temperatures were maintained at 250 and 200 °C, respectively. The oven temperature was maintained at 90 °C. The samples were introduced by splitless injection with a split flow of 50 mL/min starting 1 min after injection. The concentration of each SCFA was determined in comparison to a known concentration of acetate, propionate, and butyrate solutions (from 0.2 to 2 mM), which were analyzed under the same chromatographic conditions.

Measurement of Biomarkers of the Gut Barrier Function. *Western Blot Analysis of HSP-25, HSP-72, and ZO-1.* Jejunum samples from 12-week-old mice belonging to the combined study were homogenized in a solution containing TRIzol (50−100 mg of tissue in 1 mL) for 5 min at room temperature. The homogenate was suspended in chloroform, vigorously vortexed for 15 s, and incubated for 2−3 min at room temperature. The solution was then centrifuged at 12000*g* and 4 °C for 15 min. The organic phase was washed twice with acetone at 4 °C, dried, and resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail). The proteins were determined in duplicate with a colorimetric assay (Bio-Rad Laboratories, Munich, Germany) using albumin as the protein standard. The electrophoresis was performed using a 12% SDS−polyacrylamide gel. The separated proteins were electrophoretically transferred onto nitrocellulose membranes. Before immunostaining, the nonspecific binding sites were blocked with 5% cow's milk in Tris-buffered saline (TBS) containing 0.1% Tween-20. The membranes were incubated for 1 h with the respective detection antibody: HSP-25 (SPA-801, Stressgen, Victoria, Canada) diluted 1:500; HSP-72 (SPA-810, Stressgen) diluted 1:500; ZO-1 (2-R1, Invitrogen, Paisley, UK) diluted 1:500; and β-actin (C4, sc-47778, Santa Cruz Biotechnology) diluted 1:2000. After several washes in TBS containing 0.1% Tween-20, the membranes were incubated with the appropriate secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG; Jackson, West Grove, PA, USA), which were diluted 1:5000. A chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate, Pierce, Rockford, IL, USA) was added, and the luminescence was measured with a CCD camera (Luminescent Image Analyzer LAS 3000, Fujifilm, Tokyo, Japan).

MUC-2 Secretion in Jejunum by Immunohistochemistry. The samples used to determine the MUC-2 secretion levels were fragments of jejunum collected from 12-week-old mice belonging to the combined study. Sections of the paraffin-embedded tissue arrays were deparaffinized, successively rehydrated in decreased concentrations of alcohol baths, and microwave-treated three times for 3 min in a citrate buffer (pH 6.0). The endogenous peroxidase activity was blocked with PBS containing 2% hydrogen peroxide. The nonspecific binding sites were blocked with 3% bovine serum albumin in PBS. The arrays were incubated overnight at 4 °C with a MUC-2 detection antibody (SC-15334, Santa Cruz Biotechnology) that was diluted 1:100. The arrays were then incubated with a secondary antibody (goat anti-rabbit IgG-FITC, Invitrogen, Paisley, UK) that was diluted 1:500. The fluorescence intensity (emission at 520 nm and excitation at 490 nm) was measured.

Statistical Analyses. The values are expressed as the mean ± SD. GraphPad Prism version 5.02 for Windows software (GraphPad Software, San Diego, CA, USA) was used to perform the statistical

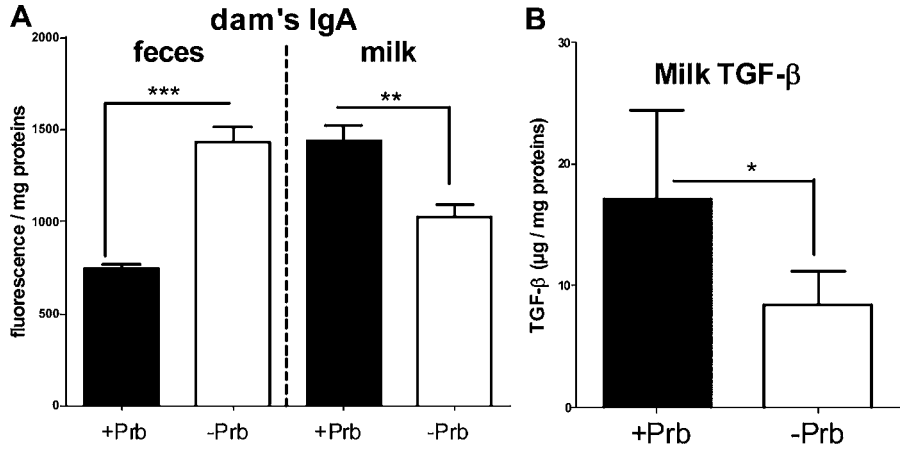

Figure 1. Effect of a prebiotic diet on the tolerance biomarker expression in dams. The total IgA (A) production was assayed in the feces and milk of dams that were exposed or not exposed to prebiotics for 8 weeks. The amount of TGF-β (B) in the milk of dams that were exposed (black bar) or not exposed (white bar) to prebiotics for 8 weeks was assayed (+Prb, $n = 13$; −Prb, $n = 12$). The data represent the mean ± SD; (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.005$.

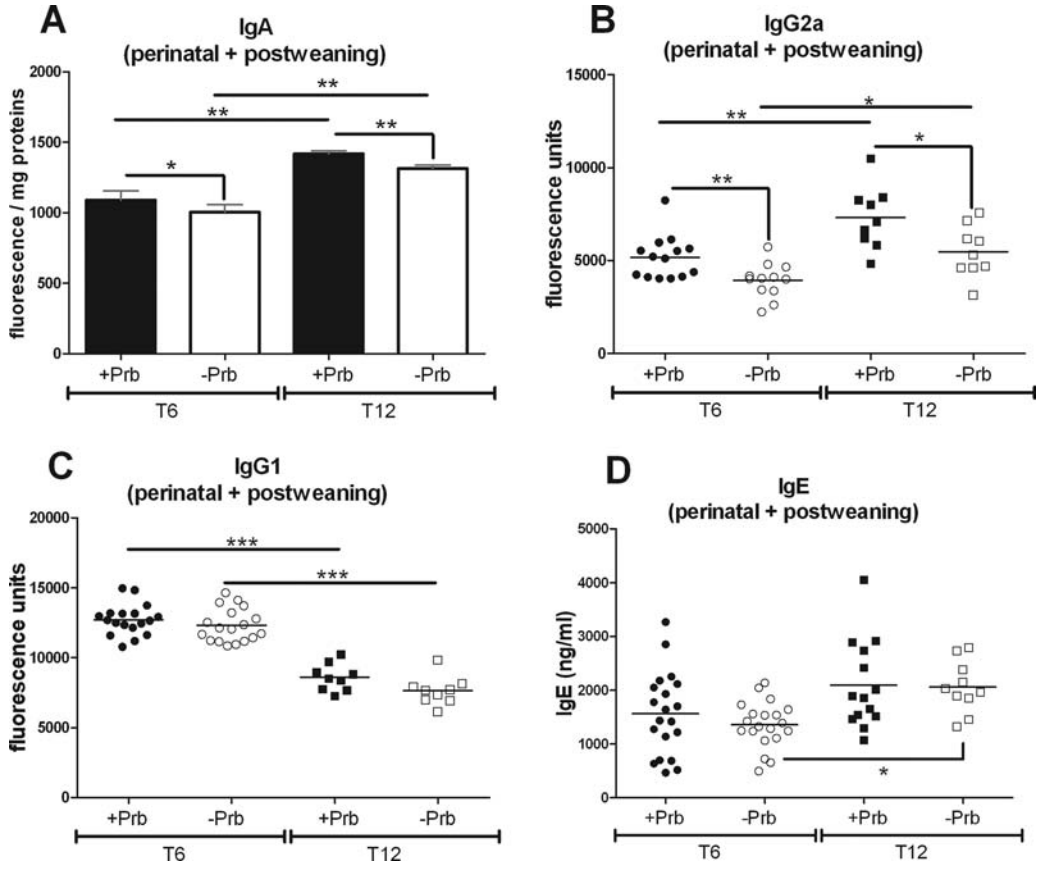

Figure 2. Effects of the combined (perinatal + postweaning) exposure period to prebiotics on the immunoglobulin expression in the offspring. The total fecal IgA (A) (Treg-related biomarker), blood IgG2a (B) (Th1-related biomarker), and total IgG1 (C) and IgE (D) (Th2-related biomarkers) were measured in the 6- (T6, circles) and 12-week-old (T12, squares) offspring. The +Prb mice are represented by black shapes and the −Prb mice by white shapes. The mothers had been or had not been in contact with prebiotics during reproduction, gestation, and lactation, and their offspring did or did not receive prebiotics for 3 (T6) or 9 (T12) weeks after weaning (+Prb, $n = 15$; −Prb, $n = 10$); (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.005$.

analyses. A nonparametric Mann−Whitney test was used to compare the production of total IgE, IgG1, and IgG2a and gut markers between the groups of mice. When the samples were pooled (for the determination of fecal IgA and plasma cytokines), Student's t test was used. Any differences with p values that were strictly <0.05 were considered to be statistically significant.

RESULTS

Study of the Perinatal and Postweaning Periods. *Analysis of Fecal and Milk IgA and Milk TGF-β Concentrations in Lactating Dams (Figure 1).* The feces from lactating females (from the second week of lactation) that were fed the prebiotic (+Prb) and control diets (−Prb) were analyzed for the

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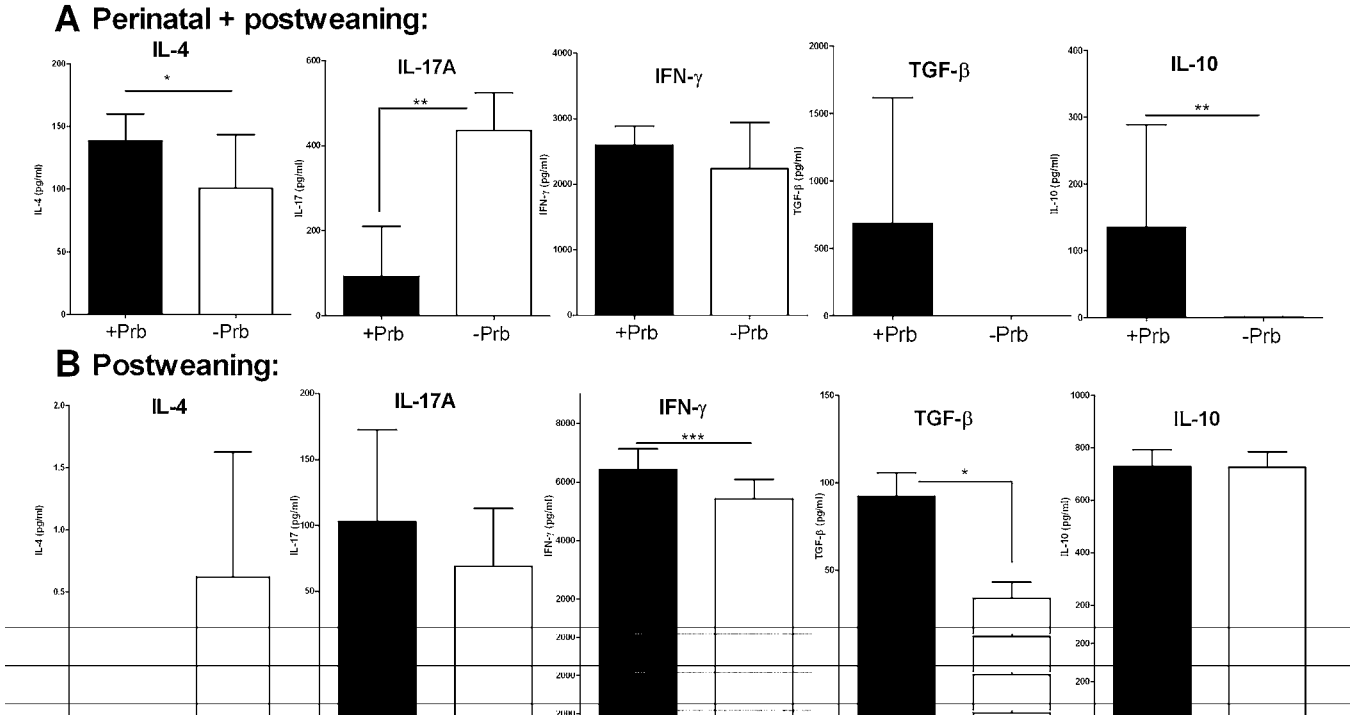

Figure 3. Effects of the two exposure periods to prebiotics on cytokine production in the offspring. The IL-4, IL-17A, INF-γ, IL-10, and TGF-β levels were measured in the supernatant of pooled splenocytes, which were activated with concanavalin A, from 12-week-old offspring (T12): +Prb, black bars; −Prb: white bars. The results correspond to the perinatal + postweaning exposure (+Prb, n = 15; −Prb, n = 10) (A) and the postweaning exposure (+Prb, n = 18; −Prb, n = 18) (B). The data represent the mean ± SD; (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.005$.

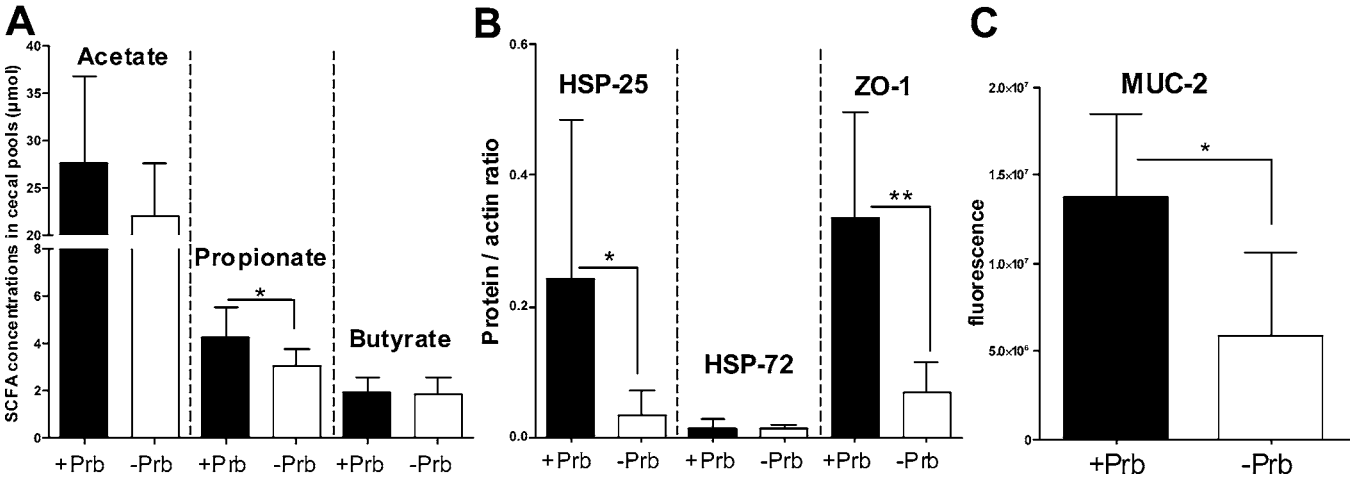

Figure 4. Effects of the combined (perinatal + postweaning) exposure period to prebiotics on the microbiota metabolism and on several gut markers that are potentially targeted by these metabolites in the offspring. The acetate, propionate, and butyrate levels in the cecum were measured (A) (+Prb, n = 10; −Prb, n = 8). The HSP-25, HSP-72, and ZO-1 protein expressions in the jejunum were measured (B) (HSP-25, +Prb, n = 6; −Prb, n = 5) (HSP-72, +Prb, n = 4; −Prb, n = 4) (ZO-1, +Prb, n = 6; −Prb, n = 4). The MUC-2 protein (C) was measured in the jejunum samples (+Prb, n = 8; −Prb, n = 6). The dams had or had not been in contact with prebiotics during reproduction, gestation, and lactation, and their offspring did or did not receive prebiotics for 9 weeks after weaning. The +Prb mice are represented by black bars and the −Prb mice by white bars. The data represent the mean ± SD; (∗) $p < 0.05$ and (∗∗) $p < 0.01$.

production of fecal IgA. The +Prb lactating dams displayed fecal IgA levels that were significantly lower than those observed in the −Prb dams ($p < 0.005$) (Figure 1A). In contrast, the milk collected from the +Prb dams contained higher IgA levels than those from the −Prb dams ($p < 0.01$) (Figure 1B). Similarly, the TGF-β levels were more pronounced in the +Prb dam milk than in the −Prb dam milk ($p < 0.05$) (Figure 1C).

Thus, the prebiotic-enriched diet appeared to increase the secretion of IgA and TGF-β in the milk of dams. These two important markers are involved in the acquisition of tolerance in newborns.

Immune Responses of Mice. *Fecal IgA Excretion.* The +Prb mice displayed IgA concentrations that were significantly higher than those observed in the −Prb mice ($p < 0.05$) at both T6 and T12 (Figure 2A). The IgA levels increased in both groups from T6 to T12 ($p < 0.01$). Therefore, the prebiotic exposure during both the perinatal and postweaning periods appeared to stimulate IgA excretion.

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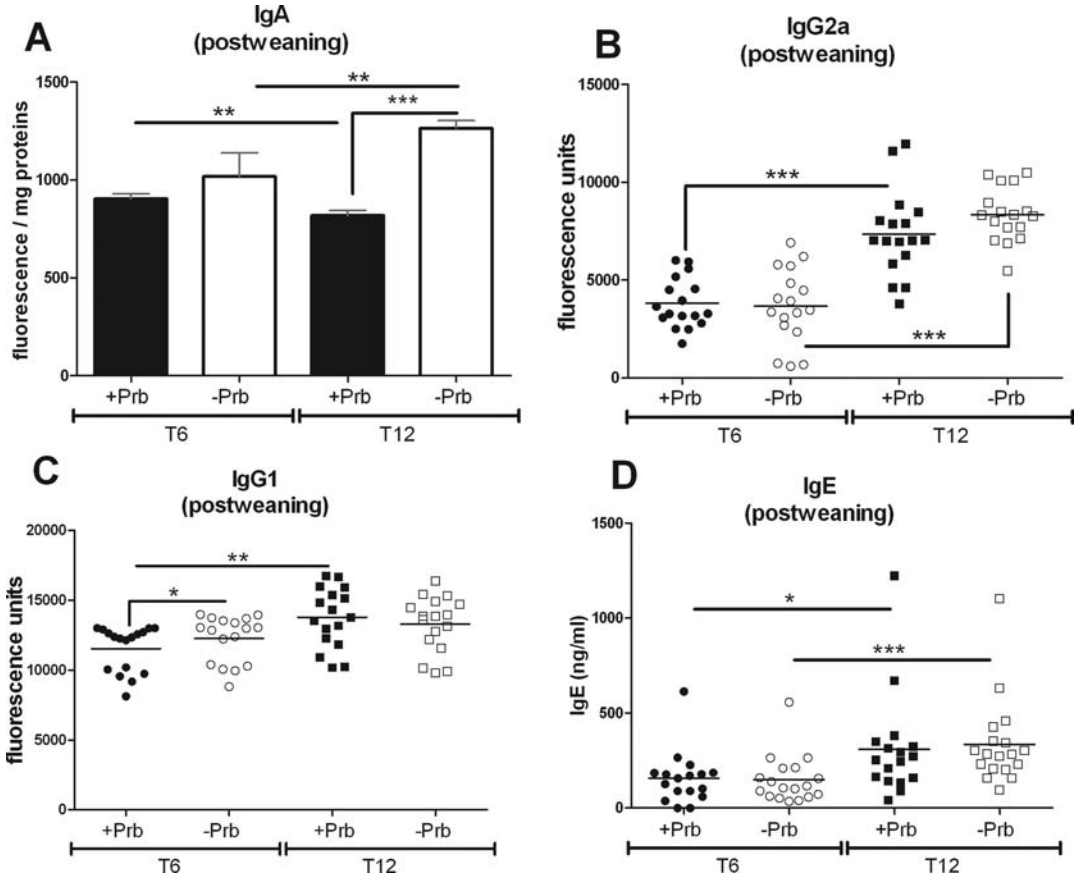

Figure 5. Effects of postweaning exposure to prebiotics on the immunoglobulin of the offspring. The total fecal IgA (A) (Treg-related biomarker), blood IgG2a (B) (Th1-related biomarker), and total IgG1 (C) and IgE (D) (Th2-related biomarkers) were measured in the 6- (T6, circles) and 12-week-old (T12, squares) offspring (+Prb, $n = 18$; −Prb, $n = 18$). The +Prb mice are represented by black shapes and the −Prb mice by white shapes; (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.005$.

Serum Immunoglobulins. The +Prb mice displayed significantly higher serum concentrations of total IgG2a than the −Prb mice ($p < 0.01$) at both T6 and T12 (Figure 2B). The IgG2a levels also increased during this 6 week period in both groups ($p < 0.01$ for +Prb mice and $p < 0.05$ for −Prb mice). At T6 and T12, all of the mice, regardless of their exposure to prebiotics (+Prb or −Prb), had similar serum concentrations of total IgG1 (Figure 2C); in addition, the IgG1 levels decreased within this period ($p < 0.005$). The serum concentrations of total IgE were also equivalent in all of these mice (Figure 2D). Only the IgE levels of the −Prb mice increased significantly during this 6 week period ($p < 0.05$). As a result, the prebiotic exposure during both perinatal and postweaning periods appears to increase the IgG2a production but has no effect on the secretion of either IgG1 or IgE.

Analysis of Cytokine Production in Activated Spleen Cells from T12 Mice: IL-4, IL-17A, IL-10, IFN-γ, and TGF-β (Figure 3A). Activated spleen cells from mice fed the prebiotic diet were able to produce higher levels of IL-10 than cells obtained from mice that were fed the control diet ($p < 0.01$); in addition, these cells were the only ones that were able to secrete detectable amounts of TGF-β. All of these cytokines constitute tolerance biomarkers. We also observed a slightly higher secretion of IL-4 with the prebiotic diet ($p < 0.05$), but the level of this secretion remained very low compared to the IFN-γ secretion. We also detected lower secretion of IL-17A ($p < 0.01$) in the spleen cells of these mice.

The perinatal and postweaning exposure to prebiotics did not appear to modify the Th2-related biomarkers. Indeed, we observed similar levels of IgG1 and IgE production and a low level of IL-4 in the +Prb mice compared with the levels of IFN-γ and IL-10. The same prebiotic exposure appeared to decrease a key inflammatory biomarker related to the Th17-response, as illustrated by the lower secretion of IL-17A in +Prb mice compared with control mice. In contrast, this nutritional approach appeared to result in short- and long-term stimulation of the tolerance biomarkers (increases in IgA excretion and TGF-β and IL-10 secretion) and Th1-related immunoglobulins (more IgG2a) in mice.

Analysis of Bacterial Metabolites and Markers of Gut Barrier Integrity (Figure 4). These analyses were performed at the end of the study (at T12). The cecum contents from the +Prb mice displayed higher levels of propionate than those of the −Prb mice ($p < 0.05$, Figure 4A). The other SCFA levels (acetate and butyrate) were the same, regardless of the diet. The jejunum from +Prb mice contained higher levels of HSP-25 ($p < 0.05$) and ZO-1 ($p < 0.01$) proteins than the jejunum from −Prb mice, whereas the HSP-72 protein was expressed at the same amount regardless of the diet (Figure 4B). The jejunum from +Prb mice also displayed a higher level of mucin MUC-2 secretion in the intestinal lumen ($p < 0.05$) than in the jejunum in −Prb mice (Figure 4C).

These results showed that the mice exposed to prebiotics during both perinatal and postweaning periods displayed a modification of the cecal microbiota metabolism, which is

characterized by a higher production of propionate. The higher expressions of HSP-25, ZO-1, and MUC-2 in the jejunum of these mice suggest a stronger integrity of the gut barrier.

Postweaning Study: Immune Responses of Mice. *Fecal IgA Excretion.* After 3 weeks of the diet, the T6 mice receiving the prebiotic supplement (+ Prb) displayed levels of fecal IgA that were similar to those observed in mice fed the control diet (−Prb, Figure 5A). After 9 weeks of the diet, the T12 mice receiving the prebiotic supplement displayed lower fecal IgA concentrations than mice on the −Prb diet ($p < 0.005$, Figure 5A). These mice also had lower fecal IgA levels than the T6 mice fed the prebiotic diet. In contrast, the T12 mice that were not exposed to the prebiotic mix had higher fecal IgA levels than the T6 mice. Therefore, a longer period of exposure (9 weeks) to the prebiotic diet caused a decrease in the IgA concentration. It appears that in the absence of prebiotic exposure, fecal IgA levels increase over time.

Serum Immunoglobulins. The total IgG2a concentrations in the serum were similar in the T6 and T12 mice, regardless of diet (+Prb or −Prb, Figure 5B). A rise in the IgG2a concentration was observed in both groups of mice during the experimental period. The T6 mice receiving the +Prb diet exhibited serum concentrations of total IgG1 that were slightly lower ($p < 0.05$) than those exhibited by the −Prb mice (Figure 5C). In the absence of prebiotics, the IgG1 levels were sustained during the diet period (3−9 weeks), whereas the supplementation of the diet with prebiotics increased the IgG1 levels to levels similar to those observed in mice that were not exposed to prebiotics. The same total IgE concentrations were observed in the T6 and T12 mice, regardless of diet (Figure 5D). Both groups of T12 mice (exposed and not exposed to prebiotics) exhibited higher IgE levels than the T6 mice ($p < 0.05$ for +Prb mice and $p < 0.005$ for −Prb mice). As a result, the exposure to prebiotics during the postweaning period did not appear to have any effect on the secretion of IgG2a, IgG1, and IgE.

Analysis of Cytokine Production by Activated Spleen Cells from T12 Mice (Figure 3B). Activated spleen cells from mice fed both diets (+Prb or −Prb) produced similar levels of IL-10. We did not detect any production of IL-4, and the IL-17A levels were the same, regardless of diet. The spleen cells from mice that were fed the prebiotic diet secreted more IFN-γ than the cells from mice fed the control diet ($p < 0.01$). A higher production of TGF-β was also observed in the spleen cells from +Prb-fed mice ($p < 0.05$), although the difference remained very slight.

To conclude, the postweaning exposure to prebiotics did not stimulate the production of biomarkers related to the Th2 or the Th17 responses (similar levels of IgE, IgG1, IL-4, and IL-17A). This exposure appears to have a moderate effect on the biomarkers related to the Th1 response (similar levels of IgG2a and higher secretion of IFN-γ in +Prb mice). Surprisingly, long-term exposure to prebiotics appeared to have contradictory effects on two tolerance biomarkers: fecal IgA production was decreased, and increased levels of TGF-β were secreted by the +Prb mice.

DISCUSSION

The aim of this study was to evaluate the effect of perinatal and postweaning exposure to prebiotics on immune responses and gut barrier functions. We first investigated the impact of a GOS/inulin mix on the immune profile of mice exposed to prebiotics during both the perinatal (gestation, delivery, and lactation) and postweaning periods. We also compared their responses to those of mice that were exposed to prebiotics only after weaning. Our results showed that the combination of perinatal and postweaning exposures to the GOS/inulin mix induced higher expression levels of regulatory and Th1-related biomarkers (IgA excretion, IgG2a secretion, and IL-10 and TGF-β production). The observed increase in IgA levels is in agreement with the results reported by Nakamura et al.,[34] who observed that the administration of some prebiotics, such as FOS, during lactation and for 3 weeks after weaning increased the production of fecal IgA. Moreover, prebiotics also act long term (12 weeks) and maintain the induced levels of some of these biomarkers, such as IgA and IgG2a. We also observed that prebiotics might be able to down-regulate the inflammatory Th17 response involved in many pathologies, as suggested by the decrease in IL-17A secretion. Altogether, our results suggest that an exposure to prebiotics during both the perinatal and postweaning periods may modulate the immune response toward key mechanisms that may be used in the prevention of some immune pathologies (allergies, autoimmune diseases and inflammatory bowel diseases) through the inhibition of IL-17A secretion and induction of Treg- and Th1-related biomarkers, which may in turn affect the Th1/Th2 balance.

The postweaning exposure to prebiotics was conducted to evaluate the influence of the intake of prebiotics shortly after weaning on the immune responses. This experimental procedure did not affect the Th2- and the Th17-related biomarkers. However, it did exert a moderate and variable effect on certain Th1-related biomarkers, as illustrated by the higher production of IFN-γ and the unchanged IgG2a concentration. The existence of an IFN-γ-independent IgG2a secretion was demonstrated by Markine-Goriaynoff et al., who found that this secretion was dependent on IL-12 or TNF-α.[53] The postweaning exposure to prebiotics also had a moderate and contradictory effect on certain Treg-related biomarkers. Indeed, this exposure caused a long-term unexpected decrease in the fecal IgA excretion, which is a crucial biomarker of intestinal tolerance. However, prebiotics were also able to slightly increase the production of TGF-β, a cytokine that is typically secreted by Treg cells. Other researchers[54] observed a similar decrease in the total IgA content of feces from 4-week-old rats that were fed lactulose for 3 weeks.

In both studies (postweaning and combined exposure), the duration of the exposure to prebiotics through solid foods was the same (3 weeks for the short period and 9 weeks for the longer period). However, in the combined approach, the pups were exposed to the milk and fecal microbiota of dams that received the prebiotic diet. In our study, the concentrations of TGF-β and IgA were higher in the milk of dams fed a prebiotic-supplemented diet. These two molecules are known to promote regulatory T cells in the infant gut.[16,55,56] Therefore, during our experiments, the increased expression of tolerance biomarkers in mice exposed to prebiotics during both perinatal and postweaning periods may be linked to an exposure to immune factors, such as IgA and TGF-β, from the milk of the dam. Roux et al.[57] observed that IgA-secreting plasmocytes in the mammary gland originated from the gut and were able to migrate from one place to the other. We can thus suggest that IgA-secreting B cells are more present in the mammary gland than in the gut of lactating dams. On the basis of our study, we can conclude that the prebiotic effect on the immune system that was observed in the combined approach is largely mediated by the mother during gestation and lactation.

To explain the effects of prebiotics on the pups that were observed in the combined study, we formulated the hypothesis that the microbiota of the dams was modified before delivery

through the consumption of the +Prb diet. We then suggest that this modification is most likely transmitted to the pups during some key periods: pregnancy (metabolites and/or bacteria[3] are transmitted through the cord blood), delivery (vaginal and perineal micro-organisms[58]), and lactation (milk bacteria,[19] milk oligosaccharides,[59] and/or microbiota metabolites). The inherited microbiota then modulate the immune profile of the pups. To assess the potential modification of the microbiota in mice fed the +Prb diet, we analyzed the release of SCFAs in the cecum of 12-week-old pups. The results showed that propionate was elevated in mice fed the +Prb diet (during both exposure periods). These data demonstrate for the first time that the combined exposure period has an impact on the microbiota metabolism of mouse pups. A similar impact on SCFA production (higher production of propionate and butyrate) has been obtained in rats fed fructans shortly after weaning,[39] during growth,[60] and during adulthood.[38] Because this analysis was performed at the end of the study, it can be assumed that the respective amount of each SCFA in the cecum would have been modified over time. In fact, Le Blay et al. showed that a prolonged exposure (2, 8, and 27 weeks) of growing rats to fructans induced a higher production of butyrate (but not propionate) over time and differentially modulated the amount of other SCFAs (acetate and lactate) throughout the duration of the exposure.[61] Due to recent data on the increased secretion of galectin-9 in the serum of both infants and mice fed a GOS/inulin diet, we can also suggest that the exposure to the GOS/inulin prebiotic mix during both the perinatal and postweaning periods might exert a direct effect on the immune responses via the formation of prebiotics/galectin-9 complexes and their fixation on T cell receptors.[35,36]

In the combined study, the effect of prebiotic consumption on the gut barrier integrity was tested. We observed that the expression of both ZO-1 and MUC-2 was higher in the jejunum of T12 mice fed the +Prb diet compared to the jejunum of T12 mice fed a −Prb diet. These two proteins constitute key modulators of the gut mucosal barrier: ZO-1 exerts an important role in the structure of tight junctions,[62] and MUC-2 is the major protein of the mucus, which constitutes the first line of defense against micro-organisms.[63] Most heat-shock proteins (HSP) are constitutively expressed and protect cells against oxidative stress. In particular, two HSPs, that is, HSP-25 and HSP-72, are notably induced by stress.[64] HSP-25 is particularly important for cell integrity because its phosphorylation results in the reorganization of actin microfilaments. HSP-25 can also be regulated by SCFAs.[65] In our study, HSP-25 was expressed at higher levels in the jejunum of mice fed the prebiotic-supplemented diet; this result was not observed with HSP-72. We can thus hypothesize that the higher expression of HSP-25 is not linked to stress but may be a consequence of the higher amount of propionate in +Prb mice. We propose the same explanation for the differential expressions of ZO-1 and MUC-2. Indeed, Peng et al.[66] showed that SCFAs can modulate the assembly of tight junctions and that the MUC-2 promoter possesses regulatory elements that are specific for SCFAs.[67] Numerous in vivo studies conducted with adult humans[40] or animals (adult or early weaned rats and piglets)[37−39,41,43−45] have tested the effect of prebiotic oligosaccharides (GOS[37] and fructans[38−40]) and dietary fibers (cellulose,[38] pectin,[38] arabinoxylans,[41] β-glucans,[42,43] resistant starch,[38,44] and other fibers[45]) on gut barrier function. However, this type of analysis has not been performed with an exposure to the GOS/inulin mix during both the perinatal and postweaning periods. Consequently, this study is the first to provide data that prove the effectiveness of this approach on both the modification of the microbiota metabolism and the strengthening of the gut barrier functions.

In this study, we demonstrate that perinatal and postweaning exposures to prebiotics is effective in modulating immune responses toward a tolerance state in mice and in strengthening the integrity of the intestinal barrier. This strategy is highly promising in the context of allergy prevention. To date, the impact of prebiotics on allergy has not been assessed under these conditions. In fact, the two human clinical studies that have been performed were conducted in infants receiving prebiotics in a cow's milk-based formula.[47,68] These studies demonstrated a reduction in the symptoms of atopic dermatitis in these children. To date, the four mouse studies that have been performed[32] administered prebiotics to 5−8-week-old adults and recorded a reduction in the allergy severity. All of these findings are extremely exciting because if such an effect is demonstrated during the postweaning period in both infants and adult mice, the effect might be even stronger following perinatal administration, as suggested by our results.

In conclusion, the perinatal combined to postweaning administrations of the GOS/inulin mix was shown to stimulate the tolerance-related pathways (Treg and Th1 responses and the inhibition of IL-17A production) and to strengthen the gut barrier (HSP-25, MUC-2, and ZO-1 expression). These responses may prove helpful in the prevention of allergy. Pregnancy, delivery, and lactation seemed to be the key periods during which the immune responses can be significantly modulated.

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Notes

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ABBREVIATIONS USED

F-ELISA, fluorometric enzyme-linked immunosorbent assay; FOS, fructooligosaccharides; GOS, galactooligosaccharides; HPAEC, high-performance anion-exchange chromatography; HSP, heat shock protein; IFN, interferon; IL, interleukin; MUC-2, mucin-2; PBS, phosphate-buffered saline; Prb, prebiotic; RPMI, Roswell Park Memorial Institute medium; SCFA, short-chain fatty acid; T6, 6 weeks old; T12, 12 weeks old; TBS, Tris-buffered saline; TGF, transforming growth factor; Treg, regulatory T cells; ZO-1, *zonula occludens* protein-1

REFERENCES

(1) Pfefferle, P. I.; Buchele, G.; Blumer, N.; Roponen, M.; Ege, M. J.; Krauss-Etschmann, S.; Genuneit, J.; Hyvarinen, A.; Hirvonen, M. R.; Lauener, R.; Pekkanen, J.; Riedler, J.; Dalphin, J. C.; Brunekeef, B.; Braun-Fahrlander, C.; von Mutius, E.; Renz, H. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: the PASTURE study. *J. Allergy Clin. Immunol.* **2010**, *125* (1), 108−115 e1−3.

(2) Jimenez, E.; Fernandez, L.; Marin, M. L.; Martin, R.; Odriozola, J. M.; Nueno-Palop, C.; Narbad, A.; Olivares, M.; Xaus, J.; Rodriguez, J. M. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr. Microbiol.* **2005**, *51* (4), 270−274.

(3) Jimenez, E.; Marin, M. L.; Martin, R.; Odriozola, J. M.; Olivares, M.; Xaus, J.; Fernandez, L.; Rodriguez, J. M. Is meconium from healthy newborns actually sterile? *Res. Microbiol.* **2008**, *159* (3), 187−193.

(4) Holt, P. G.; Jones, C. A. The development of the immune system during pregnancy and early life. *Allergy* **2000**, *55* (8), 688−697.

(5) Prescott, S. L.; Macaubas, C.; Smallacombe, T.; Holt, B. J.; Sly, P. D.; Holt, P. G. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* **1999**, *353* (9148), 196−200.

(6) Renz, H.; Brandtzaeg, P.; Hornef, M. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. *Nat. Rev. Immunol.* **2012**, *12* (1), 9−23.

(7) Brandtzaeg, P.; Johansen, F. E. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunol. Rev.* **2005**, *206*, 32−63.

(8) Mosmann, T. R.; Coffman, R. L. Th1-cell and Th2-cell - Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **1989**, *7*, 145−173.

(9) Mucida, D.; Kutchukhidze, N.; Erazo, A.; Russo, M.; Lafaille, J. J.; Curotto de Lafaille, M. A. Oral tolerance in the absence of naturally occurring Tregs. *J. Clin. Invest.* **2005**, *115* (7), 1923−1933.

(10) Chehade, M.; Mayer, L. Oral tolerance and its relation to food hypersensitivities. *J. Allergy Clin. Immunol.* **2005**, *115* (1), 3−12.

(11) Barnes, R. M. IgG and IgA antibodies to dietary antigens in food allergy and intolerance. *Clin. Exp. Allergy* **1995**, *25* (Suppl. 1), 7−9.

(12) Kim, P. H.; Kagnoff, M. F. Transforming growth factor-β-1 is a costimulator for IgA production. *J. Immunol.* **1990**, *144* (9), 3411−3416.

(13) Kunkel, E. J.; Butcher, E. C. Plasma-cell homing. *Nat. Rev. Immunol.* **2003**, *3* (10), 822−829.

(14) Afzali, B.; Lombardi, G.; Lechler, R. I.; Lord, G. M. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin. Exp. Immunol.* **2007**, *148* (1), 32−46.

(15) Komatsu, T.; Okao, M.; Miyamoto, H.; Chen, T.; Shinka, S. Effects of early antigen exposure through lactation on later specific antibody responses in mice. *J. Immunol.* **1988**, *141* (9), 2895−2906.

(16) Verhasselt, V. Neonatal tolerance under breastfeeding influence. *Curr. Opin. Immunol.* **2010**, *22* (5), 623−630.

(17) Verhasselt, V. Oral tolerance in neonates: from basics to potential prevention of allergic disease. *Mucosal Immunol.* **2010**, *3* (4), 326−333.

(18) Lehmann, A. D. L. A. D.; Daum, N.; Bur, M.; Lehr, C. M.; Gehr, P.; Rothen-Rutishauser, B. M. An in vitro triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier. *Eur. J. Pharm. Biopharm.* **2011**, *77* (3), 398−406.

(19) Collado, M. C.; Delgado, S.; Maldonado, A.; Rodriguez, J. M. Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. *Lett. Appl. Microbiol.* **2009**, *48* (5), 523−528.

(20) Mazmanian, S. K.; Liu, C. H.; Tzianabos, A. O.; Kasper, D. L. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* **2005**, *122* (1), 107−118.

(21) Shen, T. Y.; Qin, H. L.; Gao, Z. G.; Fan, X. B.; Hang, X. M.; Jiang, Y. Q. Influences of enteral nutrition combined with probiotics on gut microflora and barrier function of rats with abdominal infection. *World J. Gastroenterol.* **2006**, *12* (27), 4352−4358.

(22) Zyrek, A. A.; Cichon, C.; Helms, S.; Enders, C.; Sonnenborn, U.; Schmidt, M. A. Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKC zeta redistribution resulting in tight junction and epithelial barrier repair. *Cell Microbiol.* **2007**, *9* (3), 804−816.

(23) Mack, D. R.; Michail, S.; Wei, S.; McDougall, L.; Hollingsworth, M. A. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am. J. Physiol.* **1999**, *276* (4 Part 1), G941−G950.

(24) Tao, Y.; Drabik, K. A.; Waypa, T. S.; Musch, M. W.; Alverdy, J. C.; Schneewind, O.; Chang, E. B.; Petrof, E. O. Soluble factors from *Lactobacillus* GG activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. *Am. J. Physiol. Cell. Physiol.* **2006**, *290* (4), C1018−C1030.

(25) Husby, S.; Mestecky, J.; Moldoveanu, Z.; Holland, S.; Elson, C. O. Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding. *J. Immunol.* **1994**, *152* (9), 4663−4670.

(26) Lowney, E. D. Suppression of contact sensitization in man by prior feeding of antigen. *J. Invest. Dermatol.* **1973**, *61* (2), 90−93.

(27) Koenig, J. E.; Spor, A.; Scalfone, N.; Fricker, A. D.; Stombaugh, J.; Knight, R.; Angenent, L. T.; Ley, R. E. Succession of microbial consortia in the developing infant gut microbiome. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *108* (Suppl. 1), 4578−4585.

(28) Schrezenmeir, J.; de Vrese, M. Probiotics, prebiotics, and synbiotics−approaching a definition. *Am. J. Clin. Nutr.* **2001**, *73* (2 Suppl.), 361S−364S.

(29) del Giudice, M. M.; Brunese, F. P. Probiotics, prebiotics, and allergy in children: what's new in the last year? *J. Clin. Gastroenterol.* **2008**, *42* (Suppl. 3 Part 2), S205−S208.

(30) Baldwin, R. L. Energy metabolism in anaerobes. *Am. J. Clin. Nutr.* **1970**, *23* (11), 1508−1513.

(31) Campbell, J. M.; Fahey, G. C.; Wolf, B. W. Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *J. Nutr.* **1997**, *127* (1), 130−136.

(32) Gourbeyre, P.; Denery, S.; Bodinier, M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. *J. Leukocyte Biol.* **2011**, *89* (5), 685−695.

(33) Hosono, A.; Ozawa, A.; Kato, R.; Ohnishi, Y.; Nakanishi, Y.; Kimura, T.; Nakamura, R. Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. *Biosci., Biotechnol., Biochem.* **2003**, *67* (4), 758−764.

(34) Nakamura, Y.; Nosaka, S.; Suzuki, M.; Nagafuchi, S.; Takahashi, T.; Yajima, T.; Takenouchi-Ohkubo, N.; Iwase, T.; Moro, I. Dietary fructooligosaccharides up-regulate immunoglobulin A response and polymeric immunoglobulin receptor expression in intestines of infant mice. *Clin. Exp. Immunol.* **2004**, *137* (1), 52−58.

(35) Rabinovich, G. A.; Toscano, M. A. Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. *Nat. Rev. Immunol.* **2009**, *9* (5), 338−352.

(36) de Kivit, S.; Saeland, E.; Kraneveld, A. D.; van de Kant, H. J.; Schouten, B.; van Esch, B. C.; Knol, J.; Sprikkelman, A. B.; van der Aa, L. B.; Knippels, L. M.; Garssen, J.; van Kooyk, Y.; Willemsen, L. E. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. *Allergy* **2012**, *67* (3), 343−352.

(37) Zhong, Y.; Cai, D. L.; Cai, W.; Geng, S. S.; Chen, L. Y.; Han, T. Protective effect of galactooligosaccharide-supplemented enteral nutrition on intestinal barrier function in rats with severe acute pancreatitis. *Clin. Nutr.* **2009**, *28* (5), 575−580.

(38) Hedemann, M. S.; Theil, P. K.; Knudsen, K. E. B. The thickness of the intestinal mucous layer in the colon of rats fed various sources of non-digestible carbohydrates is positively correlated with the pool of SCFA but negatively correlated with the proportion of butyric acid in digesta. *Br. J. Nutr.* **2009**, *102* (1), 117−125.

(39) Paturi, G.; Butts, C. A.; Stoklosinski, H.; Ansell, J. Effects of early dietary intervention with a fermentable fibre on colonic microbiota activity and mucin gene expression in newly weaned rats. *J. Funct. Food* **2012**, *4* (2), 520−530.

(40) Ten Bruggencate, S. J. M.; Bovee-Oudenhoven, I. M. J.; Lettink-Wissink, M. L. G.; Katan, M. B.; van der Meer, R. Dietary fructooligosaccharides affect intestinal barrier function in healthy men. *J. Nutr.* **2006**, *136* (1), 70−74.

(41) Niewold, T. A.; Schroyen, M.; Geens, M. M.; Verhelst, R. S. B.; Courtin, C. M. Dietary inclusion of arabinoxylan oligosaccharides (AXOS) down regulates mucosal responses to a bacterial challenge in a piglet model. *J. Funct. Food* **2012**, *4* (3), 626−635.
(42) Smith, A. G.; Ryan, M.; O'Doherty, J. V.; Reilly, P.; Bahar, B.; Sweeney, T. Effects of dietary supplementation with laminarin derived from *Laminaria hyperborea* and *Laminaria digitata* on colonic mucin gene expression in pigs. *Livest. Sci.* **2010**, *133* (1−3), 204−206.
(43) Volman, J. J.; Mensink, R. P.; Buurman, W. A.; Plat, J. In vivo effects of dietary (1→3), (1→4)-*β*-D-glucans from oat on mucosal immune responses in man and mice. *Scand. J. Gastroenterol.* **2011**, *46* (5), 603−610.
(44) Bauer-Marinovic, M.; Florian, S.; Muller-Schmehl, K.; Glatt, H.; Jacobasch, G. Dietary resistant starch type 3 prevents tumor induction by 1,2-dimethylhydrazine and alters proliferation, apoptosis and dedifferentiation in rat colon. *Carcinogenesis* **2006**, *27* (9), 1849−1859.
(45) 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.
(46) Vos, A. P.; van Esch, B. C.; Stahl, B.; M'Rabet, L.; Folkerts, G.; Nijkamp, F. P.; Garssen, J. Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. *Int. Immunopharmacol.* **2007**, *7* (12), 1582−1587.
(47) Moro, G.; Arslanoglu, S.; Stahl, B.; Jelinek, J.; Wahn, U.; Boehm, G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch. Dis. Child.* **2006**, *91* (10), 814−9.
(48) Coulier, L.; Timmermans, J.; Bas, R.; Van Den Dool, R.; Haaksman, I.; Klarenbeek, B.; Slaghek, T.; Van Dongen, W. In-depth characterization of prebiotic galacto-oligosaccharides by a combination of analytical techniques. *J. Agric. Food Chem.* **2009**, *57* (18), 8488−8495.
(49) Desbuards, N.; Gourbeyre, P.; Haure-Mirande, V.; Darmaun, D.; Champ, M.; Bodinier, M. Impact of perinatal prebiotic consumption on gestating mice and their offspring: a preliminary report. *Br. J. Nutr.* **2012**, *107* (9), 1245−1248.
(50) Chatel, J. M.; Langella, P.; Adel-Patient, K.; Commissaire, J.; Wal, J. M.; Corthier, G. Induction of mucosal immune response after intranasal or oral inoculation of mice with *Lactococcus lactis* producing bovine *β*-lactoglobulin. *Clin. Diagn. Lab. Immunol.* **2001**, *8* (3), 545−551.
(51) Bodinier, M.; Leroy, M.; Ah-Leung, S.; Blanc, F.; Tranquet, O.; Denery-Papini, S.; Wal, J. M.; Adel-Patient, K. Sensitization and elicitation of an allergic reaction to wheat gliadins in mice. *J. Agric. Food Chem.* **2009**, *57* (4), 1219−1225.
(52) Gourbeyre, P.; Denery-Papini, S.; Larre, C.; Gaudin, J. C.; 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.* **2012**, *56* (2), 336−344.
(53) Markine-Goriaynoff, D.; van der Logt, J. T.; Truyens, C.; Nguyen, T. D.; Heessen, F. W.; Bigaignon, G.; Carlier, Y.; Coutelier, J. P. IFN-*γ*-independent IgG2a production in mice infected with viruses and parasites. *Int. Immunol.* **2000**, *12* (2), 223−230.
(54) Kudoh, K.; Shimizu, J.; Ishiyama, A.; Wada, M.; Takita, T.; Kanke, Y.; Innami, S. Secretion and excretion of immunoglobulin A to cecum and feces differ with type of indigestible saccharides. *J. Nutr. Sci. Vitaminol.* **1999**, *45* (2), 173−181.
(55) Brandtzaeg, P. Mucosal immunity: integration between mother and the breast-fed infant. *Vaccine* **2003**, *21* (24), 3382−3388.
(56) Saarinen, K. M.; Juntunen-Backman, K.; Jarvenpaa, A. L.; Klemetti, P.; Kuitunen, P.; Lope, L.; Renlund, M.; Siivola, M.; Vaarala, O.; Savilahti, E. Breast-feeding and the development of cows' milk protein allergy. *Adv. Exp. Med. Biol.* **2000**, *478*, 121−130.
(57) Roux, M. E.; McWilliams, M.; Phillips-Quagliata, J. M.; Weisz-Carrington, P.; Lamm, M. E. Origin of IgA-secreting plasma cells in the mammary gland. *J. Exp. Med.* **1977**, *146* (5), 1311−1322.
(58) Fanaro, S.; Chierici, R.; Guerrini, P.; Vigi, V. Intestinal microflora in early infancy: composition and development. *Acta. Paediatr. Suppl.* **2003**, *91* (441), 48−55.
(59) Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu. Rev. Nutr.* **2000**, *20*, 699−722.
(60) Juskiewicz, J.; Wroblewska, M.; Jaroslawska, J.; Balinski, P.; Matusevicius, P.; Zdunczyk, P.; Biedrzycka, E.; Zdunczyk, Z. Effects of inulin supplemented to cellulose-free or cellulose-rich diets on caecal environment and biochemical blood parameters in rats. *J. Anim. Feed Sci.* **2009**, *18* (4), 709−722.
(61) Le Blay, G.; Michel, C.; Blottiere, H. M.; Cherbut, C. Prolonged intake of fructo-oligosaccharides induces a short-term elevation of lactic acid-producing bacteria and a persistent increase in cecal butyrate in rats. *J. Nutr.* **1999**, *129* (12), 2231−2235.
(62) Fanning, A. S.; Jameson, B. J.; Jesaitis, L. A.; Anderson, J. M. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J. Biol. Chem.* **1998**, *273* (45), 29745−29753.
(63) Deplancke, B.; Gaskins, H. R. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am. J. Clin. Nutr.* **2001**, *73* (6), 1131S−1141S.
(64) Petrof, E. O.; Ciancio, M. J.; Chang, E. B. Role and regulation of intestinal epithelial heat shock proteins in health and disease. *Chin. J. Dig. Dis.* **2004**, *5* (2), 45−50.
(65) Ren, H.; Musch, M. W.; Kojima, K.; Boone, D.; Ma, A.; Chang, E. B. Short-chain fatty acids induce intestinal epithelial heat shock protein 25 expression in rats and IEC 18 cells. *Gastroenterology* **2001**, *121* (3), 631−639.
(66) Peng, L.; Li, Z. R.; Green, R. S.; Holzman, I. R.; Lin, J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J. Nutr.* **2009**, *139* (9), 1619−1625.
(67) Burger-van Paassen, N.; Vincent, A.; Puiman, P. J.; van der Sluis, M.; Bouma, J.; Boehm, G.; van Goudoever, J. B.; Van Seuningen, I.; Renes, I. B. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. *Biochem. J.* **2009**, *420*, 211−219.
(68) Gruber, C.; van Stuijvenberg, M.; Mosca, F.; Moro, G.; Chirico, G.; Braegger, C. P.; Riedler, J.; Boehm, G.; Wahn, U.; Grp, M. W. Reduced occurrence of early atopic dermatitis because of immunoactive prebiotics among low-atopy-risk infants. *J. Allergy Clin. Immunol.* **2010**, *126* (4), 791−797.