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Maternal and postnatal dietary probiotic supplementation enhances splenic regulatory T helper cell population and reduces peanut allergen-induced hypersensitivity responses in mice

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ABSTRACT

Neonatal to early childhood is the critical period for establishing a balance of T helper 1 (Th1) versus T helper 2 (Th2) cellular immunity within the gut, which is strongly influenced by the source and establishment of gut microflora. Probiotic administration has been shown to attenuate Th2-biased cellular immunity and predisposition to food allergies. To test this hypothesis we provided *ad libitum* a probiotic-supplemented (Primalac 454 Feed Grade Microbials) or control diet to lactating dams with suckling pups and weaned pups until 10 weeks of age. Weaned mice were sensitized/challenged with peanut extract, saline or adjuvant at 6, 8 and 10 weeks of age. At 3, 6, 8 and 10 weeks, fecal samples were collected for microbial analysis, while blood samples were analyzed for total plasma IgE levels. At termination (10 weeks of age), splenic T lymphocyte population subtypes were determined using FACS analysis and Th1/Th2/Th17 gene expression by PCR array. Mice given the probiotic-supplemented diet had significantly enhanced probiotic fecal counts compared to controls at 3, 6, 8 and 10 weeks. Moreover, mice fed the probiotic-supplemented diet had enhanced splenic naturally occurring T regulatory cell populations, and reduced splenic gene expression of allergic mediator IL-13 compared to controls. These results provide evidence that early probiotic supplementation may provide host protection to hypersensitivity reactions to food allergens by attenuating food allergen inflammatory responses.

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Introduction

Immunological responses to environmental allergens are initiated very early in life in most individuals. T cell cloning and subsequent genotyping studies have demonstrated that T cell responsiveness from cord blood samples is of fetal origin and exhibits a Th2 polarized and/or naïve Th0 cytokine profile (Prescott et al. 1998; Piccinni et al. 1996), which may have been primed by processed antigen crossing the placenta or bound to maternal IgG

(Holt et al. 1995; Miles et al. 1996; Casas and Björkstén 2001). Moreover, multiple studies have shown that genetic predisposition to allergy is associated with delayed postnatal maturation of T helper cell function (Holt et al. 1992), with reduced capacity to secrete Th1 cytokine, IFN γ or Th2 cytokine IL-4. This reduction is more notable for IFN γ in infants with a positive atopic family history, indicating a Th2-bias (Holt et al. 1992; Rinas et al. 1993; Liao et al. 1996; Martinez et al. 1995). In genetically predisposed individuals, the sensitization/priming process appears to occur with exposure to food allergens in the gut causing IgE-mediated food allergy during infancy or early childhood. During the sensitization process to food allergens, T cell responses promoting B cell class switching to IgE occur in response to IL-4 secretion by Th2 lymphocytes (Eigenmann et al. 1998).

The neonatal and early childhood periods are critical for the establishment of Th1–Th2 cellular immunity. Postnatal T helper cell maturation and function is primarily driven by the interaction of the gut associated lymphoid tissue and gut microflora, which affects Th1 polarization (Holt 1995; Holt et al. 1997). Th1-biased immunity may lead to pro-inflammatory responses and enhanced

Abbreviations: LA, *Lactobacillus acidophilus*; PN, peanut; PBS, phosphate buffered saline; CT, cholera toxin B; FACS, fluorescence activated cell sorter analysis; PCR, polymerase chain reaction; FoxP3, Forkhead box protein 3.

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resistance to enteric pathogens, while preventing the induction of pro-allergic Th2 immunity. Th1–Th2 balance may be strongly influenced by the source and establishment during infancy of the gut microflora which may determine the predisposition to food allergies. Children developing food allergies within the first two years of life have fewer *bifidobacteria* and *enterococci* within the gut and more *Staphylococcus aureus* and *Clostridium*, in comparison to non-allergy developing infants and toddlers (Björkstén et al. 2001).

Studies have shown that prenatal oral *Lactobacillus* GG supplementation reduced allergy in infants/toddlers (birth to two years of age) born to mothers genetically predisposed to allergy (Kalliomäki et al. 2001), suggesting that probiotic microorganisms may play a role in early development of oral tolerance and the balance of Th1/Th2 cell function during postnatal development. Maternal transfer of commensal microorganisms (Fanaro et al. 2003), the regulatory cytokine TGF β (Saito et al. 1993), prebiotic non-digestible oligosaccharides (Newburg et al. 2005; Newburg, 2009) and immunoglobulins (Brandtzaeg 2010) in maternal milk provides the young neonate with enhanced resistance to enteric pathogens (Foye et al. 2012) while attenuating the development of allergy (Isolauri and Salminen, 2008; Lodinová-Zádníková et al. 2010). Moreover, pre-inoculation of the murine gut with probiotic *Lactobacillus acidophilus* early in life has been shown to enhance host defense against enteric pathogen, *Citrobacter rodentium*, which correlated with enhanced colonic gene expression of anti-inflammatory cytokines IL-10 and TGF β , and Th1-skewed pathogen-mediated pro-inflammatory cell signaling in pathogen exposed mice (Foye et al. 2012).

Currently, there are no proven treatment methods available to sufferers of food allergies other than dietary avoidance. However, it has been hypothesized that probiotic supplementation may prevent and/or treat type I hypersensitivity reactions to food allergens (Kim et al. 2005; Kim et al. 2008; Kuitunen et al. 2009). Nevertheless most studies using probiotic microorganisms have utilized mature mice (post-weaning) and have been mainly observational; whereas the precise cellular and molecular mechanisms within the immune system governing the effects of probiotics are not known with certainty. Therefore, this study aimed to determine if probiotic administration to the maternal diet of lactating dams with suckling pups (birth to 3 weeks of postnatal age), or postnatal (3–10 weeks of age) dietary probiotic administration will abrogate type I hypersensitivity reactions to peanut proteins, using a validated murine model to parallel human infantile responses to food allergy. We propose that maternal (nursing dams) and postnatal (3–10 weeks of age) dietary probiotic supplementation will attenuate Th2-biased phenotype, therefore providing a balanced Th1/Th2 cytokine profile and provide host protection against food allergy development.

Moreover, in contrast to most probiotic studies utilizing intra-gastric probiotic administration, we aimed to determine the effects of prolonged dietary probiotic administration on the attenuation of hypersensitivity reactions to food allergens. While the use of intra-gastric administration is the predominate method utilized in most probiotic supplement studies, we utilized a standard mouse chow diet with the inclusion of feed grade microbials (Primalac 454) prior to pelleting to overcome the challenges encountered with oral gavage of neonatal/young mice (i.e. animal stress/mortality/and morbidity due to handling and oral gavage, bi-weekly dosing of animals up to 10 weeks of age). Thus, with feed grade microbials incorporated within the diet, the mice were orally administered the probiotic microorganisms daily without undue stress for a prolonged experimental period (10 weeks) in this study. We elected to utilize Primalac 454 feed grade microbials due to the thermal stable, high viability under a variety of storage conditions after incorporation within the diet post-pelleting, and extended shelf life at room temperature (24 months) of Primalac feed grade microbial additives.

Materials and methods

Mice and reagents

Two day old C3H/HeJ mice pups and lactating dams were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in an AAALAC-accredited facility at the Center for Food Safety and Applied Nutrition, Module-1 in Laurel, Maryland. Lactating dams and suckling pups were provided bi-weekly sterile water and fed either Purina Mouse Lab Diet 5015 (Control diet) or Purina Mouse Lab Diet 5015 supplemented with Primalac 454 Feed Grade Microbials (Star-Labs/Forage Research) at 0.1% inclusion (Probiotic diet) *ad libitum* from week 1 to week 6 prior to peanut sensitization. Primalac 454 Feed Grade Microbials (Star-Labs/Forage Research) freeze-dried probiotic, was supplemented within the Purina Certified Mouse Diet 5015 prior to the pelleting at an inclusion of 0.05714 g of Primalac 454 feed grade microbials per gram of Certified Mouse Diet 5015. The probiotic-supplemented diet was formulated such that each mouse received 2.0×10^9 *L. acidophilus* microorganisms per week based upon the average daily intake for rodents (National Research Council, Nutrient Requirements for the Mouse 1995). This level of supplementation was based upon previous reports demonstrating immuno-enhancing effects of weekly *L. acidophilus* supplementation with 1.0×10^9 microorganisms using a murine model (Chen et al. 2005; Foye et al. 2012). Primalac 454 Feed Grade Microbials utilized contained a proprietary blend of *L. acidophilus*, *Lactobacillus casei*, *Bifido bacterium bifidum*, and *Enterococcus faecium*, (primarily *L. acidophilus* and *L. casei*). Peanut extract was purchased from Greer Source Materials (Lenoir, North Carolina). Cholera toxin B (CT) utilized as an adjuvant was purchased from List Biological Laboratories (Campbell, California).

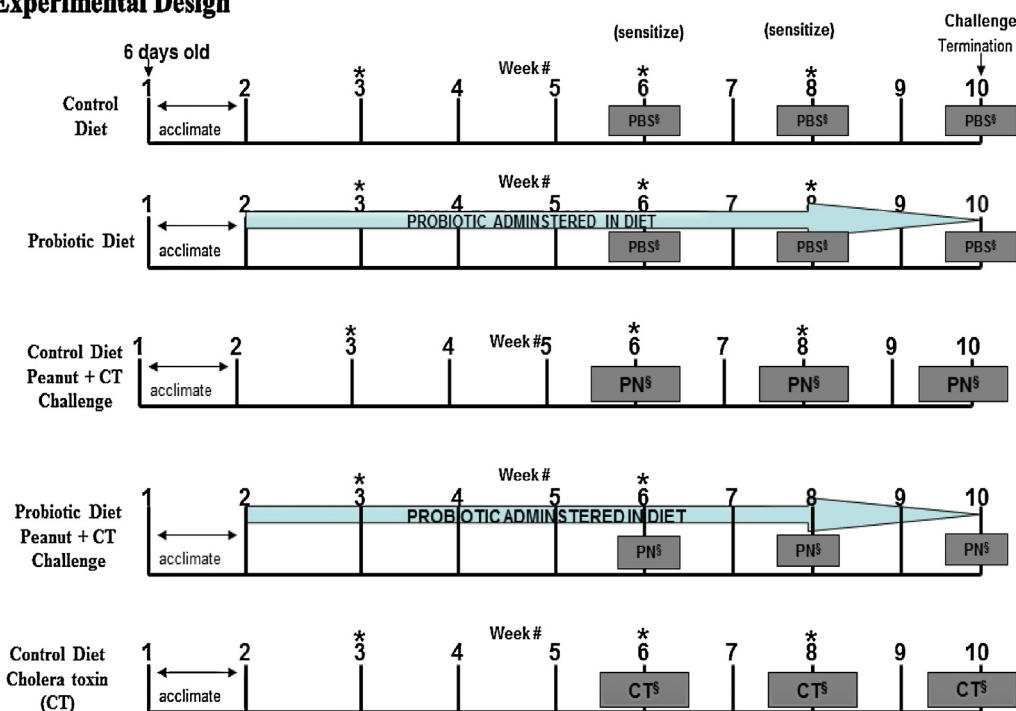
In vivo experimental design

Mice pups were housed with approximately 5–10 suckling pups per lactating dam from 2 to 21 days of postnatal age. Three independent *in vivo* experiments were conducted in which 2 day old suckling pups were divided into 5 treatment groups (Experimental Design), with each treatment group containing 10–15 suckling pups each: Control Diet-PBS challenge, Probiotic Diet-PBS challenge, Control Diet-Peanut + CT challenge, Probiotic Diet-Peanut + CT challenge, Control Diet-CT challenge. Lactating dams with suckling pups were fed experimental diets from postnatal day 2 of age until weaning. Mouse pups were weaned at 3 weeks of age and individually caged and fed aforementioned experimental diets. At 6 weeks of age, mice in treatment groups Control Diet-Peanut + CT and Probiotic Diet-Peanut + CT were orally sensitized with 1 mg of Peanut extract + 10 μ g CT in 0.2 mL of sterile phosphate buffered saline (PBS). Alternatively, mice of treatment groups Control Diet-PBS and Probiotic Diet-PBS were orally administered 0.2 mL of sterile PBS, while mice of treatment group Control Diet-CT were orally administered 10 μ g of CT in 0.2 mL of sterile PBS.

At 8 weeks of age, mice were orally challenged with 2 mg of peanut extract + 10 μ g of CT in 0.2 mL of sterile PBS. Negative and positive controls were orally administered 0.2 mL of sterile PBS (treatment group Control Diet-PBS) or 0.2 mL of sterile PBS consisting 10 μ g of CT (treatment group Control Diet-CT), respectively. After allergen, PBS or CT challenge, mice were observed twice daily for allergic symptoms. At 10 weeks of age, mice were orally challenged with 2 mg of peanut extract + 10 μ g of CT in 0.2 mL of sterile PBS, 0.2 mL of sterile PBS (treatment group Control Diet-PBS) or 0.2 mL of sterile PBS containing 10 μ g of CT (treatment group Control Diet-CT). Four hours after oral challenge, mice were sacrificed and tissues collected for immunological analysis. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility and experiments

were approved by the Institutional Animal Care and Use Committee.

Experimental Design



Experimental Design. *submandibular blood samples taken; PN=Peanut extract, PBS=phosphate buffered saline, CT=cholera toxin B. Mice were sensitized at 6 and 8 weeks of age with PBS alone, Peanut extract + CT, or CT alone. At 10 weeks of age, mice were orally challenged with PBS alone, Peanut extract +CT, or CT alone and euthanized 4 hours post oral challenge. CT was utilized as an adjuvant in PN challenge.

Fecal quantitation of *L. acidophilus*

Fecal pellets were weighed, homogenized, serially diluted with sterile phosphate buffered saline, plated on MRS agar plates and grown anaerobically at weaning, 6 and 10 weeks of age. *L. acidophilus* (LA) colonies were enumerated after overnight incubation at 37 °C and reported as colony-forming units (CFU) per gram.

Measurement of total plasma IgE levels

Plasma was obtained from blood collected from the submandibular vein using heparin as an anticoagulant at 3 weeks of age (pre-sensitization), 6 weeks of age (initial sensitization), 8 weeks of age (2nd sensitization) and 10 weeks of age (oral challenge). Total plasma IgE was determined using manufacturer's instructions for Mouse IgE ELISA Set (BD Biosciences, San Diego, CA).

Fluorescence activated cell sorter (FACS) analysis

Surface staining

Splenocytes were harvested by pressing through a nylon mesh at 10 weeks of age. Red blood cells were lysed using Red Blood Cell Lysis Buffer (Promega, Madison, Wisconsin). Lymphocyte populations were identified using PE Rat Anti-mouse CD8a, FITC Rat Anti-mouse CD4, PE Hamster Anti-mouse CD3e, APC Rat Anti-mouse CD25 and PE Rat anti-mouse FoxP3 antibodies (BD Pharmingen) and respective PE, FITC, and APC labeled isotype controls. The stained samples were analyzed on a BD Biosciences FACSscan with Cell Quest software. Dead cells and debris were excluded from analysis by gates set on forward and side angle light scatter.

Intracellular staining

Cell suspensions were activated for 3 h with Leukocyte Activation Cocktail with BD Golgi Plug (BD Pharmingen). BD Pharmingen Stain Buffer (FBS) was used to wash cells, followed by Mouse BD Fc Block purified anti-mouse CD16/CD32 mAb. Lymphocyte populations were identified using APC Rat anti-mouse CD4⁺ and PE-Cy7 Rat anti-mouse CD25⁺ labeled antibodies. PE Rat anti-mouse FoxP3⁺ labeled antibody was used for intracellular staining (BD Pharmingen, Franklin Lakes, New Jersey). Fixation and permeabilization procedures were followed as recommended by BD Pharmingen Mouse FoxP3 Fixation/Permeabilization Buffer Set. Approximately 60,000 events were collected. A sample containing unstained cells and single stained cell samples for PE CD4, APC CD4⁺ and PE-Cy7 CD25⁺ respectively, were used to establish the compensation for the analysis. Dead cells and debris were excluded from analysis by gates set on forward and side angle light scatter. Results from events within the CD4⁺ subpopulation gate were expressed as percentages of CD25⁺FoxP3⁺ from CD4⁺ parent events and analyzed using BD FACSDiva software.

PCR array analysis

Total RNA was isolated from fresh spleen sections using manufacturer's instructions described for RNeasy Mini Kit (Qiagen). A column containing DNase I digest was utilized to digest and remove contaminating genomic DNA before elution of total RNA. The concentration and A260/A280 ratio of total RNA were determined using NanoDrop 2000 (Thermo Scientific) and subsequently stored at –80 °C. First strand cDNA was generated per manufacturer's

instructions using RT² First Strand Kit (Qiagen) using 1.0 µg of total RNA. Th1 and Th2 Pathway-Focused Gene Expression profiling was conducted using a 96 well format per manufacturer's instructions and analysis for RT² Profiler™ PCR Array Mouse Th1 & Th2 Responses with diluted first strand cDNA (SA Biosciences/Qiagen).

Statistical analysis

All data (bodyweights, *L. acidophilus* fecal counts, total plasma IgE, and FACS data) were analyzed at the $p < 0.05$ significance level. In addition, total plasma IgE levels were analyzed at both $p < 0.05$ and $p < 0.10$ significance levels. All results were expressed as the mean $\pm$ standard error of the mean. Each animal served as an experimental unit for statistical analysis, and treatment effects were evaluated by age (i.e. 3 weeks, 6 weeks, 8 weeks and 10 weeks of age).

For the bodyweights, statistical differences in the means were determined using a nested ANOVA model in which three independent *in vivo* experiments were pooled and aggregated for all bodyweights in SAS (version 9.4, 2013). Nested ANOVA tests were significant at $p < 0.05$, and subsequent least squares means (model-predicted means) were compared using *t*-tests to ascertain which treatment groups had significantly different means. Treatment variables were grouped by age and significantly different treatment variables were assigned differing subscripts at $p < 0.05$ significance level.

Statistical differences between the means of total plasma IgE (ng/mL) were ascertained using a gamma log model in SAS (9.4 version, 2013) at $p \leq 0.05$ and $p \leq 0.10$ significance levels. Statistical differences in the means of fecal LA colony forming units were determined using a log Poisson model to adjust for any over dispersion present in the data set using SAS (9.4 version, 2013) at $p \leq 0.05$ significance level. Lastly, statistical differences in the means of FACS analysis data of lymphocyte populations were determined using ANOVA (SAS 9.4 version, 2013) at $p \leq 0.05$ significance level.

PCRArray statistical analysis was conducted using manufacturer's software available at: <http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>. Fold regulation was calculated using the $\Delta\Delta Ct$ method: fold change = $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta Ct = [(\Delta Ct)_{\text{experimental group}}] - [(\Delta Ct)_{\text{control group}}]$. The ΔCt for each tissue-treatment combination was calculated using: $\Delta Ct = (\text{average } Ct \text{ gene of interest} - \text{average } Ct \text{ endogenous housekeeping gene})$. Average *Ct* values represent the mean *Ct* values of data pooled for Mouse 1, Mouse 2 and Mouse 3 experiments. The *p* values were calculated based on a Student's *t*-test of the replicate $2^{(-\Delta Ct)}$ values for each gene in the control group and treatment groups, at $p \leq 0.05$ and $p \leq 0.10$ significance level.

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Results

Maternal and postnatal dietary probiotic supplementation enhances bodyweight and commensal gut colonization

During the experimental period, the clinical symptoms of anaphylaxis, body weight and survival of the animals were monitored. Mice orally sensitized (6 weeks of age) and challenged (8 and 10 weeks of age) with peanut extract did not exhibit observable clinical symptoms of hypersensitivity. The bodyweights of mice fed the probiotic diet and PBS-challenged were significantly greater than mice fed the control diet and PBS-challenged at 8 and 10 weeks of age ($p < 0.0001$, Fig. 1), while there were no significant differences

in the bodyweights between mice challenged with peanut extract + CT and fed either the control or probiotic-supplemented diet at 8 and 10 weeks of age (Fig. 1).

Upon comparisons between the PBS challenged mice, mice fed the probiotic-supplemented diet had significantly greater LA fecal counts in comparison to mice fed the control diet at 3 weeks of age ($p = 0.0074$, Fig. 2A), 6 weeks of age ($p < 0.0001$, Fig. 2B), 8 weeks of age ($p < 0.0001$, Fig. 2C), and 10 weeks of age ($p = 0.0021$, Fig. 2D). Moreover, mice challenged with peanut extract + CT and fed the probiotic-supplemented diet had significantly greater LA fecal counts in comparison to mice challenged with peanut extract + CT and fed the control diet at 3 weeks of age ($p < 0.0001$, Fig. 2A), 6 weeks of age ($p < 0.0001$, Fig. 2B), 8 weeks of age ($p = 0.0009$, Fig. 2C), and 10 weeks of age ($p < 0.0001$, Fig. 2D). Interestingly, mice fed the probiotic-supplemented diet and challenged with peanut extract + CT had significantly greater LA fecal counts than all other treatment groups at 3 weeks of age (Fig. 2A), 8 weeks of age (Fig. 2C), and 10 weeks of age (Fig. 2D) at $p < 0.05$.

Elevation of total plasma IgE levels are often used as an indicator of clinical allergy, therefore in this study we aimed to use this as an indices to hypersensitivity responses to allergenic peanut proteins. Total plasma IgE levels were determined over the course of 10 weeks (3, 6, 8 and 10 weeks of age). Total plasma IgE levels were significantly enhanced in peanut extract + CT challenged mice fed the probiotic-supplemented in comparison to mice fed the control diet and peanut extract + CT challenged at 3 weeks of age ($p = 0.0101$, Fig. 3A). Also, mice fed the probiotic diet and PBS challenged had significantly greater total plasma IgE levels than all other treatment groups with the exception of control diet-PBS challenged treatment group at 3 weeks of age ($p < 0.05$, Fig. 3A). However, there were no significant differences in total plasma IgE levels between treatment groups at any of the remaining time points measured (6 weeks of age, Fig. 3B; 8 weeks of age, Fig. 3C; 10 weeks of age, Fig. 3D) at $p < 0.05$ or $p < 0.10$ significance levels.

Maternal and postnatal dietary probiotic supplementation alters splenic T cell populations and function

The effect of probiotic supplementation and sensitization to peanut proteins on splenic lymphocyte populations was determined using surface and intracellular cell staining procedures and fluorescence activated cell sorter analysis (FACS). We utilized surface staining of CD3⁺ glycoprotein to identify the total T cell population of treatment groups. Mice fed the probiotic-supplemented diet and challenged with peanut extract + CT had a significantly larger splenic total T cell population in comparison to all other treatment groups ($p = 0.0140$, Table 1). T helper cells are key players of adaptive immunity and critical to almost all adaptive immunological responses. Hence, we used CD3⁺CD4⁺ surface staining procedures and FACS analysis to identify this T helper cell sub-population. Mice fed the probiotic-supplemented diet and sensitized with peanut extract + CT had significantly more splenic CD3⁺CD4⁺ lymphocytes in comparison to mice fed the control diet and sensitized with peanut extract + CT ($p = 0.0176$, Table 1), while mice fed the probiotic-supplemented diet and sensitized with PBS had significantly more CD3⁺CD4⁺ lymphocytes in comparison to mice fed the control diet and sensitized with PBS ($p < 0.0001$, Table 1). Moreover, mice fed the probiotic-supplemented diet and sensitized with PBS had significantly more splenic CD3⁺CD4⁺ lymphocytes in comparison to all other treatment groups ($p < 0.05$, Table 1). CD8⁺ glycoprotein cell surface staining procedures were utilized to identify and quantify splenic cytotoxic T cells and natural killer cells. Mice fed the probiotic diet and challenged with PBS, had a significantly greater splenic lymphocyte CD3⁺CD8⁺ population in comparison to mice of all other treatment groups ($p < 0.05$, Table 1), while mice fed the control diet and challenged with CT had a

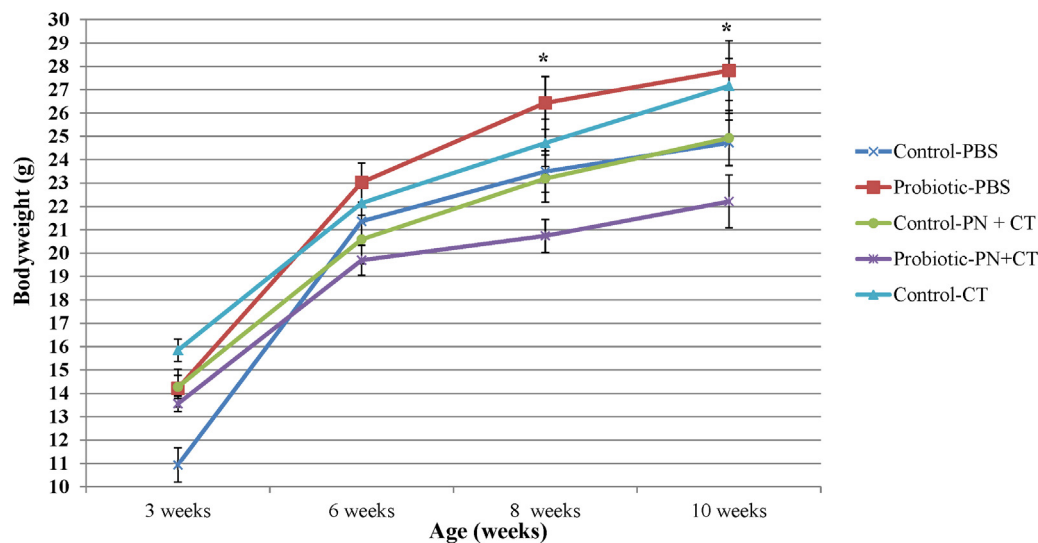


Fig. 1. Maternal and postnatal dietary probiotic supplementation enhances bodyweight in treated mice. All data represent the mean value $\pm$ standard error of data pooled for 3 independent experiments. *Bodyweights probiotic diet and PBS-challenged mice were significantly greater than mice fed the control diet and PBS-challenged at 8 and 10 weeks of age ($p < 0.0001$). There were no treatment differences between peanut challenged treatment groups (probiotic diet versus control diet).

significantly smaller CD3⁺CD8⁺ splenic lymphocyte population compared to all other treatment groups ($p < 0.0001$, Table 1).

We utilized CD4⁺CD25⁺ surface staining to identify and quantify this subtype of T cells, which serves as a biomarker for regulatory or suppressor cells (Lan et al. 2012). Mice fed the

probiotic-supplemented diet and sensitized with PBS had a significantly enhanced CD4⁺CD25⁺ splenic lymphocyte population in comparison to mice fed the control diet and sensitized with PBS ($p < 0.0001$, Table 1). In parallel, mice fed the probiotic-supplemented diet and sensitized with peanut extract + CT had a

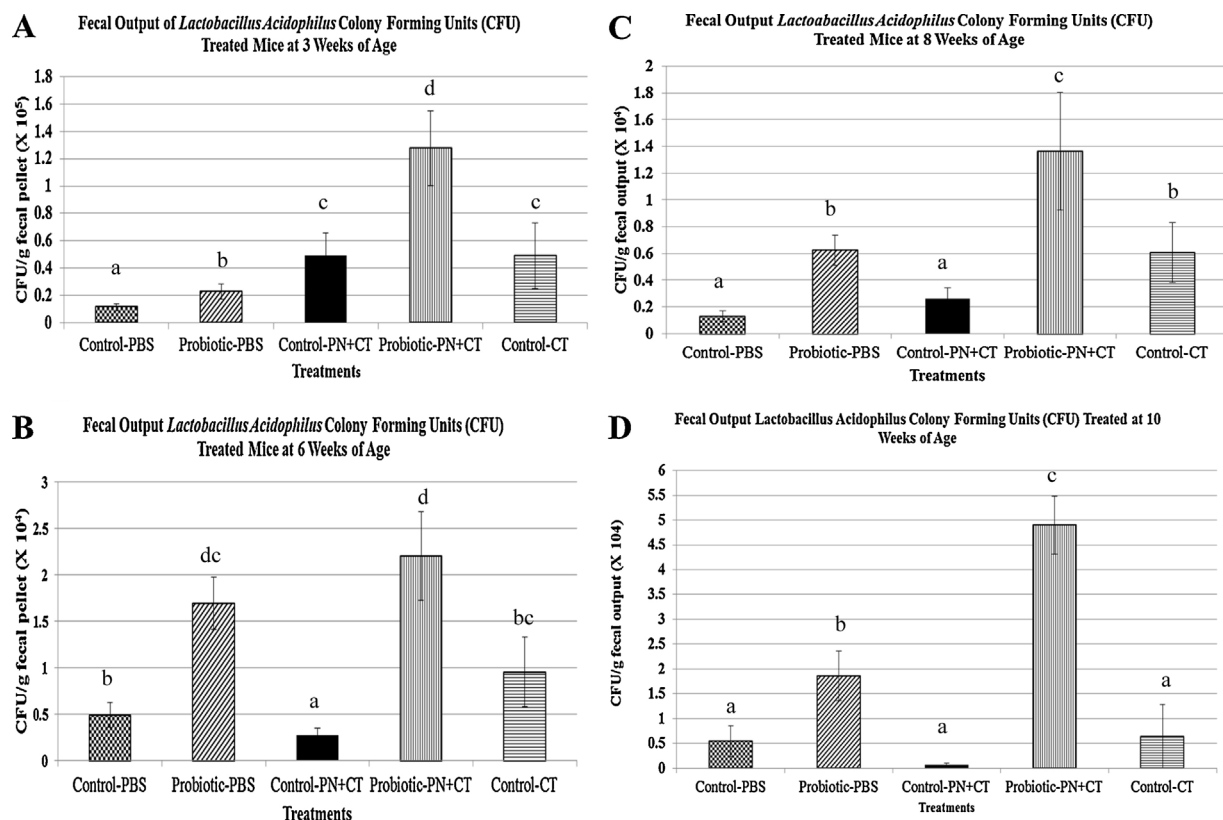


Fig. 2. Maternal and postnatal dietary probiotic supplementation enhances *Lactobacillus acidophilus* fecal colonization in treated mice. All data represent the mean value $\pm$ standard error of data pooled for 3 independent experiments, and treatment effects were evaluated by age (i.e. 3 weeks, 6 weeks, 8 weeks and 10 weeks of age). Fecal Output of *Lactobacillus acidophilus* (LA) (A) 3 weeks of age, (B) 6 weeks of age, (C) 8 weeks of age and (D) 10 weeks of age. ^{a,b,c,d} Statistical differences were determined using a nested ANOVA model in SAS (version 9.4, 2013). Nested ANOVA tests were significant at $p < 0.05$, and subsequently treatments were separated by least squares means (t -test) for comparisons between each treatment group. At each age, bar graphs with differing superscripts were significantly different ($p < 0.05$).

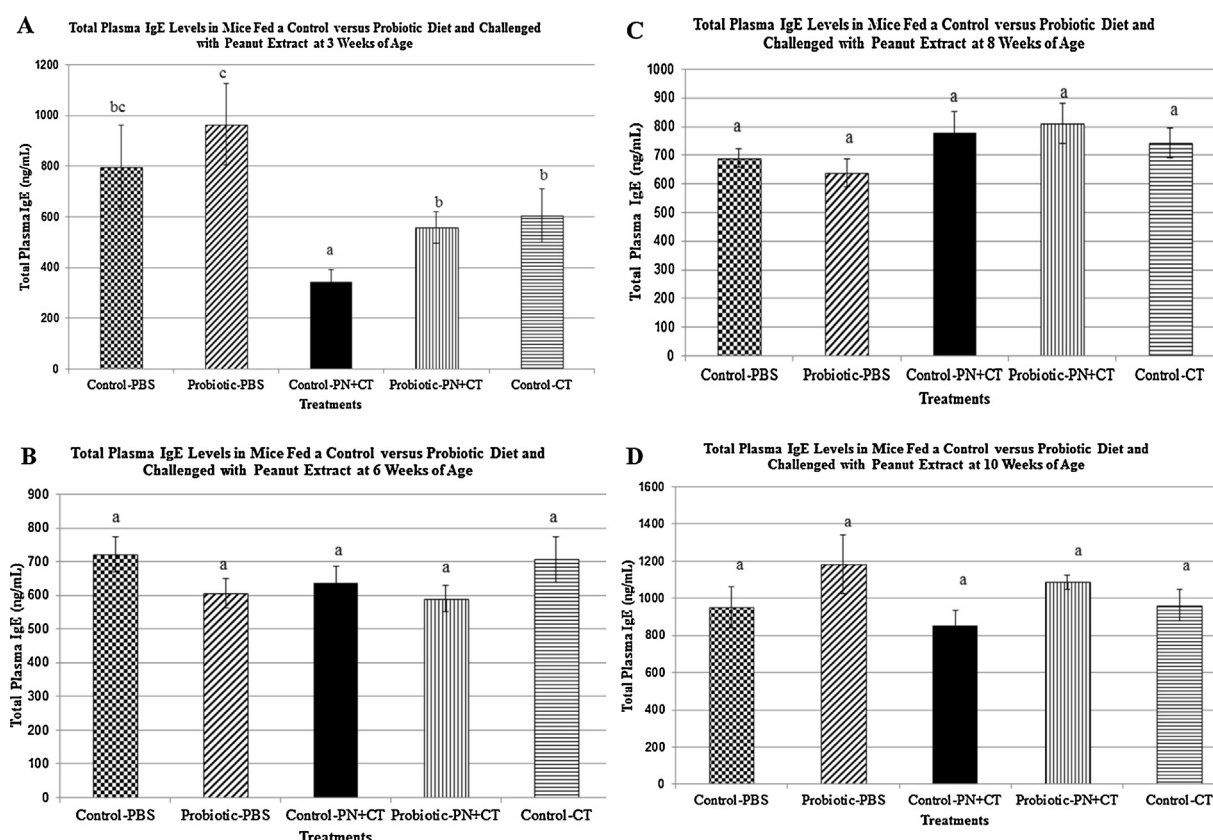


Fig. 3. Maternal and postnatal dietary probiotic supplementation enhances early total IgE plasma levels in peanut sensitized mice. All data represent the mean value $\pm$ standard error of data pooled for 3 independent experiments and treatment effects were evaluated by age (A) 3 weeks of age, (B) 6 weeks of age, (C) 8 weeks of age and (D) 10 weeks of age). ^{a,b,c,d}Statistical differences were determined using a nested ANOVA model in SAS (version 9.4, 2013). Nested ANOVA tests were significant at $p < 0.05$, and subsequently treatments were separated by least squares means (t -test) for comparisons between each treatment group. Bar graphs with differing superscripts were significantly different at $p < 0.05$ level.

significantly enhanced CD4⁺CD25⁺ splenic lymphocyte population in comparison to mice fed the control diet and sensitized with peanut extract + CT ($p = 0.0076$, Table 1).

Nevertheless, because CD25⁺ is an activation marker for lymphocytes the utility of CD25 surface expression as cell population biomarker for T regulatory cells is limited since it does not discriminate between activated T effector cells and regulatory T cells. Hence, in our final *in vivo* mouse experiment we aimed to more specifically identify naturally occurring and adaptive T regulatory cell populations, using intracellular FoxP3⁺ and CD4⁺CD25⁺ and CD4⁺CD25⁻ surface staining procedures, respectively (Lan et al. 2012; Su et al. 2012; Fontenot et al. 2003). There were no significant differences in the induced splenic T regulatory cell population (CD4⁺FoxP3⁺

positive) between mice challenged with peanut + CT fed the control diet or probiotic-supplemented diet (Fig. 4A). However, mice fed the probiotic-supplemented diet and challenged with peanut extract + CT had a significantly enhanced (~4-fold) splenic naturally occurring T regulatory cells (CD4⁺CD25⁺FoxP3⁺ positive) in comparison to the corresponding control group and all other treatment groups ($p = 0.0067$, Fig. 4B).

Maternal and postnatal dietary probiotic supplementation alters Th1/Th2/Th3 associated splenic gene expression

Our final objective was to determine the effect of maternal and postnatal dietary probiotic supplementation on splenic expression

Table 1
Maternal and postnatal dietary probiotic supplementation enhances splenic T cell populations in peanut sensitized mice.¹

	Dietary treatments ²				
	Control diet-PBS	Probiotic diet-PBS	Control diet-PN + CT	Probiotic diet-PN + CT	Control diet-CT
Lymphocytes	% Marker-positive of parent population				
CD3 ⁺	59.02 $\pm$ 2.13 ^a	62.51 $\pm$ 1.2 ^a	61.11 $\pm$ 5.37 ^a	76.28 $\pm$ 2.92 ^b	57.62 $\pm$ 5.93 ^a
CD3 ⁺ CD4 ⁺	28.29 $\pm$ 0.84 ^a	51.59 $\pm$ 2.4 ^c	27.80 $\pm$ 2.93 ^a	35.58 $\pm$ 1.67 ^b	24.25 $\pm$ 1.90 ^a
CD3 ⁺ CD8 ⁺	14.91 $\pm$ 0.34 ^b	17.2 $\pm$ 0.71 ^c	12.19 $\pm$ 1.13 ^b	13.98 $\pm$ 0.78 ^b	8.27 $\pm$ 1.52 ^a
CD4 ⁺ CD25 ⁺	1.83 $\pm$ 0.15 ^a	3.81 $\pm$ 0.34 ^b	3.90 $\pm$ 0.46 ^b	5.31 $\pm$ 0.28 ^c	5.03 $\pm$ 0.53 ^c

Splenic T cell lymphocyte populations were determined using surface cell staining procedures and fluorescence activated cell sorter analysis.

^{a,b,c,d,e} Statistical differences were determined using a nested ANOVA model in SAS (version 9.4, 2013). Nested ANOVA tests were significant at $p < 0.05$, and subsequently treatments were separated by least squares means (t -test) for comparisons between each treatment group.

¹ All data represent the mean value $\pm$ standard error of data pooled for 3 independent experiments of 45–60 animals per treatment.

² Dietary treatments = lactating dams and weaned pups were fed a control mouse chow or probiotic-supplemented mouse chow diet for 10 weeks (2days to 10 weeks of age), and sensitized (6 weeks of age) with 0.2 mL of sterile PBS, or 10 μ g of cholera toxin (CT) in 0.2 mL of PBS, or 1 mg of peanut extract (PN) + 10 μ g CT in 0.2 mL of PBS. At 8 and 10 weeks of age, mice were orally challenged with 0.2 mL of sterile PBS, or 10 μ g CT in 0.2 mL of PBS, or 2 mg of PN + 10 μ g of CT in 0.2 mL of PBS.

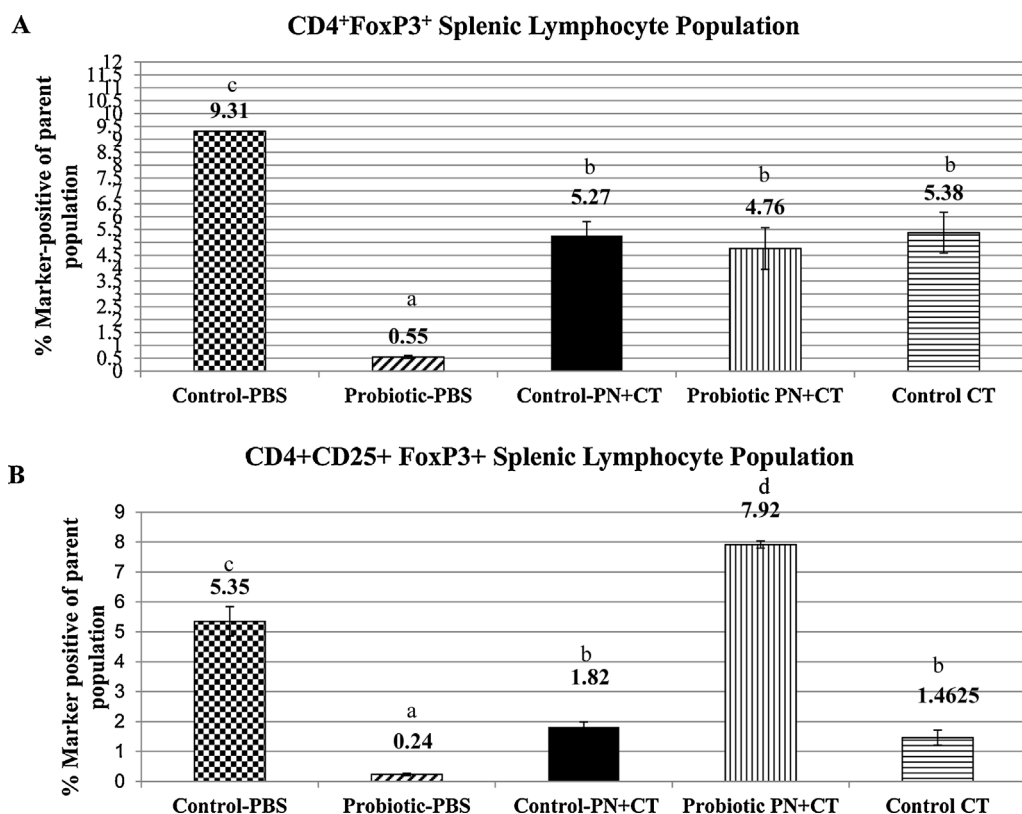


Fig. 4. Maternal and postnatal dietary probiotic supplementation and peanut challenge enhances naturally-occurring splenic T regulatory cell populations in peanut sensitized mice. Splenic T cell lymphocyte populations were determined using surface and intracellular cell staining procedures and fluorescence activate cell sorter analysis. All data represent the mean value $\pm$ standard error. ^{a,b,c,d}Statistical differences were determined using a nested ANOVA model in SAS (version 9.4, 2013). Nested ANOVA tests were significant at $p < 0.05$, and subsequently treatments were separated by least squares means (t -test) for comparisons between each treatment group. Bar graphs with differing superscripts are significantly different ($p < 0.01$).

of Th1, Th2 and Th17 associated gene expression utilizing the Mouse Th1 & Th2 RT² Profiler PCR Array profiling the expression of 84 genes involved in T helper cell immune responses using real-time PCR. Splenic gene expression of chemokine CCL11 and cytokine IL-13 (mediators of allergic inflammatory responses) were down-regulated by approximately 4- and 7-fold, respectively in mice fed the probiotic-supplemented diet and challenged with peanut + CT relative to gene expression in mice fed the control diet and PBS challenged (Table 2, $p \leq 0.001$).

While there was a significant reduction in splenic gene expression of Th2-associated cytokines IL-6 and IL-9 in mice of the following treatment groups: Probiotic Diet-PBS, Control Diet-Peanut + CT, Probiotic Diet-Peanut + CT and Control Diet-CT relative to control mice (Control Diet-PBS), mice fed the probiotic-supplemented diet and challenged with peanut + CT had a 19-fold reduction in IL-6 splenic gene expression relative to the controls ($p < 0.05$, Table 2). Among the Th1-associated cytokines analyzed (IFN- γ , IL-27 and TNF- α) only TNF- α splenic expression

Table 2

Maternal and postnatal dietary probiotic supplementation alters Th1/Th2/Th17-associated splenic gene expression.¹

Gene	Probiotic diet-PBS	Control diet-PN + CT	Probiotic diet-PN + CT	Control diet-CT
	Fold regulation comparing to control diet-PBS (p -value) ²			
TGF β	$\downarrow 1.174(0.941)$	$\downarrow 1.674(0.161)$	$\downarrow 1.700(0.037)$	$\downarrow 1.344(0.298)$
IL-4	$\downarrow 1.234(0.111)$	$\downarrow 1.412(0.890)$	$\downarrow 1.185(0.681)$	$\downarrow 1.739(0.620)$
IL-17	$\downarrow 2.237(0.010)$	$\downarrow 3.547(0.001)$	$\downarrow 3.475(0.0003)$	$\downarrow 3.283(0.922)$
IFN γ	$\downarrow 1.181(0.431)$	$\downarrow 2.618(0.178)$	$\downarrow 2.122(0.186)$	$\downarrow 2.404(0.384)$
TNF α	$\downarrow 2.704(0.007)$	$\downarrow 3.252(0.008)$	$\downarrow 4.949(0.001)$	$\downarrow 2.780(0.440)$
IL-27	$\downarrow 1.236(0.667)$	$\downarrow 1.791(0.230)$	$\downarrow 1.435(0.200)$	$\downarrow 1.095(0.603)$
CCL11	$\downarrow 1.383(0.819)$	$\downarrow 3.270(0.031)$	$\downarrow 4.406(0.001)$	$\downarrow 3.176(0.026)$
CCL7	$\downarrow 6.451(0.016)$	$\downarrow 10.300(0.005)$	$\downarrow 21.965(<0.001)$	$\downarrow 14.952(0.008)$
IL-13	$\downarrow 2.705(0.018)$	$\downarrow 5.451(0.004)$	$\downarrow 6.886(<0.001)$	$\downarrow 8.283(0.014)$
IL-6	$\downarrow 4.634(0.008)$	$\downarrow 9.011(0.001)$	$\downarrow 18.668(<0.001)$	$\downarrow 13.349(0.006)$
IL-7	$\downarrow 2.237(0.010)$	$\downarrow 3.547(0.001)$	$\downarrow 3.475(<0.001)$	$\downarrow 3.283(0.922)$
IL-7R	$\downarrow 3.494(0.010)$	$\downarrow 3.858(0.005)$	$\downarrow 5.830(<0.001)$	$\downarrow 6.815(0.009)$
IL-9	$\downarrow 2.793(0.010)$	$\downarrow 5.986(0.005)$	$\downarrow 10.546(<0.001)$	$\downarrow 11.266(0.010)$

Splenic gene expression was determined using total RNA isolated from fresh spleen sections. First strand cDNA was generated and Th1/Th2/Th17 pathway-focused gene expression profiling was conducted using RT² ProfilerTM PCR Array. The ΔCt for each tissue-treatment combination was calculated using: $\Delta Ct = (\text{average } Ct \text{ gene of interest} - \text{average } Ct \text{ endogenous housekeeping gene})$. Average Ct values represent the mean Ct values of data pooled for 3 independent experiments. p -Values < 0.05 were significant.

¹ All data represent pooled data for 3 independent *in vivo* experiments.

² Fold regulation was calculated using the $\Delta\Delta Ct$ method: fold change = $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = [(\Delta Ct)_{\text{experimental group}}] - [(\Delta Ct)_{\text{control group}}]$.

levels were significantly reduced in mice of treatments: Probiotic Diet-PBS, Control Diet-Peanut+CT, and Probiotic-Diet-Peanut+CT relative to mice of the control group ($p < 0.05$, Table 2). Most interestingly, this effect was more pronounced in mice fed the probiotic-supplemented diet and challenged with peanut+CT with a 5-fold reduction in TNF- α splenic gene expression relative to mice of the control group (Control Diet-PBS).

Splenic gene expression of cytokine IL-17 (pro-inflammatory cytokine secreted by associated with the Th17 subpopulation of T helper cells) and IL-7 were significantly down-regulated in mice of treatment groups Probiotic Diet-PBS, Control Diet-Peanut+CT, and Probiotic Diet-Peanut+CT relative to mice of the control group, Control Diet-PBS ($p < 0.05$, Table 2). While there was significant reduction in splenic gene expression of CCL7 in mice of treatments Probiotic Diet-PBS, Control Diet-Peanut+CT, and Probiotic Diet-Peanut+CT, mice fed the probiotic-supplemented diet and challenged with peanut+CT had a 22-fold reduction in splenic gene expression of chemokine CCL7 relative to control mice, Control Diet-PBS ($p < 0.05$, Table 2).

Discussion

The neonatal and early childhood periods are critical for the establishment of Th1/Th2 cellular immunity. Differentiation of T helper cells within the Th1 lineage promotes cell-mediated immunity, and secretion of cytokines IFN- γ , TNF- α , IL-12, IL-18, and IL-27, whereas Th2 cells promote IgE-mediated humoral immunity and secretion of cytokines, IL-4, IL-9, IL-13, IL-5, and IL-6. Excessive Th1 polarization enhances inflammatory responses and risk of autoimmunity, while Th2 polarization enhances predisposition to food allergies. The induction of pro-allergic Th2 immunity may be influenced by the source and establishment during infancy of the gut microflora. Therapeutic probiotic supplementation has been shown to balance the Th1/Th2 cytokine profile (Kidd 2003) during early development and to enhance host protection from disease development (Foye et al. 2012) and oral tolerance (Moussu et al. 2012). Therefore, in this study we hypothesized that maternal (nursing dams with suckling pups) and postnatal (3–10 weeks of age) dietary probiotic supplementation will attenuate a Th2-biased phenotype, therefore providing a more balanced Th1/Th2 cytokine profile and provide host protection against food allergen sensitization.

Studies using C3H/HeJ mice have reported numerous benefits of therapeutic probiotic treatment to prevent food allergy to the shellfish allergen tropomyosin, (Schiavi et al. 2011), egg allergen ovalbumin (Kim et al. 2005, 2008) and milk allergen β -Lactoglobulin (Thang et al. 2013). However, most of these studies have explored the immunological effects of acute probiotic administration (bi-weekly oral gavage) using weaned animals. In contrast, the current study aimed to address the immunological effects of early and prolonged probiotic administration (suckling to 10 weeks of age) on peanut sensitized and challenged mice by feeding lactating dams with suckling pups a probiotic-supplemented diet daily for the maternal transfer of commensal microorganisms prior to weaning (postnatal 2–21 days of age) through 10 weeks of age postnatally. Moreover, we aimed to minimize distress that might be experienced with oral gavage procedures by incorporating probiotic microorganisms into the diet and therefore providing probiotic supplementation daily over an extended experimental timeline.

Studies with probiotic supplementation of the maternal diet have demonstrated that maternally derived commensal microorganisms, which colonize the intestine of vaginally delivered and breast-fed infants and persist up to 2 years of age (Tannock et al. 1990) may have protective effects against allergy development (Kim et al. 2010; Abrahamsson et al. 2007; Kalliomäki et al. 2001;

Wickens et al. 2008). In this study we demonstrate that at weaning, pups suckled by dams fed the probiotic-supplemented diet had enhanced fecal counts of *L. acidophilus* (predominate probiotic strain) relative to treatment controls, suggesting the maternal transfer of the probiotic microorganisms to suckling pups during early development. However, in our study, we could not ascertain maternal transfer of commensals with certainty due to the limitations of sampling imposed by suckling pups co-housed with lactating dams, which prevent microbiological analysis of maternal milk and excreta. Nonetheless, after weaning, enhanced *L. acidophilus* fecal counts were maintained in these offspring up to 10 weeks of age in the probiotic-supplemented treatment groups (Probiotic Diet-PBS, Probiotic Diet-Peanut+CT) relative to treatment controls.

T regulatory cells are a component of the immune system that suppresses immune responses critically needed for immunological tolerance of innocuous antigens and preventing autoimmunity (Lan et al. 2012). “Naturally-occurring” T regulatory cells arise within the thymus and have intracellular molecule, transcription factor forkhead box protein 3 (FoxP3⁺) and surface glycoproteins CD4⁺CD25⁺ (Su et al. 2012; Lan et al. 2012). Induced T regulatory cells develop outside the thymus after antigen presentation and activation under a variety of conditions (non-inflammatory, inflammatory, infections) distinguishable as CD4⁺CD25⁺FoxP3⁺ (Su et al. 2012; Lan et al. 2012). In this study we demonstrate that maternal and post-weaning probiotic dietary supplementation provide immunological enhancement by enhancing activated T helper cell, cytotoxic T cell and/or natural killer cell and naturally-occurring T regulatory cell populations which may attenuate and/or regulate hypersensitivity responses to food allergens. Our results are supported by previous reports demonstrating that acute probiotic supplementation enhances activated Th17 helper (Donkor et al. 2012; Kobayashi et al., 2012) and T regulatory cell populations (Toomer et al. 2014; Lavasani et al. 2010; Schiavi et al. 2011) and secretion and/or expression of regulatory cytokine TGF β (Chen et al. 2005; Schiavi et al. 2011; Foye et al. 2012). Induced regulatory T cells have been shown to inhibit macrophage function *in vitro* and to attenuate established macrophage-driven skin contact hypersensitivity after *in vivo* transfer via TGF- β mediated mechanisms (Su et al. 2012).

In contrast to other probiotic feeding studies demonstrating a reduction in total plasma IgE levels in allergen sensitized subjects, we demonstrate that probiotic dietary supplementation does not reduce total plasma IgE levels in peanut challenged mice. Kim et al. (2010) reported that prenatal and postnatal supplementation with a mixture of probiotic strains reduced the incidence of offspring atopic eczema during the first year, while there were no differences in serum total IgE and food allergen specific IgE between probiotic-supplemented and placebo groups in infants at high risk for allergy development. While total plasma IgE levels are often utilized as a potential predictor of allergic sensitization in animal (Thang et al. 2013) and human studies (Satwani et al. 2009) measurement of total plasma IgE levels have been shown to be of questionable diagnostic value, due to the potential of numerous mitogenic factors (virus, bacteria, parasites, environmental adjuvants), which may enhance the production of total plasma IgE levels not associated with allergen-specific IgE sensitization. Therefore, it is our aim in all future probiotic feeding studies to analyze peanut-specific plasma IgE levels in addition to total plasma IgE levels to more accurately access hypersensitivity responses to peanut allergens.

The overall objective of this study was to determine the effect of maternal and postnatal probiotic dietary supplementation (2 days of age to 10 weeks of age) on gut *L. acidophilus* colonization, total plasma IgE levels, the dynamics of the T helper cell splenic population, and splenic expression profile of Th1/Th2/Th17 associated genes using a validated mouse model to parallel hypersensitivity

reactions to peanut in humans. In summary, our data demonstrate that probiotic dietary supplementation enhances gut colonization of the predominate commensal microbe, *L. acidophilus*, and enhancement of regulatory T helper and cytotoxic/natural killer T cell populations in mice challenged with peanut allergens.

Moreover, this study suggest that early probiotic dietary supplementation may attenuate food allergen hypersensitivity reactions by the reduction in pro-inflammatory cytokines (TNF- α , IL-17) and immunological mediators of allergy (IL-13 and CCL11), as indicated by reduced splenic gene expression.

In summary, our data parallel the findings of other reports utilizing oral probiotic supplementation protocols, suggesting that prolonged probiotic supplementation (2 days of age to 10 weeks of age) in the diet may provide additive immunological benefits by preventing host development of hypersensitivity reactions to food allergens. While the majority of probiotic microorganisms have not been shown to cause illness, additional research is needed to determine the safety, dose responses and efficacy of probiotic supplementation especially in high risk populations, such as infants and young children. Nevertheless, numerous studies imply that probiotic supplementation may serve as an effective therapeutic strategy in the prevention and treatment of food allergies. In conclusion, these results suggest that the probiotic strains present in the feed grade microbials utilized in this study may be good candidates for clinical research in food allergy.

Conflict of interest

None declared.

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