



Maternal and postnatal dietary probiotic supplementation enhances splenic regulatory T helper cell population and reduces ovalbumin 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 egg allergen ovalbumin, 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 ovalbumin-IgE and total plasma IgE levels. At termination, splenic T helper cell lymphocyte population subtypes were determined using FACS analysis and Th1/Th2/Th17 gene expression by PCR array. At 21 days of age, pups suckled by lactating dams fed the probiotic supplemented diet had significantly enhanced *Lactobacillus acidophilus* fecal counts compared to controls. Moreover, mice fed the probiotic supplemented diet had enhanced splenic naturally occurring and induced regulatory T cell populations, enhanced TGFβ gene expression and reduced expression of allergic mediator IL13 compared to controls. These results provide evidence that early probiotic supplementation may provide host protection from 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, IFNγ or Th2 cytokine IL-4. This reduction is more notable for IFNγ 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 of food allergy, T cell responses promoting B cell class switching to IgE occurs with Th2 lymphocytes secreting IL-4.

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 resistance to enteric pathogens, while preventing the induction of pro-allergic Th2 immunity. Th1–Th2 balance may be strongly

Abbreviations: OVA, ovalbumin; 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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influenced during infancy by the source and establishment 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 otherwise healthy infants and toddlers (Björkstén et al., 2001).

Studies have shown that prenatal oral *Lactobacillus* GG supplementation reduced allergy in infants/toddlers born to mothers genetically predisposed to allergy (atopic eczema, allergic rhinitis, asthma) during the first two years of life (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) 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 (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 the 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, 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 early (pre-weaning) administration of probiotic microorganisms orally to lactating dams with suckling pups (2–21 days of age), and during postnatal development (3–10 weeks of age) will abrogate type I hypersensitivity reactions to the food allergen ovalbumin, 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

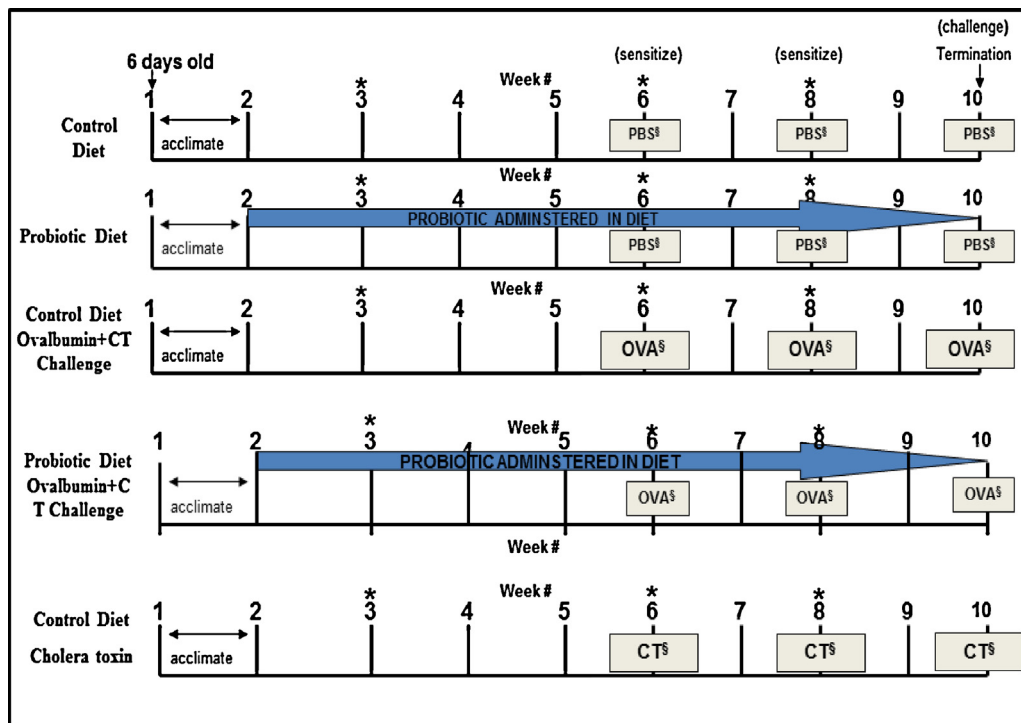
Mice and reagents

To test this hypothesis, 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 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 ovalbumin 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, such that each mouse received 2.0×10^9 *L. acidophilus* microorganisms per week. 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*). Ovalbumin was purchased from Greer Source Materials (Lenoir, NC). Cholera toxin B (CT) utilized as an adjuvant was purchased from List Biological Laboratories (Campbell, CA).

In vivo experimental design

Animals were housed with approximately 5–10 suckling pups per lactating dam from 2 to 21 days of age. Three independent experiments were conducted in which 2-day-old suckling pups were divided into 5 treatment groups, with each treatment group containing 10–15 suckling pups each: Control Diet-PBS challenge, Probiotic Diet-PBS challenge, Control Diet-Ovalbumin + CT challenge, Probiotic Diet-Ovalbumin + CT challenge, Control Diet-CT challenge. Lactating dams with suckling pups were fed experimental diets from 6 days 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-Ovalbumin + CT and Probiotic Diet-Ovalbumin + CT were orally sensitized with 50 μ g of Ovalbumin + 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 100 μ g of Ovalbumin + 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 100 μ g of Ovalbumin + 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 at 10 weeks of age, mice were sacrificed and tissues collected for immunological analysis. All animal experiments were approved by the Institutional Animal Care and Use Committee.



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* colonies were enumerated after overnight incubation at 37 °C and reported as colony-forming units (CFU) per gram.

Measurement of total plasma IgE and ovalbumin-IgE levels

Plasma was obtained from blood collected from the sub-mandibular 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 and ovalbumin-specific IgE was determined using manufacturer's instructions for mouse IgE ELISA Set (BD Biosciences, San Diego, CA) and mouse ovalbumin-specific IgE ELISA assay kit (AbD Serotec, Raleigh, NC).

Fluorescence activated cell sorter (FACS) analysis

Surface staining

The mice were sacrificed at 10 weeks of age and splenocytes were removed and harvested by pressing through a nylon mesh. Red blood cells were lysed using Red Blood Cell Lysis Buffer [Promega (Madison, WI)]. 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, Franklin Lakes, NJ) with PE, FITC, and APC labeled isotype controls, respectively. 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 stain

Cell suspensions were activated for 3 h with Leukocyte Activation Cocktail with BD Golgi Plug (BD Pharmingen, Franklin Lakes,

NJ). BD Pharmingen Stain Buffer (FBS) was used to wash cells, followed by Mouse BD Fc Block purified anti-mouse CD16/CD32 mAb to block cells from binding to non-specific antigens. 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, NJ). 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, and expressed as percentages of CD25⁺Foxp3⁺ from CD4⁺ events. The stained samples were 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, Valencia, CA). 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, Wilmington, DE) and the RNA was subsequently stored at –80 °C. First strand cDNA was generated per manufacturer's instructions RT² First Strand Kit (Qiagen, Valencia, CA) 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, Frederick, MD).

Statistical analysis

All data (bodyweights, *L. acidophilus* fecal counts, total plasma IgE, ovalbumin-specific plasma IgE, and FACS data) were analyzed at the $p < 0.05$ significance level. In addition, total plasma IgE levels and plasma ovalbumin-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 *in vivo* experiments were pooled (3 experiments: Mouse 1, Mouse 2, Mouse 3) and were 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 grouped by age, with differing subscripts were significantly different at $p < 0.05$.

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 *L. acidophilus* 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 plasma ovalbumin-IgE (ng/mL) and FACS analysis data of lymphocyte populations were determined using ANOVA model (SAS 9.4 version, 2013) at $p \leq 0.05$ and $p \leq 0.10$ significance levels for plasma ovalbumin-IgE, and at $p \leq 0.05$ significance level for FACS analysis data.

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

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 egg allergen ovalbumin 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). Interestingly, the body weights of both ovalbumin challenged treatment groups (fed control diet or probiotic supplemented diet) were significantly smaller than mice in the three control groups: Control-PBS, Probiotic-PBS, and Control-CT at 8 ($p = 0.0037$) and 10 ($p = 0.0012$) weeks of age (Fig. 1).

At weaning (3 weeks of age), mice pups of lactating dams fed the probiotic supplemented diets Probiotic-PBS and Probiotic-OVA + CT had significantly greater *L. acidophilus* fecal counts than

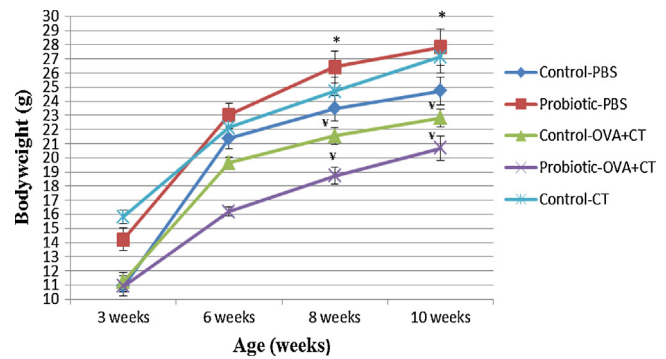


Fig. 1. Maternal and postnatal dietary probiotic supplementation enhances bodyweights in control mice. All data represent the mean value $\pm$ standard error of data pooled for 3 independent experiments. *Bodyweights probiotic diet and PBS-challenged mice were significantly greater than mice fed the control diet and PBS-challenged at 8 and 10 weeks of age ($p < 0.0001$). ^vBody weights of ovalbumin challenged treatment groups (control or probiotic supplemented diet) were significantly smaller than mice in the three control groups: Control-PBS, Probiotic-PBS, and Control-CT ($p \leq 0.01$).

mice pups of lactating dams fed the control diets Control-PBS and Control-OVA + CT, respectively ($p < 0.0001$, Fig. 2A). After 6 weeks of probiotic supplementation, mice fed the probiotic diet, Probiotic Diet-PBS and Probiotic Diet-Ovalbumin had significantly greater *L. acidophilus* fecal counts in comparison to all other treatment groups ($p < 0.05$, Fig. 2B). At 8 weeks of age ($p < 0.0001$, Fig. 2C) and 10 weeks of age ($p = 0.0416$, Fig. 2D), mice fed the probiotic supplemented diet and challenged with ovalbumin, had significantly greater *L. acidophilus* fecal output in comparison to all other treatment groups. By 10 weeks of age, fecal colonization of *L. acidophilus* in the probiotic fed groups was greater than the control groups, however only *L. acidophilus* fecal counts in Probiotic Diet-Ovalbumin group were significantly greater than the other treatment groups ($p = 0.0416$, Fig. 2D).

Maternal and postnatal dietary probiotic supplementation enhances splenic T cell population and function

The effect of probiotic supplementation and ovalbumin sensitization on splenic lymphocyte populations was determined using surface and intracellular cell staining procedures and fluorescence activated cell sorter (FACS) analysis. We utilized surface staining of CD3⁺ glycoprotein to identify the total T cell population of treatment groups. There were no significant differences in the total T cell population between any of the treatment groups (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 orally supplemented with probiotic and/or sensitized with ovalbumin (Probiotic Diet-PBS, Control Diet-Ovalbumin, Probiotic Diet-Ovalbumin) had significantly greater splenic CD3⁺CD4⁺ lymphocyte populations in comparison to mice of control groups Control Diet-PBS and Control Diet-CT ($p < 0.0001$, 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 control diet and challenged with CT, had a significantly reduced splenic lymphocyte CD3⁺CD8⁺ population in comparison to mice of treatment groups Control Diet-PBS, Probiotic Diet-PBS, Control Diet-Ovalbumin, and Probiotic Diet-Ovalbumin ($p < 0.0001$, Table 1).

We utilized CD4⁺CD25⁺ surface staining to identify and quantitate this subtype of T cells, with CD4⁺CD25⁺ as a cell population biomarker for regulatory or suppressor cells (Lan et al., 2012). Interestingly, mice fed the probiotic-supplemented diet,

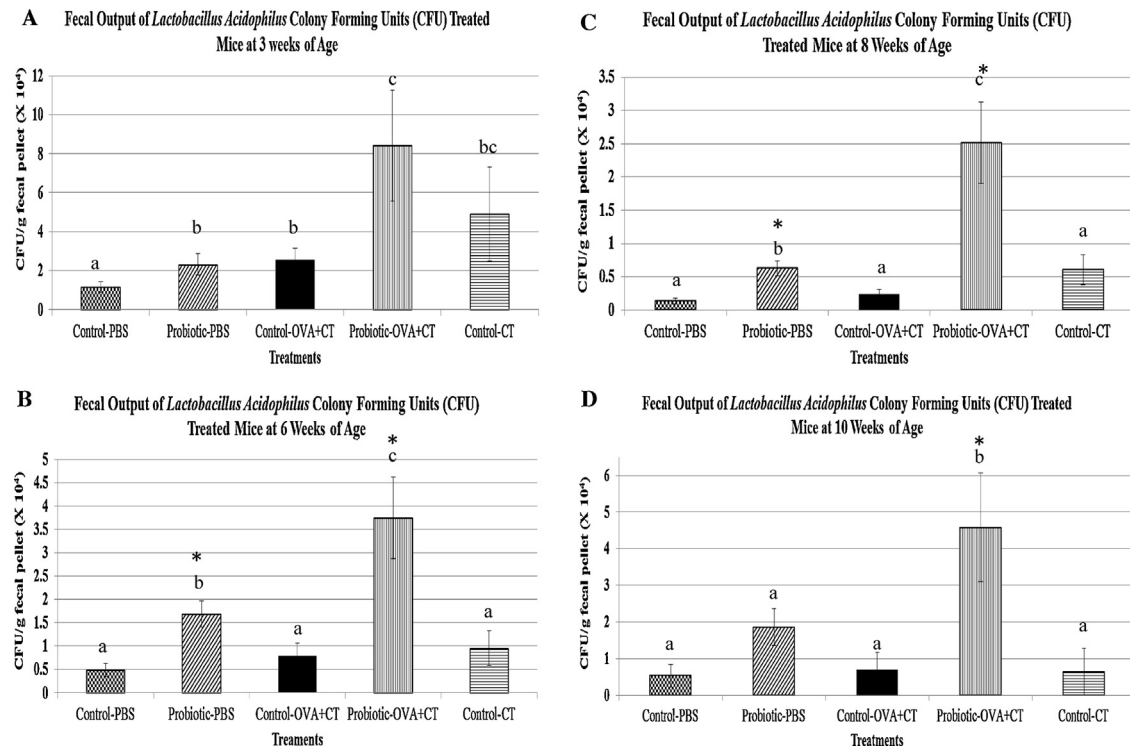


Fig. 2. Maternal and postnatal dietary probiotic supplementation enhances *Lactobacillus acidophilus* fecal colonization in treated mice. All data represent the mean value $\pm$ standard error of data pooled for 3 independent experiments. Fecal output of *Lactobacillus acidophilus* (LA) (A) 3 weeks of age, (B) 6 weeks of age, (C) 8 weeks of age and (D) 10 weeks of age. At each age, bar graphs with differing superscripts were significantly different at $p < 0.05$ significance level. *Mice had greater LA fecal counts in comparison to other treatment groups ($p < 0.05$).

Probiotic Diet-PBS and Probiotic Diet-Ovalbumin, had a significantly enhanced CD4⁺CD25⁺ splenic lymphocyte population in comparison to the respective control groups, Control Diet-PBS and Control Diet-Ovalbumin ($p < 0.0001$, Table 1). However, 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). Mice fed the probiotic-supplemented diet and sensitized with ovalbumin had enhanced induced CD4⁺FoxP3⁺ ($p < 0.0001$, Fig. 3A) and naturally occurring CD4⁺CD25⁺FoxP3⁺

($p < 0.0001$, Fig. 3B) T regulatory cell populations in comparison to mice of treatment groups Control Diet-PBS, Probiotic Diet-PBS, Control Diet-Ovalbumin, and Control Diet-CT.

Maternal and postnatal dietary probiotic supplementation reduces plasma ovalbumin-IgE levels in adult mice sensitized with ovalbumin

The total plasma IgE levels were determined over the course of 10 weeks (3, 6, 8 and 10 weeks of age). The data were analyzed at $p < 0.05$ and $p < 0.10$ significance levels at all-time points. Mice fed the control diet and sensitized with ovalbumin + CT had the lowest total IgE plasma levels in comparison to Control Diet-PBS, Probiotic Diet-PBS, and Control Diet-CT at weaning ($p < 0.05$, Fig. 4A). Mice fed the probiotic supplemented diet and sensitized with PBS

Table 1

The effect of probiotic dietary supplementation on splenic T cell populations in ovalbumin challenged mice.^a

	Dietary treatments ^b				
	Control Diet-PBS	Probiotic Diet-PBS	Control Diet-OVA + CT	Probiotic Diet-OVA + CT	Control Diet-CT
Lymphocytes	% Marker-positive of parent population				
CD3 ⁺	59.02 $\pm$ 2.13a	62.51 $\pm$ 1.2a	57.21 $\pm$ 1.54a	58.70 $\pm$ 2.05a	57.62 $\pm$ 5.93a
CD3 ⁺ CD4 ⁺	28.29 $\pm$ 0.84a	51.59 $\pm$ 2.4b	43.22 $\pm$ 3.44b	47.69 $\pm$ 4.35b	24.25 $\pm$ 1.90a
CD3 ⁺ CD8 ⁺	14.91 $\pm$ 0.34a	17.2 $\pm$ 0.71a	18.17 $\pm$ 1.57a	15.78 $\pm$ 0.31a	8.27 $\pm$ 1.52b
CD4 ⁺ CD25 ⁺	1.83 $\pm$ 0.15a	3.81 $\pm$ 0.34b	8.17 $\pm$ 0.96d	18.96 $\pm$ 1.38e	5.03 $\pm$ 0.53c

Splenic T cell lymphocyte populations were determined using surface cell staining procedures and fluorescence activate 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. Means within a row with differing alphabets are significantly different ($p < 0.0001$).

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

^b Dietary treatments=lactating dams and weaned pups were fed a control mouse chow or probiotic-supplemented mouse chow diet for 10 weeks (2days of age to 10 weeks of age), and sensitized (6 weeks of age) with 0.2 mL of sterile PBS, or 10 μ g CT in 0.2 mL of cholera toxin (CT) in 0.2 mL of PBS, or 50 μ g of egg allergen ovalbumin (OVA) + 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 100 μ g of OVA + 10 μ g of CT in 0.2 mL of PBS.

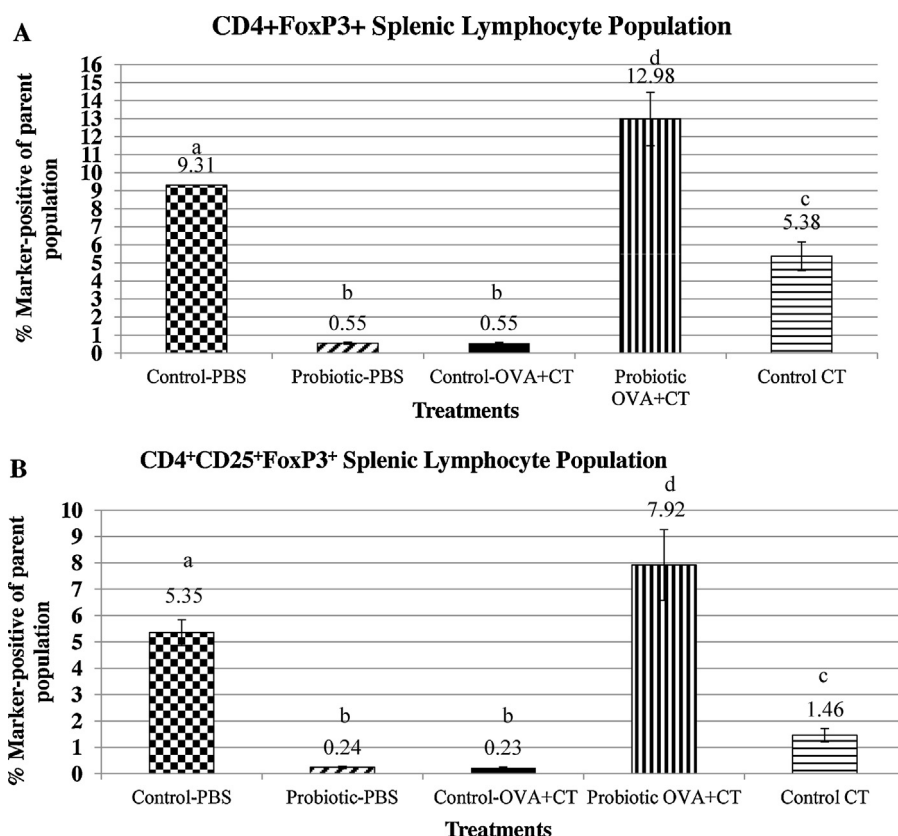


Fig. 3. Maternal and postnatal dietary probiotic supplementation and ovalbumin sensitization enhances induced and naturally occurring T regulatory splenic cell populations in mice. Splenic T cell lymphocyte populations were determined using surface and intracellular cell staining procedures and fluorescence 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 ($p < 0.0001$).

had significantly greater total IgE plasma levels in comparison to mice fed the probiotic supplemented diet and sensitized with ovalbumin + CT at 3 weeks of age ($p < 0.05$, Fig. 4A).

There were no significant differences in total plasma IgE levels between the treatment groups at 6 weeks of age at the $p < 0.05$ significance level. However, at the $p < 0.10$ significance level mice of control groups, Control Diet-PBS and Control Diet-CT had significantly enhanced total plasma IgE levels in comparison to mice of Probiotic Diet-PBS and Control Diet-ovalbumin + CT treatment groups at 6 weeks of age ($p = 0.0865$, Fig. 4B). While a significance level of $p < 0.05$ is conventionally used in the sciences, a probability of $p < 0.10$ may be of research value and/or biological importance. Therefore, we elected to include the statistical analysis of this data set at the $p < 0.10$ significance level.

At 8 weeks of age, mice fed probiotic-supplemented diet and sensitized with ovalbumin + CT had significantly greater total plasma IgE levels in comparison to mice of all other treatment groups Control Diet-PBS, Probiotic Diet-PBS, Control Diet-Ovalbumin + CT, and Control Diet-CT ($p = 0.0083$, Fig. 4C). There were no significant differences in total IgE plasma levels between any of the treatment groups at 10 weeks of age at either $p < 0.10$ or $p < 0.05$ significance level (Fig. 4D).

While total IgE plasma levels are used as an indicator of clinical allergy, there is considerable overlap between total IgE plasma levels of food allergic and non-allergic patients. Therefore, in this study we additionally analyzed ovalbumin specific IgE plasma levels for better comparison of allergic sensitization to ovalbumin between treatments. ovalbumin-IgE plasma levels were determined using only Mouse 3 experimental samples from 10-week-old animals, due to the limited availability of plasma from animals younger than

10 weeks of age required for total IgE and ovalbumin-IgE ELISA analysis. While not statistically significant at the $p < 0.05$ significance level, there was a reduction in ovalbumin specific-IgE plasma levels in ovalbumin sensitized mice fed the probiotic supplemented diet in comparison to mice fed the control diet and sensitized with ovalbumin + CT at 10 weeks of age ($p = 0.0560$, Fig. 5). Interestingly, mice fed the control diet and sensitized with adjuvant cholera toxin B had significantly enhanced plasma ovalbumin-IgE levels in comparison to mice of Control Diet-PBS and Probiotic Diet-PBS at 10 weeks of age ($p = 0.0173$, Fig. 5).

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

Our final objective was to determine the effect of maternal and postnatal dietary probiotic supplementation on splenic expression 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 for pro-inflammatory cytokines, IL-17a and IL-23a, associated with the Th17 (IL-17 producing T helper cells) lineage were up-regulated in mice of the Probiotic Diet-Ovalbumin + CT treatment group in comparison to gene expression in mice of the Control Diet-PBS and Control Diet-ovalbumin + CT treatment groups, respectively (Table 2, $p < 0.05$).

Splenic gene expression of cytokine CCL11 (eosinophil recruiter and mediator of allergy) and Sftpd (pattern recognition receptors that bind microorganism surface oligosaccharides or lipids) was up-regulated in mice of the probiotic treatment groups:

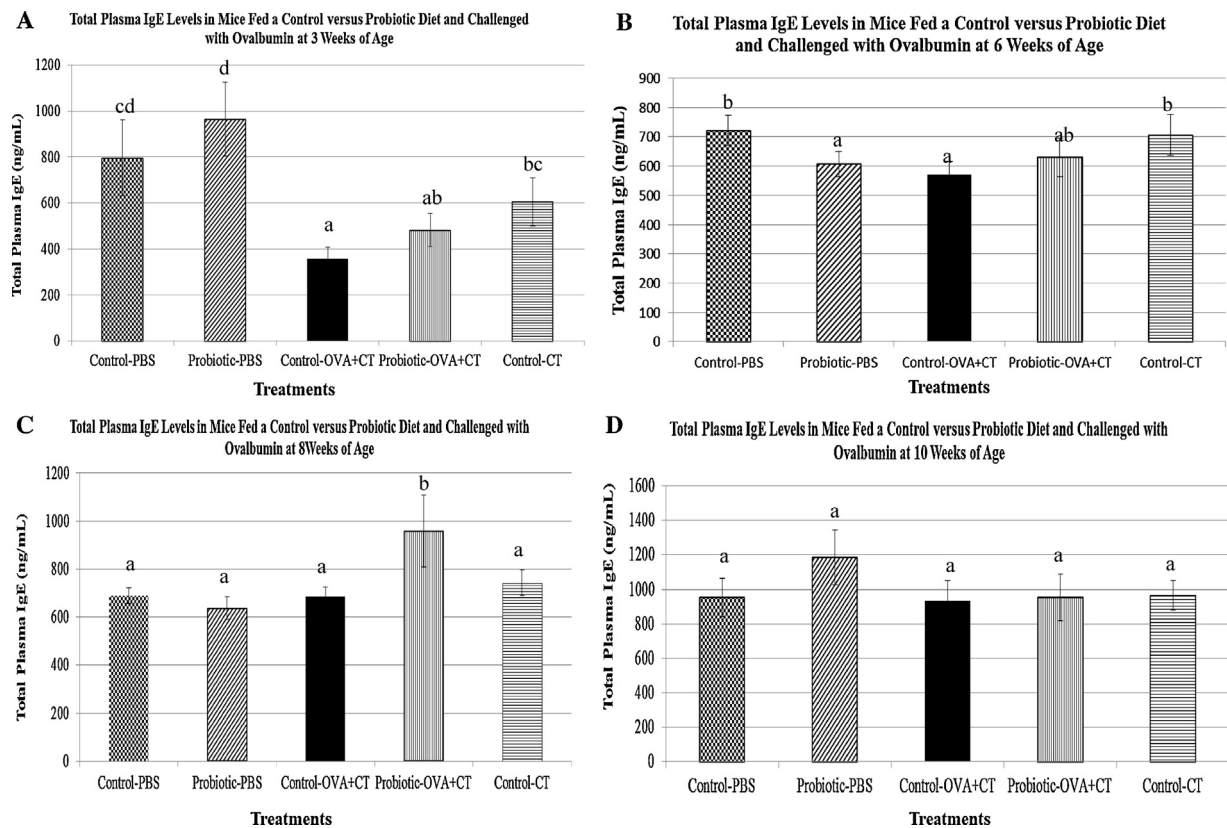


Fig. 4. Maternal and postnatal dietary probiotic supplementation alters plasma total IgE levels in ovalbumin sensitized mice at 8 weeks of age. All data represent the mean value $\pm$ standard error of data pooled for 3 independent experiments and treatment effects were evaluated by age (*i.e.* 3 weeks, 6 weeks, 8 weeks and 10 weeks of age). *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/or $p \leq 0.10$, treatments were separated by least squares means (*t*-test) for comparisons between each treatment group. Bar graphs with differing superscripts were significantly different: (A) $p < 0.05$, (B) $p \leq 0.10$, (C) $p = 0.01$, (D) there were no significant differences between treatment groups at $p < 0.05$ or $p \leq 0.10$.

Probiotic Diet-PBS, Probiotic Diet-Ovalbumin + CT in comparison to mice in the control diet groups: Control Diet-PBS and Control Diet – ovalbumin + CT, respectively (Table 2). Moreover, splenic gene expression of TGF β , (potent regulatory and anti-inflammatory cytokine), Nfatc2ip (transcription factor of Th2 associated cytokines), and T cell receptor CTLA-4 (down-regulates the immune system) were significantly up-regulated in mice fed the probiotic supplemented diet and ovalbumin + CT challenged in comparison to mice fed Control Diet-PBS and control diet and challenged with ovalbumin + CT (Table 2, $p \leq 0.05$).

Most importantly, splenic gene expression of the Th2-associated cytokine receptor, IL-13, and Th1 associated cytokines, IL-18, IL-27 (receptor) and IFN γ (Irf1) were significantly down-regulated in probiotic treated groups (Probiotic Diet-PBS and Probiotic Diet-Ovalbumin + CT) in comparison to the control group (Table 2), Control Diet-PBS ($p \leq 0.004$, Table 2). Similarly, mice of the probiotic treatment groups (Probiotic Diet-PBS and Probiotic Diet-Ovalbumin + CT) had significantly less splenic gene expression of CCL5 (recruits leukocytes to inflammatory sites), CD86 (co-stimulatory molecule for T cell activation and

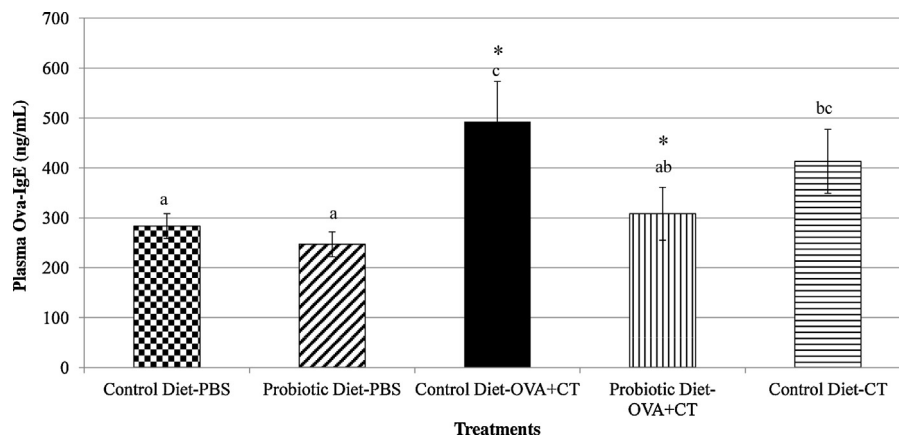


Fig. 5. Maternal and postnatal dietary probiotic supplementation reduces plasma ovalbumin-specific IgE levels in ovalbumin sensitized mice. All data represent the mean value $\pm$ standard error. *a,ab,bc,c* Statistical differences were determined using a nested ANOVA model in SAS (version 9.4, 2013), treatments were separated by least squares means (*t*-test) for comparisons between each treatment group at significance levels $p < 0.05$ and/or $p \leq 0.10$. * $p = 0.0560$: *p*-value for treatment comparison between Control Diet-OVA + CT and Probiotic Diet-OVA + CT treatment groups.

Table 2The effect of probiotic dietary supplementation on Th1/Th2/Th17-associated splenic gene expression in ovalbumin sensitized and challenged mice.^a

Gene	Probiotic Diet-PBS	Control Diet-OVA + CT	Probiotic Diet-OVA + CT	Control Diet-CT
Fold regulation comparing to Control Diet-PBS ^b (p-value)				
IL-17a	↑2.794 (0.700)	1.116 (0.477)	↑7.100 (0.043)	↑5.299 (0.027)
CCL11	↑3.599 (0.130)	1.205 (0.335)	↑9.318 (0.001)	↑2.963 (0.027)
IL23a	↑2.539 (0.786)	1.000 (0.461)	↑6.056 (0.040)	↑3.175 (0.0730)
Sftpd	↑3.338 (0.634)	−1.075 (0.490)	↑10.332 (0.010)	↑3.220 (0.070)
Ctla4	1.803 (0.677)	1.082 (0.553)	↑4.378 (0.032)	↑2.320 (0.257)
TGFβ3	1.392 (0.987)	−1.376 (0.766)	↑2.370 (0.047)	↑2.669 (0.152)
Gene	Probiotic Diet-PBS	Control Diet-OVA + CT	Probiotic Diet-OVA + CT	Control Diet-CT
Fold regulation comparing to Control Diet-PBS (p-value)				
CCL5	↓7.179 (0.013)	1.478 (0.076)	↓35.469 (0.003)	↓14.940 (0.008)
Cd86	↓3.122 (0.005)	1.593 (0.452)	↓6.225 (0.003)	↓6.808 (0.006)
IL13ra1	↓2.893 (0.014)	1.257 (0.115)	↓13.226 (0.002)	↓8.283 (0.014)
IL18	↓2.351 (0.019)	1.773 (0.093)	↓4.776 (0.004)	↓6.668 (0.006)
IL27ra	↓2.678 (0.016)	1.534 (0.085)	↓15.584 (0.002)	↓6.031 (0.024)
IFNγ	↓5.087 (0.005)	1.689 (0.063)	↓29.680 (0.001)	↓13.471 (0.006)
Stat1	↓3.895 (0.009)	1.989 (0.097)	↓11.811 (0.003)	↓6.082 (0.007)

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 Profiler™ PCR Array.

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

^b 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: $\Delta Ct = (\text{average Ct gene of interest} - \text{average Ct endogenous housekeeping gene})$. Average Ct values represent the mean Ct values of data pooled for 3 independent experiments. *p*-Values ≤ 0.05 were significant.

survival), and STAT1 (transducer involved in IFN γ cell signaling) in comparison to mice of the control treatment groups (Control Diet-PBS and Control Diet-Ovalbumin + CT), respectively ($p < 0.02$, Table 2).

Discussion

During the early neonatal period the intestine is colonized with approximately 100 trillion bacteria (Ogra and Welliver, 2008). Early exposure to environmental microbes promotes the maturation and development of the infant's gut and gut-associated immunity. This may determine the outcome to induced mucosal inflammation (Sjögren et al., 2009), resistance to enteric pathogens, disease development (Hoque et al., 1994), autoimmunity and allergic disorders (Isolauri and Salminen, 2008) in later life. The diversity of acquired neonatal microbiota is dependent upon the external environment microflora, breastfeeding (Kaplan et al., 2011), use of antibiotics and the presence of non-digestible sugars (prebiotics) in the maternal milk (Newburg, 2009; Newburg et al., 2005).

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 ovalbumin 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 (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 10 weeks.

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 show that at weaning, pups suckled by dams fed the probiotic-supplemented diet had enhanced fecal counts of *L. acidophilus* (predominate probiotic strain; Fig. 2) 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 ovalbumin + CT treatment groups (Fig. 2D).

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 which may attenuate hypersensitivity responses to food allergens, by enhancing both splenic population of “natural-occurring” and induced regulatory T helper cells (Fig. 3). Our results are supported by previous reports demonstrating that acute probiotic supplementation enhances activated Th17 helper (Donkor et al., 2012) and T regulatory cell populations (Lavasani et al., 2010; Schiavi et al., 2011) and secretion and/or expression of regulatory cytokine TGFβ (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).

Barletta et al. (2013) demonstrated that probiotic-induced TGF-β secretion and expression within the gut had TGFβ-mediated protective effects via the induction of regulatory T cells expressing FoxP3⁺, reducing food allergen inflammatory responses. In this study we demonstrate that maternal and post-weaning probiotic dietary supplementation significantly enhances splenic gene expression of anti-inflammatory and regulatory cytokine TGFβ, and Th17 associated cytokines IL-17 and IL-23, while reducing expression Th2 associated cytokine IL-13 (mediator of allergic responses) and Th1 associated cytokines IL-18, IL-27 and IFNγ (Table 2). IL-23, normally produced by antigen-presenting cells stimulates naïve CD4⁺ T cells to differentiate into Th17 subset of T helper cells which secreted IL-17. Th17 cells play a critical role in pathogen host defense and the induction of tissue inflammation associated with autoimmunity (Lin et al., 2005). Other studies have demonstrated that acute probiotic supplementation enhances activated Th17 and T helper regulatory cell populations and IL-17 secretion (Donkor et al., 2012; Kobayashi et al., 2012) and mRNA expression (Kobayashi et al., 2012) *in vitro* and *in vivo*. These reports imply that probiotic-induced TGFβ mRNA expression and secretion play a regulatory role in the differentiation of T helper regulatory and Th17 effector cells, balancing pro-inflammatory and anti-inflammatory responses to facilitate enhanced host protection against pathogens without self-reactive T-cell responses.

Patients with inflammatory bowel disease have been shown to have excessive IL-17 and pro-inflammatory Th1 associated IFNγ secretion within the gut with reduced TGFβ expression and secretion (Rovedatti et al., 2009). Significant concentrations of regulatory cytokines, IL-10 and TGFβ function to inhibit excessive expression and secretion of Th1-associated pro-inflammatory cytokines by antigen presenting cells and by inducing expression of other co-stimulatory surface markers and soluble cytokines (Donkor et al., 2012; Meyaard et al., 1996). Similarly, we demonstrate in this study enhanced splenic gene expression of TGFβ and Th17 associated cytokines IL-17 and IL-23, with reduced splenic gene expression of Th1 associated cytokines IL-18, IL-27 and IFNγ in probiotic treated mice challenged with ovalbumin + CT (Table 2).

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. In contrast, other reports have shown a reduction in total plasma IgE levels in allergen sensitized and challenged subjects given probiotic supplementation (Kim et al., 2005). In the current study total plasma IgE levels in ovalbumin + CT-challenged mice fed the probiotic-supplemented diet were not significantly reduced in comparison to mice fed the control diet and challenged with ovalbumin + CT over the course of development at 3, 6, 8 and 10 weeks of age (Fig. 4). However,

allergen specific plasma IgE levels at 10 weeks of age were reduced in ovalbumin + CT challenged mice fed the probiotic-supplemented diet in comparison to those mice receiving the control diet at 10 weeks of age (Fig. 5). This is similar to several reports demonstrating reduction in allergen-specific plasma IgE levels with probiotic supplementation given therapeutically (Ivory et al., 2008; Kuitunen et al., 2009; Adam et al., 2010). 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 little 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.

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, plasma IgE levels (total and ovalbumin-specific), 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 food allergen ovalbumin in humans. In summary, our data demonstrate that probiotic dietary supplementation enhances gut colonization of the predominate commensal microbe, *L. acidophilus*, in parallel with reduced plasma ovalbumin-IgE levels, balancing the splenic expression of pro-inflammatory, anti-inflammatory and regulatory Th1/Th2/Th17 associated cytokines and enhancement of regulatory T helper cell populations in mice sensitized and challenged with egg allergen ovalbumin.

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 the management and/or prevention of food allergy.

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