

**EFFECTS OF PROBIOTICS AND PREBIOTIC MILK OLIGOSACCHARIDES  
IN MURINE MODELS OF FOOD ALLERGY**

EFFECTS OF PROBIOTICS AND PREBIOTIC MILK OLIGOSACCHARIDES IN  
MURINE MODELS OF FOOD ALLERGY

By

LUCRECIA MARIA CASTILLO, B. Sc., M. Sc

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AUTHOR: Lucrecia Maria Castillo, B. Sc., M. Sc.

SUPERVISOR: Dr. Paul Forsythe

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## **ABSTRACT**

**Introduction:** Incidence of food allergy has increased in the recent years yet no treatment is available except for allergen avoidance; therefore, new therapies are in development involving probiotic bacteria and human milk oligosaccharides. Food allergy results from an unbalanced T helper cell population favouring Th2/Th1 cells. Human milk oligosaccharides (HMOs) and probiotic bacteria have been shown to modulate the immune system; therefore they might be proposed as potential therapies against allergic diseases. In this study, HMOs 2' fucosyllactose and 6' sialyllactose, and probiotic strains *Lactobacillus reuteri* DSM17938 and *Lactobacillus rhamnosus* JB-1 were used as treatments against ovalbumin-induced food allergy in mice.

**Results:** The effects of oral treatment with probiotics and prebiotics on anaphylactic symptoms induced by oral ovalbumin (OVA) challenge in sensitized mice were investigated. Mast cell functions in response to oral HMO treatment were also measured in the passive cutaneous anaphylaxis model and direct effects on IgE mediated degranulation of mast cells were assessed. Daily oral treatment with 2' fucosyllactose or 6' sialyllactose attenuated food allergy symptoms including diarrhea and hypothermia. Treatment with HMOs also suppressed antigen induced increases in mouse mast cell protease-1 in serum and mast cell numbers in the intestine. These effects were associated with increases in the CD4<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> cell populations in the Peyer's patches and mesenteric lymph nodes, while 6' sialyllactose directly inhibited mast cell degranulation *In-vitro*, at high concentrations. Effects of direct probiotic treatment were less dramatic.

*Lactobacillus reuteri* DSM17938 decreased diarrhea and mMCP-1 in the colon while increasing IL-10 production. While *Lactobacillus rhamnosus* JB-1 increased IL-10 and IFN- $\gamma$  while decreasing TNF- $\alpha$  from stimulated splenocytes but had no effect on symptoms.

**Conclusion:** Our studies suggest that 2' fucosyllactose and 6' sialyllactose reduce the symptoms of food allergy through the induction of IL-10<sup>+</sup> T regulatory cells and indirect stabilization of mast cells. Thus, human milk oligosaccharides may have greater therapeutic potential in food allergy than probiotic treatment.

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## **ABBREVIATIONS**

- 2' FL: 2' Fucosyllactose
- 6' SL: 6' Sialyllactose
- Ag: Antigen
- BMDC: Bone marrow-derived mast cells
- CD: Cluster of differentiation
- CFU: colony forming units
- CX3CR1: CX3C chemokine receptor 1
- DCs: Dendritic cells
- DNP: Dinitrophenyl haptenic group
- EC-PAD: Pulsed amperometric detection

- ELISA: Enzyme-linked immunosorbent assay
- FCER1A: Alpha subunit of the high affinity IgE receptor
- FCER1G: Gamma subunit of the high affinity IgE receptor
- FCεRI: high-affinity receptor for the Fc region of immunoglobulin E
- Fig: Figure
- Foxp3<sup>+</sup>: Forkhead box P3
- GI: Gastrointestinal
- HAS: human serum albumin
- HMOs: Human milk oligosaccharides
- IEC: Intestinal epithelial cells
- IFN-γ: Interferon gamma
- Ig: Immunoglobulin
- IKCa: Membrane calcium-potassium channel
- IL-: Interleukin
- ILC: Innate lymphoid cell
- iTregs: Induced regulatory T cell
- KOH: Potassium hydroxide
- LAL: Limulus amoebocyte lysate
- LPS: Lipopolysaccharide
- MEM: Minimum essential medium
- MHC: Major histocompatibility complex
- MLN: Mesenteric lymph node

- mMCP-1: Mouse mast cell protease 1
- MRS: Man-agarose-sharpe medium
- NLR: Nod-like receptor
- nTregs: Natural regulatory T cell
- OCT: Optimal cutting temperature compound for frozen tissues
- OVA: Ovalbumin
- PBS: Phosphate-buffered saline
- PCA: Passive cutaneous anaphylaxis
- PP: Peyer's patches
- PRR: Pattern recognition receptor
- RPM: revolutions per minute
- T cell: Thymus cell
- TCR: T cell receptor
- TGF- $\beta$ : Transforming growth factor beta
- Th (1 or 2): T helper cell
- TLR: Toll-like receptor
- TLRs: Toll-like receptors
- TNF- $\alpha$ : Tumor necrosis factor alpha
- Tregs: Regulatory T cells
- TSLP: Thymic stromal lymphopoietin

## **BACKGROUND INFORMATION**

### **Food allergy and its prevalence**

In the recent years, incidences of food allergy have increased worldwide and 25% of the population experience adverse reactions to food (Bischoff & Crowe, 2004). Children occupy up to 6% of this prevalence (Bischoff, 2006; Yamamoto et al., 2009).

Food allergy is most commonly seen early in the life of an individual. Food allergy results from the inability to establish oral tolerance while foods are being consumed or from the exposure in the gastrointestinal (GI) tract or respiratory tract resulting in sensitization by the allergen. An ideal immunological response would develop immunotolerance to food, yet, when the GI tract is exposed to such allergens in early stages of GI development, the body is not capable to recognize these particles as harmless (Bischoff, 2009).

Most food allergens are usually proteins consumed from the normal diet of the individual which are not degraded completely in the stomach or intestine and are seen as foreign by the body when they reach the GI tract (Bischoff, 2006). Allergens encountered by infants or children with an immature immune system result in adverse food reactions when consumed or exposed to them (Chehade & Mayer, 2005).

The hygiene hypothesis proposes that one of the causes of food allergy is due to the lack of microbial exposure of the immune system at early stages of life (Feleszco et al., 2006; Kim et al., 2005). This suggests that the lack of exposure to microbes and bacteria during

early stages of life have shifted the body response to a Th2/Th1 cells, from which, immune responses from Th2 cells are the compellers of allergic reactions (Bufford & Gern, 2005; Garn & Renz, 2007)

### **Effects of food allergens in the body**

The clinical characterization of food allergy ranges from gastrointestinal symptoms, such as colic, vomiting and diarrhea, to anaphylaxis (Robbie-Ryan & Brown, 2002; Sampson, 2004). Allergens activate cellular pathways through IgE-antigen that lead to inflammation and other immunological responses (Sicherer et al., 2006). Food allergy is mucosal mast cell dependent and the site of sensitization is the intestinal tract but the sites of manifestations can also be seen in the skin and the respiratory tract depending on the individuals and the allergens, causing atopic dermatitis and asthma respectively (Brant et al., 2003; Yamaki & Yoshino, 2012).

The most common food allergens in children are cow's milk, soy, wheat and egg including ovalbumin, which is the major protein component of hen eggs (Kim et al., 2013; Wood, 2003). These allergies usually resolve with age (Wood, 2003). While peanut and seafood allergies can resolve by age five, they more usually persist to adulthood (Fleischer et al., 2003).

## **Oral Tolerance**

Oral tolerance is the “state of local and systemic immune unresponsiveness that is induced by oral administration of innocuous antigen such as food proteins” (Pabst & Mowat, 2012). Oral tolerance is able to reduce immune responses against self-antigens. In addition, it reduces delayed type hypersensitivity, T-cell proliferation, cytokine production and serum antibodies in response to antigens. Oral tolerance also regulates commensal bacteria and prevents intestinal disorders such as Crohn’s disease (Pabst & Mowat, 2012).

Failing to the induction of oral tolerance usually results in autoimmune diseases and food allergy (Pabst & Mowat, 2012). This tolerogenic response can be maintained after several months of antigen encounter but it involves a constant process of regulatory T cell generation in response to intestinal antigens from the daily food diet (Mowat et al., 2005).

Oral tolerance can be induced by administering a single high dose of antigen known as clonal anergy, or a continuous low dose of the antigen called “Adaptive tolerance” or “In vivo anergy” (Friedman, 1994; Schwartz, 2003). Lymphocyte anergy is defined by Schwartz (2003) as the inactivation of a lymphocyte function after antigen encounter. Clonal anergy is characterized by growth arrest. Adaptive tolerance Oral tolerance is characterized by inhibition of proliferative and effector functions of T cell, where T cells are incompletely activated leading to loss of function (Schwartz, 2003).

### **Mast cells in food allergy**

Mast cells are derived from hematopoietic progenitors, start differentiating in the bone marrow in the presence of IL-3 and the stem cell factor c-kit ligand and do not circulate in the blood but instead they establish in tissues around the body (Rodewal et al., 1996).

They are found in mucosal surfaces of the respiratory and intestinal tract, as well as in the skin and near peripheral nerves and pose distinct differentiation according to their location (Galli & Hammel, 1994).

Mast cells are directly involved in inflammatory processes of allergic diseases which are dominated by Th2 cell type and are dependent on the IgE-specific FcεRI receptor on their surface (Williams & Gali, 2000). When antigen-specific IgE crosslink with the antigen, mast cells activate and initiate signal transduction for the release of hallmark cytokines such as IL-4 and TNF- $\alpha$ , histamine, proteases, heparin, serotonin and kinins. Upon mast cells activation they then synthesize mediators such as IL-1-IL-8, IL-12, IL-15, IL-16, leukotrienes, prostaglandins, chemokines and growth factors among others (Robbie & Brown, 2002).

Mast cells release chymases (IE mMCP-1) and histamine, which promotes mucus production and electrolyte release (Bischoff & Kramer, 2007). Mast cells also secrete cytokines such as TNF- $\alpha$ , IL-13 and IL-8 involved in intestinal epithelial cells (IEC) permeability, which allows the leakage of the allergen (Perrier & Corthesy, 2011).

Mast cells can be activated by the cross-linking of IgE receptors, Toll-like receptors (TLRs) in mast cells, complement system molecules and neuropeptides such as substance



P (Ansel et al., 1993; el-Lati et al., 1994). TLRs on mast cells recognize molecules from pathogens, allergens or gut bacteria. Initially, mast cells are activated via TLR on their surfaces (Supajatura et al., 2002). Upon activation, cytokines are released which determines if the response to the reaction would be mediated by Th1 or Th2 cells (Supajatura et al., 2002). At the same time chemokines and cytokines recruit inflammatory cells and amplify the inflammatory response.

### **Gut barrier**

The intestinal mucosa is composed of a single layer of epithelial cells that serve as a barrier for the external environment (Chehade & Mayer, 2005). This mucosal surface is very permeable to antigens and is here where commensal bacteria inhabit, and collectively with immune cells, they activate immunoregulatory pathways in response to pathogens and foreign substances, such as allergens (Peterson & Artis, 2014). Mucus, glycosylated mucins, and other antimicrobial proteins such as C-type lectin proteins, defensins, cathelicidins and lysozymes are secreted by the epithelium to the lumen and serve as a biochemical line of defence against pathogenic bacteria (Gallo & Hooper, 2012).

### **Permeability and food allergy**

A characteristic of the intestinal mucosa is the restriction of large molecules such as proteins or allergenic particles, while allowing nutrients through the gut barrier (Perrier & Corthesy, 2011). When oral tolerance is not well established by the immune system, allergen-specific IgE (Ag-IgE) is produced by B cells upon antigen encounter and that specific antigen is presented to T cells for T cell activation. T cells respond by inducing cytokines responsible for inflammatory lymphocytes recruitment to the gut barrier, including mast cells found in peripheral tissues (Perrier & Corthesy, 2011; Wang et al., 1998). Cross-linking of Ag-IgE and IgE receptors on the mast cells leads to activation, degranulation and release of inflammatory mediators, such as cytokines and proteases, which increase the permeability of the gut barrier leading to an increase of foreign substances and a cascade of allergic reaction in the body (Perrier & Corthesy, 2011).

Intestinal permeability is increased in allergic reactions and it might also be a cause for allergic reactions in the body (Andre et al., 1987; Perrier & Corthesy, 2011). Small amounts of intact antigenic protein can trespass through the barrier transcellularly and crosslink to IgE and activate mast cells (Bet al., 1987). A few minutes after allergen exposure, mast cells degranulate causing alterations of tight junctions due to the inflammatory mediators such as TNF- $\alpha$ , IFN- $\gamma$ , prostaglandins and proteases.

Subsequently is initiate a cascade of response by recruiting and activating a major number of mast cells and allowing the entrance of more antigenic proteins paracellularly leading to an allergic reaction (Scudamore et al., 1995). It has been demonstrated that mouse mast

cell protease 1 (mMCP-1) increase leakiness of the gut and exposure to antigens by degradation of the tight protein junction occludin (McDermott, 2003)

### **Immunoregulatory functions of the gastrointestinal tract**

Intestinal epithelial cells (IECs) are primed to initiate innate and adaptive immune responses. They regulate inflammation in the body and play a role in the homeostasis of the intestine (Peterson & Artis, 2014). IECs express pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nod-like receptors (NLRs) that serve as sensors to test the environment of the gut for further immunological responses such as inflammation or immune tolerance (Peterson & Artis, 2014).

IEC secreted thymic stromal lymphopoietin (TSLP), transforming growth factor- $\beta$  (TGF- $\beta$ ), Interleukin-10 (IL-10) and retinoic acid is produced in response to commensal bacteria-derived signals which promote dendritic cells (DCs) and macrophages with tolerogenic properties (Peterson & Artis, 2014; Taylor et al, 2009; Zeuthen et al., 2008).

Intestinal mononuclear phagocytes, which include dendritic cells (DCs) and macrophages, have been characterized in two populations: pre-DC-derived CD11c<sup>+</sup>CD103<sup>+</sup> DCs and monocyte-derived CD11c<sup>low</sup>F4/80<sup>+</sup>CX<sub>3</sub>CR1<sup>hi</sup> intestine-resident macrophages (Varol et al., 2009). CD103<sup>+</sup> DCs are migratory cells that sample secondary lymphoid tissues such as the mesenteric lymph nodes (MLN) and the Peyer's patches (PP), capturing antigens (Ag) and bacteria for Ag presentation to T cells (Schulz et al.,

2009). These migratory cells are induced by IEC's secretory TGF- $\beta$  and retinoic acid to induce the differentiation of Foxp3<sup>+</sup> regulatory T cells (Tregs) which allow immune tolerance (Coombes et al., 2007). CD103<sup>+</sup> DCs also imprint gut-homing on T cells for further encounter with an allergen in the intestinal epithelial barrier (Coombes et al., 2007)

CD103<sup>+</sup> CX<sub>3</sub>CR1<sup>hi</sup> DC's are stationary and collaborate with IECs through TLRs for pathogen and commensal bacteria riddance through the epithelial barrier. These DCs' dendrites are long which allow them to sample the external surface of the barrier for allergens (Varol et al., 2009). They are also responsible for immunological tolerance by maintaining the survival of Foxp3<sup>+</sup> Tregs at the gut barrier (Hadis et al., 2011).

IECs may also induce T helper 2 (Th2) cell class by the release of TSLP and IL-25 cytokines which induce the differentiation of hematopoietic progenitor cells into Th2 cytokine-producing cells at the mucosal sites in response to allergens (Siracusa et al., 2013)

Homeostasis of the intestinal mucosal barrier is also maintained by innate lymphoid cells (ILC) by initiating inflammation and innate immune responses upon bacterial and antigen exposure. The approximate number of lymphoid cells in the small intestine corresponds to 1012 per meter (van der Jeidjen et al., 1987).

They are classified in three categories: subset 1 does not rearrange their Ag receptor and is composed of ILCs subset 1 and natural killer cells which produce Th1 associated cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ) and tumour necrosis factor (TNF) in response to

IL-12 and IL-15 (Spit et al., 2013). ILCs 2 cells produce cytokines similar to Th2 cells, such as IL-5 and IL-13 in response to IL-25, IL-13 and TSLP, and they protect and promote repairs in tissue in allergic responses (Moro et al., 2009). ILCs3 cells produce cytokines similar to Th17 and Th22 cells, such as IL-17 and IL-22 in response to IL-23 (Spit et al., 2013).

### **T cells and food allergy**

T cells are double faced in relation to food allergy. They may aggravate and cause allergic symptoms due to autoimmunity responses or they induce oral tolerance (Kim & Samson, 2012). T cells are found in several locations such as Peyer's patches, mesenteric lymph nodes, the intestinal mucosa and peripheral blood (Kim & Samson, 2012).

### **Th1, Th2 and their balance in allergy**

T cells can be divided in cytotoxic T cells ( $CD8^+$ ) and T helper cells ( $CD4^+$ ). T helper cells (Th) can be subdivided according to their cytokines (Berin & Sampson, 2013). Th1 cells express the transcriptional factor T-bet and secrete interferon-gamma ( $IFN-\gamma$ ), IL-2 and tumor necrosis factor-beta ( $TNF-\beta$ ) which activates macrophages responsible inflammatory responses, cell mediated immunity and phagocyte-dependent immunity (Romagnani, 1999). They are activated upon intracellular bacterial or viral infection (Romagnati, 2000).

Th2 cells produce IL-4, IL-5, IL-10 and IL-13 for antibody production, especially IgE, eosinophil activation and inhibition of macrophages responses. They are activated during parasite or nematode infection in the GI tract and in allergic reactions (Romagnati, 2000).

Allergy is the result of an unbalanced T helper cell population, with Th2 dominant population over Th1 type cells in response to allergens. Th2 cells are able to recognize the allergen directly via T cell receptor (TCR). In response, they release cytokines (Romagnani, 1994).

These cytokines include IL-4, which is responsible for IgE isotype switching in response to allergens, recruiting and activating mast cells and eosinophils (Romagnati, 2000). IL-13 stimulates IgE production in B cells and IL-5 for eosinophil recruitment. IL-10, and also IL-4, is produced for mast cell proliferation, and IL-9 and IL-13 is produced for mucus hypersecretion, as mucus serves as a line of defense against foreign particles (Romagnati, 2000).

It has been shown in many studies that immunotherapy can redress the cytokine imbalance in allergy resulting in higher concentrations of the Th1 cytokine IFN- $\gamma$ , and lower Th2 cytokine expression. Allergen immunotherapy also results in higher IL-10 expression, which is an immunoregulatory cytokine that down regulates Th1 and Th2 cytokine responses and induce Tregs production (Hansen, 1999).

## **Regulatory T cells**

Regulatory T cells (tregs) are a subpopulation of the T cell lymphocytes, those which express the cluster of differentiation 4 (CD4) on their surface (Corthay, 2009). A specific marker for Tregs is lacking but many regulatory T cells express the Forkhead Box p3 (Foxp3) transcriptional factor. Foxp3 is able to suppress or activate genes involved in T cell stimulation, especially the genes activated by the T cell receptor (TCR) interaction (Marson et al., 2007). In addition, Treg cells express  $\alpha\beta$ TCR that confer antigen specificity which is important for the suppression of antigen-specific immune responses (von Boehmer, 2005). This conveys Tregs to recognize self/non-self-antigens for immune tolerance (Corthay, 2009). Tregs are able to suppress the responsiveness of other lymphocytes (Itoh et al., 1999).

CD103<sup>+</sup> DCs are able to induce gut homing T cells and Foxp3<sup>+</sup> Tregs. Foxp3<sup>+</sup> Tregs can be classified as natural Tregs (nTregs), which are generated in the Thymus. Their phenotype is described as CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>low</sup> and they usually target antigen presenting cells (APCs) and effector T cells (Curotto & lafaie, 2009).

Induced Tregs (iTregs) are induced in the Mucosal Associated Lymphoid Tissues (MALT) and are IL-10 and TGF- $\beta$  mediated and they target effector T cells. iTregs are differentiated under inflammatory conditions (Curotto & Lafaie, 2009). When inflammation occurs, Tregs and other effector T cells extravasate and reach the site of inflammation and is there where Tregs determine the specificity of immunosuppression.

Then Tregs suppress neighbouring or bystander recruited cells that are at the same site of inflammation and possess the antigen specificity (Klein et al., 2003)

Tregs suppresses T-cell proliferation and down-regulate pro-inflammatory cytokine profiles such as TNF- $\alpha$  and IFN- $\gamma$  and inhibit IL-2 transcription, which is involved in the expansion of effector T cells and memory T cells (Tiemessen, 2007). In addition, Tregs secrete IL-9, IL-10 and TGF- $\beta$  for immunosuppression of other lymphocytes (Tiemessen, 2007).

### **The immune response to an allergen**

Foreign particles are detected by mature B-cells, dendritic cells or by other antigen presenting cells (APCs). Specific antibodies recognize and bind the foreign particles and APCs uptake and process the foreign particle into specific epitopes or short proteins to be displayed in the Major histocompatibility complex II (MHC-II) for T-cell interaction. Upon activation of T-cell by epitope displayed, T cells release cytokines which would consequently activate B cells for formation and release of specific antibodies such as IgE (Madigan et al., 2009).

The initial sensitization stage of mucosal mast cells in food allergy consists in the leakage of the allergen particle into the intestinal mucosa. Allergen-specific IgE is created by B-cells and is loaded into the high affinity IgE receptor (Fc $\epsilon$ RI) which is naturally expressed on the surface of mast cells (Rivera & Olivera, 2008). This mast cell-IgE dependent



process is classified as type I hypersensitivity reaction (Coombs, 1992). Cross-linking of FcεRI bound IgE with allergen triggers Ca<sup>2+</sup> intake into the cells which initiates activation of mast cells, and also basophils, leading to signal transduction by degranulation and release of many molecular mediators that contribute to inflammation and allergic reactions in the mucosa, the latter classified as “early reaction” (Bischoff, 2009).

The production of IL-13 by mast cells induces the production of IgE by B cells and IL-13 is increased by the presence of IL-4 (Brandt et al., 2009; Munitz et al., 2008). IL-4 is an important regulatory cytokine which prompt the switch of mast cell released pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF), IL-1 and IL-6 to T-helper 2 cells (Th2) such as IL-13 (Lorentz et al., 2000). Subsequently, the “late reaction” takes place by the release of TNF-α, IL-5, IL-3 and IL-4 from mast cells which attract and activate pro-inflammatory cells such as neutrophils, Th2 cells and eosinophils to the epithelium resulting in loss of barrier function, inflammation and foreign bacterial infiltration into the mucosa (Bischoff, 2009).

When FcεRI bound antibody detect food allergens, the transmission of this signal is translated as a threat and secretory and propulsive behaviour in the intestine is engaged and is expressed in symptoms such as abdominal pain, cramping, diarrhea and fecal urgency which are common in food allergy (Demaude et al., 2007).

## **Therapies**

The mechanisms the body uses to regulate allergy symptoms still remain unclear (Yamamoto et al., 2009). At this time, the most efficient therapies for food allergies is allergen avoidance (Yamamoto et al., 2009). However, new therapeutic approaches have been proposed based on the Hygiene Hypothesis and focus in the gastrointestinal bacterial balance by the introduction of beneficial bacteria in the gut (Forsythe et al., 2007; Forsythe, 2011). Others focus on immunological cells and the ways to counteract their effects in allergy by shifting and balancing the population Th1/Th2 cytokines (Mosmann & Coffman, 1989).

There is increased interest in the potential protective effect of bacteria in allergy and in nutraceutical approaches to therapy using probiotics and prebiotics (Eiwegger et al., 2004; Mosmann & Coffman 1989). In this regard in human milk oligosaccharides is on the rise as these prebiotic substances are been shown to modulate anti-allergic responses (Eiwegger et al., 2004).

## **Human milk oligosaccharides**

Human milk oligosaccharides (HMOs) were initially classified as non-digestible metabolic substrates that enhanced the survival and growth of gut microbiota (Schrzenmeir & Vrese, 2001). They were called “Bifidus factor” as they enhanced the survival of bifidobacteria in the gut (György et al., 1954). Now, HMOs are classified as

unconjugated glycans with a variety of structures found in exuberant quantities in milk of several mammals although human milk is known to pose the highest structural diversity at higher concentrations (5-15 g/L) (Bode, 2012; Kunz et al., 2000). 2' fucosyllactose levels in human milk can range from 0.06-4.65 g/L (Erney et al., 2000).

The proportion of milk composition such as lipids, protein and carbohydrates varies among species; yet, carbohydrates are the highest component of human milk (Kunz et al., 2000). In addition, the profile of human milk oligosaccharides varies from woman to woman (Kobata, 2010). 2' fucosyllactose is a neutral oligosaccharide and is the most abundant oligosaccharide in human milk with the exception of lactose, yet not every woman produces it as some do not possess the genes encoding the enzyme FUT responsible for fucosylation or express them in lower levels (Erney et al., 2000; Thurl et al., 1997). 6' sialyllactose is an acidic oligosaccharide and is considered a good bifidofactor. Other species such as mouse milk contain 3' SL and 6' SL but none of the fucosylated or elongated oligosaccharides found in human milk; yet, 2' FL can be produced by transgenic mice containing the enzyme FUT (Fuhrer et al., 2010; Prieto et al., 1995).

The structure of human milk oligosaccharides consists in three to five of the following monosaccharides: glucose, L-fucose, galactose, N-acetylglucosamine and sialic acid, from which, each HMO have lactose (glucose + galactose) at their reducing end (Bode, 2012). More than a hundred oligosaccharides have been isolated and classified up to this date yet any biological effect of these are known to be structure-specific (van Hoffen et al., 2009).

HMOs have been proposed to have many beneficial effects to human health. They are considered to be prebiotics. Prebiotics are defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Gibson et al., 2004). Prebiotics must be resistant to gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption, which HMOs meet those criteria (Bode, 2012). Prebiotics favour the population growth of gut bacteria, which overrides the population of pathogenic bacteria (Gibson & Wang, 1994).

There is evidence that HMOs can modulate the immune system. They are able cross the epithelial barrier and act directly on the immune system via specific receptors in immune cells (Eiwegger et al., 2010). They also are able to attenuate T cell responses, to inhibit cell-cell interactions of lymphocytes via selectins and to inhibit leukocyte rolling and adhesion to endothelial cells (Bode et al., 2004, 2004)

HMOs are also beneficial to the host as they act as antiadhesive antimicrobials from which pathogens attach instead of attaching to glycans on the cell surfaces. Therefore, they are capable of reducing microbial infections (Newburg et al., 2005). Pathogen adhesion is usually through lectin-glycan interactions, such as the case of *Escherichia coli* with type 1 fimbria and *Helicobacter pylori* (Firon et al., 1983; Ruiz-Palacios et al., 2003). HMOs act as decoys not only for bacteria but also for viruses and protozoans and they might not only protect against GI infections but also against respiratory infections (Bode, 2012). When babies are breastfed, the milk covers the mucosal surfaces of the nasopharyngeal region and it has been shown that these babies are less likely to develop

otitis media infections cause by *Haemophilus influenza*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* (Abrahams & Labbok, 2011).

In addition, HMOs may protect the host against urinary tract infections caused by enterourogenic *E. coli* as HMOs are absorbed and excreted in the urine (Martin-Sosa et al., 2002). Also, it has been shown that HMOs directly interact with intestinal epithelial cells, influence gene expression and reprogram cell cycle and cell surface glycosylation (Kuntz et al., 2009).

HMOs may also induce direct anti-inflammatory effects (Schrzenmeir & Vrese, 2001).

Studies suggest that HMOs modulate the immune system such as cytokine production leading to a balanced Th1/Th2 response and could act locally in mucosal-associated lymphoid tissues or systemically (Rudloff et al., 1996). IFN- $\gamma$  and IL-13 produced by lymphocytes increase when they are exposed to sialylated HMOs (Eiwegger et al., 2004). Sialylated HMOs also diminish the production of IL-4 in patients with peanut allergy, suggesting they may be capable of preventing allergy (Eiwegger et al., 2010).

Fucosylated HMOs are able to stimulate macrophages and to increases levels of prostaglandin E<sub>2</sub>, IL-10 and TNF- $\alpha$  *in-vitro* (Atochina & Harn, 2005).

Due to all the benefits that HMOs provide to the immune system, it is possible that HMOs might be able to reduce food allergy symptoms by decreasing inflammatory responses and balancing Th1/Th2 cell populations characteristics of allergic reactions; hence the importance of study them for its characterization (Rudloff et al., 1996; Eiwegger et al., 2010).

## **Probiotics**

Microbial intestinal colonization occurs in the early life and it influences the immune system development, maturation, and function. Introduction or changes in probiotic bacterial populations during infancy might protect against allergic reactions (Feleszko et al., 2006).

For a bacteria to be classified as probiotic it must meet specific criteria. They must be able to survive in the GI tract and must confer a benefit to the host; therefore, probiotics are defined as live bacteria that when given in adequate numbers have beneficiary effects on the host (Guarner & Schaafsma, 1998). These beneficial bacteria improve the microbial balance and modulate immune functions (Noverr & Huffnagle, 2004). Bacterial species classified as probiotics are largely as *Lactobacillus* and *Bifidobacterium*; although there are lactic acid bacteria and some non-pathogenic species of *Escherichia coli* that has been classified as probiotics as well (Forsythe & Bienenstock, 2010). Most of these species are commensal and are naturally found in the gut.

There is evidence that probiotics help maintain the gut barrier and diminish signs of inflammation in GI diseases such as colitis, pouchitis, irritable bowel syndrome and food allergy (Giochenti et al., 2003; Kim et al., 2005; O' Mahony et al., 2005). Although the mechanisms of action remain unclear, certain probiotic bacteria induce Tregs which have anti-inflammatory functions because they produce cytokines with immunoregulatory properties, such as IL-10 (Smits et al., 2005). Probiotics also maintain a balance between Th1 and Th2 cells, in which, the drive for Th2 cells and pro-inflammatory cytokines is

attenuated (Torii et al., 2007). Probiotics administration to pregnant and lactating mothers have shown improved immunoprotective effects of breast milk by lowering the immunosuppressive cytokine TGF- $\beta$  and atopic disease (Rautava et al., 2002)

More recently, evidence suggests that certain probiotic bacterium, such as *Lactobacillus rhamnosus* strain JB-1 , stabilize mast cells by inhibiting the membrane potential in the membrane potassium channels (IKCa), which in turn inhibit mast cell degranulation (Forsythe et al., 2012). *L. rhamnosus* JB-1 has also been shown to be immunomodulatory by increasing the population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the spleen and mediastinal lymph nodes of mice. In addition, adoptive transfer of these Tregs in donor mice have led to protective effects such as the ones produced by the bacteria itself (Karimi et al., 2009). Furthermore, *L. rhamnosus* species have been shown to suppress murine allergic airway responses when administrated in the neonatal period (Blümer et al., 2007).

*L. reuteri* has been shown to reduce, colic, constipation and diarrhea in infants, as well as effectively treating *Helicobacter pylori* infections (Francavilla et al., 2014 Gutierrez-Castrellon et al., 2014; Indrio et al., 2013)

### **Microbial transplantations**

Microbiota plays an important role in the immunity of the individual therefore it has been targeted as potential therapy for infections and allergy (Willing et al., 2011).

The “Microflora Hypothesis” of allergic diseases proposes that modifications in the diet of an individual can lead to disruptions of the normal gut microbiota population and functional dysregulations which increases the susceptibility to immunological disorders including allergy (Shreiner et al., 2008). Reductions in the populations of lactobacilli and bifidobacteria in the gut has been linked to increase incidence of allergic diseases (Stsepetova et al., 2007; Suzuki et al., 2007).

The protective effect of certain bacteria has led to determine a microbial-based therapy to protect and treat allergic disorders such as atopic dermatitis, hay fever and asthma (Riedler et al., 2001).

Microbial transplantations are also known as fecal transplantations and the procedure gained recognition as a treatment for *Clostridium difficile* infections (Matila et al., 2012). Faecal microbial transplantations is a procedure consisting in taking stool samples from a donor, mixed with saline and strained with the purpose to isolate the gut bacteria in the donor to transfer it to the recipient (FTF, 2014). This procedure has also been proposed as being potentially beneficial in digestive and autoimmune diseases such as chronic diarrhea, food poisoning, Crohn’s disease, ulcerative colitis and irritable bowel syndrome and it can be potentially used for balancing the immune system in food allergy patients (FTF, 2014; Zhang et al., 2012)



## **HYPOTHESIS**

The immunomodulatory actions of specific prebiotics and/or probiotics will reduce the severity of food allergy in a mouse model.

## **OBJECTIVES OF THIS STUDY**

1. To investigate the effects of prebiotic milk oligosaccharides, 2' FL and 6' SL and probiotic bacteria, *L. reuteri* DSM17938 and *L. rhamnosus* JB-1, in murine models of food allergy
2. To characterize immunomodulatory changes associated with beneficial effects of these treatments in OVA-induced food allergy

## **METHODS (Fig S2)**

### **Human milk oligosaccharides**

Test oligosaccharides were obtained as a gift from Abbott Nutrition (Columbus, OH, USA). HMO "purity" was established by high performance ion chromatography with pulsed amperometric detection (IC-PAD) using relative peak area comparisons. Moisture content was determined separately using the Karl Fischer method for moisture determination. 6'Sialyllactose (6'SL) and 2'Fucosylactose (2'FL) were derived from bacterial synthesis. Endotoxin levels were estimated by limulus assay (Limulus Amebocyte Lysate (LAL) QCL-1000, Lonza catalogue number 50-647U, Wilmington,

MA, USA) and lipopolysaccharide (LPS) 500,000 EU/mg from Sigma (catalogue number L2637, St. Louis, MO, USA) (Table S1).

### **Probiotic bacteria**

*Lactobacillus rhamnosus* JB-1 from in house were grown in Man-Rogosa-Sharpe (MRS) medium, harvested at 48 h, washed in PBS, and stored at -20°C in aliquots of 1.1 ml at 1E10 Colony Forming Units (CFUs/ml). *Lactobacillus reuteri* DSM17938 was supplied by BioGaia AB (Stockholm, Sweden) and were also grown in MRS medium. Probiotic bacteria stored at -20°C in aliquots of 1.8 ml at 5E9 CFUs/ml. Bacterial stocks were thawed at room temperature and centrifuged at 3000 RPM for 5 minutes. Pellet was centrifuged, washed and resuspended 2 more times prior usage.

### **Food allergy model**

Experiments were performed in strict accordance with guidelines of the Canadian Council for Animal Care. Protocols were approved by the Animal Research Ethics Board of McMaster University. Adult male Balb/c mice (20–25 g) were maintained in an automatic light/dark cycle (light periods of 12 h) and provided water and chow ad libitum. Mice were acclimatized to the animal facility for 1 week before experimentation. Age-matched (8–9 week old) animals were used in all experiments.

Mice were sensitized with 50µg of OVA (Sigma, St. Louis, MO) and 2 mg of alum (Sigma, St. Louis, MO) in 200 µl of Phosphate Buffered Saline (PBS) on day 0 and 14 (Figure 1). Treatments of HMOs; 2'FL, 6'SL and lactose (1 mg in 200 µl PBS); and probiotic bacteria, *Lactobacillus reuteri* DSM17938 and *Lactobacillus rhamnosus* JB-1 (1E9 cells in 200 µl); were administrated via oral gavage to OVA sensitized mice from day 27 to day 43 of the protocol. From day 28, mice were orally challenged with 50 mg OVA in saline every 3 days, for a total of six times. On the challenge day, the treatment was administered 1 h before the challenge. Sham-sensitized mice served as controls.

Rectal temperature was measured before and after final OVA challenges using a rectal probe (Physitemp, Clifton, NJ). Diarrhea symptoms were scored by visually monitoring mice for 60 min after challenge as follows [22]: diarrhea – 0, normal stools; 1, a few wet and unformed stools; 2, a number of wet and unformed stools with moderate perianal staining of the coat; 3, severe, watery stool with severe perianal staining of the coat. Serum was obtained 1 hour following the final antigen challenge for measurement of mouse mast cell protease (mMCP-1) and immunoglobulins using commercial ELISA (eBioscience, San Diego, CA).

### **Splenocyte culture for Cytokine measurement**

Spleens were obtained 1 hour following the final antigen challenge and splenocyte suspension prepared as described previously (Yamaki & Yoshino, 2012). A total of  $5 \times 10^6$  cells were cultured for 3 days in RPMI1640 medium containing 10% fetal bovine

serum, penicillin (100 units/ml)–streptomycin (100 µg/ml), 2-mercaptoethanol (50 µM), with or without OVA (100µg/ml) for 72 hours. Supernatants were then collected and stored in -20°C prior to cytokine measurement. IL-6, IL-10, IL-13 and TNF from culture supernatants of splenocytes were assessed by ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA).

### **Culture of mouse bone-marrow derived mast cells**

Bone marrow derived mast cells from male Balb/c mice were cultured for up to 10 weeks in 50 % enriched medium (RPMI 1640 containing 100 U/mL of penicillin, 0.1 mM MEM nonessential amino acid solution, and 10 % fetal bovine serum (FBS)) and 20 % pokeweed mitogen-stimulated spleen cell conditioned medium as a source of IL-3. Non-adherent cells were hemi-depleted twice each week with enriched medium containing the cytokines mentioned above. After 3 weeks, >98% of the cells in the culture were mast cells, as determined by staining with toluidine blue and alcian blue/safranin.

### **Mast cell degranulation assay**

To assess the effect of 2'FL and 6'SL on IgE mediated degranulation of mast cells *in-vitro*, mouse bone marrow derived mast cells ( $1 \times 10^6$  cells/ml) were resuspended in Heps-Tyrode's buffer (HTB) and passively sensitized for 2 hours at 37°C with 100 µg/ml of monoclonal IgE antibody against dinitrophenyl haptenic group (anti-DNP)

(Sigma, St. Louis, MO). Subsequently, mast cells were washed with HTB and incubated for 30 minutes at 37°C with a range of concentrations of 2' FL and 6' SL prior to challenge with DNP conjugated to Human Serum Albumin (DNP-HSA) for 30 mins at the stated concentrations. The release of  $\beta$ -hexosaminidase from mast cells was measured in the cell supernatant. 100  $\mu$ l of supernatant was mixed with 50  $\mu$ l of  $\beta$ -hexosaminidase substrate (80  $\mu$ l of 1mM 4-methylumbelliferyl-N-acetyl- $\beta$ -D-glucosaminide diluted in 5 ml of glycine buffer pH 10.7) for 2 hours at 37°C. The reaction was stopped with 100 $\mu$ l citrate buffer (pH 4.5) and read using CytoFluor 2350 fluorescent spectrophotometer at 450 nm (excitation 356 nm).  $\beta$ -hexosaminidase release was presented as a percentage of the total  $\beta$ -hexosaminidase release determined by cell lysis.

### **Intestinal permeability**

Intestinal permeability was measured after treatments of HMOs; 2'FL, 6'SL and lactose (1 mg in 200  $\mu$ l PBS); and probiotic bacteria, *Lactobacillus reuteri* DSM17938 and *Lactobacillus rhamnosus* JB-1 (1E6 cells in 200  $\mu$ l); were administrated via oral for 5 days. At day 5 of the protocol, 0.6 mg/kg of FITC-Dextran 4000 beads were given to each mouse (at exception of the sham group). Mice were fasted for 4 hours, at the exception of water (Sigma, St. Louis, MO). Serum samples were then obtained after 4 hours and fluorescence was measured at 525 nm (Excitation 490 nm) using a CytoFluor 2350 fluorescent spectrophotometer.

### **Assessment of intestinal mucosal mast cell numbers by immunofluorescent microscopy**

One hour after the 6th oral OVA challenge, the proximal colon was excised and flushed with ice-cold saline. The proximal colon was fixed by immersion in 4% paraformaldehyde (w/v) in 0.1 M sodium phosphate buffer (pH 7.3) at 4°C for 12–18 h. The tissue was washed with 0.01 M PBS (pH 7.3), cryoprotected by infiltration with 30% sucrose in 0.01M PBS and then embedded in OCT compound (Tissue-Tek). Frozen sections (20 µm) were cut at –20°C using a cryostat. The sections were incubated for 30 min with normal donkey serum (1:10; Jackson Immunoresearch Laboratories, West Grove, Pa., USA) to block nonspecific binding of antisera and then washed in 0.01 M PBS. The sections were exposed for 12–18 h to antiserum against mouse mast cell protease (mMCP)-1, a marker of mouse mucosal mast cells (1:5,000; Moredun Scientific, Scotland, UK), washed with 0.01 M PBS, then incubated for 2 h with Texas Red-conjugated sheep anti-donkey IgG (1:200). Sections were then washed in 0.01 M PBS and mounted in sodium carbonate-buffered glycerol (pH 8.6). The immune-stained sections were examined using a fluorescence microscope (Zeiss AxioObserver.Z1). Five non-consecutive sections of jejunum and proximal colon were assessed for each of five mice per treatment group and mMCP-1 positive cells enumerated per unit area in the digital images using Fiji image analysis software.

### **Passive cutaneous anaphylaxis**

To induce the IgE- and mast cell dependent passive cutaneous anaphylaxis (PCA) reaction, mice were injected intra-dermally in the left ear with 0.5 mg of anti-DNP IgE. After 24 h, each mouse received an injection of 10 µg DNP-BSA containing 1% Evans blue via the tail vein. Thirty minutes after challenge, the mice were euthanized and the ears were removed. The amount of dye in the ear was determined colorimetrically after extraction with 1 ml of 1 mM KOH and 9 ml of mixture of acetone and phosphoric acid (5:13). The intensity of absorbance was measured at 620 nm and difference in absorbance between the sensitized and non-sensitized ears calculated for each mouse. To assess the effect of oligosaccharide treatment, mice were administered 2'FL, 6'SL or lactose (1 mg in 200 µl PBS) by oral gavage daily for 5 days, beginning 3 days prior to sensitization or given a single dose of the oligosaccharides i.p (30 mg/kg) 1 hour prior to antigen challenge.

### **Flow cytometry**

Single cell suspensions isolated from Peyer's patches (PP) and mesenteric lymphnodes (MLN) were stained for FACS analyses as described previously (Karimi et al., 2012; Al-Nadawi et al., 2014). Cells were first stained for surface markers including CD4-FITC, CD3-PE-Cy7, CD25-APC (BD Pharmingen, San Diego, CA, USA). Cells were then fixed and permeabilized by BD Cytotfix/Cytoperm reagent (BD Bioscience, Mississauga, ON, Canada) and stained for intracellular expression markers, Foxp3-PerCP-Cy5.5 and IL-10-

PE (eBioscience, San Diego, CA, USA). Data were acquired with FACSCanto (Becton Dickinson, Oakville, ON, Canada) and analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

### **Statistical analysis**

Experimental results are expressed as means  $\pm$  the standard errors of the means. Statistical analyses were performed by means of one-way analysis of variance (ANOVA), followed by Tukeys test for comparing all pairs of groups. Significant differences between two groups were determined using the unpaired Student's *t* test. A statistical software package (GraphPad PRISM™ version 5.0) was used for the analysis. A *p* value of less than 0.05 was considered statistically significant.

### **Microbial transplantations**

Mice were divided into two groups, donors and recipient mice (Fig S3). Treatments of HMOs; 2'FL, 6'SL and lactose (1 mg in 200  $\mu$ l PBS); were administrated to the non-sensitized-non-challenged donor mice via oral gavage for 30 consecutive days. Recipient mice were sensitized with 50 $\mu$ g of OVA (Sigma, St. Louis, MO) and 2 mg of alum (Sigma, St. Louis, MO) in 200  $\mu$ l of Phosphate Buffered Saline (PBS) on day 0 and 14 (Fig. 9).



On day 15 of the protocol, recipient mice were depleted of their gut microbiota by administration of a single dose of streptomycin (20mg/mice) prior first transplantation. Fresh fecal pellets from 3-4 donor mice were collected and placed in 1 mL of transfer buffer (phosphate buffered saline containing 0.05% cysteine HCl (Sigma-Aldrich; Missouri, USA) on ice. The fecal pellets were homogenized by vortexing, centrifuged at 3000 RPM for 2 min and the supernatant was collected and diluted (1:3) in transfer buffer. One hundred  $\mu$ l of diluted fecal supernatant were orally inoculated in recipient mice 6 times prior the first OVA challenge (Willing et al., 2011).

From day 28, mice were orally challenged with 50 mg OVA in saline every 3 days, for a total of six times. On the challenge day, the treatment was administered 1 h before the challenge. Sham mice received PBS only and served as a negative control. Sensitized and PBS fed mice served as OVA control. Rectal temperature was measured before and after the last final OVA challenges using a rectal probe (Physitemp, Clifton, NJ). Diarrhea symptoms were scored by visually monitoring mice for 60 min after challenge as follows [22]: diarrhea – 0, normal stools; 1, a few wet and unformed stools; 2, a number of wet and unformed stools with moderate perianal staining of the coat; 3, severe, watery stool with severe perianal staining of the coat. Serum was obtained 1 hour following the final OVA challenge for measurement of mouse mast cell protease (mMCP-1) using commercial ELISA (eBioscience, San Diego, CA).

## **RESULTS**

### **Preventative effect of HMOs and probiotics in OVA-induced food allergy**

OVA sensitization and challenge lead to diarrhea symptoms that became marked after the fourth challenge (Fig. 2A and 2B). OVA challenge also lead to a dramatic increase in mMCP-1 levels in the serum. Administration of 2' FL and 6'SL markedly reduced OVA-induced diarrhea symptoms and circulating mMCP-1 at the last challenge (Fig. 2A, 2B and 2E). On the contrary, mice administrated with lactose did not alter diarrhea or the release of mMCP-1 induced by OVA.

Treatments with *L. reuteri* DSM17938 but not with *L. rhamnosus* JB-1 lead to a significant decrease in diarrhea symptoms (Figure 2B). Conversely, treatment with *L. rhamnosus* JB-1 reduced circulating mMCP-1 but *L. reuteri* DSM17938 did not (Fig. 2F).

Hypothermia is a common characteristic of anaphylaxis induced by food allergens such as OVA. OVA sensitized and challenged mice presented a significant decrease in core temperature. 2' FL and 6' SL markedly attenuated core temperature drop (Fig. 2C), whereas lactose had no effect on antigen induced hypothermia. Neither treatment with *L. reuteri* DSM17938 nor with *L. rhamnosus* JB-1 significantly altered hypothermia in response to OVA (Fig. 2D).

### **Intestinal mast cell numbers**

The number of mast cell protease positive cells was assessed in the jejunum and colon of OVA sensitized and challenged mice following treatment with the oligosaccharides. As has previously been described there was a significant increase in mast cell (mMCP-1+ve) numbers in both the jejunum and colon of the sensitized and challenged mice compared to control ( $224.6 \pm 32.2$  vs  $53.4 \pm 11.3$  and  $256.3 \pm 36.8$  vs  $10.2 \pm 0.8$  cells/mm<sup>2</sup> in jejunum and colon respectively). Treatment with 2' FL reduced the mast cell hyperplasia in the jejunum by 40% ( $133.9 \pm 27.4$  vs  $224.6 \pm 32.2$  mMCP-1 +ve cells/mm<sup>2</sup>), while 6'SL treatment elicited more dramatic effects, reducing mast cell numbers almost to control levels (Figure 3B). In the colon, a similar pattern was observed with both 2'FL and 6'SL treatments resulting in a significant reduction of mast cell numbers (Figure 3A).

However, Lactose treatment did not significantly alter mast cell numbers compared to OVA sensitized and challenged controls. Likewise, treatment with *L. reuteri* DSM17938 ( $8.720 \pm 2.334\%$ ) and *L. rhamnosus* JB-1 ( $66.58 \pm 5.680\%$ ) significantly reduced mast cell numbers compared to the OVA control ( $4.861 \pm 0.2135\%$ ) in the colon but none of the treatments reduced mast cells in the jejunum (Fig. 3F).

### **Intestinal permeability**

To determine if the mechanism of protection against food allergy involves increased mucosal barrier, intestinal permeability was assessed to identify if HMO and probiotic treatments could block antigen passage to the mucosa and could subsequently inhibit

activation of mucosal mast cells by antigen. Lower emission readings of FITC-dextran beads were identified in the serum of mice treated with *L. reuteri* DSM17938; yet, treatments with 2' FL and lactose presented higher emission suggesting that the use of these as treatments may not decrease but may increase intestinal permeability (Figure 8).

### **Immunoregulation of HMOs and probiotic treatments**

Treatment with HMOs (Fig. 5A-5C) and probiotic bacteria (Fig. 6A-6D) did not alter the production of Th2-dependent IgE, OVA-IgE, OVA-IgG1 and Th1-dependent IgG2A. Nonetheless, 6'SL significantly increased the levels of IgG2A compared to sensitized control ( $209.2 \pm 46.4$  ng/ml vs  $90 \pm 46.4$  ng/ml) (Fig. 5D)

Cytokines released from spleen cells were measured with ELISA to assess if treatment with probiotic bacteria and HMOs could modulate cytokine profile of activated cells. Splenocytes exhibited a different response to OVA exposure. Treatments with 6'SL lead to low TNF- $\alpha$  levels and a three-fold increase in IFN- $\gamma$  production; although, spleen cell response of 2' FL treated mice presented significant lower levels of IFN- $\gamma$  (Fig. 5F). Similarly, treatment with lactose also resulted in low TNF- $\alpha$  levels (Fig. 5E). Treatments with *L. reuteri* DSM17938 significantly increased TNF- $\alpha$ , iL-10 and IL-13 levels but lowered IFN- $\gamma$  compared to the OVA control group (Fig. 6E, 6G, 6I and 6F respectively). In addition, *L. rhamnosus* JB-1-exposed splenocytes secreted lower levels of TNF- $\alpha$  and higher concentrations of IL-10 and IFN- $\gamma$  compared to the OVA group (Fig. 6E, 6G and 6F respectively).

To assess the immunoregulatory effects of probiotics and prebiotic human milk oligosaccharides, induction of Foxp3<sup>+</sup> and IL-10<sup>+</sup> Tregs was assessed from the mesenteric lymph nodes (MLN) and Peyer's patches (PP) from treated mice.

OVA challenged mice exposed higher population of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells in the PP than the non-sensitized controls. However, there was no clear difference in the population of Tregs in mice treated with prebiotics and probiotics neither in the PP nor in the MLN (Fig. 7B, 7C, 7F and 7G). Nonetheless, the population of IL10<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Tregs significantly increased in the PP and MLN of mice treated with 6' SL and 2' FL compared to controls ( $17.0 \pm 0.9\%$  and  $18.5 \pm 1.5\%$  vs  $10.3 \pm 1.5\%$  respectively) (Fig. 7D and 7E); although 2' FL only increased IL10<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Tregs in the PP only ( $14.3 \pm 1.5\%$  vs  $9.4 \pm 0.6\%$ ) (Fig. 7D). Treatment with *L. rhamnosus* JB-1 also increased the population of IL10<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> in the PP and MLN in comparison to *L. reuteri* DSM17938 ( $11.70 \pm 1.175\%$  and  $23.64 \pm 1.266\%$ ), which only increased it in the MLN ( $18.94 \pm 1.417\%$ )(Fig. 7H and 7I respectively).

In addition, separated experiments performed by feeding non-sensitized-non-challenged mice with HMOs showed no effect in the population of Foxp3<sup>+</sup> or IL10<sup>+</sup> T cells in the PP or MLN. On the contrary, feeding non-sensitized mice with *L. reuteri* DSM17938 significantly increased of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells in the PP and MLN compared to the control ( $49.63 \pm 3.667\%$  vs  $32.23 \pm 1.445\%$  and  $44.57 \pm 0.7688\%$  vs  $31.90 \pm 5.5\%$ ) (Fig. S1E and S1F).

### **Mast cell stabilization**

As previously mentioned, mast cells are one of the main effector cells in food allergy and the ability of 2' FL and 6' SL to reduce circulating mMCP-1 levels in the serum triggered us to conduct further investigation of these treatments on mast cells. To determine if these oligosaccharides are capable of mast cell stabilization, experiments in the PCA response were conducted. Feeding of 2' FL or 6' SL reduced the PCA response suggesting the treatments are able to stabilize mast cells systemically. In addition, administration of a single dose (30 mg/kg) of these HMOs i.p one hour prior challenge also significantly reduced the PCA response (Fig 8A and 8B).

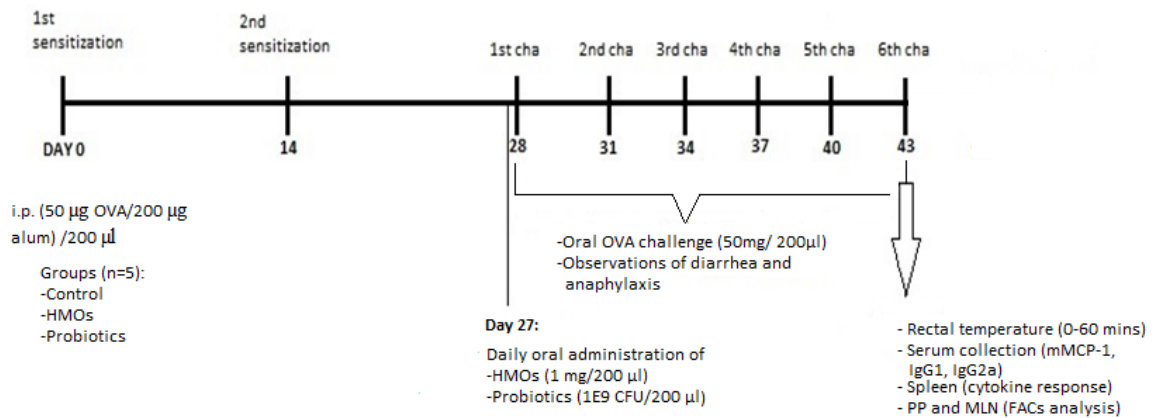
The assessment of degranulation by  $\beta$ -hexosaminidase release is a way to measure mast cell stability. Incubation of BMMC and 6' SL directly inhibited IgE mast cell activation *in-vitro* at relatively high concentrations (1 mg/ml); yet, 2' FL did not have any effect on mast cell stability (Fig. 8C and 8D)

### **Microbial transplantations**

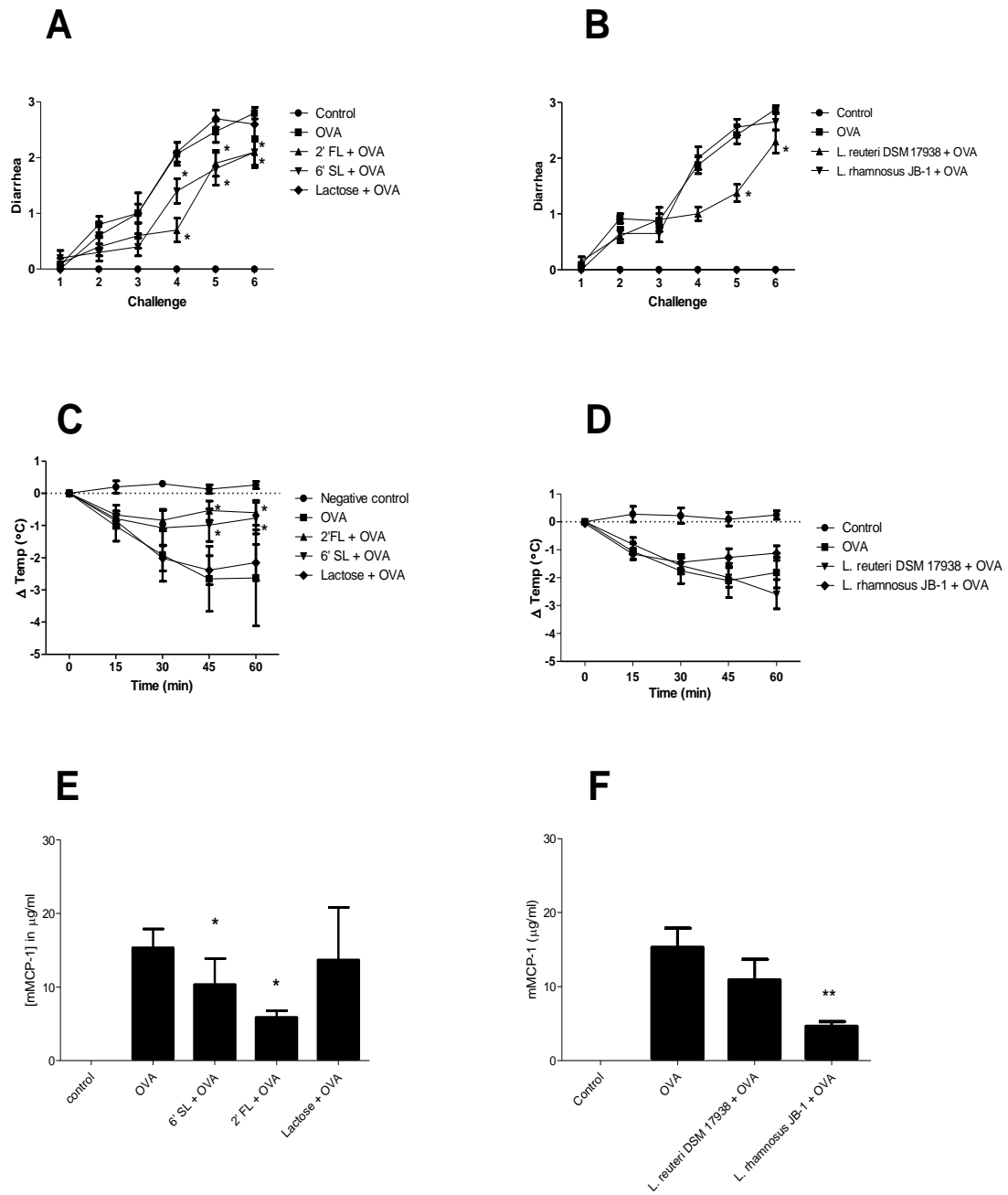
Levels of mMCP-1 in the serum of recipient mice were not affected with the transplantation of gut microbiota from donor mice. At the same time, diarrhea and anaphylaxis symptoms in recipient mice did not show any significant change compared to the controls (Fig 9B-9D).

However, microbiota analysis suggests that the recipient microbiota did not reflect that of the donor indicating that the transfer of microbiota was unsuccessful or incomplete.

## FIGURES AND TABLES

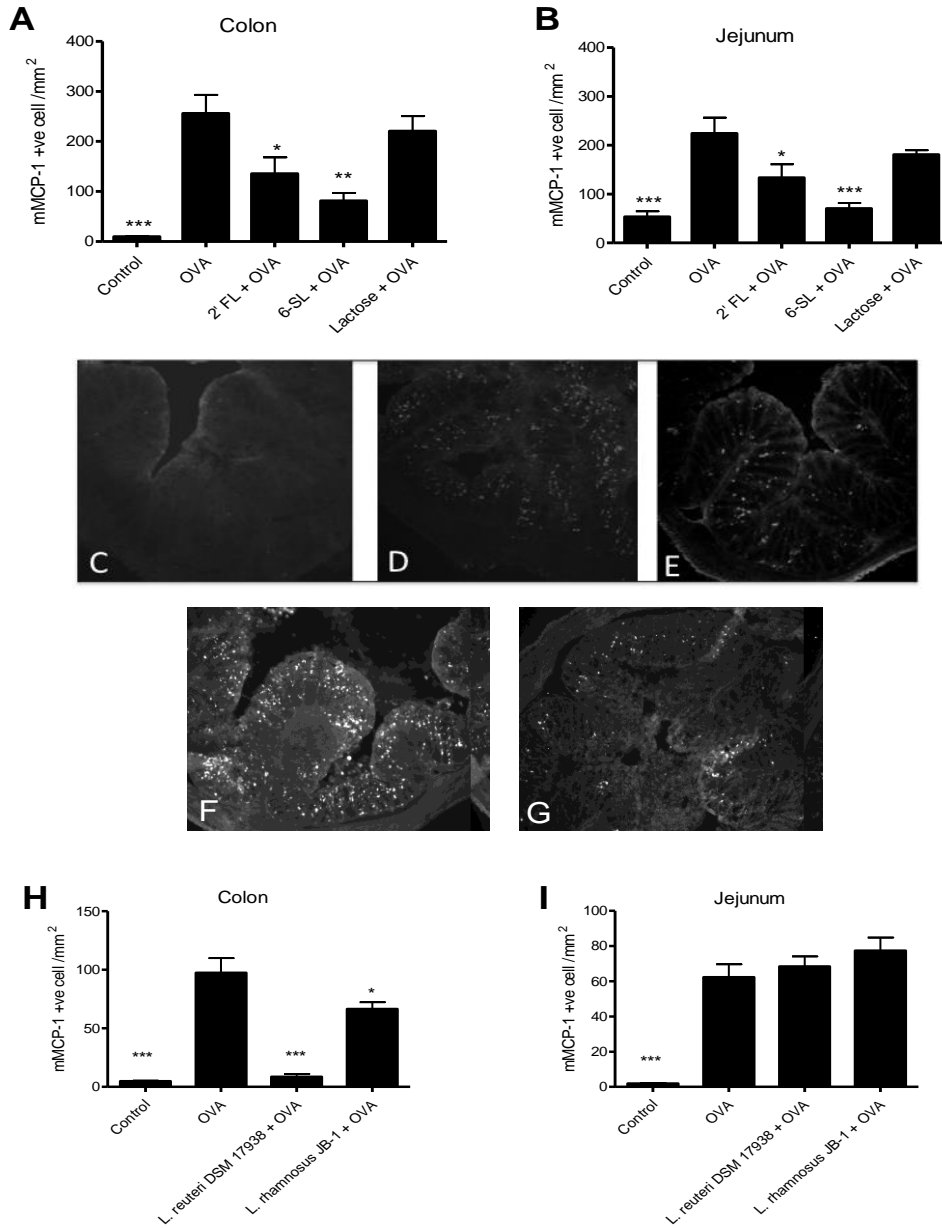


**Figure 1:** Protocol for the food allergy model. Mice were sensitized with 50µg of OVA and 2 mg of alum in 200 µl of PBS, orally challenged with 50 mg OVA and treatments of 2’FL, 6’SL or lactose (1 mg in 200 µl PBS) or probiotics *L. reuteri* DSM17938 and *L. rhamnosus* JB-1 (1E9 CFU in 200 µl PBS) were administrated via oral gavage in saline.

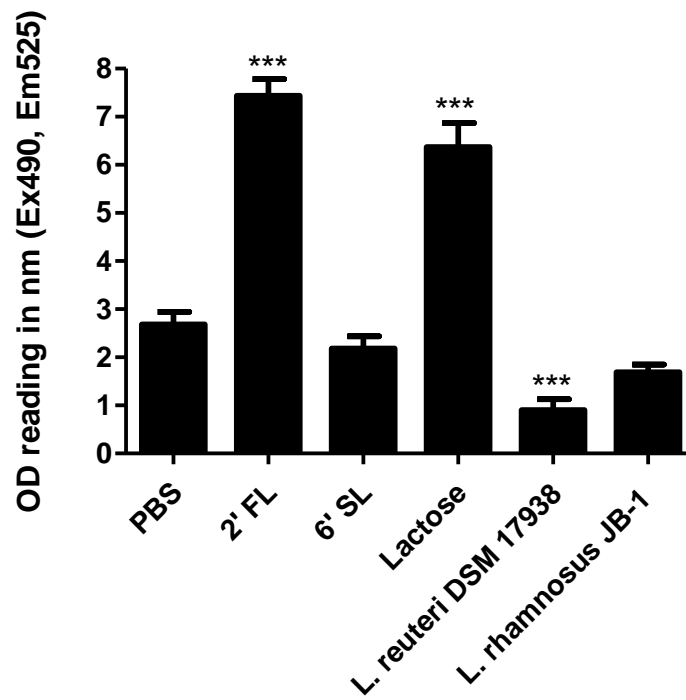


**Figure 2:** The effect of daily oral treatment with 2'FL, 6'SL or lactose or probiotics *L. reuteri* DSM17938 and *L. rhamnosus* JB-1 on diarrhea severity (A, B), rectal temperature (C, D) and serum mast cell protease-1 levels (E, F) following repeated ovalbumin (OVA) challenge in sensitized mice. (n=15, \*=p<0.05 compared to PBS treated OVA sensitized and challenged controls).

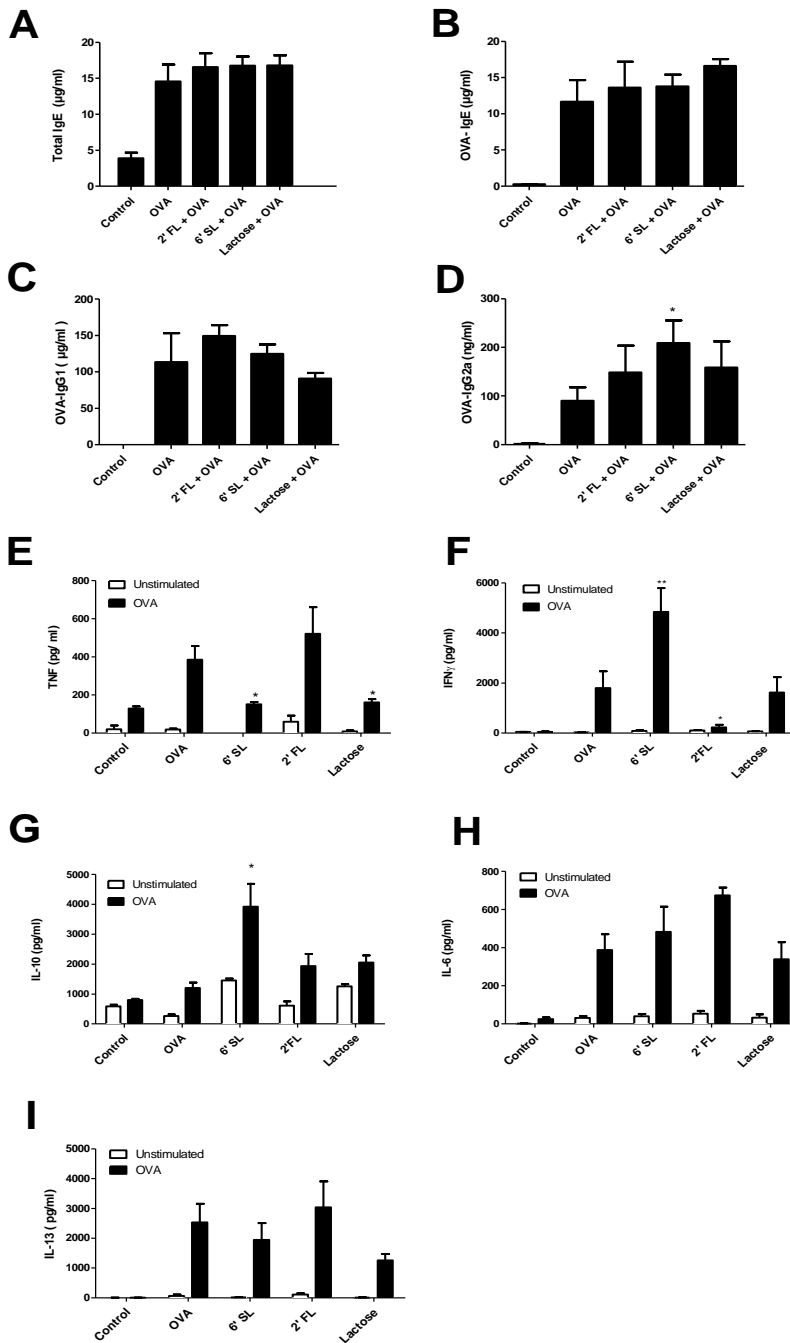




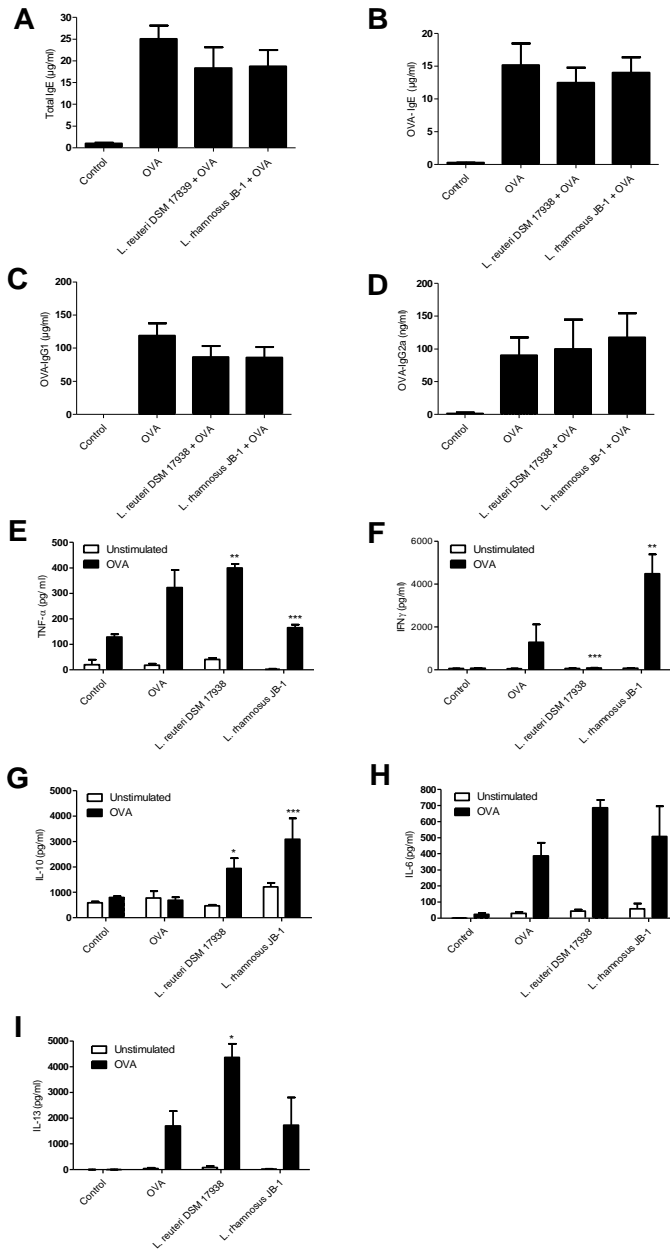
**Figure 3:** The effect of daily oral treatment with 2'FL, 6'SL or lactose or probiotics *L. reuteri* DSM17938 and *L. rhamnosus* JB-1 on mast cell numbers per unit area of the colon (A, H) and jejunum (B,I) of ovalbumin (OVA) challenge in sensitized mice. Representative sections showing mast cell staining in the colon of non-sensitized (C) OVA sensitized (D) 6'SL treated sensitized mice (E) *L. rhamnosus* JB-1 (F) *L. reuteri* DSM17938 (G). (\*= $p < 0.05$ , \*\*= $p < 0.01$ , \*\*\*= $p < 0.001$  compared to PBS treated OVA sensitized and challenged controls).



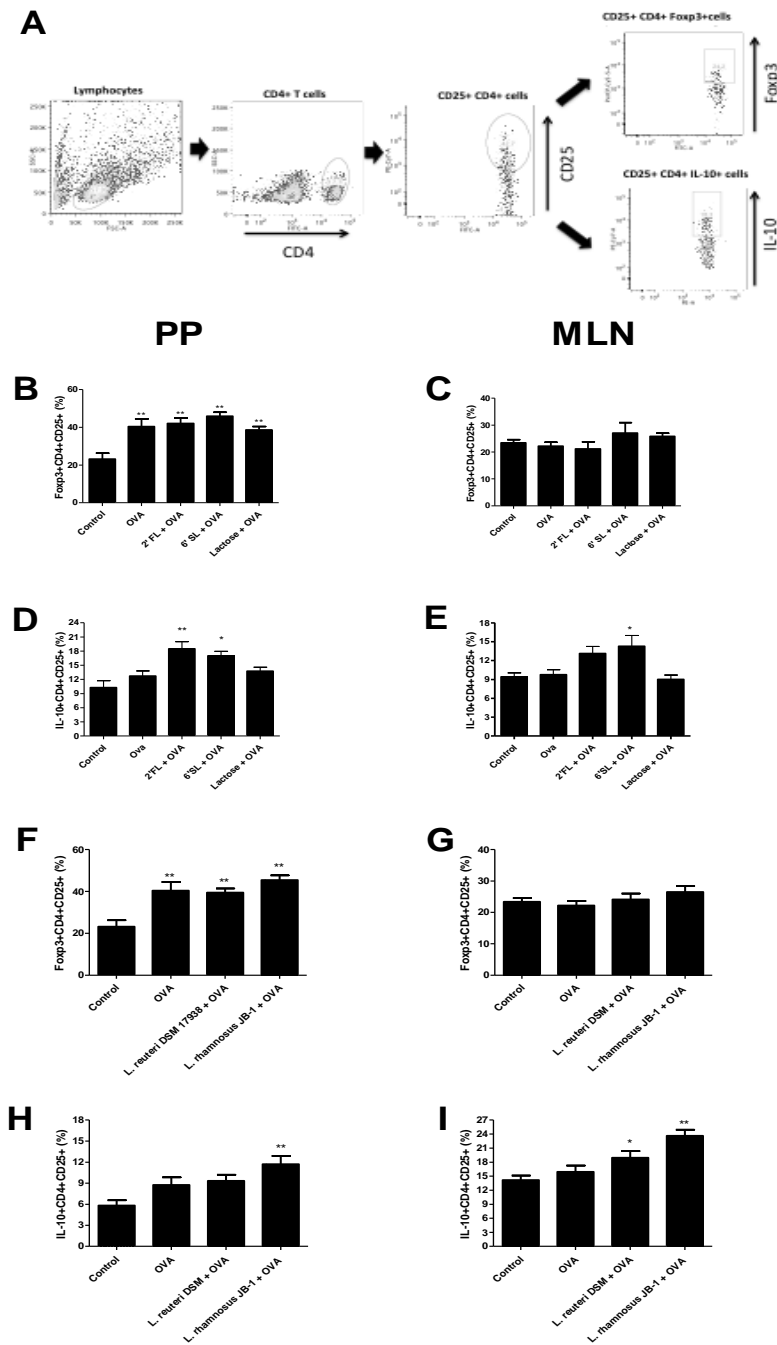
**Figure 4:** The optical reading of FITC-Dextran levels on serum of mice administrated oral treatment with 2'FL, 6'SL or lactose or probiotics *L. reuteri* DSM17938 and *L. rhamnosus* JB-1 in intestinal permeability compared to PBS fed mice (n=10, \*\*\*=p<0.0001)



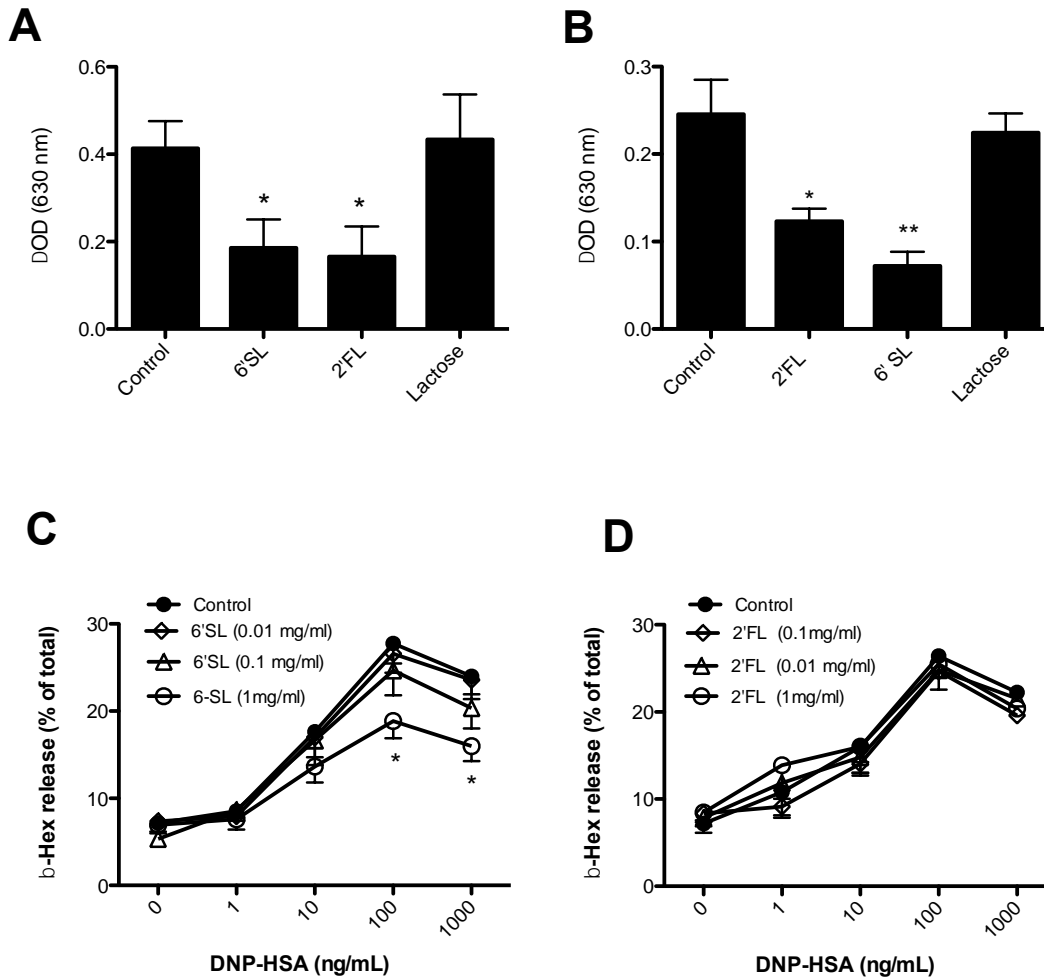
**Figure 5:** The effect of daily oral treatment with 2'FL, 6'SL or lactose on serum levels of total IgE (A) and OVA specific IgE (B), IgG1 (C) and IgG2a (D) following repeated ovalbumin (OVA) challenge in sensitized mice. The baseline and OVA stimulated release of cytokines from isolated splenocytes for each treatment group are also shown (E-I). (n=10, \*=p<0.05 compared to PBS treated OVA sensitized and challenged controls).



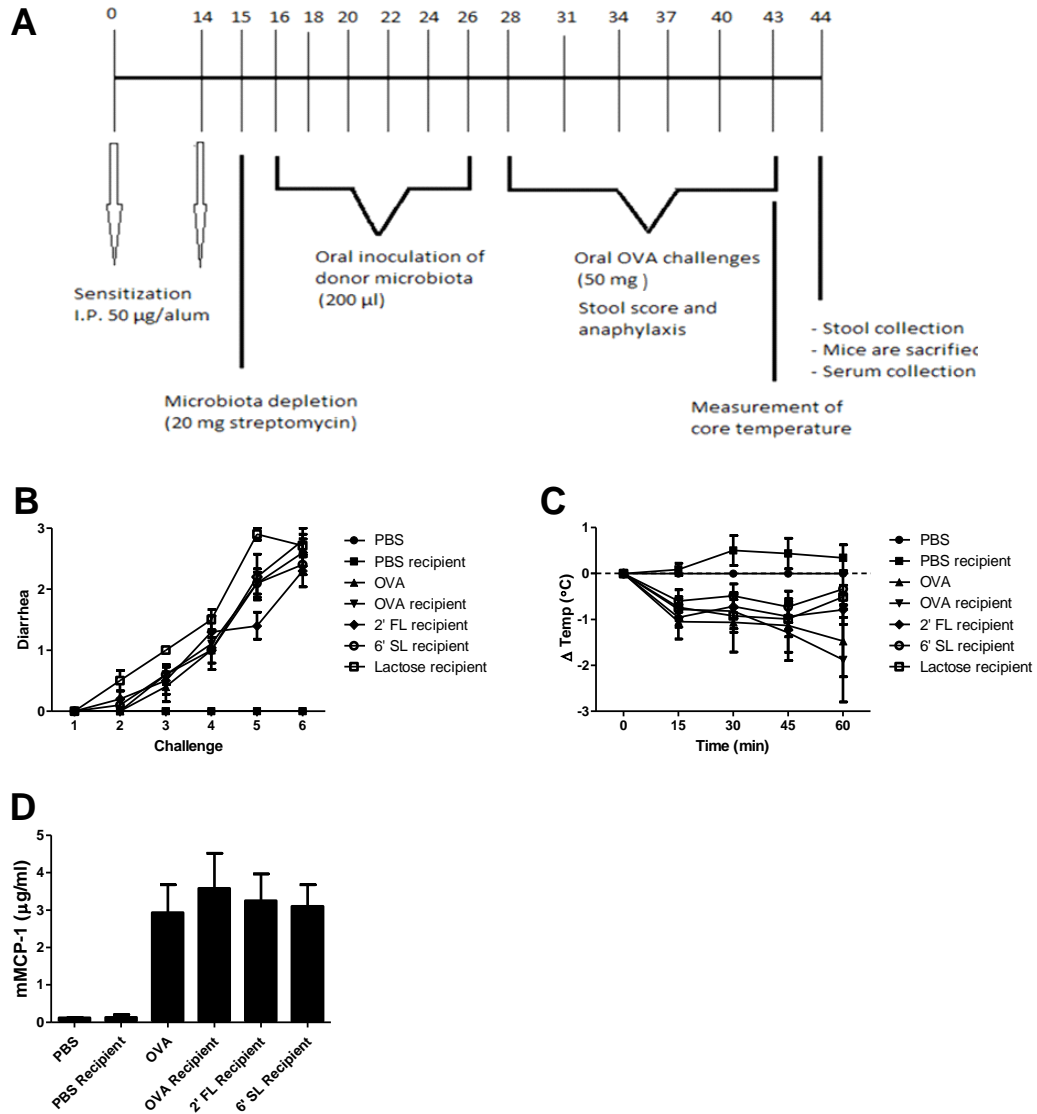
**Figure 6:** The effect of daily oral treatment with *L. reuteri* DSM17938 or *L. rhamnosus* JB-1 on serum levels of total IgE (A) and OVA specific IgE (B), IgG1 (C) and IgG2a (D) following repeated ovalbumin (OVA) challenge in sensitized mice. The baseline and OVA stimulated release of cytokines from isolated splenocytes for each treatment group are also shown (E-I). (n=10, \*p<0.05, \*\*p<0.001, \*\*\*p<0.0001 compared to PBS treated OVA sensitized and challenged controls).



**Figure 7:** The gating strategy (A) and effect of daily oral treatment with 2'FL, 6'SL or lactose on populations of CD4<sup>+</sup>CD25<sup>+</sup>Fosp3<sup>+</sup> (B&C, F&G) and CD4<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> (D&E, H&I) cells in the Peyer's patches (PP) and mesenteric lymph nodes (MLN) of ovalbumin sensitized and challenged mice. (n=10, \*=p<0.05, \*\*=p<0.01 compared to non-sensitized controls).



**Figure 8:** The effect of 5 days oral treatment (A) or single i.p. injection (30 mg/kg) (B) of 2'FL, 6'SL or lactose on the passive cutaneous anaphylaxis response in mice (n=15, \*=p<0.05, \*\*=p<0.01) and the direct effect of 2'FL, 6'SL and lactose on IgE mediated degranulation of mouse bone marrow derived mast cells *in vitro* (n=10, \*=p<0.05 compared to control)



**Figure 9:** Protocol for microbial transplantation (A). The effect of daily oral treatment on diarrhea severity (B), rectal temperature (C) and serum mast cell protease-1 levels (D) following repeated ovalbumin (50 mg OVA) challenge in recipient sensitized mice orally administrated with gut microbiota from non-sensitized donor mice treated with 2'FL, 6'SL, lactose (1 mg in 200 µl PBS) or PBS.

## **DISCUSSION**

### **Prebiotics**

This study demonstrates that specific oligosaccharides found in human milk, 2' FL and 6' SL, are capable of reducing symptoms of food allergy in a mouse model.

As mucosal mast cells are directly involved as the main effectors in food allergy, an increase in number, activation and degranulation can be correlated to antigen-induced diarrhea (Bischoff, 2009; Yamaki & Yoshino, 2012). Individuals suffering food allergy have elevated mucosal mast cell degranulation (Bischoff et al., 1997). This correlates with our results as mucosal mast cell degranulation was also elevated in OVA-induced allergic mice; yet, treatments with 2' FL and 6' SL lowered serum levels of secreted mMCP-1. This suggests 2' FL and 6' SL reduce mediator release from mucosal mast cells. Previous studies have shown reduced allergic disease and acute allergic symptoms such as asthma and atopic dermatitis with the use of plant derived non-digestible oligosaccharides and probiotics as dietary supplementation (Arslanoglu et al., 2008; Moro et al., 2006, Shouten et al., 2009, 2010; van der Aa et al., 2011). This is the first demonstration of protective effects of human milk oligosaccharides in a model of allergy.

When an allergic response is triggered, Ag-specific IgE and mast cells are recruited to the mucosa. Mast cells degranulate and release cytokines and proteases that provoke gut leakage and increase intestinal permeability (Perrier & Cortes, 2011). In this study, treatments with 2' FL and lactose increased permeability. It has been previously reported that formula milk might increase permeability in infants, yet human milk has been shown



to decrease permeability in infants (Shulman et al., 1998; Taylor et al., 2009; Rouwet et al., 2002). Westerbeek et al., (2010) demonstrated that treatments with a mixture of fructo- and galacto-oligosaccharides did not enhance the postnatal decrease in intestinal permeability in pre-term infants. However, the oligosaccharides tested are not found in human milk. Therefore, we hypothesized that specific oligosaccharides found in human milk may reduce intestinal permeability as reported breast feeding; yet our results showed that 2' FL and lactose increased intestinal permeability while 6' SL had no effect; suggesting that additional factors in human milk are responsible for decreasing intestinal permeability. Decreased permeability obtained in this experiment does not explain the protective effect of the oligosaccharides in food allergy, as 2' FL increased permeability but decreased OVA-induced allergy symptoms.

Treatment with 6' SL did significantly increase levels of IgG2. IgG2a is induced through the action of Th1 cells in mice in response to stimuli (Lefeber et al., 2013). IgG2a has high affinity to Fc receptors and might compete with FcεRI receptors in mast cells or might be able to intercept allergen before IgE binding though acting as blockers and inhibiting IgE-mediated allergy as demonstrated by Strait et al., (2006). These results may suggest a shift to a Th1 type response as Th1-producing IFN-γ levels were elevated. In addition, spleen cells exposed to OVA from mice treated with 6' SL produced higher levels of IL-10 and IFN-γ while decreased TNF levels also suggesting that 6' SL might be able to modulate T cell response towards Th1 profile. The data obtained in this study is supported by studies with the milk oligosaccharide lacto-*N*-neotetraose where IL-10

levels were increased and inflammatory cytokines were decreased in peritoneal cells (Terrazas et al., 2001).

While it has previously been shown that lactose has prebiotic and immunomodulatory activity, a decrease in TNF production from OVA stimulated splenocytes was the only significant effect of lactose treatment observed in this study (Daly et al., 2014; Paasela et al., 2014).

In this study, OVA sensitization lead to an increase of  $CD4^+CD25^+Foxp3^+$  T cells in the Peyer's patches of treated mice compared to the control but there was no change following milk oligosaccharides treatments. However, treatments with milk oligosaccharides did increase  $CD4^+CD25^+IL-10^+$  cell population in the Peyer's patches of 2' FL and 6' SL treated mice; and in the mesenteric lymph nodes of 6' SL treated mice. Hence it is suggested that the treatments might enhance a T regulatory response in OVA-induced food allergy. Increased  $CD4^+CD25^+IL-10^+$  cell population in this study is supported by previous reports on suppression of allergic inflammation by Tregs via IL-10 production and the induction of IL-10 by regulatory T cells (Kearley et al., 2005; Zhang et al., 2014).

HMOs can cross the epithelial barrier to act directly on immune cells (Eiwegger et al., 2010). Therefore it was hypothesized that 2' FL and 6' SL would directly act on mast cells for their stabilization. Our results showed that 6' SL inhibited IgE-activation and degranulation of bone marrow derived mast cells *In-vitro* but only at very high concentrations (1mg/ml), meanwhile 2' FL had no effect on bone marrow derived mast

cells *In-vitro*. In contrast, 2' FL and 6' SL inhibited PCA response suggesting indirect inhibition of IgE-mast cell activation systemically possibly through involvement of other immune cells. During an allergic response, Tregs migrate to the site of mast cell activation and may be able to regulate mast cells as shown by Kashyap et al., (2008). IL-10 can inhibit the expression of FcεRI receptor and mast cell activation. Furthermore, Tregs inhibit IgE receptor expression by direct contact through OX40/OX40L (Gillespie et al., 2004; Gri et al., 2008). Therefore, it is possible that mast cell stabilization in the PCA response was by means of IL-10<sup>+</sup> Tregs as IL-10<sup>+</sup> Tregs population was increased with 2' FL and 6' SL treatments. It is also suggested that 2' FL and 6' SL might act on the PCA response independently of any prebiotic effect on bacterial growth as mast cells were stabilized with the administration of these milk oligosaccharides within an hour prior challenge. The decreased serum mMCP-1 levels observed following antigen challenge in 2' FL and 6' SL treated animals may be due to a combination of indirect mast cell stabilization as shown in the PCA, and mast cell activation as lowered numbers of activated mast cells were present in the colon.

Attempts to determine a causal relationship between microbiota changes induced by 2' FL and 6' SL and the attenuation of allergy symptoms failed as symptoms of OVA-induced allergy were not altered by microbial transplantations in this study. However, donor gut bacteria was not established sufficiently in the recipient mice so no firm conclusion can be drawn from this experiment. Development of a more efficient transfer methodology with a mixture of antibiotics for deletion of recipient microbiota and an

increased number of microbial transfers is used to increase microbial transplantation establishment.

### **Probiotics**

Studies using certain putative probiotic strains have shown the potential of these organisms to modulate immune responses involved in inflammatory and allergic diseases (Noverr & Huffnagle 2004; Özdemir et al., 2010). Ingestion of probiotic bacteria also modulates gut motility and might be able to provide benefits to gastrointestinal diseases (Wu et al., 2013). Our study demonstrated that *L. reuteri* DSM17938 significantly reduced diarrhea in the food allergy model.

These are in concordance with previous studies showing the ability of *L. reuteri* species to reduce frequency and duration of diarrhea episodes in preschool children (Gutierrez-Castrellon et al., 2014). In specific, *L. reuteri* DSM17938 has been reported to increase bowel frequency in infants suffering chronic constipation, reduce colic in infants and reduce the frequency and duration of acute diarrhea in children (Coccorulo et al., 2010; Francavilla et al., 2012; Savino et al., 2010). Yet, *L. reuteri* DSM17938 have no effect in nosocomial induced diarrhea in children (Wanke et al., 2012). Studies done in colon and jejunum exposed to *L. reuteri* DSM17938 showed that the frequency and velocity of propulsion of gut motility is decreased in the jejunum but increased in the colon (Wu et al., 2013). Therefore, *L. reuteri* DSM 17938 might act differently depending on the type and severity of gastrointestinal diseases and the modulation provoked in colon might differ from the jejunum.

It has also been reported that *L. rhamnosus* species may reduce diarrhea or constipation yet the strain JB-1 had no effect on the reduction of OVA-induced diarrhea (Moayyedi et al., 2010). The inability of *L. rhamnosus* JB-1 in reducing diarrhea could be related to previous findings where *L. rhamnosus* JB-1 increases the velocity of gut motility in the jejunum, therefore increasing diarrhea episodes (Wu et al., 2013). In addition, *L. rhamnosus* JB-1 was unable to decrease hypothermia. However the level of hypothermia even in positive controls in these experiments was very low suggesting it may not be a reliable parameter in this model. The reason for reduced hypothermia in positive controls for this set of experiments compared to previous experiments with prebiotic oligosaccharides is unknown. Though, diarrhea is a better indicator of the severity of food allergy in this particular model, any beneficial inhibitory effect of *L. rhamnosus* JB-1 in OVA-induced diarrhea could have been masked by the ability of this particular strain in increasing gut motility in mice (Wu et al., 2013).

Mucosal mast cell degranulation was also elevated in OVA-induced allergic mice yet treatments with *L. rhamnosus* JB-1 lowered serum levels of secreted mMCP-1. This suggests that *L. rhamnosus* JB-1 is able to inhibit mucosal mast cell activation. Forsythe et al., (2012) have previously demonstrated the ability of *L. rhamnosus* JB-1 to stabilize mast cells in a rat model. Furthermore, the results of this study also concord to previous studies where certain probiotic bacteria reduced clinical allergic manifestations and lowered serum mMCP-1 in mice (Kim et al., 2005; Sagar et al., 2014).

Both of the probiotic treatments decreased mast cell hyperplasia in the colon. This was particularly marked following treatment with *L. reuteri* DSM17938, however neither

strain significantly altered mast cell numbers in the jejunum. It has been previously demonstrated a reduction of mast cell hyperplasia in the colon through the action of probiotic bacteria (Schouten et al., 2008). Oksaharju et al., (2011) also demonstrated that *L. reuteri* DSM17938 is capable of down-regulate the expression of the allergy related high affinity IgE receptor subunits  $\alpha$  (FCER1A) and  $\gamma$  (FCER1G) and histamine H4 receptor in human peripheral blood-derived mast cell.

In addition to reducing mast cell numbers in the colon, *L. reuteri* DSM17938 also decrease permeability in the gut. This result concur in a study has been shown previously the ability of *L. reuteri* strains in decreasing gut permeability rats, humans and in piglets fed with formula milk (Forsyth et al., 2009; White et al., 2006; Yang et al., 2015). The means of these are by inducing the expression of tight junction proteins claudin-1 and occludin ZO-1 in the intestinal epithelium (Yang et al., 2015).

Treatments with *L. reuteri* DSM17938 and *L. rhamnosus* JB-1 had no significant effects on levels of IgE and OVA specific IgE, IgG1 and IgG2a in sensitized mice in this Ova-induced allergy model. However, spleen cells exposed to OVA from mice treated with *L. rhamnosus* JB-1 produced higher levels of IL-10 and higher IFN- $\gamma$  with decreased TNF levels suggesting that *L. rhamnosus* JB-1 might be able to modulate T cell response towards Th1 profile. Likewise, studies using *L. rhamnosus* species as treatments have shown increased levels of IL-10 and IFN- $\gamma$  with decrease TNF- $\alpha$  in respiratory infections and allergy (Marschan et al., 2008; Prescott et al., 2008; Thomas et al., 2011; Villena et al., 2012).

*L. reuteri* DSM17938 treatment showed variable results in the production of cytokine levels in this study. *L. reuteri* DSM17938 increased the production of the immunoregulatory cytokine IL-10 and effectively reduced OVA-induced diarrhea, mast cell hyperplasia in the colon and intestinal permeability. In addition, *L. reuteri* DSM 17938 failed to decrease the pro-inflammatory cytokine TNF- $\alpha$  in this food allergy model, yet studies using *L. reuteri* DSM 17938 reduced TNF-  $\alpha$  levels in rats with LPS-induced intestinal inflammation (Liu et al 2010). In contrast to treatments with HMOs *L. reuteri* DSM 17938 decreased the Th1 derived cytokine IFN- $\gamma$  which have also been reported to decrease IFN- $\gamma$  levels in the intestine of rats with induced intestinal inflammation (Liu et al., 2010).

Certain putative probiotic bacteria are able to induce Tregs population and regulatory cytokines, such as IL-10 (Kanjarawi et al., 2011; karimi et al., 2012; Schouten et al., 2010; Smits et al., 2005). OVA sensitization lead to an increase of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in the Peyer's patches of treated mice compared to the control but probiotic treatments did not modulate this response. However, only the *L. reuteri* DSM17938 and *L. rhamnosus* JB-1 treatment groups showed significantly increased CD4<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> cell population in the mesenteric lymph nodes compared to the controls. Hence, it is suggested that the treatments might enhance a T regulatory response against food allergy but each of them act differently depending on the disease and location in the intestine. Increased CD4<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> cell population in this study is supported by previous reports on suppression of allergic inflammation by Tregs via IL-10 production and the induction of IL-10 by other immune cells (Kearley et al., 2005; Zhang et al 2014). In

addition, it has been shown that IL-10-producing cells is increased in Foxp3<sup>-</sup> Treg population, but not in Foxp3<sup>+</sup> Treg population of mice treated with the probiotic *Bifidobacterium breve* (Jeon et al., 2012).

## **CONCLUSION**

These studies suggest that 2' fucosyllactose and 6' sialyllactose reduce the symptoms of food allergy perhaps through the induction of IL-10<sup>+</sup> regulatory T cells and indirect stabilization of mast cells, though *L. reuteri* DSM17938 reduced diarrhea and mucosal mast cell hyperplasia in the colon. Therefore it is suggested that *L. reuteri* DSM17938 may act more locally in the colon during food allergic responses. *L. rhamnosus* JB-1 did not reduce food allergy symptoms while also induced IL-10<sup>+</sup> regulatory T cells and stabilized mast cells indirectly; thus, human milk oligosaccharides may have greater therapeutic potential in food allergy than the probiotic treatments tested.

Future studies may test the functionality of regulatory T cell to add further support to their role in prebiotic mediated protection against allergy. Enhance ability of these cells to modulate mast cells would also support their role in the observed indirect inhibition of mast cell degranulation. At the same time, evaluate the effects of 2' FL and 6' SL in the population of CD11c<sup>+</sup>CD103<sup>+</sup> dendritic cells as they have been proven to induce systemic oral tolerance of food allergens through the induction of regulatory T cells (Oozer et al., 2013).

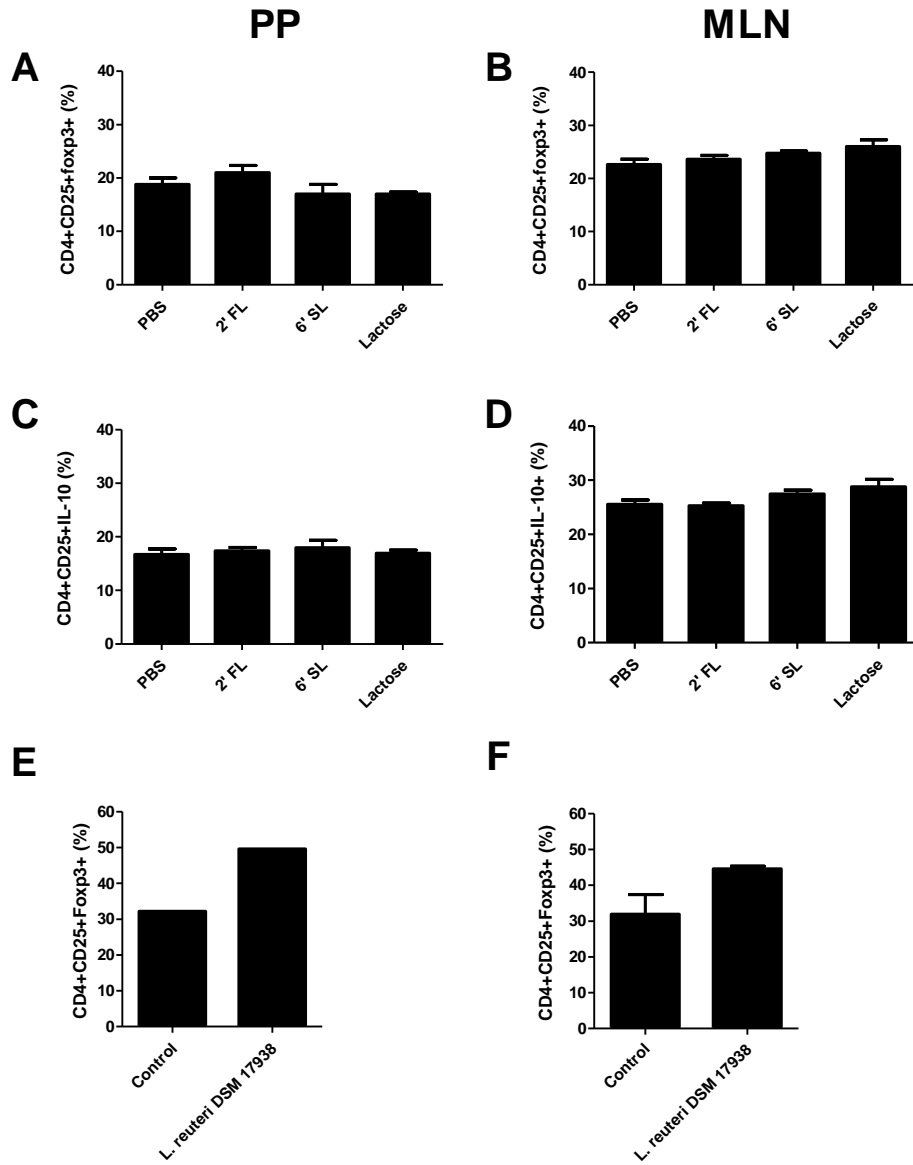


**SUPPLEMENTARY**

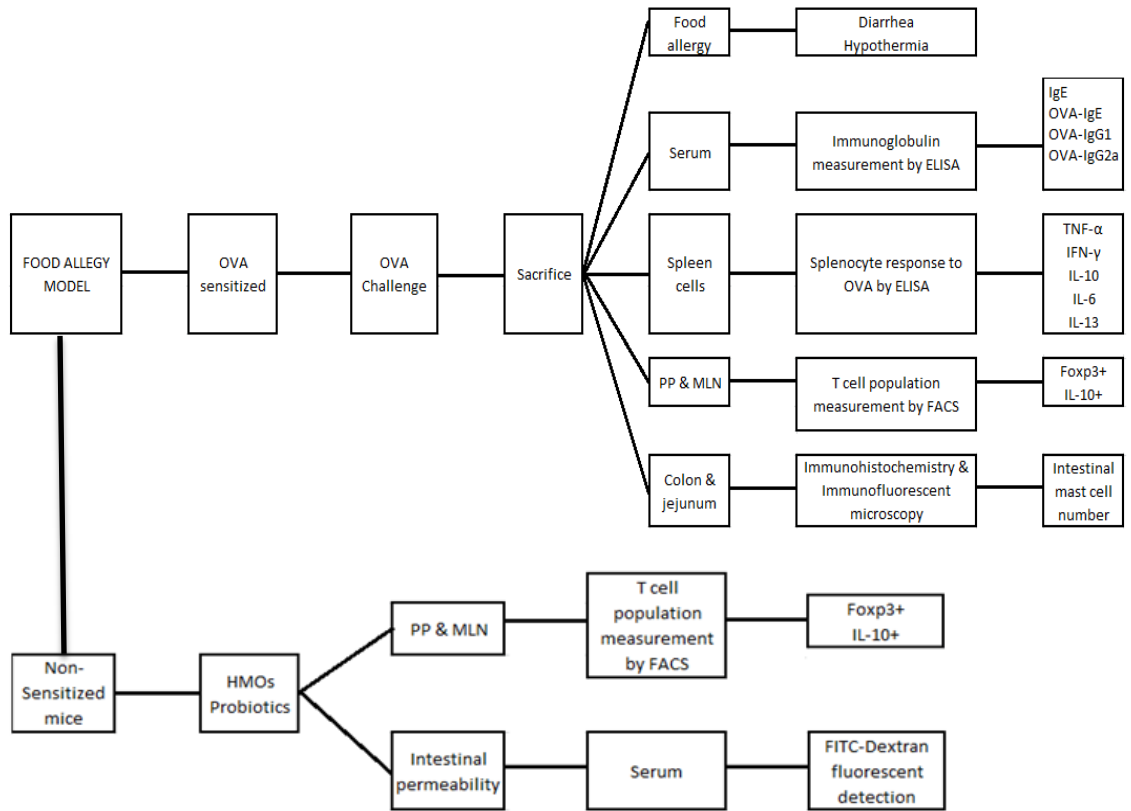
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<b>Saccharides</b>	<b>% Purity</b>	<b>Endotoxin EU/mg</b>
Lactose	99 %	0.002
2'-Fucosylactose (2'FL)	95.3 %	0.375
6'Sialyllactose (6'SL)	96.6 %	0.496

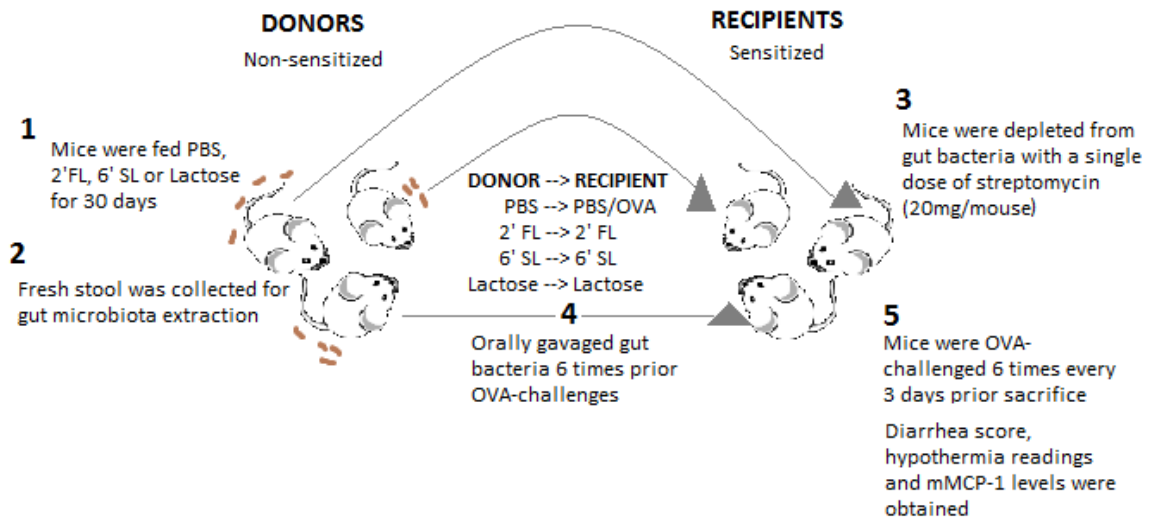
**Table S1.** Characteristics of saccharides utilized in the study



**Figure S1:** The effect of daily oral treatment (10 days) with 2'FL, 6'SL, lactose and *L. reuteri* DSM17938 on populations of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (A,C, E) and CD4<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> (B, D, F) in the Peyer's patches (PP) and mesenteric lymph nodes (MLN) of non-sensitized mice.



**Figure S2:** Schematic representation of the food allergy protocol and techniques used in this study.



**Figure S3:** Schematic representation of the microbial transplantation protocol.

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