Probiotic modulation of mast cells *in vitro*

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List of Abbreviations

AHL – Acyl homoserine lactone

APC – Antigen-presenting cell

BB - *Bifidobacterium bifidum*

BI - *Bifidobacterium infantis*

DNP-HSA – Dinitrophenyl-human serum albumin

DSM - *Lactobacillus reuteri*

FBS – Fetal Bovine Serum

Ig – Immunoglobulin

IL – Interleukin

JB-1 - *Lactobacillus rhamnosus* JB-1

LH - *Lactobacillus helveticus*

LPS – Lipopolysaccharide

MV – Microvesicles

OMV – Outer membrane vesicles

OVA – Ovalbumin

PAMP – Pathogen-associated molecular pattern

p-NAG - P-nitrophenyl N-acetyl-β-D-glucosamide

SCF – Stem Cell Factor

TLR – Toll-like receptor

TNFα – Tumour necrosis factor alpha

Chapter 1: Introduction

1.1 Food Allergy

Food allergy is a widespread condition that lowers the quality of life for individual sufferers, with differing degrees of severity. Food allergy occurs as result of immune hypersensitivity in response to substances in food that are normally tolerated, subsequently resulting in a response that may produce uncomfortable symptoms such as diarrhoea, or even life-threatening symptoms such as airway closure due to inflammation. While the exact cause of food allergy is unclear, many factors appear to contribute to the disease, such as genetic background, diet and environmental exposure to antigens. The prevalence of food allergy, particularly among those below 17 years of age, has increased in recent years (Sicherer and Sampson, 2014). Currently, no effective therapies exist to treat food allergies, and affected individuals must largely rely on avoidance of food allergens and managing symptoms in the case of accidental exposure.

Risk factors are widespread with respect to food allergy, including race, maternal care, dietary fat consumption and exposure to microbes (Sicherer and Sampson, 2014). An elevated level of circulating IgE antibodies may be an indication of increased risk for the development of allergies. IgE antibodies are a mediator of mast-cell activity and have been used to predict food allergy severity in children with peanut allergies, showing a strong association between IgE levels and allergy severity (Neuman-Sunshine et al., 2012). Early environmental exposure to microbes is also believed to contribute to the risk of allergy development, in which it is theorized that limited environmental microbe exposure may increase the risk of developing allergies; the so-called hygiene hypothesis (Schaub et al., 2006).

The management of food allergy symptoms differ based on severity of allergy and symptoms. In severe cases, individuals must carry injectable epinephrine in the form of an Epi-pen, used to transiently treat anaphylactic shock before seeking immediate medical attention. By intervening the process of inflammation before symptoms arise, potentially life-threatening situations caused by accidental exposure to food allergens may be avoided, and subsequent costs to the individual and health care system.

Food allergy symptoms result from several immunological responses upon exposure to food allergens. Upon initial exposure, antigen-presenting cells (APC) such as dendritic cells detect the antigen. Dendritic cells then signal for B-cells to differentiate into plasma cells and produce antigen-specific IgE antibodies. These IgEs will bind to FcεRI receptors on mast cells to initiate degranulation (Galli and Tsai, 2012). T-cells also recognize the antigen presented by APCs and subsequently provide signals to B-cells in the form of the first signal, interleukin (IL)-4 and IL-13 to activate transcription of immunoglobulin genes (Prussin and Metcalfe, 2006). The second signal is the ligation of CD40 on B cells, activating DNA switch recombination, switching the isotype of immunoglobulins, such as IgE, or IgA that is produced by B-cells (Prussin and Metcalfe, 2006). Food allergy primarily acts through mast cells, being the sentinels of the immune system, as the IgE cross-linking cascade is necessary to mount the immunological response to food antigens. There are several models of food allergy, which include the use of mast cells *in vitro* and animal models of food allergy. Potential therapies for food allergy may be used in these model systems to determine if there is an ameliorating effect on the presence of inflammation or symptoms.

1.2 Models of Food Allergy

1.2.1 Mast Cells

Mast cells are granulocytes that do not recognize antigens directly, instead bind antigen-specific IgE antibodies by their constant regions by the extracellular FcεRI receptor. Mast cells are found throughout the body, but are particularly concentrated in areas that interact with the external environment, such as the mucosa of the lungs, skin and intestines and are not found in circulation. Two types of mast cells exist, mucosal and connective tissue mast cells (Amin, 2012). The two types of mast cells can be differentiated visually by their staining patterns, such that mucosal mast cell granules stain blue with copper phthalocyanin dyes, while connective tissue mast cell granules stain red (Amin, 2012). The two types of mast cells differ from one another based on the proteases contained in their granules as well as in their structure and function (Amin, 2012). Connective tissue mast cells contain both tryptase and chymase proteases, while mucosal mast cells contain only chymases (Abraham and St John, 2010). Mucosal mast cells in the gut lie in close proximity to enteric neurons, and consequently may alter their firing patterns and affect gut motility (Barbara et al., 2004). Connective tissue mast cells may be located superficially, such as in the epithelium lining the airways and gut or in deeper structures such as the smooth muscle layer (Oskeritzian et al., 2005). This allows mast cells to be exposed to pathogens that make direct contact with regions of the body exposed to the external environment such that a quick immune response can be mounted.

Mast cells respond to many signals that may indicate the presence of pathogens or pathogenic substances in the body, hence they are referred to as the sentinels of the immune system. Their most notable role is the recognition of antigen-specific IgE and

IgA antibodies produced by B-cells or plasma cells upon the first exposure to an antigen, eliciting the immune response by means of degranulation and cytokine release. Activated T cells also have been shown to cause mast cell degranulation upon direct contact possibly through activated microparticles produced by T cells involving the MAPK signalling pathway (Shefler et al., 2010). In addition, mast cells express toll-like receptors (TLRs) that recognize pathogen-specific particles directly such as lipopolysaccharide from gram negative bacteria, inducing different responses based on which specific TLR receptor is bound by a ligand (Abraham and St John, 2010). This pathway enables mast cells to initiate a response to the presence of pathogens and recruits other immune cells to defend the host against disease.

Mast cells also respond to quorum-sensing molecules produced by bacteria, as is the case with N-3-oxo-dodecanoyl-L-homoserine lactone, belonging to a class of acyl homoserine lactones (AHL), produced by *Pseudomonas aeruginosa* (Khambati et al., 2016). AHLs have been shown to activate downstream effectors associated with bitter taste transduction, the same means by which irritants are detected in the airway (Tizzano et al., 2010). Bitter taste receptors were once thought to be expressed exclusively on the tongue, however they have been found in other tissues of the body including on mast cells. Adding agonists of the TAS2R receptor to mast cells *in vitro* inhibited IgE-dependent mast cell activation (Ekoff et al., 2014). Taken together, many ligands may alter mast cell signalling and ultimately affect their ability to activate in the presence of antigens.

Antigen-specific IgE antibodies are the most notable mast cell activator and primarily mediate food allergy symptoms. IgE antibodies differ in their variable regions

for specific antigens and are synthesized during an allergic response or initial sensitization. During sensitization to an antigen, the antigen is taken up by dendritic cells or other antigen-presenting cells such as B-cells (Galli and Tsai, 2012). IgE antibodies are produced by plasma cells or other antigen-presenting cells upon the first exposure to an antigen and are produced during subsequent exposures (Galli and Tsai, 2012). Sensitization, or the first exposure is asymptomatic, while symptoms develop upon subsequent exposures. Mast cells recognize antigen-bound IgE antibodies by FcεRI receptors on their surface and undergo crosslinking when two IgE antibodies are linked together by a single antigen molecule, thereby initiating the inflammatory cascade (Burton and Oettgen, 2011; Galli et al., 2011). IgE is a relatively short-lived molecule in its free form in the serum, however its stability increases upon binding to mast cells, making this a potentially therapeutic target for the treatment of food allergies (Fried and Oettgen, 2010).

Upon activation, mast cells undergo degranulation, in which storage granules inside the cell release their contents into the extracellular environment (Metcalfe et al., 2009). Factors such as histamine, and proteases are released from mast cells to recruit and activate surrounding immune cells to mount a response (Figure 1).

Histamine is a mediator of vascular permeability, vasodilation and is responsible for producing the flu-like symptoms associated with airborne allergies to dust and pollen (Metcalfe et al., 2009). Histamine also interacts with enteric neurons, binding to H_1 and H_2 receptors and thereby altering neuronal firing patterns (Barbara et al., 2004), along with tryptase, which also alters enteric neuron firing by its proteolytic activity. Mast cell activation also relies on the increase of intracellular calcium concentration, such that an

influx of calcium or release of intracellular stores elicits mast cell degranulation (Pearce, 1985). Consequently, mast cell activation has profound effects on gut activity and thereby may produce gastrointestinal distress during food antigen-associated inflammation. β-hexosaminidase is another enzyme that is released, involved in glycoprotein metabolism and cellular homeostasis, and can be measured as an index of mast cell degranulation and activation (Fukuishi et al.,2014).

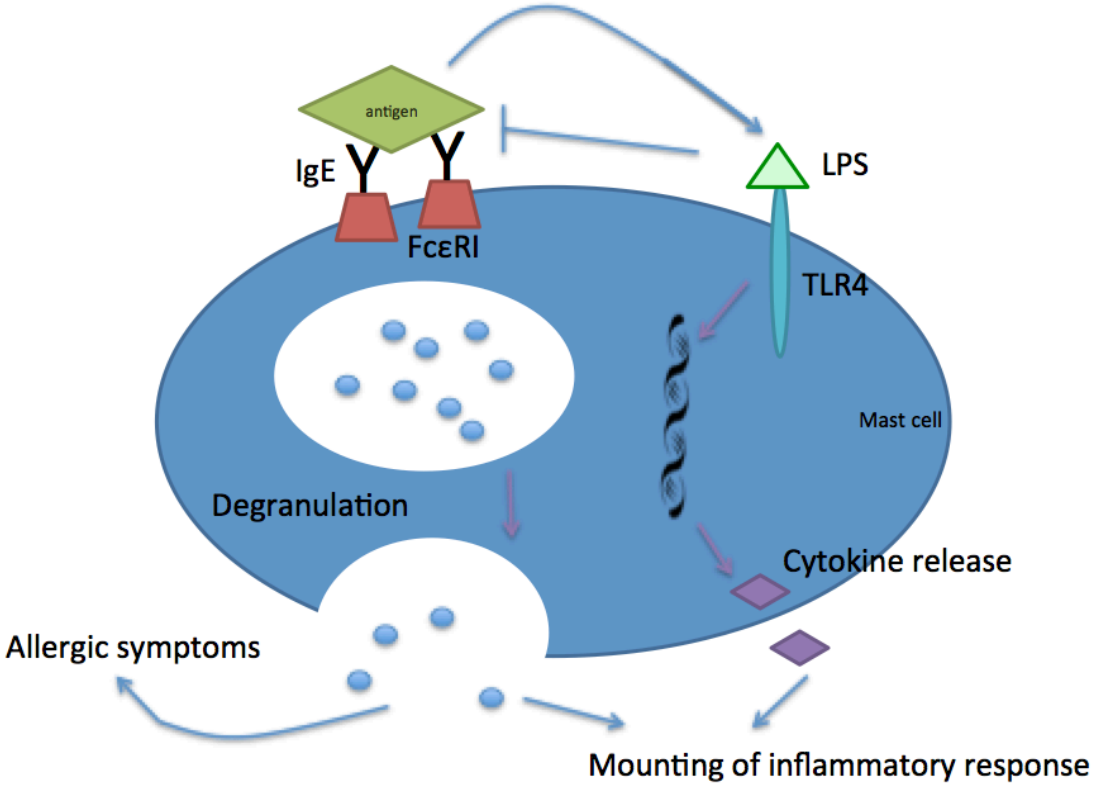

Figure 1. Mast cells recognize IgE antibodies that are specific for a particular antigen by FcεRI receptors on their surface. When the antigen crosslinks 2 IgE antibodies recognized by the FcεRI receptors, the mast cell is activated and degranulation occurs, as shown above. This initiates the signalling cascade that releases chemical mediators from the mast cell that recruit other cells to mount an inflammatory response.

During inflammation, the immune system seeks to eliminate the antigen-presenting cell or antigen itself to prevent harm to the host. In cases of bacterial and viral

infection, bacterial and viral particles are attacked and destroyed by the immune system, thereby preventing disease in the host. This is done through the recognition of these particles as pathogenic, usually through the TLR-mediated signalling pathway. Many symptoms experienced during sickness are not a result of the pathogen itself, but rather the result of the inflammation mounted by the immune system, as is the case with histamine release by mast cells (Galli et al., 2008). It is therefore believed that preventing degranulation of mast cells and inhibiting the release of granule contents would ultimately result in mitigated symptoms of allergy. The TLR-mediated pathway, however can act to reduce IgE-mediated inflammation in addition to promoting inflammation in the presence of pathogens. LPS, a TLR-4 ligand is associated with the release of many cytokines, notably IL-6 and IL-10. When LPS is treated with mast cells and the IgE-mediated allergic pathway is subsequently activated, there is a decrease in the presence of surface FcεRI and a decreased sensitivity to IgE (Wang et al., 2017). To mitigate food allergy symptoms, probiotics may act directly on the IgE-mediated pathway or indirectly, in a modulatory role through the TLR-mediated pathway. This thesis will focus on the IgE-mediated signalling pathway, as well as the TLR-mediated signalling pathways. While the IgE-mediated pathway is initiated directly during episodes of allergy, there is crosstalk between the IgE and TLR pathways, in which each modulate the effects of the other pathway, to alter response to both allergens and pathogens (Figure 1).

1.3 Current Food Allergy Therapies

The only known method of preventing food allergy-associated symptoms is to avoid the antigen-containing food. Individuals with food allergies must take caution to avoid the ingestion of the food allergen and adjusting their diets accordingly if certain

food groups must be avoided entirely. A link between early life exposure to allergens and the prevention of food allergy development in children was previously studied, such that a delayed first exposure to egg in a child's diet was associated with an increase risk of egg allergy (Koplin et al., 2010). Breastfeeding may also be linked to an altered risk of food allergy, as breast milk contains immunoregulatory factors that promote immunoregulation and subsequently may be implicated in the prevention of food allergy development in children (Field, 2005). In addition, human breast milk contains probiotic oligosaccharides that promote a healthy gut microbiota, and has attenuated food allergy symptoms in a mouse model (Castillo-Courtade et al., 2015).

When an allergic response to food occurs, however, treatment options to manage the symptoms are available in the form of injected catecholamines or corticosteroid treatments. Epi-Pens contain epinephrine, a catecholamine acting as a potent vasodilator that reduces airway swelling when injected intramuscularly during anaphylaxis (Kemp et al., 2008). In asthma patients, inhaled corticosteroids are administered to prevent airway closure due to inflammation during an episode, and corticosteroids can also be used to treat skin inflammation and other symptoms (Neuman-Sunshine et al., 2012). The symptoms following an allergic response may be treated, but treatments do not lessen the severity of subsequent responses should re-exposure to the antigen occur.

The permanent treatment of food allergy would involve altering immunity on a systemic level, such that the recognition of a food antigen is either prevented, or fails to elicit the associated symptoms. As a result, most readily available drugs target the symptoms of food allergy, to manage, rather than cure the condition. Immunotherapy focuses on taming the immune system to prevent autoimmunity or allergic responses to

non-pathogenic food allergens. Targeting the IgE-mast cell binding step is one venue of immunotherapy, using the drug Omalizumab, a monoclonal antibody that binds to the Cε3 region of IgE and competes with FcεR1 for binding (Fried and Oettgen, 2010; Hayashi et al., 2007). As a result, Omalizumab targets free and membrane-bound IgE but not IgEs already bound to mast cells (Hansel et al., 2010). In a clinical population of asthma patients, treatment with Omalizumab has been associated with a decrease in both serum IgE levels and IgE^+ cells in the airway, consistent with a decrease in mast cell-mediated inflammation (Djukanović et al., 2004). The drug, however, may compromise the individual's immunity against pathogens, as the Cε3 region of IgE antibodies is shared among all IgE antibodies, regardless of antigen specificity. Other side effects of this treatment may include anaphylaxis, as IgE production is increased in the presence of an antigen, and in the absence of a downstream mast cell response (Hansel et al., 2010).

Probiotic bacterial strains have been implicated in the treatment of food allergy, acting on mast cells to attenuate inflammation to suppress the allergic response (Forsythe, 2016). In addition to promoting digestive health, probiotics have been demonstrated to promote immunoregulation and consequently may be used as a potential treatment for food allergy symptoms. As a result, it is of interest to develop a deeper understanding of the function of mast cells and study their interactions with probiotic bacteria to elucidate a mechanism by which inflammation is attenuated.

1.4 Probiotic Bacteria, Microvesicles and Food Allergy

1.4.1 Probiotic Bacteria

Probiotics bacterial strains are organisms that confer benefits on the host organism when consumed in sufficient quantities (Marteau and Shanahan, 2003). There is

a wide range of bacterial strains that are classified as probiotic, but their function in the body either on their own or in combination with other strains has yet to be fully classified (Senol et al., 2011). Probiotics have been previously shown to mitigate local inflammation in the host gut, and can treat inflammation-associated disorders (Sartor, 2004). In addition to local inflammation, there is evidence suggesting that probiotic bacteria may interact with mast cells either locally or systemically to attenuate inflammation, promoting immunoregulation in the host (Al-Nedawi et al., 2015; Forsythe, 2016; Kim et al., 2016).

1.4.2 Bacteria Interaction with Mast Cells

There are several ways in which bacteria interact with host immune cells. Bacteria can interact with immune cells such as mast cells directly, by releasing factors that directly affect immune cell function or indirectly through interacting with other cells that in turn affect immune cells. Bacteria are able to modulate the host immune system, and the ability to attenuate inflammation and suppress immune cell function is beneficial to bacteria survival. By lowering the host's ability to mount an inflammatory response as a defense against bacteria, this augments the ability of microbes to propagate and thrive in the host. For pathogenic bacteria, this augments their ability to cause host disease, but for non-pathogenic bacteria, this enables them to thrive in the host.

Bacteria may undergo quorum-signalling, in which they communicate with chemical messengers that enable them to coordinate their behaviour and gene expression while in the host (Forsythe, 2016; Khambati et al., 2016). Gram-negative bacteria use small molecules, of which the most notable class being the N-acyl-homoserine lactone system, based in fatty acids, while in contrast, gram-positive bacteria use small peptides

(Forsythe, 2016). These quorum-signalling molecules have been shown to inhibit mast cell activation *in vitro* and suppress activation *in vivo* (Khambati et al., 2016). Mast cells in particular, also express receptors for self-associated molecular pattern molecules, which suppress mast cell activation to maintain a baseline non-activated state (Forsythe, 2016; Varki, 2011). Some bacteria and other human pathogens have developed the ability to mimic these self-associated molecular patterns to bypass the host immune response. In addition, some bacteria, such as *Salmonella typhurium* are able to directly synthesize factors that inhibit mast cell function (Forsythe, 2016).

Mast cells can also be indirectly modulated through bacteria. Probiotic bacteria and prebiotic supplements that enhance probiotic bacteria survival have been associated with a decrease in mast cell degranulation through increasing endogenous galectin-9 expression and secretion (de Kivit et al., 2012). Galectin-9 binds to IgE and prevents binding to the FcεRI receptors on the surface of mast cells, forming a complex that prevents subsequent activation (Forsythe, 2016). Mast cell activation is also augmented with the opening of the KCa3.1 K^+ channel (Forsythe, 2016). Oral feeding with probiotic *Lactobacillus rhamnosus* JB-1 has also been associated with inhibition of the KCa3.1 channel *in vivo*, although the mechanism has not been identified and cannot be replicated with direct treatment of mast cells and probiotics *in vitro* (Forsythe et al., 2012).

Probiotic bacteria have been shown to interact with mast cells both directly and indirectly to affect mast cell activation and behaviour. In a murine model of food allergy, mast cell activation and infiltration of intestinal mucosa was shown to be attenuated by *Lactobacillus plantarum* when delivered gastrically prior to an allergenic challenge (Eun et al., 2017). In a separate study, *Bifidobacterium longum* was shown to attenuate food

allergy symptoms when orally fed to mice prior to allergenic challenge and was associated with mast cell apoptosis *in vitro* (Kim et al., 2016). While attenuation of mast cell activation and inflammation has been observed when mast cells or animal models have been treated with whole bacteria, studies have also aimed to elucidate the mechanism by which this occurs.

1.4.3 Microvesicles

One potential mechanism by which mast cells and probiotics interact may be through extracellularly shed microvesicles (MVs) originating from the bacterial cell membrane. Bacteria are classified as gram positive or gram negative based on their ability to retain the Gram stain after being washed. Gram positive bacteria retain the stain due to their thick peptidoglycan layer outside the cell membrane, while the thin peptidoglycan layer between the inner and outer membranes of gram negative bacteria allows the gram stain to leak out. MVs from gram positive bacteria are considered true MVs, while gram negative bacteria produce outer membrane vesicles (OMVs) (Al-Nedawi et al., 2015). These microvesicles are approximately 150nm in size and are constantly shed by the bacterium, containing cellular components and can only be visualized by electron microscopy (Al-Nedawi et al., 2015).

Previous work shows that OMVs can modulate the host immune system, suppressing immunity and promoting virulence (Ellis and Kuehn, 2010). By suppressing the host immune system, non-pathogenic bacteria may be implicated in promoting immunoregulation. A probiotic strain, *Bifidobacterium longum*, results in decreased mast cell activation and numbers in the mouse intestine following oral antigen challenges, and the effect of the parent bacterium on mast cells was replicated with MV isolates (Kim et

al., 2016). The authors suggest that the bacteria elicit apoptosis in mast cells, thereby reducing the measured mast cell degranulation and mast cell numbers in the intestine after an oral challenge; however, one objective of this thesis is to elucidate whether the bacteria have a stabilizing effect on mast cells without causing apoptosis and the mechanism by which this occurs.

Previous work in our lab has also demonstrated that co-incubation of bone marrow-derived mast cells from mice with probiotic strains, *Lactobacillus rhamnosus*, *Lactobacillus helveticus, Bifidobacterium infantis* and *Bifidobacterium bifidum* decreased mast cell protease release after an antigen challenge, therefore it is concluded that co-incubation of mast cells with probiotic bacteria or their MVs would be able to decrease mast cell activation (Figure 2). The specific effect of each strain is unclear, and we are interested in studying and comparing the effect of different species and strains to determine which probiotics are effective in inhibiting mast cell activation.

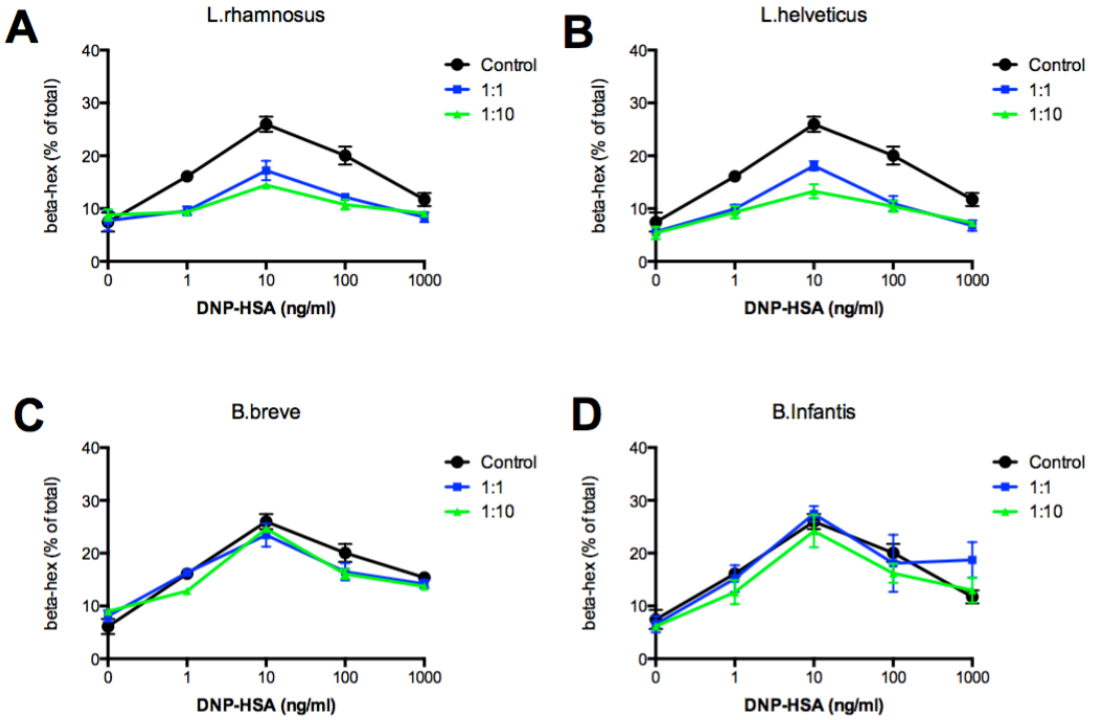

Figure 2. Co-incubation of mast cells with bacteria for 24h inhibits mast cell degranulation and release of β-hexosamindase when mast cells are exposed to an antigen (DNP) to which they have previously been sensitized (n=4).

1.5 Objectives

I. To determine if direct interaction of probiotics or their MVs with mast cells is associated with suppression of degranulation.

It was previously shown that co-incubating mast cells with MVs prior to stimulation with an antigen to which the mast cells have been sensitized decreases the release of β-hexosamindase, a marker of mast cell degranulation (Kim et al., 2016). We will study the extent to which mast cell degranulation is attenuated by either MVs or their whole parent bacteria *in vitro*. There are several bacteria that are classified as probiotics, and we plan to study if they interact with mast cells *in vitro* and if they prevent mast cell degranulation and inflammation. We will also measure the cytokine release of mast cells

both during and post-treatment with probiotics when stimulated with different TLR ligands such as peptidoglycan or lipopolysaccharide (LPS), to see if probiotics have an attenuating effect on proinflammatory cytokines and upregulate the release of anti-inflammatory cytokines.

II. To determine if probiotic bacteria or their MVs modulate cytokine release and expression profiles of mast cells during pathogen-associated molecular pattern (PAMP) challenge.

Mast cells contain TLR1-10 receptors on their surface, which each respond to their own, or a combination of PAMP molecules, which are produced by potentially pathogenic bacteria or organisms (Sandig and Bulfone-paus, 2012). The ability to recognize pathogenic components enables mast cells to mount an inflammatory response against pathogens. Interleukin cytokines are released by mast cells when a PAMP molecule binds its respective TLR. Cytokines are either classified as pro- or anti-inflammatory, and the cytokine release profile of mast cells can be assessed to determine mast cell phenotype. We plan to assess if probiotic treatment of mast cells attenuates the inflammatory phenotype shift of mast cells during PAMP exposure.

Chapter 2: Methods

2.0 Selected Probiotic Strains

The probiotic strains that we have selected to use are *Lactobacillus rhamnosus* (JB-1), *Lactobacillus helveticus* (LH), *Bifidobacterium infantis* (BI) and *Bifidobacterium bifidum* (BB) and *Lactobacillus reuteri* (DSM). Work has been previously done on the first four strains, with JB-1 and LH producing decreased degranulation of mast cells when challenged with low and intermediate doses of antigen (Figure 2). We have selected

the last strain, DSM, because we have demonstrated previously in our lab that this strain may also decrease inflammation.

Probiotics were grown in Man-Rogosa-Sharpe medium and harvested at 48h and stored in 1ml aliquots at 1×10^{10} CFU/ml. MVs were isolated and purified as described in previous work (Al-Nedawi et al., 2016).

2.1 *In Vitro* Effects on Mast Cell Activation

2.1.1 β-hexosaminidase Assay

To determine the effect of probiotic bacteria and their microvesicles on mast cells *in vitro*, probiotics were co-incubated with mast cells at different time points to assess their effect on mast cell activity. How mast cells respond to co-incubation *in vitro* may predict how they behave in an *in vivo* model. Primary mast cells derived from murine bone marrow were grown to 5-8 weeks in culture, to a density of approximately $2x10^6$ cells/ml. Mast cells were suspended in HEPES-Tyrode buffer and sensitized for 2 h at 37°C with 100 μg/ml of monoclonal IgE antibody against dinitrophenyl-human serum albumin conjugate (DNP-HSA) before co-incubation. Subsequently, mast cells were co-incubated with either the whole bacteria or MVs from each bacterial strain at MOIs of 1 and 10 for 30 minutes.

After co-incubation, increasing concentrations of DNP-HSA protein were added to the mixture, thereby binding to the anti-DNP IgE antibodies incorporated onto the mast cell surface and eliciting mast cell activation. 1% Triton-X 100 were added to wells containing sensitized mast cells to cause mast cells to release their contents and subsequently, all of the β-hexosamindase present in the cell. This serves as the upper limit of β-hexosaminidase release, and is thereby labeled as, total release.

β-hexosamindase is released from mast cells during degranulation and can be measured as an index of mast cell activation. The β-hexosamindase assay was performed after the co-incubation experiment and β-hexosamindase release was measured by the after activation by stimulation the anti-DNP IgE. 0.3mg/ml of p-nitrophenyl N-acetyl-β-D-glucosamide (p-NAG) dissolved in citrate buffer (pH = 4.5) was then added and incubated with the supernatant containing released β-hexosamindase for one hour at 37°C to produce a colourimetric change in the presence of β-hexosamindase (Kuehn et al., 2011). This result was visualized by stopping the reaction with 0.05M carbonate buffer (pH=10.5). The results were read spectrophotometrically at a wavelength of 405 nm, and the absorbance of each sample was expressed as a percentage of total release.

2.1.2 Direct Measurement of Cytokines with ELISA

To determine if probiotics affect the cytokine profile of mast cells *in vitro*, mast cells were co-incubated with probiotics and their MVs with LPS from *E. coli*. TLR activation of mast cells occurs when cells are exposed to PAMPs such as LPS (Figure 3). Mast cells are found to express TLR1-10 (Sandig and Bulfone-paus, 2012) and each TLR responds to a specific ligand either alone, or coupled with another TLR. LPS is a cell wall component of gram-negative bacteria, and is detectable by host cells as an indicator of the presence of pathogenic bacteria. LPS is an activator of TLR4 signalling and is soluble in medium, so it was dissolved and diluted to the desired concentrations.

When activated either on their own or in combination with other receptors on mast cells, TLR4 and TLR2 have been shown to promote the release of both pro- and anti-inflammatory cytokines. Changes in cytokine expression and release provide a cytokine profile that is indicative of the mast cells' inflammatory state. Elevated levels

and expression of pro-inflammatory cytokines such as IL-6 and TNFα are indicative of an activated inflammatory mast cell phenotype. In contrast, increased release of anti-inflammatory cytokines such as IL-10 is indicative of an anti-inflammatory, regulatory phenotype (Galli and Tsai, 2012).

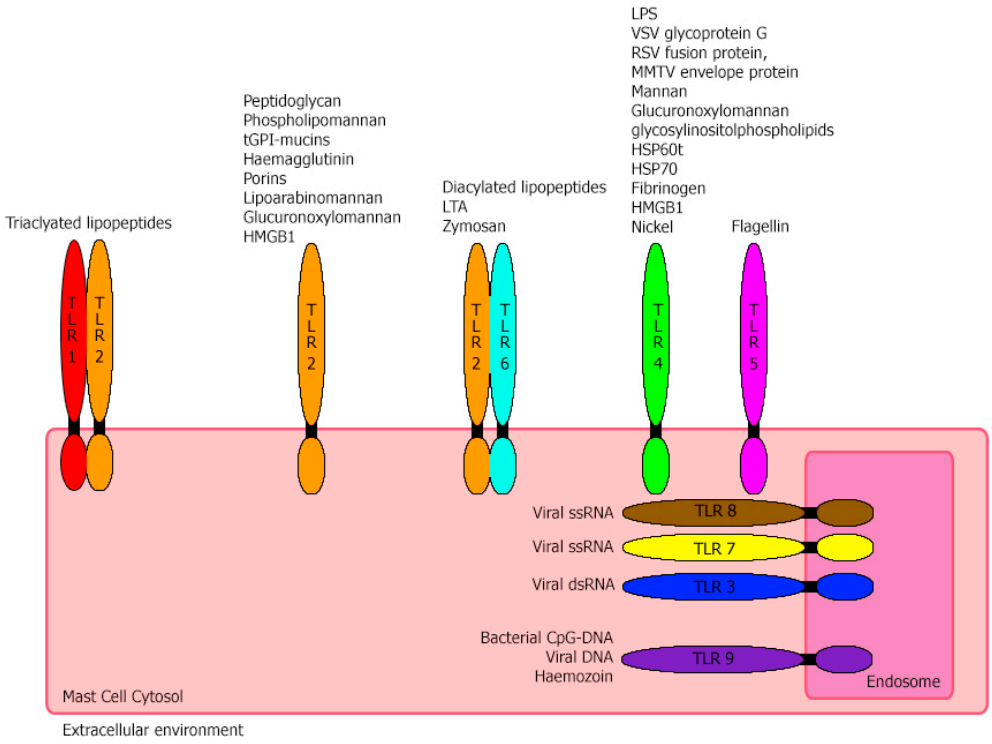

Figure 3. TLRs and their ligands are shown. Some TLRs respond to ligands coupled with other TLRs and the locations of TLRs can be both intra- and extra-cellular. Adapted from Sandig et al. (2012).

2.1.3 Quantitative Polymerase Chain Reaction (qPCR) For Cytokine Expression

To determine the relative increase of cytokines expressed by mast cells upon direct interaction with probiotic bacteria, mast cells were cultured and co-incubated with probiotics with LPS serving as a positive control. $1.5x10^6$ mast cells were co-incubated with different probiotics at MOIs of 1 and 10. At the end of the incubation period, mast cells were centrifuged and collected for RNA extraction and cDNA synthesis. Mast cell RNA was extracted using the Qiagen RNEasy Mini-Kit and cDNA was synthesized.

The SYBR Green Master Mix (Thermo Fisher) was used to supply the reagents required for qPCR. The forward and reverse primers used were previously designed by

Leclercq et al. (2017) and the β-actin primers were used from Listvanova et al. (2003) (Supplementary Table 2), with β-actin as a control gene. The reaction was run in 40 cycles, and initial quantities of mRNA were determined by rate of total mRNA increase throughout the reaction (Supplementary Figure 1). With qPCR, the differential gene expression of different cytokines relative to the constitutive gene β-actin was measured.

Chapter 3: Optimizing the Mast Cell Culture Protocol

In our studies, mast cells were derived from mouse femoral bone marrow and cultured weekly. Mice were of 6-8 weeks of age, and sacrificed by CO_2 before femurs were harvested. Experiments were performed when mast cells reached 5-8 weeks of age since the culture was started. For mast cell maturity, IL-3 is the main cytokine required for progenitor cells to differentiate into mast cells, and stem cell factor (SCF) mediates cell maturity (Amin, 2012). There are several different methods, by which BMMCs can be grown from primary cultures. SCF and IL-3 are drawn from different sources and doses are adjusted for optimal cell growth and maturity.

The first method that was attempted used supernatant derived from cultured splenoctyes stimulated with pokeweed (*Phytolacca americana*) mitogen. Pokeweed mitogen stimulates splenoctyes to produce IL-3, which will be used to grow mast cells. In this method, spleens were collected alongside femurs and homogenized with the back of a syringe. The splenoctyes were grown at a concentration of 2×10^6 cells/ml in RPMI medium containing 10% fetal bovine seriu, (FBS), 4mM L-glutamine, 1mM sodium pyruvate, 5×10^{-2} mM β-mercaptoethanol, 1% PenStrep antibiotic and 1ug/ml of pokeweed mitogen. After 5-7 days, the culture was centrifuged at 3000 rpm for 15 minutes and the supernatant was stored at -20°C for further use. The bone marrow was

grown in standard BMMC medium, containing 10% FBS, 4mM L-glutamine, 1mM sodium pyruvate, 5×10^{-2} mM β-mercaptoethanol, and 1% PenStrep antibiotic and grown at a density of $0.5\text{-}1\times10^{6}$ cells/ml at volumes of 25ml per each T175 flask. Mast cells were counted with a hemocytometer and suspended in 20ml of medium. 5ml of spleen supernatant was added to 20ml BMMCs, to comprise the full 25ml per flask. The medium was changed and new spleen supernatant was added every 7 days.

This method had an unreliable yield of BMMCs, as the concentration of IL-3 varied with each culture. Furthermore, the lack of sufficient SCF caused the cells to die prematurely, at 3 weeks of age. The mast cells at this point in their growth were only 20% mature. Maturity and purity of the culture were measured by means of FACS, by the proportion of cells that express c-Kit and FcεRI receptors on their surface (data not shown). To enhance their viability, the concentration of spleen supernatant in each flask was increased such that BMMCs were suspended in 12.5ml of medium, with the remaining 12.5ml being spleen supernatant. This method enhanced the viability of the BMMCs slightly, however, the cells still failed to reach the minimum age of maturity of 5 weeks. It was later demonstrated that mast cells were near fully mature by this age (Figure 4).

The next method that was attempted was to use WEHI cell-conditioned medium in place of spleen supernatant. WEHI-3b cells are an immortalized cell line that secrete large amounts of IL-3 (Galli and Tsai, 2012; Galli et al., 2008, 2011) and have been used to culture primary BMMCs. WEHI-3 cells were grown CMLESS medium containing 10% FBS, 2mM L-glutamine, 5×10^{-2} mM β-mercaptoethanol and 1% PenStrep antibiotic at a concentration of 10^{5} cells/ml. Cells were centrifuged at 1000 rpm for 10 minutes and

supernatant was harvested each week to be stored at -20°C for further use. WEHI-3 supernatant was added to CMLESS medium to a final concentration of 5% and used to culture bone marrow cells. This method yielded no mast cells, as the cells that grew from the CMLESS medium did not resemble mast cells morphologically and adhered to the bottom of the flask, where true BMMCs are in suspension. This was due to the fact that we could not ascertain the identity of the WEHI-3 cells and due to time restraints, did not test the composition of the WEHI-3 supernatant.

The final option that was tested was to use recombinant, commercially purchased IL-3 and SCF added directly to the standard RPMI medium described above to yield final concentrations of 2ng/ml. This dose is less than what is found in the optimal growth condition of mast cells, which is usually 100ng/ml SCF and 30ng/ml IL-3 (Desai et al., 2016; Ito et al., 2013) (Desai et al., 2016; Ito et al., 2013). However, there have been cases where lower doses have been successfully used, as low as 3ng/ml IL-3 (Mccurdy et al., 2001). BMMCs are flushed and cultured at a concentration of 1 mouse per flask. While this method generally allows mast cells to live as long as 10 weeks, as long as the medium is changed weekly, there are still a few weeks where the mast cells do not grow, and as a result, die. This is a rare occurrence; however, and thus this method has been adopted as the standard by which mast cells are grown in our lab (Figure 4).

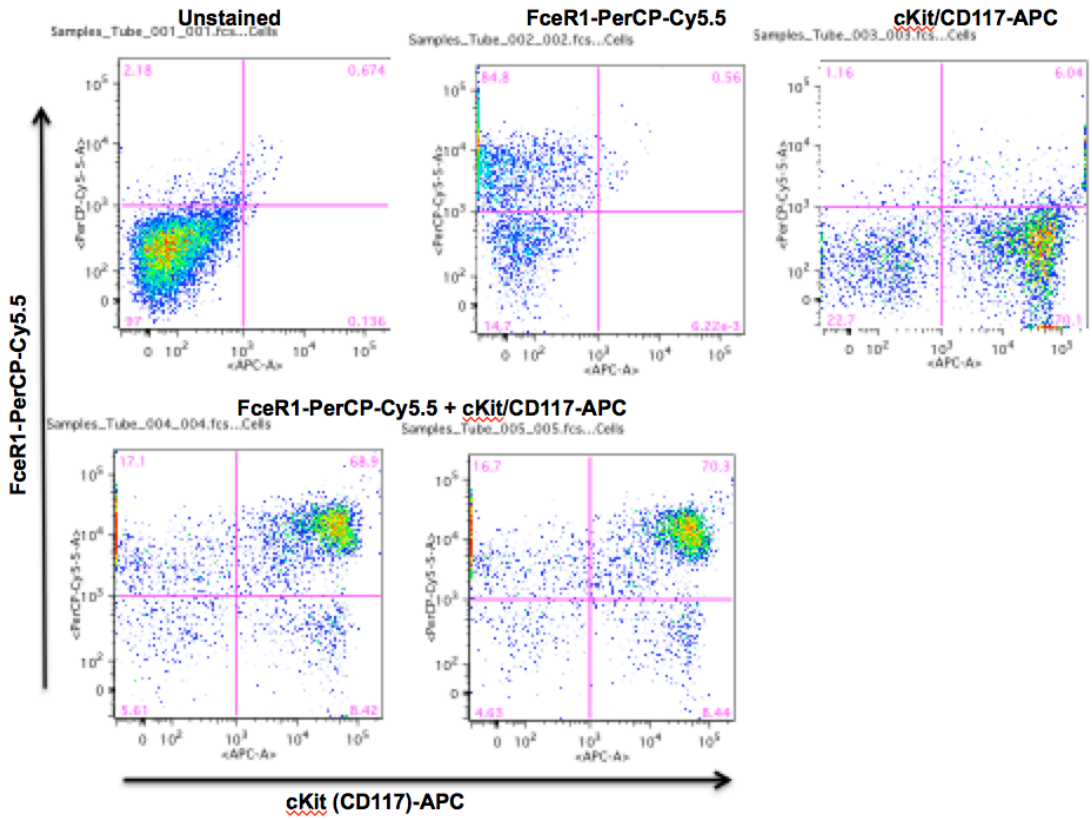

Figure 4. FACS data describes the presence of c-Kit and FcεRI receptors on the surface of 5 week-old mast cells. ~100% of the cells measured were positive for both receptors, indicating ~100% purity and maturity.

Chapter 5:

5.1 Mast Cell Cytokine Profile Analysis

5.1.1 Direct Cytokine Measurement by Enzyme-linked Immunoassay

To assess the effect of probiotics on mast cells in the absence of LPS, mast cells were cultured as described above and were co-incubated overnight with probiotics with 0.1μg/ml LPS as a positive control. Mast cells were then co-incubated with probiotic strains or their respective MVs in antibiotic-free medium for 4 hours with different concentrations of either LPS to stimulate cytokine release in 24-well tissue culture plates.

The plates were spun at 1200 rpm for 10 minutes and supernatant from the co-incubated culture was collected and cytokines were measured with the Ready-Set-Go! ELISA kit (ThermoFisher) and IL-6 and IL-10 release after co-incubation were compared to controls.

To assess whether probiotics or their MVs can reduce IL-6 release following challenge with different doses of LPS, $5x10^5$ mast cells in 1ml of medium were incubated in medium as a negative control and in the absence of probiotic strains in each concentration of LPS. LPS concentrations of 0.01 μg/ml, 0.05 μg/ml, 0.1μg/ml, 0.5 μg/ml, 1μg/ml 5 μg/ml were used. Probiotic bacteria suspended in 0.25ml of medium were added such that the each well contained a ratio of 1:1 bacterium to mast cell and 10:1 bacteria to mast cell. MVs were diluted according to the same equivalent of bacteria from which they were produced. The plates were spun at 1200 rpm for 10 minutes and supernatant from the co-incubated culture was collected and cytokines were measured with the Ready-Set-Go! ELISA kit (ThermoFisher) and IL-6 release after co-incubation were compared to controls.

We have assessed if pre-treatment with probiotics before the LPS incubation induces mast cells to shift to an immunoregulatory phenotype. Mast cells were treated with probiotics for 1 hour at MOIs of 1 and 10 with 0.01 μg/ml, 0.5 μg/ml and 1 μg/ml of LPS. We are also determining if incubating with LPS for a longer or shorter duration affects cytokine release from mast cells. After the one hour pre-treatment, mast cells were incubated for 2 or 4 hours of with LPS. The plates were spun at 1200 rpm for 10 minutes and supernatant from the co-incubated culture was collected and cytokines were

measured with the Ready-Set-Go! ELISA kit (ThermoFisher) and IL-6 release after co-incubation were compared to controls.

5.1.2 Cytokine Gene Expression Analysis by Quantitative Polymerase Chain Reaction

Mast cells were incubated with probiotics at MOI of 1 and 10, and cytokine gene expression for IL-6 was measured. 0.1 μg/ml of LPS was used as a control. We plan to assess and compare the expression of other cytokine genes such as IL-10 and TNFα.

Chapter 6: Results

6.1 Probiotics modulation of mast cell degranulation

After incubation with probiotics for 30 minutes prior to antigen challenge, mast cells did not demonstrate a significant decrease in β-hexosaminidase release compared to negative controls for all probiotics (Figure 5A, C, E). The same was observed when probiotic MVs were co-incubated with mast cells. The same was observed when probiotics were co-incubated with mast cells for 2 hours (Figure 6A-E).

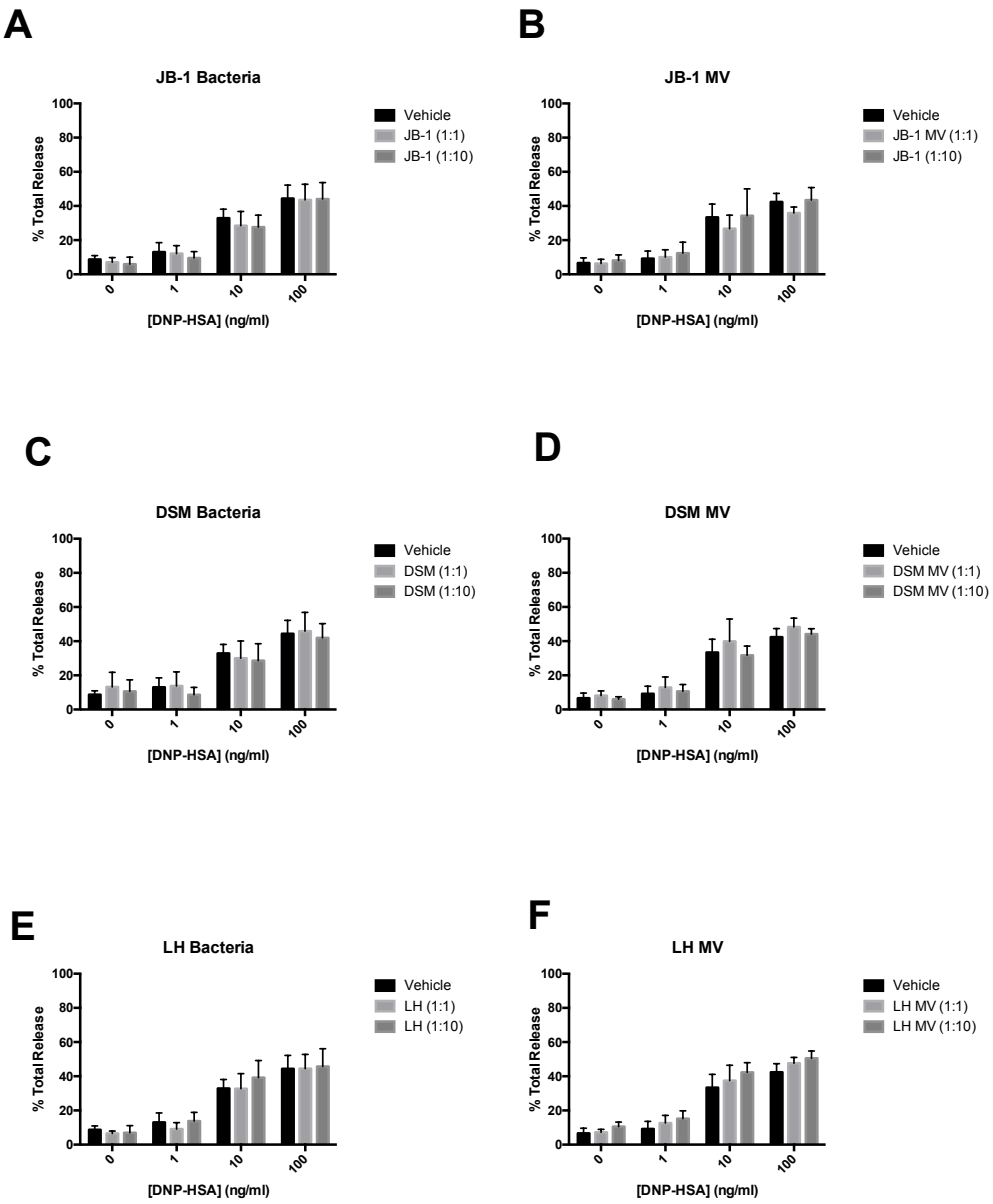

Figure 5. Mast cells were sensitized with anti-DNP IgE antibodies for 2 hours, agitating the tube every 30 minutes at 37°C. They were treated with probiotics by co-incubation or their MVs at MOIs of 1 and 10 cell to bacteria for 30 minutes at 37°C. Following co-incubation with probiotics, mast cells were stimulated for 30 minutes with DNP-HSA antigen and β-hexosaminidase release was measured by the β-hexosaminidase assay. Experiment was run in HEPES-Tyrode Buffer (pH=7.2-7.4). (N=3).

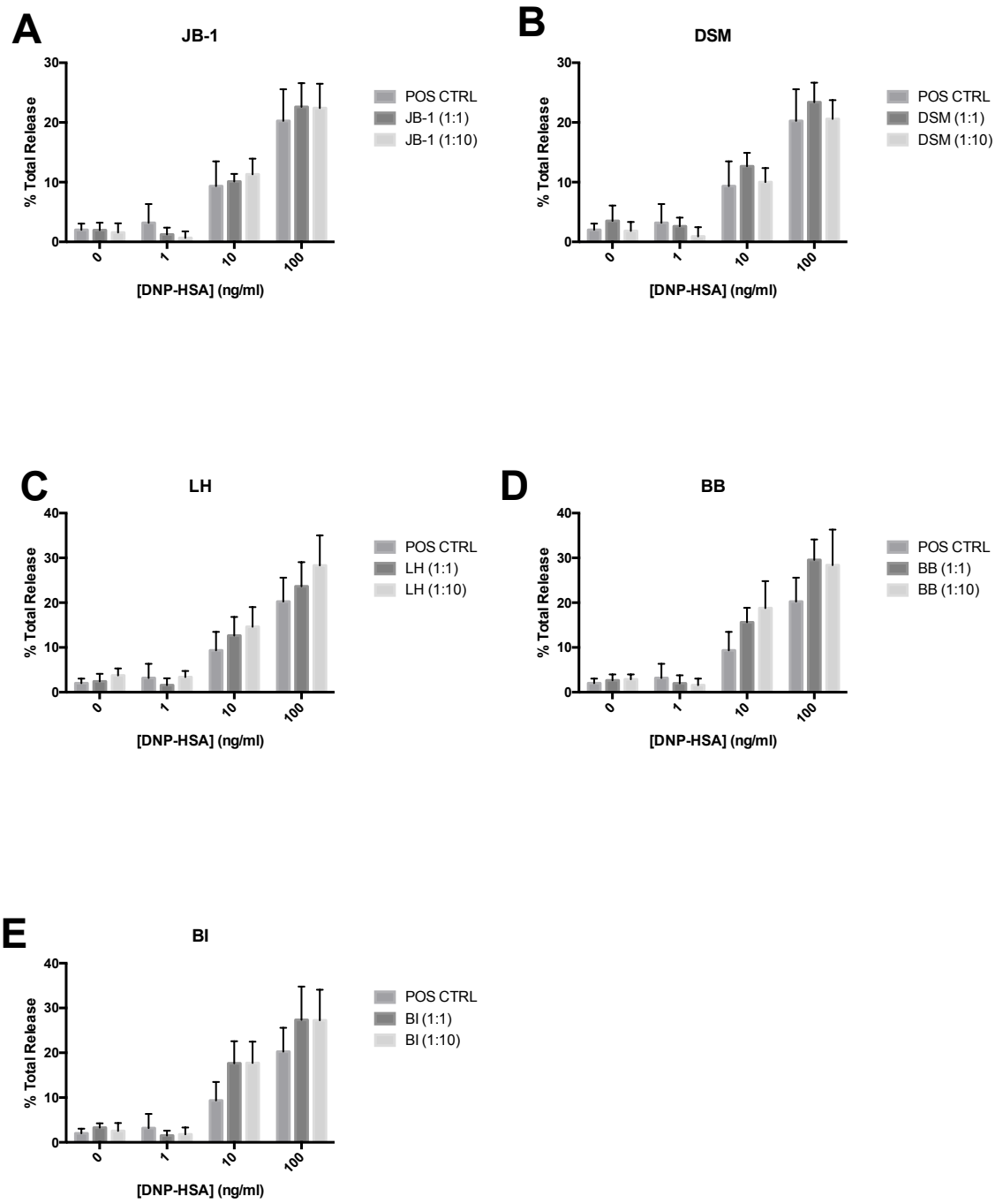

Figure 6. Mast cells were sensitized with anti-DNP IgE antibodies for 2 hours with shaking every 30 minutes at 37°C. They were treated with probiotics by co-incubation at MOIs of 1 and 10 cell to bacteria for 2 hours minutes at 37°C. Following co-incubation with probiotics, mast cells were stimulated for 30 minutes with DNP-HSA antigen and β-hexosaminidase release was measured by the β-hexosaminidase assay. Experiment was run in HEPES-Tyrode Buffer (pH=7.2-7.4). (N=3). A two-way ANOVA was performed with Tukey's multiple comparisons test.

6.2 Probiotic modulation of cytokine release profile upon PAMP challenge

Probiotics alone did not increase the relative release of IL-6 from mast cells (Figure 7A) after 24 hours of co-incubation compared to medium control. However, IL-10 release was increased upon 24h co-incubation with DSM at MOIs of 10 and 100, as well as BB and BI at all MOIs. LH showed a decreased level of IL-10 relative to control (Figure 7B), where levels of IL-10 were undetectable. The LPS positive control also produced an increase of IL-10.

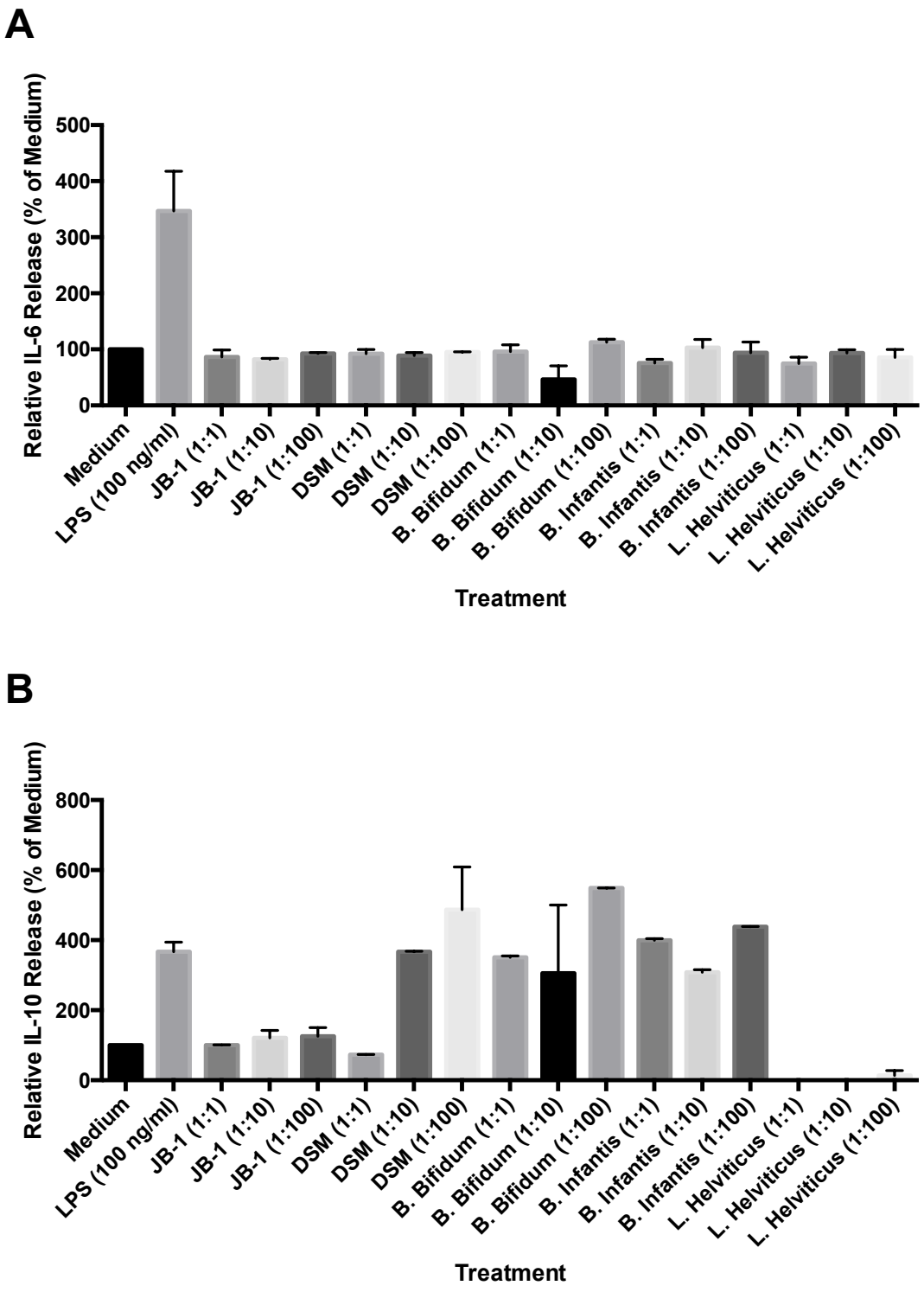

Figure 7. Mast cells were co-incubated with probiotic bacteria at MOIs of 1, 10 and 100 in antibiotic-free medium for 24 h. Supernatant from the culture was measured by ELISA for cytokine release. (N=2).

At different doses of LPS, all probiotics did not produce a significant relative decrease in IL-6 release when co-incubated with mast cells for 4 hours simultaneously with different doses of LPS compared to controls lacking probiotic treatment (Figure 8A-E). IL-6 release of each treatment was expressed as percent of untreated IL-6 release to account for an inconsistent baseline between replicates. The MVs of three strains (LH, BB and BI) that showed a decrease in LPS in initial replicates (Figure 9A-C) were co-incubated with the mast cells for 4 hours simultaneously with LPS. They did not produce any significant relative decrease in IL-6 release compared to untreated controls. Relative release is expressed as opposed to absolute IL-6 release due to the difference in baseline IL-6 release of untreated controls across replicates.

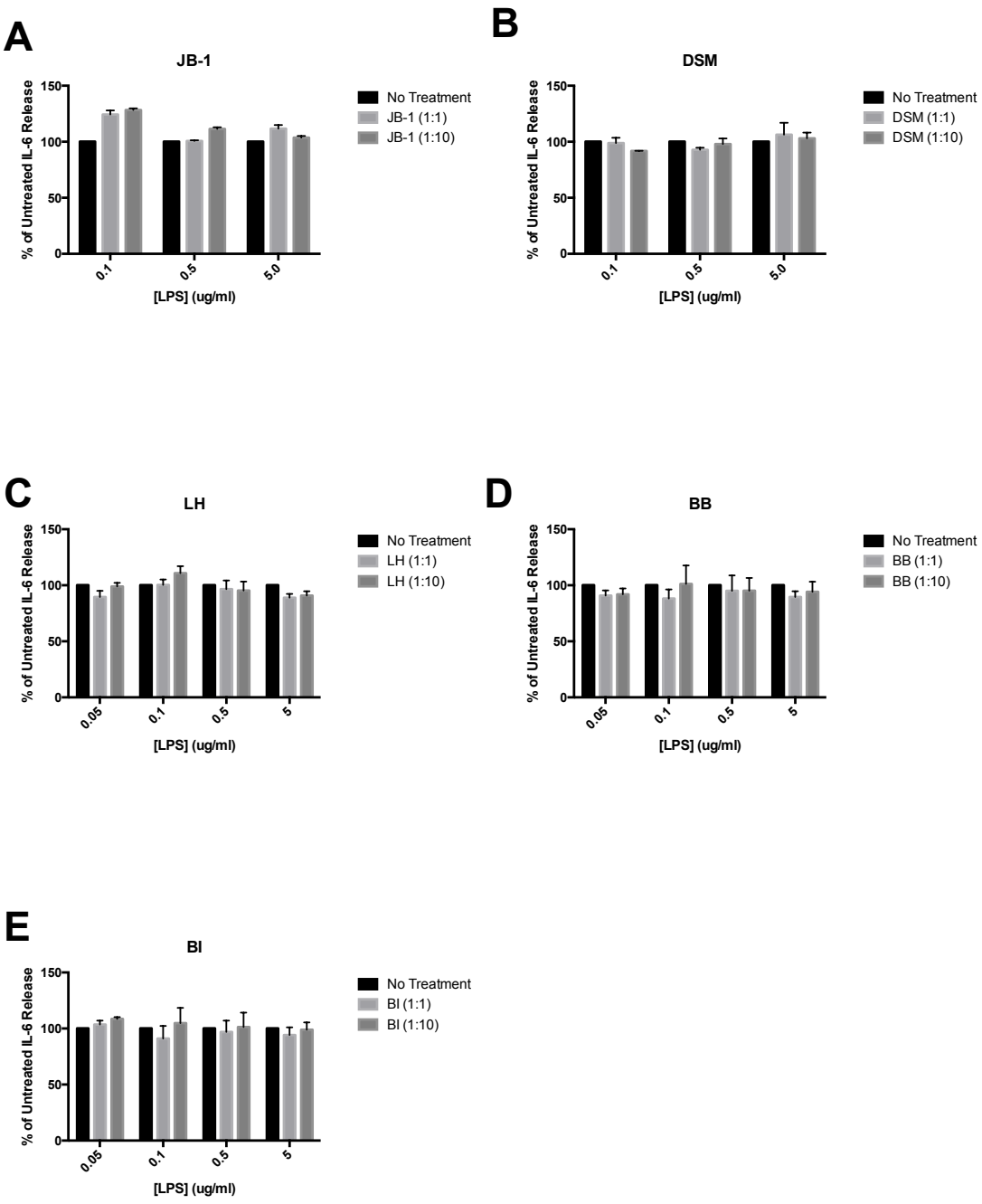

Figure 8. Probiotics were co-incubated for 4 h with mast cells and different concentrations of LPS. IL-6 release was measured by ELISA. (N=3). Absolute values of the untreated baseline are 0.28±0.17 pg/ml for 0.01 μg/ml of LPS, 15.31±0.89 pg/ml for 0.01 μg/ml of LPS, 51.37±4.54 pg/ml for 0.05 μg/ml of LPS, 50.83±5.59pg/ml for 0.5 μg/ml of LPS and 69.51±3.88 pg/ml for 5.0 μg/ml of LPS. A two-way ANOVA was performed with Tukey's multiple comparisons test.

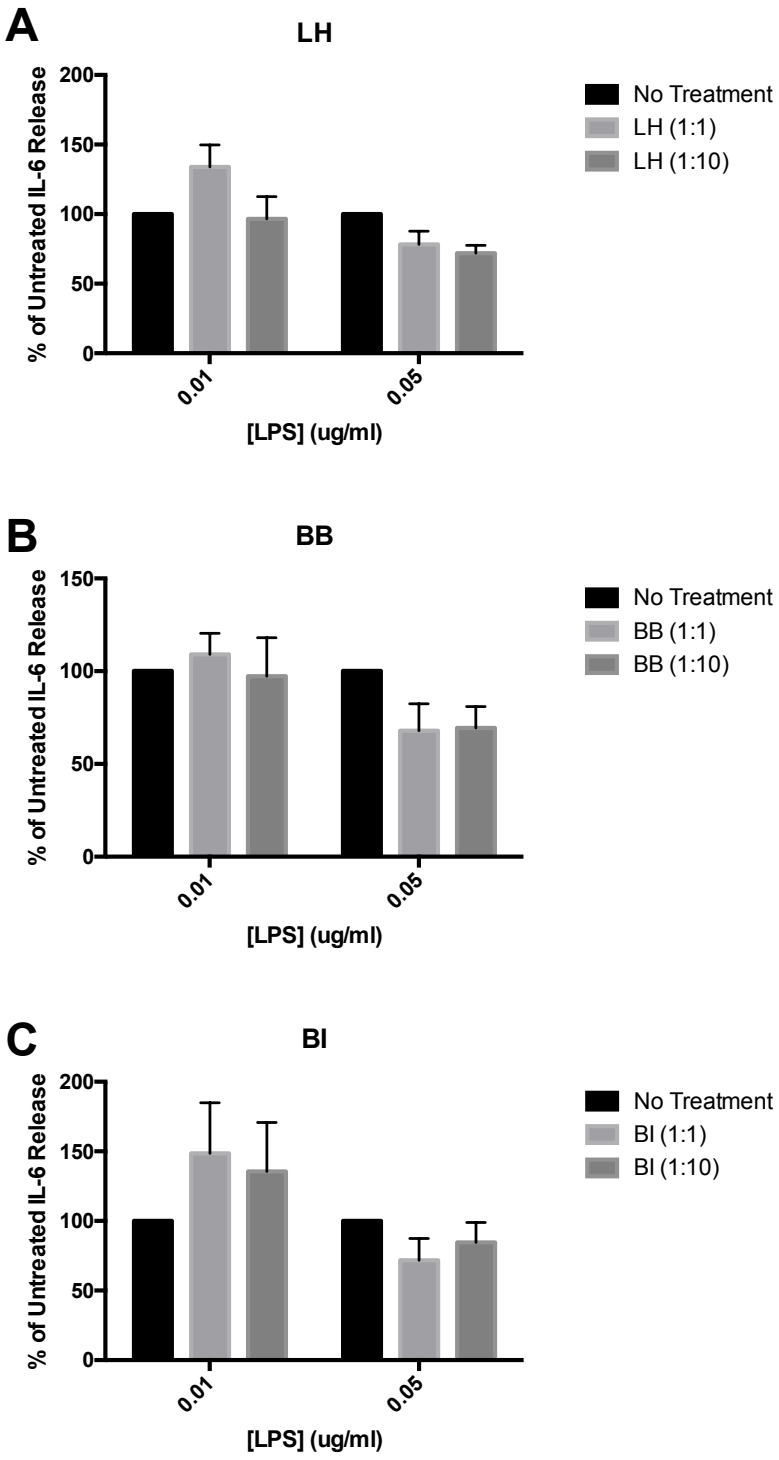

Figure 9. LH, BB and BI MVs were co-incubated for 4 h with mast cells and different concentrations of LPS. IL-6 release was measured by ELISA. A two-way ANOVA was performed with Tukey's multiple comparisons test.

Mast cells were then pre-treated with probiotics or their MVs for 1 hour prior to a 4 hour LPS challenge to determine if treatment prior to a challenge would have an effect on relative IL-6 release. A two-way ANOVA with Tukey's multiple comparisons test was performed, and it was shown that BB-treated mast cells produced a significant decrease in relative IL-6 release at an MOI of 10 at the highest dose of LPS ($p<0.05$) (Figure 10D), while BI produced a significant decrease in relative IL-6 release at all doses of LPS and at an MOI of both 1 and 10 ($p<0.0001$, $p<0.001$ and $p<0.01$) (Figure 10E). The same conditions were repeated, now treating mast cells with the MVs of LH, BB and BI, to determine if there was an effect of pre-treatment. LH, BB and BI all produced a significant decrease in relative IL-6 release at the higher dose of 0.05 μg/ml LPS at both MOIs. The high dose of 1.0 μg/ml LPS was omitted due to a lack of effect of the MVs on relative IL-6 release.

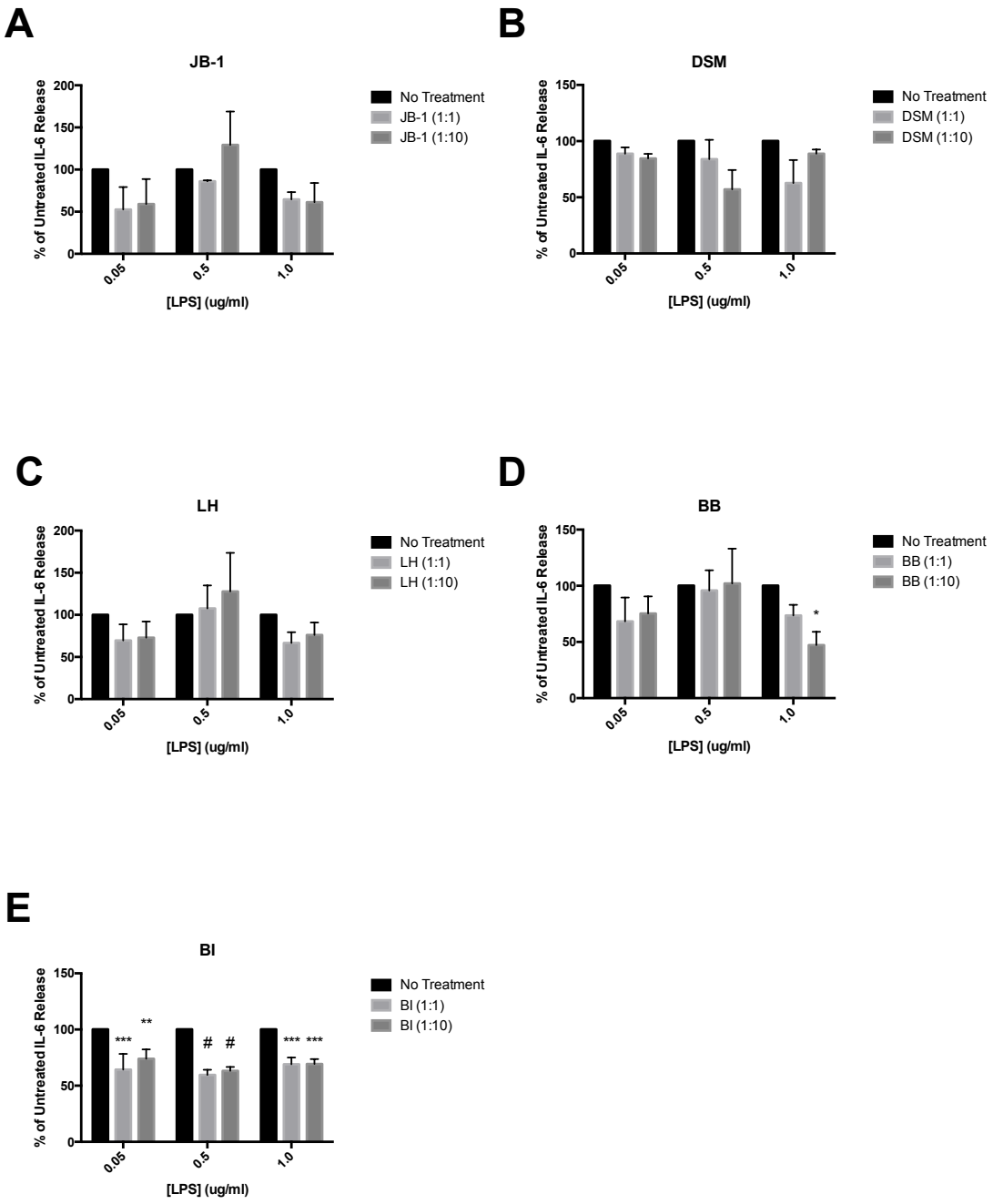

Figure 10. Mast cells were pre-treated for 1 h with probiotics and then co-incubated for 4 h with different concentrations of LPS. IL-6 release was measured by ELISA. (N=4-7) A two-way ANOVA was performed with Tukey's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001, #p<0.0001).

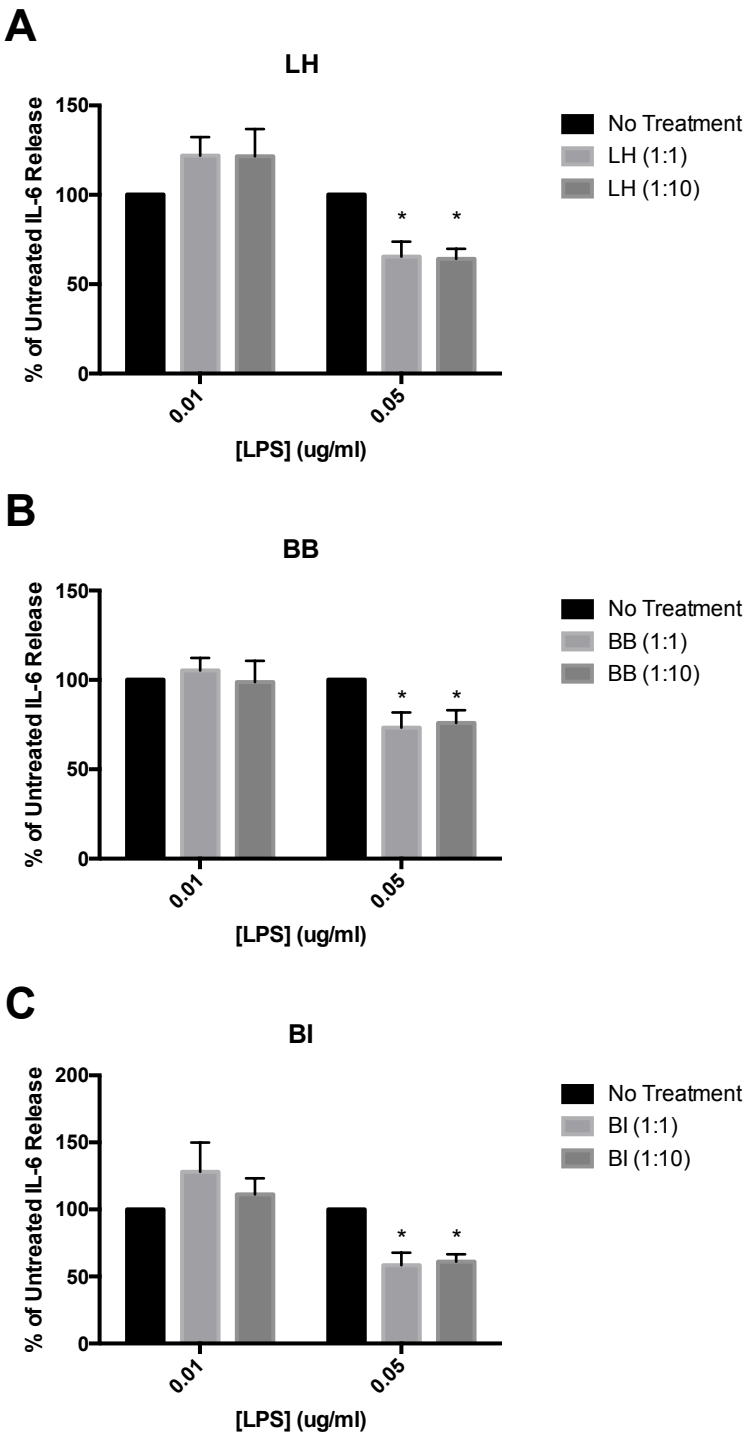

Figure 11. LH, BB and BI MVs were pre-treated with mast cells for 1hr and then co-incubated for 4 h with mast cells and different concentrations of LPS. IL-6 release was measured by ELISA. (N=3-7). A two-way ANOVA was performed with Tukey's multiple comparisons test (*$p<0.05$).

6.3 Probiotic modulation of cytokine gene expression profile upon PAMP challenge

6.3.1 Analysis of Relative IL-6 Gene Expression

When co-incubated for 4 hours with mast cells, none of the probiotics produced an increase in IL-6 gene expression relative to the constitutive reference gene, β-actin. JB-1 at both MOIs produced a slight decrease in IL-6 gene expression, as did DSM at an MOI of 1 (Figure 12).

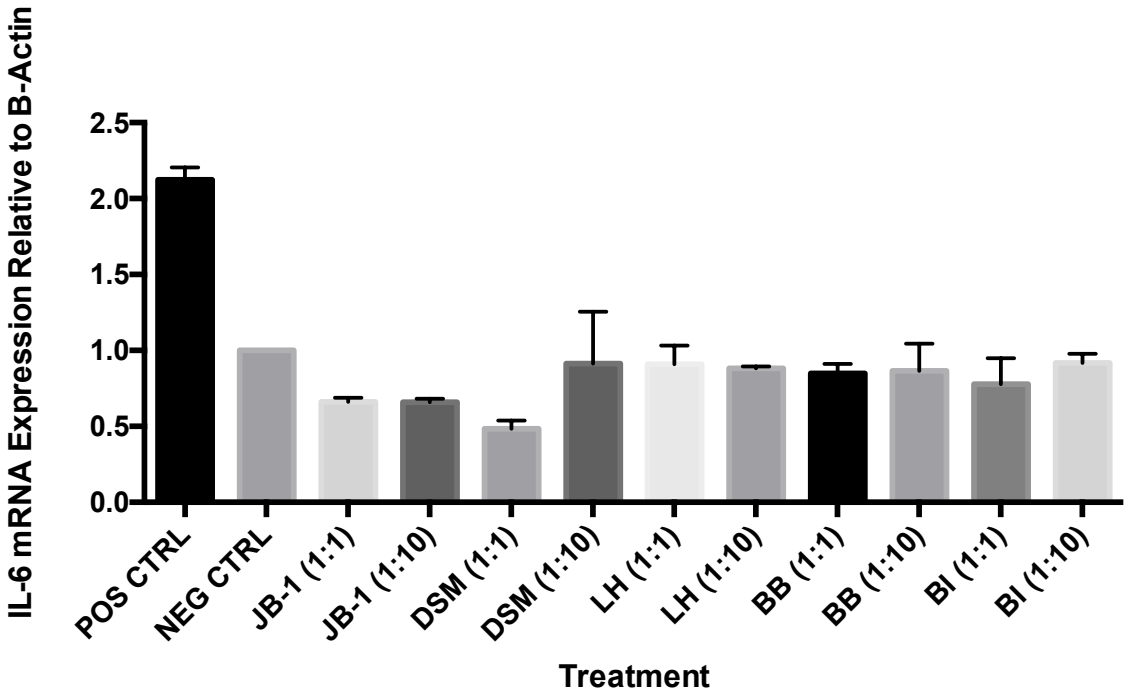

Figure 12. Mast cells and probiotics were co-incubated for 4h. Relative gene expression of IL-6 was compared to β-actin as a reference gene. Negative control consisted of medium, and positive control consisted of 0.1μg/ml of LPS incubated with the mast cells. (N=2).

The same gene expression pattern of IL-6 as in Figure 12 was observed for co-incubation of mast cells with probiotics for one hour (Figure 13), however, when the mast cells were co-incubated with probiotics in the presence of LPS for 1 hour, some strains

showed a decrease in IL-6 gene expression compared to positive control in which no probiotic treatment was given (Figure 14). In particular, BI was the only probiotic that reduced relative IL-6 gene expression at both MOI values to a greater extent compared to the others. BB reduced IL-6 gene expression at only an MOI of 10 to the levels seen with BI. JB-1 at MOI of 10 and DSM at both MOI values produced a modest decrease in IL-6 gene expression. When mast cells were pre-treated for one hour prior to a 1 hour LPS challenge, the trends seen previously disappeared, with no significant changes present (Figure 15).

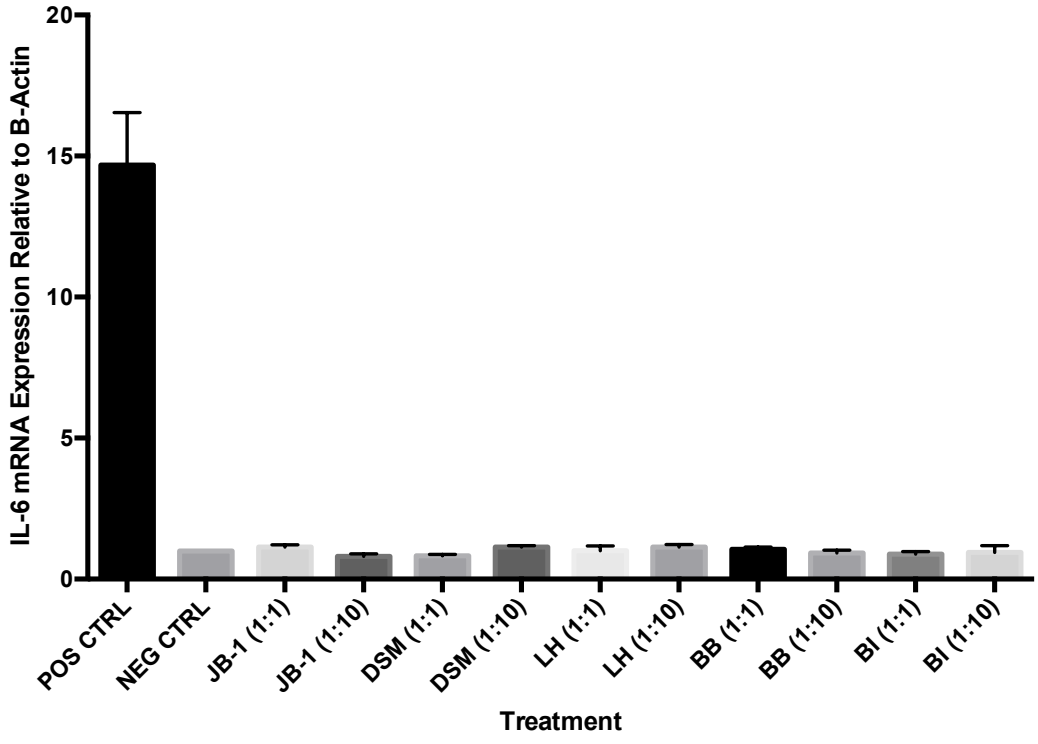

Figure 13. Mast cells and probiotics were co-incubated for 1 hour. Relative gene expression of IL-6 was compared to β-actin as a reference gene. Negative control consisted of medium, and positive control consisted of 0.1μg/ml of LPS incubated with the mast cells. (N=3).

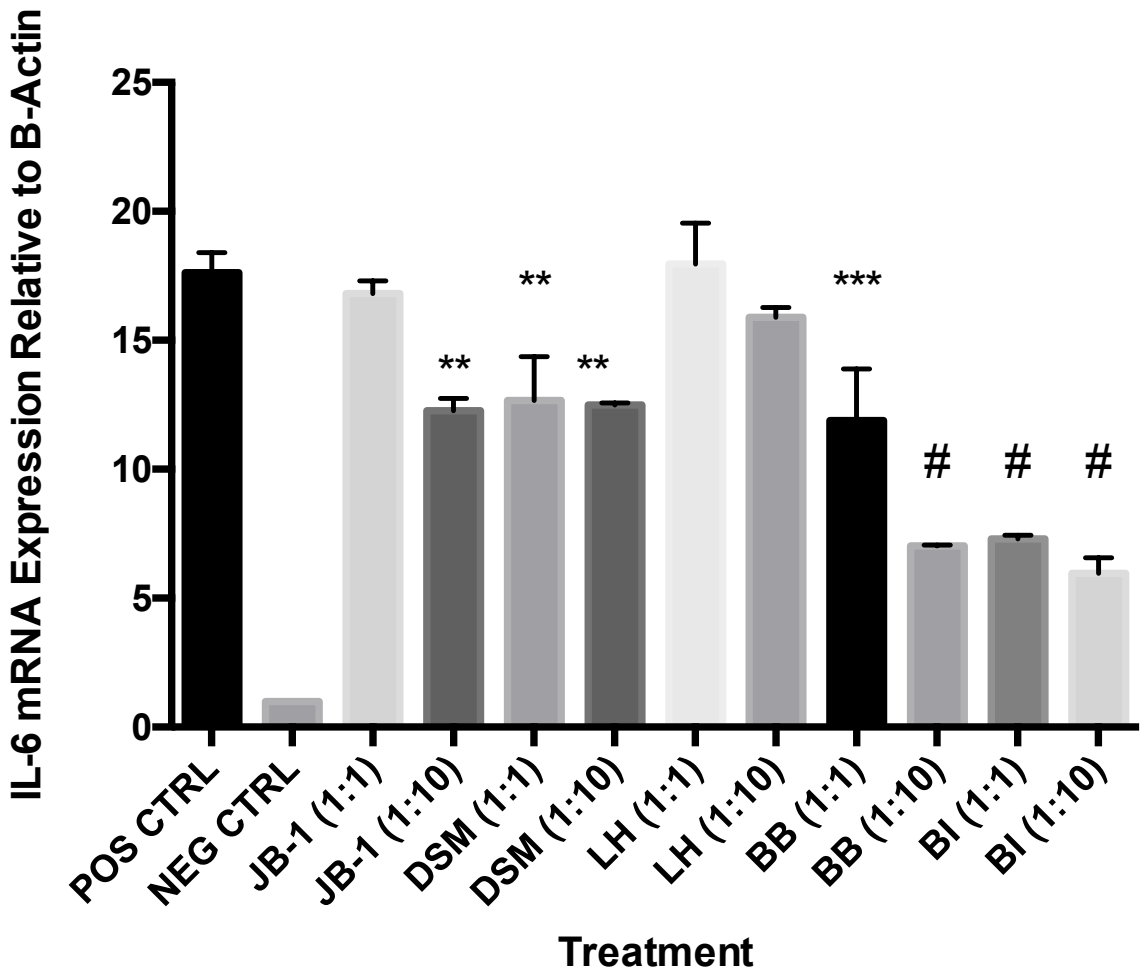

Figure 14. Mast cells and probiotics were co-incubated for 1 hour with 0.1μg/ml of LPS. Relative expression of IL-6 was compared to β-actin as a reference gene. Negative control consisted of medium, and positive control consisted of 0.1μg/ml of LPS incubated with the mast cells. (N=3). A one-way ANOVA was performed with Dunnett's multiple comparisons test (**p<0.01, ***p<0.001, #p<0.0001).

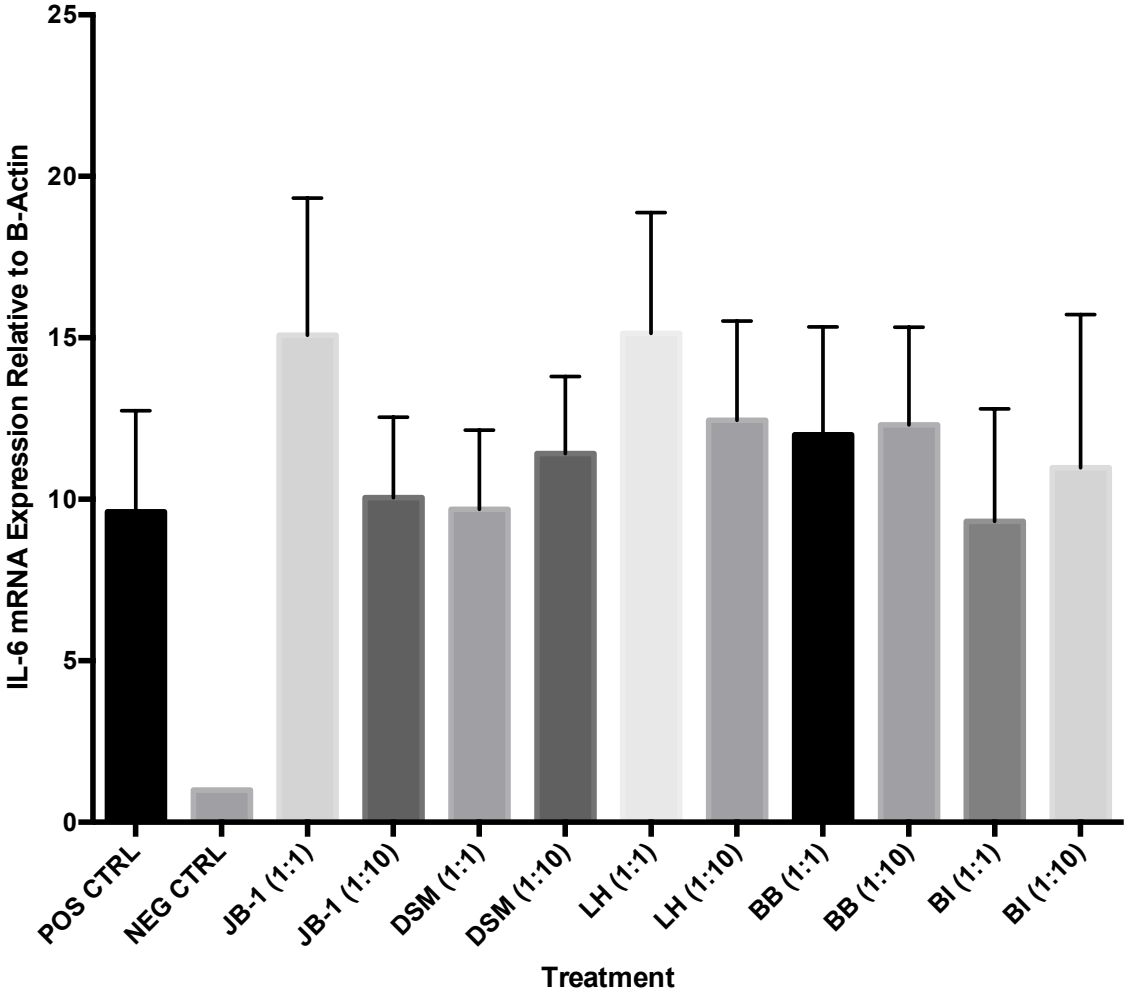

Figure 15. Mast cells and probiotics were co-incubated for 1 hour and then LPS was added for a final concentration of 0.1μg/ml. Relative expression of IL-6 was compared to β-actin as a reference gene. Negative control consisted of medium and no LPS, and positive control consisted of 0.1μg/ml of LPS with no treatment. (N=3). A one-way ANOVA was performed with Dunnett's multiple comparisons test.

6.3.2 Analysis of Relative IL-10 Gene Expression

Relative gene expression of the pro-inflammatory cytokine IL-10 was analyzed in mast cells co-incubated with probiotics in the presence of 0.1 μg/ml of LPS (Figure 16). LH at MOI of 10 as well as BB and BI at both MOI values produced an increase if IL-10 compared to both negative and positive controls. The large increase in IL-10 gene

expression observed with BI at MOI of 10 mirrors the decrease in IL-6 observed previously under the same experimental conditions (Figure 14).

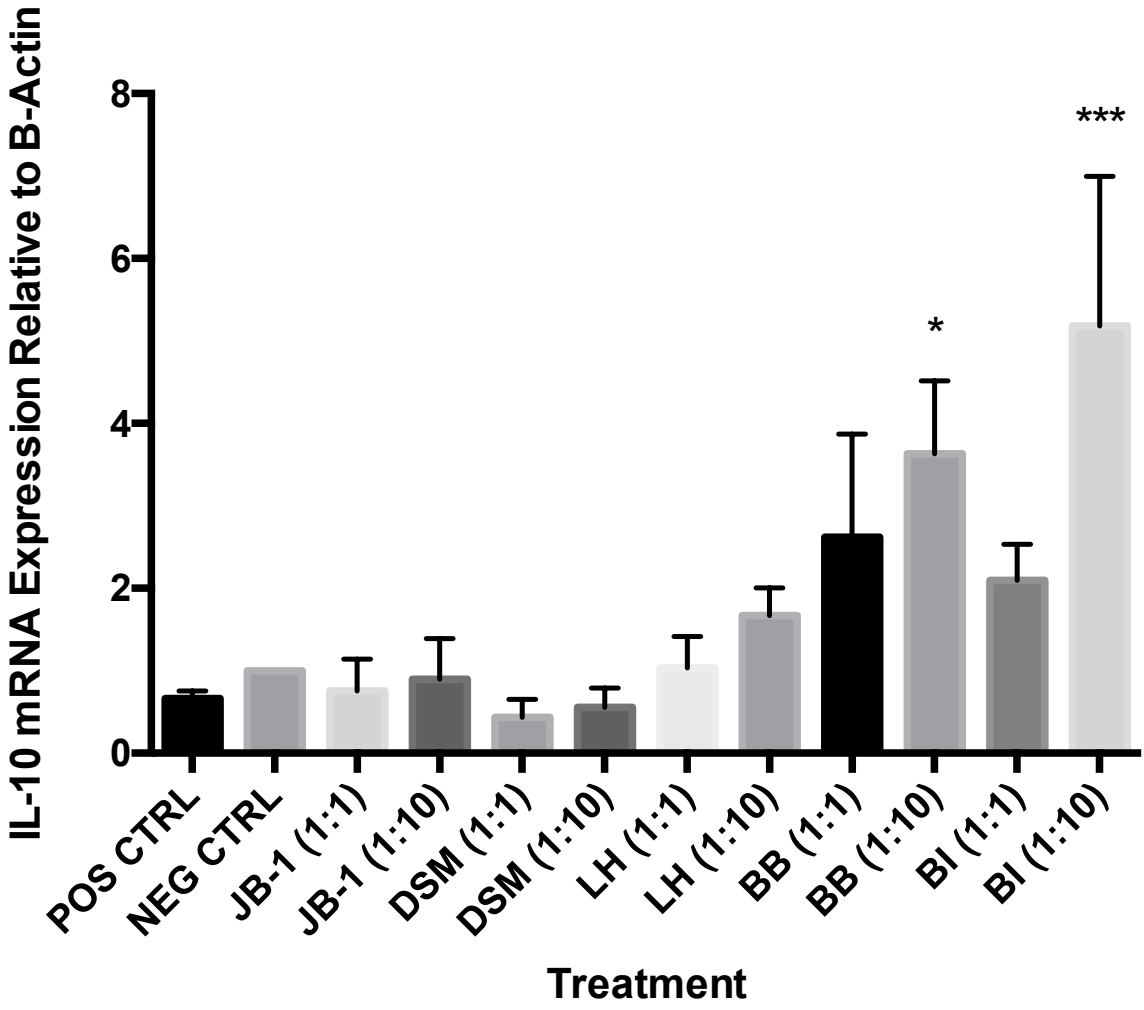

Figure 16. Mast cells and probiotics were co-incubated for 1 hour with 0.1μg/ml of LPS. Relative gene expression of IL-10 was compared to β-actin as a reference gene. Negative control consisted of medium, and positive control consisted of 0.1μg/ml of LPS incubated with the mast cells. (N=3). A one-way ANOVA was performed with Dunnett's multiple comparisons test (*$p<0.05$, ***$p<0.001$).

Chapter 7: Discussion

We have demonstrated that treating mast cells for 30 minutes with whole probiotics JB-1, DSM and LH and their MVs (Figure 5) and 2 hours of whole probiotic treatment of JB-1, DSM, LH, BB and BI prior to an allergen challenge did not mitigate IgE-mediated mast cell degranulation (Figure 6) as measured by relative β-hexosaminidase release compared to total release controls. However, while the probiotics or their MVs did not significantly decrease degranulation, they did not significantly increase degranulation either. There is no additional activation of the IgE-mediated degranulation pathway, suggesting that probiotics do not elicit any background IgE-mediated inflammation. This suggests that while mast cell degranulation may not be affected by direct interaction with probiotics or their MVs, probiotic bacteria may act on mast cells in through a different mediator *in vitro*. Previous work by Khambati et al. (2016), has demonstrated that bacterial quorum-sensing molecules N-3-oxo-dodecanoyl-L-homoserine lactone and N-dodecanoyl-L-homoserine lactone decrease β-hexosaminidase release by more than half, at doses of ≥10μM *in vitro*. The prebiotic human milk oligosaccharide, 6^{1}-sialyllactose at a dose of 1mg/ml decreases IgE-mediated β-hexosaminidase release from cultured mast cells at high concentrations of 100 and 1000ng/ml of the IgE-activating antigen, DNP-HSA (Castillo-Courtade et al., 2015). Both quorum-sensing molecules and prebiotics are conducive to probiotic microbe growth, indicating a potential role in the attenuation of mast cell activation pertaining to probiotics and/or their released components. The objective of our work was to determine if probiotics or their MVs had any effect on mast cell degranulation through directly interacting with them.

In an *in vivo* model, peritoneal mast cells isolated from rats treated orally for 9 days with whole JB-1 bacteria showed a decrease in relative β-hexosaminidase release by approximately 20% (Forsythe et al., 2012). The presence of an effect in the *in vivo* model suggests that alternate mediators may be interacting with the probiotics or MVs along with the mast cells. Different durations of treatment can additionally be tested, and this can be enabled in future experiments by adding additional SCF to the culture medium while mast cells are being treated with probiotics, allowing mast cell sviability to persist for as long as 24 hours (Oksaharju et al., 2011).

While the exact mechanism of how probiotics attenuate mast cell degranulation is unknown, there are different pathways that are hypothesized for how mast cells may respond to probiotics. It is known that mast cell degranulation is dependent on the polymerization and dynamics of microtubules, such that treatment of mast cells with the microtubule inhibitor nocodazole disrupts IgE-mediated degranulation entirely (Nishida et al., 2005), but this is unlikely, as cell viability is unaffected. Mast cells may also be affected by probiotics indirectly, through probiotic interaction with different cell types that affect mast cells. T-cells can both activate and suppress mast cell activation, as demonstrated by supernatant collected from activated, but not resting T cells (Mekori and Hershko, 2012; Shefler et al., 2010). CD4+CD25+ regulatory T cells can suppress mast cell degranulation, mediated through the OX40 ligand, OX40L expressed by mast cells that mediate T cell proliferation (Gri et al., 2009). Probiotic administration affects T cells, as well as dendritic and B cells which in turn act on mast cells to modulate mast cell activity. Administration of a probiotic mixture *in vitro* and *in vivo* demonstrated a shift in

T cell phenotype towards regulatory phenotypes and elevation in IL-10 levels by dendritic cells (Kwon et al., 2010).

Alternatively, the gene of key pathway components of the degranulatory pathway may be affected. Treatment of mast cells for 24 hours with probiotic *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* Lc705 decreased FcεRI gene expression by approximately 1.2-fold, consequently decreasing the number of available sites for mast cells to bind antigen-specific IgE (Oksaharju et al., 2011). A similar effect on FcεRI expression is observed with cytokines, such that the activation of TLR receptors results in a decreased gene expression of FcεRI. LPS stimulation of mast cells causes IL-6 and IL-10 release among other cytokines. When cultured with IL-10 at a concentration of 10ng/ml, gene expression of FcεRI decreases, and this effect grows larger the longer the exposure of the culture to IL-10 (Gillespie et al., 2004; Norton et al., 2008). While probiotics or their MVs may have no direct effect on degranulation, they may instead mediate mast cell degranulation through alternate pathways that modulate the IgE-mediated allergy pathway.

There has been work done with whole probiotic bacteria; however, there has not been much focus on the direct interaction of probiotic MV isolates with mast cells. Kim et al. (2016), treated mice orally with MVs from *Bifidobacterium longum* to demonstrate a decrease in mast cell degranulation, as measured by serum mast cell protease-1 and mast cell apoptosis. MVs from JB-1 have been shown to shift dendritic cell phenotype to a regulatory phenotype, and our objective was to determine if the effect could be replicated with mast cells. Future work on the effect of probiotic and MVs on mast cell

degranulation may focus on longer treatment periods and adjustment of the culture conditions to improve viability.

The lack of effect on mast cell degranulation does not mean that probiotics have no effect on allergy, as the TLR and IgE-mediated inflammatory pathways of mast cells interact. The TLR pathway, associated with pathogen-recognition, serves to modulate the FcεRI allergenic pathway to fine-tune the severity of the inflammatory response. The converse is also seen, that IgE crosslinking produces an increase in cytokine gene expression compared to the absence of FcεRI activation (Suurmond et al., 2015). When LPS is present, the activation of the TLR-mediated pathway inhibits the IgE-mediated pathways, such that there is less mast cell activation (Wang et al., 2017). The activation of the TLR-mediated pathway may ultimately influence how the IgE-mediated pathway behaves. In particular, we focused on cytokines IL-6, a pro-inflammatory cytokine and IL-10, a regulatory cytokine to determine the phenotype of the mast cells.

IL-6 is a cytokine associated with inflammation and pathogen recognition. It is synthesized *de novo* upon mast cell stimulation by LPS, and increased levels of IL-6 are associated with allergic disease and immune activation (Desai et al., 2016). The ability for probiotics or their MVs to suppress relative IL-6 gene expression and release suggests there may be potential therapeutic benefit in treating allergy and suppressing inflammation. LPS has been shown to produce an increase in IL-10 release despite its role in pathogen-recognition and subsequent inflammatory response (Suurmond et al., 2015; Wang et al., 2017), indicating that IL-10 may play an immunomodulatory role, such that increased release with exposure to pro-inflammatory stimuli may serve to control inflammation. Wang et al. (2017) proposed that IL-10 is associated with a

decrease in the gene expression of FcεRI receptors on the surface of mast cells upon LPS exposure prior to an allergen challenge, which may explain how the TLR-mediated pathway modulates the activity of the IgE-mediated pathway.

When directly co-incubated with mast cells in the absence of LPS challenge for 24 hours, none of the probiotic bacteria produced a relative increase in IL-6 at all MOIs of 1, 10 and 100 (Figures 7A). This absence of an IL-6 increase indicates that the probiotics do not shift mast cells to a pro-inflammatory phenotype, which is supported by the increase in IL-10 (Figure 7B). When mast cells are co-incubated with probiotics in the presence of LPS, no significant decrease in IL-6 release was observed (Figure 8). The experiment was performed with the MVs of LH, BB and BI, as these strains of probiotic had shown the most effect in earlier replicates. There was no significant decrease in IL-6 release observed (Figure 9).

However, when co-incubated with whole probiotics as a treatment for 1 hour prior to a 4 hour LPS challenge, only the *Bifidobacterium* strains, BB and BI produced a significant decrease in relative IL-6 gene expression compared to untreated controls (Figure 10D, E). The effect of BB was only observed when the MOI was 1:10, and at a high concentration of LPS. In contrast, BI produced this effect at a 1:1 and 1:10 MOIs at all concentrations of LPS. This may indicate that BI is the most robust probiotic in its ability to shift mast cell phenotype away from an inflammatory phenotype. The level of relative IL-6 suppression by both BB and BI are comparable to that of the mast cell stabilizer, dexamethasone when comparing percent inhibition of IL-6 mast cell release (Leal-Berumen et al., 1994) of ≥40%. The study by Leal-Berumen (1994) measured IL-6 release when mast cells were activated with anti-IgE, while the study we performed

stimulated mast cells with LPS. Although the modes of activation are different, the level suppression of relative IL-6 release of the probiotics is comparable to that of a current therapeutic.

When the same conditions were repeated for MVs, LH, BB and BI were associated with an attenuation of relative IL-6 release at an intermediate dose of LPS at both MOIs of 1:1 and 1:10 (Figure 11A-C). This difference in effect in MVs indicates that whole cells and MVs may act on mast cells differently (Kim et al., 2016). The same doses of LPS were not used across experiments, because it was observed that MVs did not produce a change in relative IL-6 release at high doses of LPS (≥1.0 μ g/ml). Relative release accounted for the inconsistency in absolute IL-6 release across biological replicates, confirmed among the literature, as absolute levels of IL-6 release varies between different studies in the literature (Leal-Berumen et al., 1994; Zhu and Marshall, 2017).

When treated with probiotic bacteria for 24 hours, only DSM, BB and BI produced an elevation in IL-10 release relative to medium (Figure 7B). As previously mentioned, this is consistent with a regulatory mast cell phenotype. This is consistent with both BB and BI, which, when pre-treated with mast cells showed a relative decrease in IL-6 (Figure 10). This effect is not seen for DSM, which suggests that this probiotic is not sufficient for altering the relative levels of both cytokines and that it may only affect IL-10 release. The relative amount of IL-10 release was measured for both the co-incubation and pre-treatment preceding co-incubation conditions described above, however, no detectable amounts of IL-10 were detected (data not shown). This may indicate that for measurable amounts of IL-10 release, incubation periods exceeding the

4-hour period we used may be required. This poses an additional challenge, as prolonged exposure to live bacteria results in mast cell inviability due to toxic metabolites produced by bacteria. While the classification of mast cell phenotype is not standardized throughout the literature, the ability for BB and BI strains to affect both IL-6 and IL-10 release indicate that these probiotics have a more pronounced effect on mast cell phenotype compared to other strains, which only alter one or none of the two cytokines of interest. Future work will focus on analyzing the complete relative cytokine release profile of both whole bacteria and MVs for all co-incubation durations and experimental conditions.

Comparing the effect of whole bacteria to their MV isolates, the MVs are more consistent when pre-treated with mast cells before an LPS challenge (Figure 11) than the whole bacteria (Figure 10). While not all probiotic MVs have yet been tested, LH MVs produced a significant decrease in IL-6 in contrast to the whole bacteria, which produced no significant effect. Whole bacteria still produce MVs when co-incubated with the mast cells, as MVs continuously bud off the membranes of live cells, but the concentration of MVs in the isolates are much higher, given that they are cumulatively the amount of MVs produced by a the same number of bacteria over a longer period of time than the co-incubation duration. This suggests that MVs may play a key role in how probiotics act on immune cells to modulate their function. Kim et al. (2016) visualized mast cell internalization of *Bifidobacterium longum* MVs and that this uptake was relatively unaffected by temperature. Uptake of JB-1 MVs was also observed in dendritic cells by Al-Nedawi et al. (2016), but it is unclear if this is a strain-specific effect, as

internalization of JB-1 MVs or the MVs of our probiotic selection have not been yet been observed.

We then sought to confirm our results with qPCR, and to determine if the probiotics that did not alter cytokine release had any effect on cytokine gene expression. When co-incubated with mast cells for both 4 hours (Figure 12) and 1 hour (Figure 13), there was no relative increase in gene IL-6 gene expression compared to the housekeeping gene, β-actin. The 1-hour incubation suggests that an hour is sufficient for gene expression to be observed when challenged with LPS. This is consistent with our ELISA data (Figure 7A), suggesting that the probiotics do not elevate IL-6 and inflammation even at the level of gene-expression. When co-incubated with the probiotics during a simultaneous 1-hour LPS challenge, JB-1 at MOI of 1:10, DSM, BB and BI at MOI of both 1:1 and 1:10 significantly decreased relative IL-6 gene . This effect is most pronounced in BB at MOI of 1:10 and BI at both MOIs, consistent with direct cytokine release (Figure 10). When mast cells are pre-treated for 1 hour with probiotics prior to a 1 hour LPS challenge, there is no distinct trend. This may suggest that more time may be required to fine-tune gene expression to match observed cytokine released levels (Figure 10).

The results of this thesis suggest that the potential for probiotics or their MVs to alter the cytokine profile of mast cells may play a role in ameliorating inflammation associated with allergy. Probiotics do not promote mast cell inflammation as measured by cytokine release and cytokine gene expression levels. While treatment with probiotics was concurrent with a 4-hour LPS challenge, there was no significant increase in IL-6 release, but there was a decrease in IL-6 gene expression for J B-1 at MOI of 1:10, DSM,

BB and BI at MOI of both 1:1 and 1:10. When a 1-hour treatment with probiotics was performed before the 4-hour LPS challenge, there was a significant decrease in IL-6 release for BB at MOI of 10 at 1.0μg/ml LPS and BI at MOIs and concentrations of LPS. This effect was not observed at the level of gene expression. IL-10 release was elevated for DSM, BB and BI at all MOIs, but this was only observed for BI and BB at MOI of 10 at the level of gene expression. Either indirectly through MVs, or directly interacting with mast cells, probiotics alter the inflammatory phenotype.

Chapter 8: Planned Work

8.1 Elucidation of Mast-Cell Probiotic Interaction Mechanisms

The overall goal of this thesis is to explore the mechanisms by which non-pathogenic, potentially probiotic bacteria directly interact with mast cells and thereby reduce symptoms of food allergy in addition to gaining a deeper understanding of mast cells on a functional level. Mast cells may respond to probiotic bacteria by shifting to an immunoregulatory phenotype, thereby preventing activation and downstream symptoms, or may cease the release of pro-inflammatory mediators, including cytokines. By measuring and comparing different cytokines released by mast cells, the shift in phenotype may be predictive of mast cell behaviour in a model of allergy.

In addition, Kim et al. (2016) stained MVs and showed that mast cells uptake MVs, making physical contact with MVs necessary for the process by which they inhibit mast cell activation. We are also interested in studying which proteins of MVs enable this, or if MVs may release factors into the extracellular environment that act on mast cells, allowing them to act on mast cells without direct contact.

In addition to testing the ability of bacteria and MV to attenuate IgE-mediated activation of mast cells, we plan to assess:

I) Changes in the cytokine profile produced by mast cells and the potential increase in IL-10, suggesting a shift to an immunoregulatory phenotype.

II) The requirement that bacteria must be live to attenuate mast cell function during an inflammatory challenge

III) Determine if mast cells uptake MVs through fluorescent labeling of MVs and visualization of uptake.

IV) Assess complete cytokine profile change upon different PAMP exposure.

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Supplementary Information

Supplementary Table 1: Β-hexosaminidase Experiment and Assay Buffers

HEPES-Tyrode Buffer (pH=7.2 - 7.4)	4.0g NaCl 0.5g Glucose 0.5g BSA 1.43g HEPES 0.1005g KCl 0.0735g $CaCl_2 \bullet 2H_2O$ 0.0276g $NaH_2PO_2 \bullet H_2O$ Add dH_2O up to 500ml Adjust pH with NaOH
0.05M Citrate Buffer (pH=4.5)	5.25g Citric acid 7.35g Trisodium citrate Add dH_2O up to 500ml Adjust pH to 4.5 with HCl
0.05M Carbonate Buffer (pH=10.5)	1 carbonate buffer tablet (Sigma) Add dH_2O up to 100ml/tablet Adjust pH to 10.5 with NaOH
p-NAG	15.1mg p-NAG Add 50 ml 0.05M Citrate Buffer Mix overnight at 4°C covered in foil

Supplementary Table 2: Primers to be used in qPCR (Leclercq et al., 2017; Listvanova et al., 2003)

Gene	**Forward Primer (5'-3')**	**Reverse Primer (3'-5')**
IL-6	CTGCAAGAGACTTCCATCCAGTT	GAAGTAGGGAAGGCCGTGG
IL-10	TGAACAGCCCCCCAATGT	TCAACTCTTTCCGCATAGTCAGAT
TNFα	CCACCACGCTCTTCTGTCTAC	TGGGCTACAGGCTTGTCACT
β-actin	GGATGCAGAAGGAGATCACTG	CGATCCACACGGAGTACTTG

Supplementary Figure 1. qPCR experimental parameters.