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THE ROLE OF THE PROBIOTIC *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* ON SYSTEMIC IMMUNE FUNCTION IN HEALTHY ADULTS

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ABSTRACT

Probiotic bacteria are functional ingredients that provide significant health benefits, including modulation of host immune function and attenuating the risk and severity of infections. Numerous studies have examined the influence of probiotic bacteria, either alone or combined, on innate and adaptive immune function in an effort to link probiotic use with changes in inflammatory mediators or risk of infection. However, observed results vary by genus, species and strain of organism studied. Furthermore, the use of mixtures of probiotic bacteria makes it difficult to determine the immunomodulatory properties of BB-12 or other probiotic species. Thus, careful analysis of the immune outcomes affected by each of the commonly used probiotic strains is necessary.

Bifidobacterium animalis subsp. *lactis* BB-12 is a widely used strain in the genera *Bifidobacterium*. Evidence suggests that consumption of BB-12 combined with other probiotic species can modulate both innate and adaptive immune responses in human subjects. A limited number of studies have investigated the role of BB-12 alone on immunity and disparate effects of BB-12 on immune outcomes are reported. Therefore, the primary aim of this project was to evaluate the effect of oral consumption of one strain of probiotic bacteria, BB-12, at a dose of log 10 ± 0.5 CFUs/day, on innate and adaptive immune responses in healthy adults in a randomized, partially blinded, 4-period cross-over free-living study. There is evidence that the matrix used to deliver probiotic bacteria may influence the performance and efficacy of probiotic interventions *in vivo*. BB-12 can be consumed either in dairy products, especially yogurt, or in capsule form. Furthermore, BB-12 can be

added into yogurt either prior to or following the yogurt fermentation process, which varies among manufacturers. Thus, a secondary goal of this study was to determine if the delivery matrix (yogurt smoothies vs capsule), and timing of the addition of BB-12 to the yogurt smoothies (pre- or post-fermentation) impacted the immunological responses to BB-12 in humans.

Healthy adults (n=30) aged 18-40 years old were recruited, and received 4 treatments for 4 weeks in a random order: A) yogurt smoothies alone; smoothies with BB-12 added B) before or C) after yogurt fermentation, or D) BB-12 given in capsule form. At baseline and after each 4-week treatment, peripheral blood mononuclear cells (PBMCs) were isolated, and functional immune outcomes and phenotypic marker expression on immune cells was assessed. Dietary intake of total calories and various nutrients were assessed via self-reported 3-day dietary recall. Physical activity status was evaluated via self-reported IPAQ questionnaire. Incidence and severity of cold or flu infection in the past month was assessed using established self-reported URTI questionnaire.

The goal of study 1 was to determine at baseline which endogenous and exogenous host factors contribute to the heterogeneity in innate and adaptive immune responses and cold or flu status among healthy subjects; and if these host factors also confound the relationship between phenotypic marker expression, immune function, and cold or flu status. We analyzed baseline immune function of participants. This included anti-CD3 induced T cell proliferation and secretion of IL-2 and IFN-γ, and LPS stimulated TNF-α and IL-6 secretion from PBMCs. We also examined dietary intake, physical activity level, and the incidence and severity of cold or flu symptoms in our

participants. We demonstrated that host-related factors (including age, BMI, physical activity, and daily intake of total calorie and various dietary components) contributed significantly to heterogeneity in T cell effector function and cold or flu status, and confounded the association between activation marker expression on T cells and T cell effector function. These factors also impacted the association between innate and adaptive immune response and incidence and severity of cold or flu infection. We also found that individual lifestyle and dietary variables contribute a small amount to T cell effector function and cold or flu status. However, combinations of these variables significantly improved the predictive relationship between activation marker expression and T cell proliferation and IL-2 secretion, and also strengthened the associations between IL-6 and IFN-γ secretion and the incidence and severity of cold or flu symptoms, respectively. In conclusion, these results suggest that lifestyle and dietary factors are important variables that contribute to immune responses and should be included in human clinical trials that assess immune endpoints. The analysis with baseline immune data has enabled us to understand the variability in human immune outcomes and has been insightful about how to analyze the treatment effects of BB-12 consumption on immune outcomes in our clinical trial.

The goal of study 2 was to evaluate the effect of BB-12 consumption on innate immune responses; and to determine if the innate immune response to BB-12 differed depending on the delivery matrix (yogurt smoothies vs capsule) of BB-12 and timing of addition of BB-12 to yogurt fermentation process (pre- or post-fermentation). We found that consumption of BB-12 delivered in yogurt smoothies post-fermentation significantly reduced pro-

inflammatory cytokine (TNF-α) secretion from peripheral myeloid cells stimulated with heat-inactivated BB-12 (p=0.0490) or LPS (p=0.0387) compared to baseline in young healthy adults, suggesting an anti-inflammatory effect of BB-12. We also found that BB-12 interacted with peripheral myeloid cells via Toll-like receptor 2 (TLR-2), and consumption of yogurt smoothies with BB-12 added post-fermentation for 4 weeks significantly decreased TLR-2 expression on peripheral blood derived monocytes, which may contribute to the reduction in TNF-α secretion in participants. These findings are not only the first to demonstrate anti-inflammatory properties of BB-12, but also indicate that the matrix of BB-12, and the timing of its addition to yogurt fermentation process influenced the immunological effects of BB-12.

The goal of study 3 was to evaluate the effect of BB-12 consumption on T cell and NK cell function and concurrently self-reported cold or flu incidence and severity. A sub-aim was to determine if delivery matrix (yogurt smoothies vs capsule) of BB-12 and timing of addition of BB-12 to yogurt fermentation process (pre- or post-fermentation) influenced immune responses and related cold or flu status. We found that participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form increased T cell proliferation and cytokine (TNF-α and IL-2) secretion, and NK cell cytotoxicity, and concurrently reduced number of days with cold or flu by 2-3 days. However, consumption of yogurt smoothies with BB-12 added post-fermentation did not change T cell and NK cell function; and did not alter severity of cold or flu. These findings

demonstrate that the timing of addition of BB-12 to yogurt smoothies influenced the immunomodulatory properties of BB-12.

Combined, the results from our clinical trial demonstrate that diet and physical activity variables should be assessed in clinical trials to control for variability in immune outcomes. Furthermore, consumption of 10^9-10^{10} cfu/d of BB-12 for 4 weeks was effective in modulating responses of both innate (cytokine secretion from myeloid cells and NK cell cytotoxicity) and adaptive cells (T cell proliferation and cytokine secretion) in young adults. We also found that the delivery matrix of BB-12 (e.g. yogurt smoothies vs. capsule) and timing of BB-12 addition to yogurt influences the effect of BB-12 on immune responses, and inflammatory and infection-related outcomes. Specifically, the consumption of yogurt smoothies with BB-12 added post-fermentation results in blunted innate and adaptive immune responses, suggesting that this processing technique influences the probiotic activity of BB-12, and warrants further study. Lastly, we found strong correlation between changes in T cell and NK cell function and URTI severity suggesting that immune modulation may be one mechanism underlying the link between probiotic consumption and infection-related outcomes. Results from the current studies will contribute to our understanding that BB-12 alone modulates systemic immunity in humans and sheds light on potential mechanisms by which BB-12 is exerting its effects on immunity and infection risk.

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LIST OF ABBREVIATIONS

NK: natural killer

IL: interleukin

MCP-1: monocyte chemoattractant protein-1

CTL: cytotoxic T cell

MHC: major histocompatibility complex

Ig: immunoglobin

IFN-γ: Interferon-gamma

TNF-α: Tumor necrosis factor-alpha

DCs: dendritic cells

LPS: lipopolysaccharide

URTI: upper respiratory tract infection

PBMC: peripheral blood mononuclear cell

CFS: chronic fatigue syndrome

UC: ulcerative colitis

IBD: inflammatory bowel disease

OVA: ovalbumin

KO: knock-out

DMH: dimethylhydrazine

BALF: bronchoalveolar lavage fluid

PGN: peptidoglycan

LTA: lipoteichoic acid

EPS: exopolysaccharide

BMI: body mass index

IPAQ: International Physical Activity Questionnaires

TC: cholesterol

TG: triglycerides

HDL: High density lipoprotein

LDL: low density lipoprotein

hs-CRP: high-sensitivity C-reactive protein

MFI: mean fluorescence intensity

SI: stimulation index

n-3 PUFA: n-3 polyunsaturated fatty acids

PMA: phorbol myristate acetate

PHA: phytohaemagglutinin

SEB: staphylococcal enterotoxin

TT: tentanus toxoid

Con A: Concanavalin A

OR: Odds ratio

MET: metabolic equivalent of task

TLR: Toll-like receptor

GI: gastrointestinal

MoDCs: monocyte-derived dendritic cells

COX-2: cyclooxygenase-2

IBD: inflammatory bowel disease

CFU: colony-forming unit

MRS: de Man, Rogosa, and Sharpe

NNLP: nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate

BL: baseline

YS: yogurt smoothie

PRE: pre-fermentation

POST: post-fermentation

CAP: capsule

IEC: intestinal epithelial cell

NF-κB: nuclear factor-κB

SOCS: suppressor of cytokine signaling

FOXP3: forkhead box P3

TGF-β: Transforming growth factor beta

LGG: *Lactobacillus Rhamnosus* GG

NKCC: natural killer cell cytotoxicity

APC: antigen-presenting cells

MLN: mesenteric lymph node

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CHAPTER 1

INTRODUCTION

Probiotic bacteria are functional ingredients that provide significant health benefits, including modulation of host immune function (1). A functional immune system is required to maintain health, and it is important in the clearance of foreign pathogens and tumor cells and reduction in inflammation and infection risk. Immunomodulatory properties of probiotic bacteria in the genera *Lactobacillus* and *Bifidobacterium* are extensively studied, and overall the results are mixed (2-8). It appears that large strain and species differences exist (8). In addition, variability due to dose and duration of supplementation, characteristics of the study population, and delivery matrix of probiotics have been reported (8). Thus, well-designed clinical trials using a single strain of probiotics on immune, inflammatory, and infection outcomes are needed.

Bifidobacterium animalis subsp. *lactis* BB-12, a strain in the genera *Bifidobacterium*, is widely used in food manufacturing (9). In comparison to other probiotic strains, BB-12 has numerous advantages, including maintenance of viability until consumption, resistance to degradation by gastric acid and bile, and adhesion to human intestinal epithelial cells and mucin following consumption (9). These features may allow BB-12 to survive during passage through the gastrointestinal tract and exert its probiotic effect (9-13). Evidence suggests that consumption of BB-12 combined with other probiotic species can modulate both innate and adaptive immune responses in human subjects (14-16). However, the results vary based on which probiotic bacteria were given in conjunction with BB-12 (14-16). Studies

investigating the effect of BB-12 alone on immunity also yield inconsistent results (15, 17-20). Additional studies are needed to clarify the role of BB-12 alone on innate and adaptive immune responses, and inflammation- and infection-related outcomes in humans. Therefore, the primary aim of this project was to evaluate the effect of one strain of probiotic bacteria, BB-12, on innate and adaptive immune responses in healthy adults in a randomized, partially blinded, 4-period cross-over free-living study. Specifically, we hypothesize that consumption of BB-12 will 1) increase secretion of pro-inflammatory cytokines (TNF-α and lL-6) from heat-inactivated BB-12 or lipopolysacchride (LPS) stimulated PBMCs, and alter expression of HLA-DR and Toll-like receptor 2 on peripheral blood-derived monocytes; 2) increase T cell and NK cell effector function, and concurrently reduce incidence and severity of cold or flu infection.

Probiotics are often considered to act independently of the matrix used to deliver them to the human host (21). Dairy products, especially yogurt, are the most common delivery matrix of BB-12 (22); however, BB-12 can also be consumed as dietary supplements in capsule form (23). While this expands the options for BB-12 consumption, it is not clear if BB-12 in different delivery matrices will have different immunomodulatory effects (23). Furthermore, BB-12 can be added into yogurt either prior to or following the yogurt fermentation process, which varies among manufacturers. Fermentation products produced by BB-12 during yogurt manufacturing may also have an influence on the efficacy of BB-12. Thus, a secondary goal of this study was to determine if the delivery matrix (yogurt smoothies vs capsule), and timing of the addition of BB-12 to the yogurt smoothies (pre- or post-fermentation) impacted the

immunological responses to BB-12 in humans. In particular, we hypothesize that 1) BB-12 delivered in yogurt smoothies will have greater impact on immune responses than BB-12 in capsule form; 2) timing of addition of BB-12 in yogurt fermentation process will not influence immunological responses.

Results from this project will contribute to our understanding of how BB-12, as a single organism, modulates immunity and upper respiratory tract infection (URTI) risk and severity. Additionally, the results of this dissertation provide insight on food manufacturing process that influences the probiotic effect of BB-12.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Probiotic bacteria are functional ingredients that provide significant health benefits, including modulation of host immune function (1). A functional immune system is required to maintain health, and it is important in the clearance of foreign pathogens and tumor cells. Previous studies using various lactic acid producing probiotic strains in the genera *Bifidobacterium* report alterations in both innate and adaptive immunity, including phagocytic activity of macrophages, natural killer (NK) cell cytotoxicity, serum antibody levels, and cytokine secretion from $CD4^+$ helper T cells (2, 4, 6, 24). However, different *Bifidobacterium* species have diverse effects on immune function, indicating large strain specificity in the immunomodulatory effect of *Bifidobacterium* species. Therefore, careful analyses of the immunomodulatory properties of each commonly used *Bifidobacterium* species are necessary to fully characterize the relationship between *Bifidobacterium* consumption and immunity.

2.2. Overview of *Bifidobacterium*

Probiotics are live cultures of microorganisms that, when administered in an adequate amount, can be beneficial to the health of the host (25). Lactic acid producing bacteria, including the genera *Lactobacillus* and *Bifidobacterium*, are the most widely used probiotic bacteria (1, 26). This review focuses on the characteristics of *Bifidobacterium*, which is one of the major constituents of microbiota in human intestine (26). *Bifidobacterium* is Gram-positive, non-pore-forming, non-mobile, and anaerobic bacterium (26). It was first discovered from the feces of a breast-feeding infant in 1899 (26). There are numerous *Bifidobacterium* species; however, the most common

species used are *B. lactis, B. animalis, B. longum, B. adolescentis, B. breve,* and *B. bifidum*.

2.3. Health Benefits of *Bifidobacterium*

Bifidobacterium has been found to provide significant health benefits, including many in the gastrointestinal tract (1). Evidence from human clinical studies and animal experiments demonstrate that consumption of *Bifidobacterium* can improve intestinal microbial balance, decrease bowel transit time, as well as reduce the risk and severity of diseases in the gastrointestinal tract (27-29). Previous studies report that these probiotic bacteria can be protective in animal models of spontaneous and chemically-induced colitis due to a reduction in gene expression of pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-1β and MCP-1 (29, 30). A recent animal study demonstrates that a combination of 3 *Bifidobacteria* and 4 *Lactobacilli* strains ameliorates colitis-associated colorectal carcinogenesis in mice via an increase in anti-tumor cytokine production in the gut (31). Furthermore, in animal models of food allergy, *Bifidobacterium* can decrease allergen-specific IgE production, and alter systemic cytokine production (32). In humans, probiotics have been used to treat inflammatory and infectious diseases of the intestine (e.g. inflammatory bowel disease, irritable bowel syndrome, infectious diarrhea, colorectal cancer, *H.pylori* infection) with some success (27, 28, 31, 33). Overall, these beneficial effects may be due to the modulation of host immune response by *Bifidobacterium*.

2.4. Overview of the Immune System

A functional immune system is required to maintain health, and is essential in clearance of invading foreign pathogens and tumor cells. The

immune system is typically divided into two separate but complimentary components, innate and adaptive immunity. Innate immunity provides the first line of defense against common microorganisms, and refers to non-specific defense mechanisms that may be triggered immediately or within a short period of time of a foreign pathogen (antigen) invasion in the body. These defense mechanisms include physical barriers such as skin and intestinal membranes; proteins that exert anti-microbial activities, including lysozymes, interferon and complements; and immune cells that can recognize, phagocytose or kill invading pathogens or infected cells. Major immune cells involved in innate immune system include dendritic cells (DCs), monocytes/macrophages, natural killer (NK) cells, and granulocytes, such as neutrophils, basophils, eosinophils, and mast cells. DCs, monocytes/macrophages, and mast cells are on guard in areas of entry of a broad spectrum of microorganisms. If an invading pathogen disrupts the normal tissue environment, DCs, macrophages and mast cells release cytokines (e.g. TNF-α and IL-6) and histamine that recruit other immune cells into the damaged tissue, which initiates the inflammation process. Activation of innate immunity is critical for adequate initiation of the more complicated and effective adaptive immune response, including both humoral and cell-mediated immunity.

Immune cells of the adaptive immune system, including B and T lymphocytes, differ from innate immune cells in that they express surface receptors that are specific for invading antigens. Upon recognition of an antigen, immune cells of the adaptive immune system undergo repeated rounds of clonal expansion to eradicate foreign antigens or tumor cells.

Adaptive immunity cells also have the capacity to form immunological memory for future protection against specific antigens. Humoral immune responses are mediated by B cells, which secrete immunoglobins into peripheral blood or tissues, and these immunoglobins bind to antigens to facilitate their clearance from the body. Cell-mediated immune responses are mediated by T cells, which include $CD3^+CD4^+$ T helper cells and $CD3^+CD8^+$ cytotoxic T cells (CTL). T cell recognition of antigens requires the coordination with antigen-presenting cells (APCs), including DCs, macrophages, and B cells. APCs link innate and adaptive immunity due to their ability to process and present microbial antigens to T cells following activation and maturation. Human APCs express major histocompatibility complex (MHC) HLA-DR, which is associated with intercellular recognition and antigen presentation to T cells. Ligation of T cell receptor with antigen-specific peptides bound with MHC molecules on APCs can initiate activation of T cells. Upon activation, T cells will undergo repeated rounds of cell division, or proliferation, which is accompanied by production of various cytokines (e.g. IFN-γ, TNF-α and IL-2). T cell proliferation and cytokine production are important effector functions required for adequate T cell responses. Coordination between innate and adaptive immune responses is needed for efficient elimination of invading pathogens, and is also necessary for repairing pathogen-induced tissue damage.

2.5. Immune Effect of *Bifidobacterium* in Combination with other Probiotics

Several studies have investigated the effect of consumption of *Bifidobacterium* species in combination with other lactic acid producing bacteria on innate and adaptive immunity in human subjects and experimental

animals. Distribution of immune cells, cytokine and immunoglobin production, T cell proliferation, and monocyte/macrophage phagocytic activity vary based on which probiotic bacteria were given in conjunction with *Bifidobacterium* (31, 34-73). Consumption of a single strain of *B. lactis, B. longum, B. breve*, or *B. bifidum* in combination with other *Lactobacillus* strains (e.g. *Lactobacillus rhammosus GG, Lactobacillus paracasei, and/or Lactobacillus acidophilus*) produce variable results, with some studies reporting an increase and others demonstrating no effect of probiotic on T cell proliferation and cytokine secretion, NK cytotoxiciy, leukocyte phagocytosis or immunoglobin production in human subjects and animal models (34-59). Moreover, studies with combinations of *Bifidobacterium* species alone or in conjunction with *Lactobacillus* strains have also reported inconsistent findings on T cell, B cell, and DC responses in *in vivo* and *in vitro* models (31, 60-73). The interpretation of the results from these studies is difficult because multiple probiotic strains were used. Compared to an individual strain, multiple strains may compete for the same Toll-like receptor, which can recognize and interact with various probiotic bacteria (20, 74). The competition among strains may prevent some *Bifidobacterium* species from interacting with immune cells (20, 74). In addition, certain probiotic strains may also suppress or alter the immunomodulatory effect of others by secreting inhibitory factors, which may reduce the efficacy of probiotic mixtures (74, 75). Therefore, it is essential to clarify the role of single strain of *Bifidobacterium* species on immune function in human subjects and animal models.

2.6. Immunomodulatory Properties of Various Single Strains of *Bifidobacterium* Species in Human and Animal Studies

2.6.1. Adaptive Immunity

Distribution of T cells and T cell Subsets in Peripheral Blood

Numerous studies in humans and experimental animals have explored the effect of various *Bifidobacterium* species on the distribution of T cells and T cell subsets in peripheral blood. Randomized controlled trials that investigate the effect of *B. longum and B. lactis* consumption on the distribution of T cells and T cell subsets demonstrate inconsistent findings. Subjects with a clinical history of Japanese cedar pollinosis had no change in T cell counts in peripheral blood after consuming *B. longum* BB536 for 13 weeks during pollen season (76). Similarly, healthy adults who consumed a fermented milk drink containing *B*. *lactis* BB-12 for 3 weeks or BB-12 capsules for 6 weeks had no change in the number or percent of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells and activated T cells in peripheral blood (15, 20, 77). However, consumption of another *B. lactis* strain HN019 for 3 weeks significantly increases the proportions of $CD3^+$ and $CD4^+$ T cells and activated T cells without altering the proportion of $CD8^+$ T cells in healthy older adults (2). Overall, consumption of *B. longum* or *B. lactis* BB-12 may not influence distribution of T cells and T cell subsets; however, *B. lactis* HN019 may increase the percentage of total, helper, and activated T cells. Thus, differences in the modulatory effect of *Bifidobacterium* on T cell distribution exist not only between species, but also in the sub-species level of *B. lactis.*

Animal studies that investigate the effect of various *Bifidobacterium species* on the distribution of T cells also yield mixed results depending on the model and lymphoid organ evaluated. Daily consumption of *B. lactis* HN019 for 7 days prior to *Salmonella Typhimurium* infection does not change the

percentage of $CD4^+$ and $CD8^+$ T cells in mesenteric lymph nodes and Peyer's patches of BALB/c mice (78). Oral administration of skim milk containing 0.9-1.5 x 10^9 cfu/d of *B. bifidum* BGN4 for 4 weeks does not alter the number of $CD4^+CD69^+$ activated T cells in the spleen of mice with IBD (79). The percentage of Tregs in spleen, draining lymph nodes and Peyer's patches in BALB/c mice that received 10^9 cfu of *B. longum* AH1206 for 14 days is not different from controls following challenge with OVA (80). Similarly, the percentage of Tregs in lung and T cells in bronchoalveolar lavage fluid in BALB/c mice that received 10^9 cfu of *B. longum* AH1206 for 14 days is not different from controls following challenge with OVA (80). Thus, the percentage or number of T cells and subsets, activated T cells and Tregs are not influenced by consumption of *B. lactis, B. bifidum,* or *B. longum*.

Various *Bifidobacterium* species have been examined as interventions in rodent models of lung inflammation. Oral administration of 10^9 cfu/d of *B. lactis* BB-12 for 8 weeks increases expression of FoxP3 in peribronchial lymph node cells in germ-free BALB/c mice with OVA-induced airway inflammation (81). The percentage of Tregs in lung of BALB/c mice that received 10^9 cfu of *B. longum* AH1206 for 14 days is greater compared with controls following a single challenge with OVA (80). Similarly, oral gavage with 10^8 cfu of *B. breve* for 14 days increases the number of Tregs in the intestine and spleen of OVA-sensitized mice (32). Overall, *B. lactis, B. longum*, and *B. breve* may reduce airway inflammation by increasing the percentage or number of Tregs in OVA-sensitized mice.

The infiltration of T cells into various organs in response to inflammatory stimuli has also been examined following the consumption of

various *Bifidobacterium* species. Oral consumption of 10^9 cfu of *B. animalis* for 12 weeks decreases the number of T cells infiltrating in the lung in BALB/c mice with OVA-induced respiratory allergy (82). Oral administration of skim milk containing 0.9-1.5 x 10^9 cfu of *B. bifidum* BGN4 daily for 4 weeks decreases the number of infiltrating $CD4^+$ T cells in large intestine and spleen, and number of $CD4^+CD69^+$ activated T cells in the large intestine of mice with IBD (79). Similarly, consumption of 10^9 cfu of *B. longum* AH1206 for 14 days decreases the percentage of T cells in bronchoalveolar lavage fluid in BALB/c mice following a single challenge with OVA (80). Combined, oral consumption of *B. animalis*, *B. bifidum*, and *B. longum* may lead to a reduction in the number of infiltrating T cells in tissues during the inflammation process, which may prevent inflammation-associated tissue damage.

Combined, these data suggest that the consumption of *Bifidobacterium* may influence the distribution of T cells and T cell subsets in a strain-dependent manner in both human and animal studies. In both humans and experimental animals, *B. lactis* and *B. longum* do not influence the percentage of T cells and T cell subsets. However, in animal studies, *B. lactis, B. longum*, and *B. breve* increase the percentage or number of Tregs; and *B. animalis*, *B. bifidum*, and *B. longum* reduce the number of infiltrating T cells. These alterations in T cell distribution and phenotype may contribute to a reduction in tissue damage due to inflammation, particularly in the lung.

T Cell Proliferation

No studies to date have explored the effect of any single strain of *Bifidobacterium* species on T cell proliferation in randomized, controlled

clinical trials. However, both broad-based stimuli (e.g. mitogens) and antigen-specific T cell proliferative responses have been examined following administration with *Bifidobacterium* species in experimental animals and the result vary considerably. Oral administration of skim milk containing $2x10^7$ - $2x10^{11}$ cfu of *B. adolescentis* BBMN23 or *B. longum* BBMN68 daily for 4 weeks increases Con A-induced splenic T cell proliferation in healthy pathogen-free BALB/c mice (24). Feeding BALB/c mice with *B. lactis* HN019 for 7 days prior to *Salmonella Typhimurium* infection increases the proliferative capacity of splenic and Peyer's patch T cells in response to PHA (78). In addition to murine models, feeding healthy piglets with 10^9 cfu of *B. lactis* HN019 daily for 14 days also enhances proliferation of peripheral T cells in response to Con A (83). In summary, oral consumption of *B. adolescentis*, *B. longum*, or *B. lactis* is able to enhance mitogen-induced proliferative capacity of T cells isolated from spleen, Peyer's patch, or peripheral blood in experimental animals.

In contrast, oral administration of 10^9 cfu of *B. lactis* BB-12 every second day for 8 weeks decreases OVA-specific splenocyte proliferation in germ-free BALB/c mice with OVA-induced airway inflammation (81). Daily consumption of *B. animalis* MB5 for 28 days decreases OVA-stimulated proliferative activity of T cells isolated from mesenteric lymph nodes in OVA-immunized rats (84). Similarly, oral gavage with 10^8 cfu of *B. breve* for 14 days decreases OVA-specific splenic T cell proliferation in OVA-sensitized mice (32). Overall, *B. lactis*, *B. animalis*, and *B. breve* have the ability to reduce OVA-specific T cell proliferation in murine models.

Lastly, several *Bifidobacterium* species, *B. animalis* and *B. lactis* do not influence proliferative capacity of T cells in response to either specific antigens or broad-based stimuli in mice. Daily consumption of *B. animalis* MB5 for 28 days does not change OVA-specific splenic or mesenteric lymph node T cell proliferation in OVA-tolerized rats (84). Daily consumption of *B. animalis* MB5 for 28 days also does not alter splenic T cell proliferation in response to OVA, anti-CD3 or Con A, or OVA-stimulated mesenteric lymph node T cell proliferation in OVA-immunized rats (84). Consumption of *B. lactis* HN019 every day for 7 days prior to *Salmonella Typhimurium* infection does not alter PHA-stimulated mesenteric lymph node T cell proliferation in BALB/c mice (78). Similarly, oral administration of skim milk containing 1-1.5 x 10^8 cfu/d of *B. lactis* HN001 or *B. lactis* HN019 for 5-9 weeks does not change thyroglobulin-specific splenic T cell proliferation in CBA/CaH (H-2K) mice with thyroglobulin-induced autoimmune thyroiditis (85).

In summary, these findings suggest that *Bifidobacterium* species may influence broad-based and antigen specific T cell proliferation in experimental animals in a strain dependent manner. In addition to strain specificity, variability in T cell responses following consumption of *Bifidobacterium* species may exist due to variability in dose and duration of supplementation, characteristics of the animal models used, and choice of stimuli to induce T cell proliferation.

T Cell Cytokine Secretion

Cytokine production is the most extensively studied immune outcome following *Bifidobacterium* consumption. Numerous species of *Bifidobacterium*

can augment secretion of both Th1 and Th2 cytokines from T cells. Consumption of 1 x 10^{10} cfu of *B. infantis* 35624 in malted milk for 8 weeks increases the ratio of IL-10/IL-12p40 secreted from PBMCs isolated from patients with Irritable Bowel Syndrome, indicating a potential anti-inflammatory effect of *B. infantis* (86). Consumption of fermented milk supplemented with 2 x 10^9 cfu/d of *B. longum* B6 for 8 weeks increases IFN-γ production and the IFN-γ/IL-4 ratio from OVA-stimulated splenocytes in OVA-immunized BALB/c mice (87). Furthermore, consumption of 10^9 cfu of *B. longum* AH1206 for 14 days increases the production of IL-4, IL-10, and IFN-γ from splenocytes stimulated with anti-CD3/CD28 in BALB/c mice (80). Thus, oral consumption of *B. infantis* and *B. longum may enhance* secretion of both Th1 and Th2 cytokines.

Several species of *Bifidobacterium* can reduce antigen-induced cytokine secretion from T cells. Oral consumption of 10^9 cfu of *B. animalis* for 12 weeks decreases Con A-stimulated splenic IL-13 production in mice with OVA-induced respiratory allergy (82). Consumption of fermented milk supplemented with 2 x 10^9 cfu/d of *B. longum* B6 for 8 weeks decreases IL-4 production from OVA-stimulated splenocytes in OVA-immunized BALB/c mice (87). Additionally, intranasal treatment of 5 x 10^8 cfu of *B. longum* NCC3001 for 4 hours reduces allergen-specific IL-5 and IL-10 production from splenocytes in mice that were sensitized with a mixture of allergens (88). Similarly, intranasal treatment of 0.5-1x10^9 cfu of *B. longum* NCC3001 for 10 days reduces allergen-specific IL-5 production from splenocytes in BALB/c mice that were sensitized with the same mixture of allergens (88). These findings demonstrate that *B. animals*, *B. breve, and B. longum* may reduce

secretion of Th2 cytokines from T cells stimulated with mitogens or specific antigens. However, future studies are needed to better understand additional experimental variable that contribute to the relationship between *Bifidobacterium* consumption and cytokine secretion.

Several Bifidobacterium species also decrease pro-inflammatory and Th1 cytokine secretion from T cells in both healthy adults and animals, and animals with inflammatory disease. Kekkonen *et al.* reported that oral consumption of a milk drink containing *B. lactis* BB-12 for 3 weeks decreases influenza A.H3N2-specific IL-2 secretion from T cells in healthy adults (20). Oral administration of skim milk containing 0.9-1.5 x 10^9 cfu of *B. bifidum* BGN4 daily for 4 weeks decreases basal production of TNF-α and IFN-ɣ from splenic and intestinal mononuclear cells, and IFN-ɣ and MCP-1 production from splenic and intestinal T cells cocultured with intestinal epithelial cells in mice with IBD (79). Consumption of 10^9 cfu of *B. infantis* daily for 2 weeks reduces production of TNF-α and IL-6 from anti-CD3/CD28 stimulated splenocytes in an acute DSS-induced colitis model (89). Similarly, consumption of 10^9 cfu of *B. infantis* daily for 10 weeks reduces production of IFN-ɣ, TNF-α and IL-6 from anti-CD3/CD28 stimulated splenocytes in an adoptive $CD4^+$ T cell transfer colitis model (89). Drinking water supplemented with 10^{10} cfu of *B. infantis* for 14 days also reduces Con A-stimulated IFN-ɣ and IL-6 production, and the IFN-ɣ/IL-10 ratio in PBMCs from healthy Sprague-Dawley rats (90). Oral consumption of milk with 10^8 cfu/d of *B.longum* Bb46 or 10^9 cfu of *B. longum* AH1206 for two weeks decreases Con A-induced IFN-ɣ production and CD3/CD28-induced TNF-α, respectively, from splenocytes (91). Intranasal treatment of 5 x 10^8 cfu of *B. longum*

NCC3001 for 4 hours reduces allergen-specific IFN-ɣ production from splenocytes in mice that were sensitized with a mixture of allergens (88). Mice fed with 10^9 cfu of *Bifidobacterium* Bf-1 for 8 hours have lower IL-6 and IFN-ɣ production by peritoneal T cells in response to PMA stimulation (92). In summary, a number of studies have demonstrated that consumption of *B. lactis*, *B. bifidum*, *B. infantis,* and *B.longum* (at a variety of doses and for varying duration) can reduce pro-inflammatory and Th1 cytokine secretion from both T cells and mononuclear cells.

In addition to the studies documenting a stimulatory effect of several *Bifidobacterium* species on Th1 and Th2 cytokine secretion, several studies demonstrate no effect of *Bifidobacterium consumption on* T cell cytokine secretion. Consumption of *B. lactis* NCC2818 (93) and *B. lactis* BB-12 (14, 20) does not alter secretion of both Th1 and Th2 cytokines in human subjects. In experimental animals, oral administration of *B. infantis* does not influence Th2 cytokine secretion (90), and *B. animalis* (82), *B. bifidum* (79), *B. longum* (80, 87, 91) does not change both Th1 and Th2 cytokine secretion in response to broad-based stimuli or specific antigens.

In summary, inconsistent findings were observed in studies that examined the effect of *Bifidobacterium* species on antigen or mitogen-stimulated cytokine secretion in human subjects and experimental animals. Variability in these studies may be due to strain specificity, differences in dose and duration of supplementation, characteristics of the human subjects and animal models used, delivery matrix of *Bifidobacterium,* and choice of stimuli to induce T cell response.

Distribution of B cells in Peripheral Blood

The effect of *Bifidobacterium* consumption on B cell distribution has been examined in a few human and animal studies. Consumption of *B. longum* (76) and *B.lactis* (2, 77) does not change the percentage of B cells in peripheral blood in human subjects. Intranasal treatment of *B. longum* does not alter the percentage of antigen-specific IgE releasing B cells in mice (88). In contrast, consumption of *B. animalis* (94), *B. adolescentis* (94), *B. bifidum* (95) increases the number of immunoglobin (IgA, IgG, IgM) secreting cells in intestine or spleen in mice. Combined, these data suggest that effect of *Bifidobacterium* consumption on B cell distribution may be strain dependent, since *B. animalis*, *B. adolescentis*, and *B. bifidum* increases number of B cells, whereas *B. longum* and *B. lactis* does not have an effect. Additional studies are needed to clarify the effect of *Bifidobacterium* species on B cell distribution in both randomized controlled clinical trials and animal studies.

B cell Proliferation and Antibody Production

Only one animal study investigated the effect of *Bifidobacterium* species on B cells proliferation, and reported that daily consumption of *B. lactis* HN019 for 7 days prior to *Salmonella Typhimurium* infection increases proliferative capacity of splenic and mesenteric lymph node but not Peyer's patch B cells in response to LPS in mice (78). It is difficult to summarize the effect of *Bifidobacterium species* on B cell proliferation based on a single study.

Many studies have investigated the effect of *Bifidobacterium* consumption on total or antigen-specific immunoglobin production from B cells

in both human subjects and experimental animals and the results vary considerably. Several species of *Bifidobacterium* can augment immunoglobin production in human subjects and experimental animals. Oral consumption of *B. lactis* enhances the release of total or antigen-specific immunoglobin (IgA and IgG) to serum (15, 96), saliva (15), or GI tract (97) in humans. In animal studies, consumption of several *Bifidobacterium* species increases immunoglobin levels in blood, intestinal tract, and lung. Oral administration of *B. adolescentis* BBMN23 (98), *B. longum* BBMN68 (98), *B. lactis* (78, 83, 99), *B. pseudocatenulatum* (Bp) JCM 7041 (100), and *B. bifidum* (95) increases total or antigen-specific immunoglobin (IgA, IgG, IgM) production in intestinal tract of mice and piglets. Oral consumption of *B. breve* (101), *B. lactis* (102) also increases serum antigen-specific IgG levels in mice and piglets, respectively. In addition, intranasal treatment of *B. longum* enhances antigen-specific IgA levels in the lungs of mice (88).

In contrast, a number of *Bifidobacterium* species can reduce total or antigen-specific immunoglobin production in animals. Consumption of *B. lactis* (102, 103)*, B. bifidum* (104)*,* and *B.longum* (91) reduces total or antigen-specific IgA, IgM and/or IgG production from B cells in mice and piglets. Oral consumption of *B. longum* (87), *B. bifidum* (104), and *B. lactis* (81) decreases serum OVA-specific IgE levels in mice with OVA-induced airway inflammation. In addition to the aforementioned findings, several studies demonstrate no effect of consumption of *B. longum* BB536 (76) and *B.lactis* (14, 15, 20, 96, 105) on immunoglobin levels in blood, saliva, or feces in human subjects, and *B. lactis* (85, 102, 103), *B. longum* (80, 88, 91), and *B. animalis* (82) on serum total and/or antigen-specific immunoglobin levels in animals.

In summary, these observations suggest that the effect of *Bifidobacterium* species on antibody production varies considerably. However, it is not clear if this is due to differences in strain of probiotic used, duration and dose of supplementation, and/or vaccination model, or some other host factors that may impact the relationship between *Bifidobacterium* consumption and antibody production.

2.6.2. Innate Immunity

Distribution of and Phenotypic Marker Expression on Monocytes/Macrophages

The effect of consumption of *B. longum* and *B. lactis* on distribution of monocytes/macrophages is investigated in a few human and animal studies. Consumption of either *B.longum* BB536 for 13 weeks (76) or a milk drink supplemented with *B. lactis* BB-12 for 3 weeks (20) does not change the number of monocytes in peripheral blood in healthy adults. Furthermore, the proportion of HLA-DR$^+$ APCs also remains unaltered in healthy older adults following consuming 5 x 10^9 or 5 x 10^{10} cfu/d of *B. lactis* HN019 for 3 weeks (2). In a murine allergy model, intranasal treatment of 5 x 10^8 cfu of *B. longum* NCC3001 for 4 hours does not change the number of macrophages in bronchoalveolar lavage fluid of mice that were sensitized with a mixture of allergens (88). However, a milk-based diet supplemented with 3 X 10^8 cfu/g of *B. lactis* HN019 for 7 days prior to and following *E.coli* challenge increases the percentage of active macrophages in peripheral blood and peritoneum BALB/c and C57BL/6 mice (99). The limited evidence to data suggests that the distribution of monocyte/macrophages under homeostatic conditions may not be altered by consumption of *Bifidobacterium.* However additional studies

are needed to determine if *Bifidobacterium* consumption may influence the accumulation of monocytes or tissue macrophages following inflammatory stimuli.

Cytokine Secretion from in vitro Stimulated Monocytes/Macrophages

Numerous studies in humans and experimental animals have explored the effect of various *Bifidobacterium* species on cytokine secretion from stimulated monocytes/macrophages, and the results vary depending on the model, lymphoid organ evaluated, and choice of stimuli of monocytes/macrophages. Consumption of *B. longum* (106) and *B. lactis* BB-12 (14) does not alter both pro- and anti-inflammatory cytokines from mitogen-stimulated PBMCs in healthy adults. Furthermore, oral consumption of *B. pseudocatenulatum* (100), *B. infantis* (90), and *B.longum* (91) also has no influence on monocyte/macrophage cytokine production in animal studies. However, several *Bifidobacterium* species can augment cytokine secretion from monocytes/macrophages. Consumption of milk containing 3×10^{11} cfu of *B. lactis* HN019 daily for 6 weeks increases *in vitro* IFN-α secretion from PHA-stimulated PBMCs in healthy older adults (107). Similarly, consumption of 5×10^{8} cfu/d of *B. infantis* 35624 for 5 weeks increases IL-12p40 production from *in vitro* Peyer's patch cells stimulated with *Salmonella Typhimurium* or *B. infantis* in IL-10 knock-out (KO) mice (108). Oral consumption of 10^{9} cfu/d of *B. pseudocatenulatum* (Bp) JCM 7041 for 7 days increases *in vitro* production of IL-10 and IL-12p40 from Peyer's patch phagocytes and IL-5 from cecal patch in response to Bp stimulation in mice (100).

In contrast, several species of *Bifidobacterium* decreases pro-inflammatory cytokine production from monocytes/macrophages in human subjects and experimental animals. Oral administration of *B. infantis* 35624 for 8 weeks reduces in the secretion of TNF-α and IL-6 from LPS-stimulated PBMCs in healthy adults (109). Similarly, consumption of 2 x 10^{11} cfu of *B. longum* together with fructo-oligosaccharide (FOS) and inulin for 4 weeks decreases *in vitro* TNF-α secretion from LPS stimulated (106). Administration of drinking water supplemented with 10^{10} cfu of *B. infantis* for 14 days reduces LPS stimulated TNF-α production from PBMCs in healthy Sprague-Dawley rats (90). These findings suggest anti-inflammatory effects of *B. infantis* and *B.longum* due to their ability to reduce pro-inflammatory cytokine secretion following LPS stimulation. The results from these studies suggest that multiple experimental variables (e.g. different animal model, dose, duration and species of probiotic supplementation) may influence the release of pro- and/or anti-inflammatory cytokines from monocytes/macrophages. However, no distinct patterns have emerged.

Phagocytic Activity of Monocytes/Macrophages

Monocyte/macrophage phagocytic responses have been examined following administration with *Bifidobacterium* species in both randomized controlled trials and animal studies, and the result vary considerably. All randomized controlled trials that investigate the effect of *Bifidobacterium* consumption on phagocytosis used *B. lactis* as the intervention. Consumption of milk supplemented with 5 x 10^{9} - 3 x 10^{11} cfu/d of *B. lactis* HN019 for 3-6 weeks increases phagocytic capacity of macrophages in healthy middle-aged

and older adults (2, 4, 107). Similarly, Schiffirin *et al.* found that consumption of fermented milk containing 10^{10} cfu of *B. lactis* BB-12 for 3 weeks also increases monocyte phagocytosis of *E.coli*. in healthy subjects (77). However, two additional studies observed no effect of *B. lactis* BB-12 capsule consumption on phagocytosis in healthy adults who received the probiotic at a dose of 10^{8} -- 10^{11} cfu/d for 3 weeks (14) and 10^{9} cfu/d for 6 weeks (15), respectively. An enhancement in macrophage phagocytosis is observed in participants who consumed *B. lactis* subspecies delivered in a milk drink, suggesting that dairy products may be a better delivery matrix to observe a probiotic effect of *B. lactis.*

The effect of consumption of several *Bifidobacterium* species on phagocytic activity of monocytes/macrophages is investigated in a few animal studies. Administration of non-fat milk supplemented with 10^{7} cfu/d of *B. animalis* CRL 1247 for 5 and 7 days, or *B. adolescentis* CRL 1243 for 7 days does not change peritoneal macrophage phagocytosis to *Candida albicans* in BALB/c mice (94). However, consumption of *Bifidobacterium* species also enhances phagocytic activity of monocytes/macrophages in mice and pig models. Oral administration of skim milk containing $2x10^{7}$ - $2x10^{11}$ cfu/d of *B. adolescentis* BBMN23 or *B. longum* BBMN68 for 4 weeks increases peritoneal macrophage phagocytosis in healthy pathogen-free mice (24). Similarly, oral supplementation of 1.3 x 10^{8} cfu of *B. longum* for 24 weeks enhances phagocytic activity of peritoneal macrophages to *E. coli* in mice with carcinogen-induced tumors (110). Moreover, consumption of *B. lactis* HN019 for 7 days prior to *Salmonella Typhimurium* infection increases phagocytic capacity of peritoneal and blood macrophages in response to *E. coli* in mice

(78). In addition to murine models, daily consumption of 10^9 cfu of *B. lactis* HN019 for 14 days also enhances phagocytic activity of leukocytes in peripheral blood in healthy piglets (99). Overall, these studies demonstrate that *B. adolescentis, B. longum,* and *B. lactis* increase the phagocytic activity of monocytes/macrophages. However, additional studies are needed to determine variables (e.g. delivery matrix of *Bifidobacterium*, duration of supplementation) that may influence the effect of *Bifidobacterium* consumption on monocyte/macrophage phagocytosis.

Dendritic Cells

Only one clinical study has investigated the effect of *Bifidobacterium species* on dendritic cells, and reported that consumption of 5 x 10^9 or 5 x 10^{10} cfu/d of *B. lactis* HN019 for 3 weeks does not change the proportion of HLA-DR^+ DCs in healthy older adults (2). It is difficult to summarize the effect of *Bifidobacterium species* on dendritic cells based on a single study. Additional studies are needed on this topic.

Distribution of Natural Killer (NK) Cells

Only two randomized controlled trials have investigated the effect of *Bifidobacterium* consumption on distribution of NK cells in peripheral blood, and both used *B. lactis* as the intervention. Consumption of 5 x 10^9 or 5 x 10^{10} cfu/d of *B. lactis* HN019 for 3 weeks significantly increases the proportions of NK cells in healthy older adults (2). However, consumption of fermented milk containing 10^{10} cfu of *B. lactis* BB-12 every day for 3 weeks does not influence the percentage of NK cells in peripheral blood in young healthy

adults (77). Thus, additional studies are needed to clarify the role of *Bifidobacterium species* in distribution of NK cells.

Activity of NK Cells

The effect of consumption of several *Bifidobacterium* species on NK cell cytotoxic activity has been investigated in both human and animal studies. Oral administration of low-fat milk containing 5 x 10^9 - 5 x 10^{10} cfu/d *B.lactis* HN019 for 3 weeks enhances tumor-cell-killing activity of NK cells in healthy middle-aged and older adults (2, 4). Oral administration of skim milk containing 2x10^7 - 2x10^{11} cfu/d of *B. adolescentis* BBMN23 or *B. longum* BBMN68 for 4 weeks increases NK cell cytotoxicity in healthy pathogen-free BALB/c mice (24). However, a change in NK cell cytotoxicity was not observed in healthy adults that consumed 10^9 cfu/d of *B.lactis* BB-12 capsule for 6 weeks (15). Limited evidence suggest that consumption of *Bifidobacterium* delivered in milk drink may enhance NK cell activity in both human subjects and animals, suggesting that dairy products may be a better delivery matrix to support the probiotic effect of *Bifidobacterium.* More studies are needed to determine the effect of other *Bifidobacterium* species on NK cell function in both randomized controlled trials and animal studies.

Distribution of Granulocytes in Peripheral Blood and other Tissues

Studies in humans and experimental animals have investigated the distribution of granulocytes following administration with *Bifidobacterium* species. Supplementation with B. longum (76, 80, 88) or *B.lactis* (20) does not change granulocyte distribution in human subjects and experimental animals.

In contrast, one study reported that consumption of diet containing 2 x 10^9 cfu/kg body weight of *B. lactis* NCC2818 for 8 weeks increases the number of mast cells in the small intestine of piglets (102).

However, a reduction in the infiltration of granulocytes into lung and intestinal sites, and percentage of granulocytes in peripheral blood has been observed in animals following the consumption of various *Bifidobacterium* species. Oral consumption of 10^9 cfu of *B. animalis* for 12 weeks decreases the number of infiltrating eosinophils in lung of BALB/c mice with OVA-induced respiratory allergy (82). Oral administration of 10^9 cfu of *B. lactis* BB-12 every second day for 8 weeks decreases pulmonary eosinophilia in germ-free BALB/c mice with OVA-induced airway inflammation (81). Supplementation with 10^9 cfu of *B. longum* AH1206 for 14 days decreases the bronchoalveolar lavage fluid eosinophilia in BALB/c mice following OVA challenge (80). Intranasal treatment of 5 x 10^8 cfu of *B. longum* NCC3001 for 4 hours or 10 days reduces the number of eosinophils in bronchoalveolar lavage fluid of BALB/c mice that were sensitized with a mixture of allergens (88). Oral administration of 10^9 cfu of *B. lactis* B94 for 5 weeks decreases gastric neutrophil infiltration in C57BL/6 mice infected with *H. Pylori* (103). Oral treatment with *B. bifidum* BGN4 for 5 weeks decreases serum mast cell degranulation in OVA-sensitized C3H/HeJ mice (104). These findings suggest the *B. animalis, B. lactis, B. bifidum,* and *B. longum* may have the potential to alleviate inflammation-induced tissue damage by reducing granulocyte infiltration into various tissues.

In summary, the effect of *Bifidobacterium* species on distribution of granulocytes in peripheral blood and other tissue is not consistent. Additional

studies are needed to determine if *Bifidobacterium* consumption may influence the accumulation of granulocytes in blood, lung or intestine in allergic or inflammatory conditions.

Function of Granulocytes

Only two studies have examined the effect of *B.lactis* on function of granulocytes, and found contradictory results. Consumption of fermented milk containing 10^{10} cfu/d of *B.lactis* BB-12 for 3 weeks increases granulocyte phagocytosis of *E.coli* in healthy adults (77). In contrast, consumption of *B. lactis* NCC2818 for 4 weeks decreases expression of CD63 on basophils activated with allergen in adults with history of SAR (93). Additional studies are needed to examine the role of *Bifidobacterium* species in altering granulocyte function in both randomized controlled trials and animal studies.

2.7. Rational for Current Research

It is clear that large differences exist among *Bifidobacterium* species and subspecies in their immunomodulatory properties. However, disparate immunological effects of the same probiotic also are often reported. Thus, additional studies are needed to clarify and address several key issues. First, we must identify if host factors (including age, gender, BMI, physical activity status, and dietary intake) influence immunity and confound the relationship between *Bifidobacterium* consumption and immune function. These factors have not been adequately controlled for in many clinical trials assessing the effect of *Bifidobacterium* on immune responses. An understanding of these host-related factors is necessary to evaluate the efficacy of probiotic

interventions on immune function and inflammation and/or infection-related risk and is the focus of study 1.

We chose to use *Bifidobacterium animalis* subsp *lactis* BB-12 as the probiotic intervention in this study because this organism has been extensively studied in a variety of populations with respect to its effect on gastrointestinal function (111). However significantly less work has done to determine if any of the health benefits of *Bifidobacterium animalis* subsp *lactis* BB-12 is related to changes in immune function. A number of strains of *B. animalis* subsp. *lactis* are currently in commercial use (Table 2.1) throughout the world. An example of a commercial product containing *B. animalis* subsp. *lactis* (Strain DN 173 010) is Dannon's Activia yogurt. Additionally, *B. animalis* subsp. *lactis* can be grown easily, and is tolerance to oxygen, which enhances viability throughout its shelf life.

Table 2.1. Common commercial strains of *B. animalis* subsp. *Lactis*

Strain	Supplier	Genome Sequence	Reference
BB-12	Chr. Hansen's	Complete	(111)
Bl-04	Danisco	Complete	(112)
Bi-07	Danisco	Not Available	(113)
HN-019	Fonterra	Draft	(114)
DSMZ 10140^{T}	DSMZ[1]	Complete	(112)
DN-173-010	Danone	Proprietary	(115)

[1]DSMZ=Deutsche Sammlung von Microorganismen and Zellkulturen

In humans, some *Bifidobacterium* species, including *B. lactis* BB-12, have been used to treat inflammatory diseases of the intestine (e.g., inflammatory bowel disease, irritable bowel syndrome) (18, 19). The mechanisms underlying the beneficial effect of *B. lactis* BB-12 in these inflammatory diseases are unknown. However, evidence from animal studies demonstrates that oral consumption of *B*. *lactis* NCC2818 reduces colonic inflammation in a murine colitis model via a reduction in the pro-inflammatory

markers TNF-α and IL-6 (29, 116). Therefore, the beneficial effect of *B. lactis* BB-12 in inflammatory bowel disease (IBD) patients may be mediated via its role in the modulation of innate immunity, in particular in inflammatory response. Thus, well-designed clinical trials are needed to determine the effect of *B. lactis* BB-12 consumption on innate immune function and inflammatory mediators and is the focus of study 2.

Previous studies have examined the effect of *Bifidobacterium* species, including *B. lactis* BB-12, on other health outcomes, including URTI in children and adults. These data demonstrate that *B. lactis* BB-12 supplementation can reduce susceptibility to URTI (117-120). However, the mechanisms underlying the beneficial effect of BB-12 on URTI risk and severity is unknown. Adequate immune responses, including T cell and NK cell activity, contribute to reduction in the incidence and severity of URTI (121-124). Two human studies have investigated the effect of BB-12 alone as the probiotic intervention on influenza-specific cytokine secretion from T cells, but found mixed results (15, 20). However, no study to date has investigated the effect of consumption of BB-12 alone on T cell and NK cell function concurrently with self-reported URTI questionnaire data to determine if BB-12 induced alteration in immune responses are associated with its impact on URTI. Therefore, additional studies are needed to clarify the role of BB-12 on NK and T cell function and URTI-related outcomes in humans and is the focus of study 3.

Lastly, probiotics can be given in a variety of matrices (21). Dairy products, especially yogurt, are commonly used to deliver *Bifidobacterium* (22); however, *Bifidobacterium* can also be consumed in capsule form (23). While this expands the options for probiotic consumption, it is unclear if

Bifidobacterium delivered in capsule form are as effective in modulating immunity as when delivered in a dairy food (23). Several studies found that the enhancement in macrophage phagocytosis is observed in participants who consumed *B. lactis* subspecies delivered in fermented milk drink (e.g. yogurt), indicating dairy products may be a better delivery matrix for efficacy and probiotic effect of *B. lactis.* Following consumption, the acidic environment and buffering system provided by yogurt may confer a survival advantage to *Bifidobacterium*, and the nutrient composition and buffering capacity of yogurt may also directly influence the efficacy and probiotic effect of *Bifidobacterium* (22, 125, 126). Thus, selection of delivery matrix is an important component to sufficiently evaluate the immunomodulatory effect of *Bifidobacterium*. A subcomponent to study 2 and 3 is also to determine if the delivery matrix influences the immunomodulatory effect of *B. lactis* BB-12.

CHAPTER 3

In vitro production of IL-6 and IFN-γ is influenced by dietary variables and predicts upper respiratory tract infection incidence and severity respectively in young adults

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3.1. Abstract

Assessment of immune responses in healthy adults following dietary or lifestyle interventions is challenging due to significant inter-individual variability. Thus, gaining a better understanding of host factors that contribute to the heterogeneity in immunity is necessary. To address this question, healthy adults (n=36, 18-40 years old, BMI 20-35 kg/m^2) were recruited. Dietary intake was obtained via 3-day dietary recall records, physical activity level was evaluated using the IPAQ questionnaire, and PBMCs were isolated from peripheral blood. Expression of activation markers on unstimulated immune subsets was assessed by flow cytometry. T cell proliferation and cytokine secretion was assessed following *in vitro* stimulation with anti-CD3 or LPS. Furthermore, the incidence and severity of cold or flu symptoms were obtained from self-reported URTI questionnaires. The relationship between activation marker expression on T cells and T cell effector functions; and *in vitro* cytokine secretion and URTI was determined by linear or logistic regression. CD69 and CD25 expression on unstimulated T cells was significantly associated with T cell proliferation and IL-2 secretion. Incidence and severity of cold or flu symptoms was significantly associated with *in vitro* IL-6 and IFN-γ secretion, respectively. Furthermore, host factors (e.g. age, BMI, physical activity, and diet) contributed significantly to the relationship between activation marker expression and T cell effector function, and cytokine secretion and cold and flu status. In conclusion, these results suggest that lifestyle and dietary factors are important variables that contribute to immune responses and should be included in human clinical trials that assess immune endpoints.

3.2. Introduction

Host immune function is influenced by endogenous factors (e.g. age, gender, genetics), as well as various exogenous factors (e.g. diet, physical activity, alcohol consumption) (127-132). Thus, heterogeneity in both innate and adaptive immune responses exists even among healthy adults. This inter-subject variability makes it challenging to evaluate the effect of dietary or lifestyle interventions on immune function as many of these interventions have moderate effects as compared to pharmacological intervention trials (131, 133). Additionally, cost and feasibility often limit the sample size in clinical trials. Thus, gaining a better understanding of host related factors that may contribute to the variability in innate and adaptive immune responses in healthy adults is necessary to adequately control for the contribution of these influences in clinical trials that assess immunity.

The assessment of innate and adaptive immune function often involves complex assay methodologies; thus large clinical trials frequently are not designed to quantify these types of immunologic outcomes. Many human clinical trials measure serum makers (e.g. antibody titers, cytokine levels) as indicators of immune function because of ease of collection, assessment and storage (131). However, these endpoints alone may not adequately capture the immune response. Thus, additional studies are needed to assess functional immune outcomes (e.g. T cell proliferation, *in vitro* cytokine secretion) concurrently with the phenotypic characterization of immune cell populations via flow cytometry to determine if cell surface marker expression on immune cells can serve as a biomarker for effector function (e.g. proliferation and cytokine secretion).

Numerous human clinical trials have evaluated the effect of lifestyle interventions on the incidence and severity of cold or flu symptoms captured in self-reported upper respiratory tract infection (URTI) questionnaire data. The immune response of the host is known to be an important component of the pathogenesis of cold or flu infection (121). However, very few studies have quantified immune function (inflammatory cytokine responses or T cell function) concurrently with the self-reported URTI questionnaire data to determine if any immune outcomes are correlated with cold or flu symptomology. Two studies have reported an increase in salivary IgA concentration in subjects who had a lower incidence and severity of URTI symptoms (134, 135). In a third study, the risk of URTI in athletes was associated with antigen-stimulated IL-10 production and salivary IgA secretion (136). All three studies demonstrate that changes in immune function can be correlated with reduced symptoms of URTI. However, the goal of these studies was to determine if exercise reduced URTI, so the relationship between URTI symptomology, salivary IgA and IL-10 may be confounded by the exercise intervention. To date, no studies have examined inflammatory cytokine response or T cell effector function in subjects who completed self-reported URTI questionnaire data to determine if T cell effector function or inflammatory cytokine production was related to URTI incidence or severity.

Therefore, the goals of the current study were (1) to determine which endogenous and exogenous host factors contribute to the heterogeneity in innate and adaptive immune responses among healthy subjects; (2) to determine if activation marker expression on freshly isolated T cells, macrophages or DCs is associated with functional outcomes, i.e. anti-CD3

induced T cell proliferation and cytokine (IL-2 and IFN-γ) secretion or lipopolysaccharides (LPS) stimulated cytokine (TNF-α and IL-6) secretion from PBMCs, respectively; and (3) to determine if T cell proliferation and/or *in vitro* inflammatory cytokine production is associated with self-reported incidence and severity of cold or flu symptoms collected using a validated URTI questionnaire.

3.3. Materials and methods

3.3.1. Participants

Healthy subjects (n=36, 25 women and 11 men) 18-40 years of age were recruited for the study. Exclusion criteria included: BMI greater than 40kg/m^2, smoking and/or use of other tobacco products, blood pressure ≥ 140/90 mm Hg, use of blood pressure or cholesterol lowering medications, history of myocardial infarction, stroke, diabetes mellitus, liver disease, kidney disease and thyroid disease (unless controlled by medication and blood results within the previous six months were provided), lactation, pregnancy or desire to become pregnant during the study, clinical diagnosis of Inflammatory Bowel Disease (e.g. Crohn's Disease or ulcerative colitis), excessive alcohol consumption (> 14 standard drinks per week), chronic use of anti-inflammatory medications (unless able to discontinue), refusal to agree to give blood or plasma for the length of the study. A complete blood count and standard biochemistry panel was obtained at screening to rule out the presence of illness (autoimmune disease, cancer, and immunodeficiency). Blood pressure was measured according to the Joint National Committee 7 Guidelines (137).

3.3.2. Recruitment and screening

Participants were recruited through advertisements in the local newspaper and university e-mail lists. Potential participants who called or emailed to indicate interest in participating in the study were given information about the study, and if still interested, were contacted and screened using a series of medical and lifestyle questions. Qualified participants were scheduled for clinic screening at the Penn State Clinical Research Center (CRC). After written informed consent was provided, participants' height, weight, waist circumference and blood pressure were measured, followed by a fasting blood draw for a complete blood count and metabolic and immunologic endpoints. BMI was calculated according to body weight and height measured. From the participants who were screened, 36 were eligible to participant in the study. All the experiments in this study were performed with approval of the Institutional Review Board of the Pennsylvania State University-University Park campus (University Park, PA).

3.3.3. Diet assessment

Dietary intake of the participants was obtained via 24-hour dietary recalls for 3 days, including one weekend day. Briefly, participants were asked to recall their intake of food and beverages during breakfast, lunch, dinner, and snacks in the three days according to detailed instructions provided by trained staff. Portion size of each food item was also provided. Daily Intake of total calories, macronutrients, vitamins, minerals, caffeine, and alcohol was analyzed based on the recorded food intake of participants using Food Processor SQL software (ESHA Research, Salem, OR).

3.3.4. Physical activity assessment

Physical activity level of participants was evaluated using the International Physical Activity Questionnaires (IPAQ) as previously reported (138). Briefly, the participants recorded the activities they performed during each 15-minute interval for 3 days, including one weekend day. The activities were categorized from 1 to 9 depending on their intensity as previously described (138). Daily intensity of physical activity was calculated by averaging the approximate metabolic equivalent of tasks (METs) of physical activities (categories 3 to 9) performed over a 24-hour period (96 periods of 15 min).

3.3.5. Upper respiratory tract infection questionnaire

Participants (n=34) completed a self-administered upper respiratory tract infection (URTI) questionnaire, which was developed from established, frequently used instruments on the incidence (whether or not have experienced colds or flu episodes, with symptoms including a sore throat, runny or stuffy nose, coughing sneezing, fever, headache, general aches and pains, fatigue and discomfort) and severity (total number of days with cold or flu symptoms) of cold or flu symptoms over the past month. Participants were instructed to recall the occurrence of cold or flu in the last month, and advised on questionnaire completion. Two participants did not provide any information in their URTI questionnaires for unknown reasons.

3.3.6. Blood sample collection and immunological assays

Blood (50 ml) was collected in sterile EDTA (K2)-coated blood tubes (BD Biosciences, San Jose, CA) after a 12 hour fast by trained staff in CRC of Pennsylvania State University.

Serum markers

Total cholesterol (TC) and triglycerides (TG) were measured by enzymatic procedures (Quest Diagnostics, Pittsburgh, PA; coefficient of variation CV < 2% for both). High density lipoprotein (HDL) cholesterol was estimated according to the modified heparin-manganese procedure (CV < 2%). The Friedewald equation was used to calculate low density lipoprotein (LDL) cholesterol = TC − [HDL cholesterol + TG/5]) (139). Insulin was measured by radioimmunoassay (Quest Diagnostics). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose and Lactate Analyzer (Yellow Springs Instruments). Serum high-sensitivity C-reactive protein (hs-CRP) was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

Isolation of immune cells

Human blood was diluted 1:2 with phosphate buffer saline (PBS) (Mediatech, Manassas, VA), gently layered on top of lymphocyte separation media (LSM) (Corning, Manassas, VA), and centrifuged at 1600 rpm with low speed and no brake for 30 minutes at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected at the plasma/LSM interface; washed twice with complete media RPMI 1640 (Mediatech) containing 10 mM HEPES (Mediatech), 10% heat-inactivated fetal bovine serum (Gemini, West Sacramento, CA), 2 mM L-glutamine (Mediatech), 0.1 mM nonessential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 100U/ml Penicillin/Streptomycin (Mediatech), and 55 μM 2-mercaptoethanol (Life Technologies, Grand Island, NY) at room temperature; and counted for use in functional and phenotypic analyses.

Lymphocyte proliferation assay

PBMCs (2 X 10^6/ml) were incubated with 0 or 1µg/ml plate-bound purified mouse anti-human CD3 antibody (Life Technologies) in flat-bottomed 96-well plates. After 54 hours in culture, the cells were pulsed with [^{3}H] thymidine (1 µCi/well; Perkin Elmer, Waltham, MA) and harvested 18 hours later. Following incubation, cells were harvested onto glass fiber filter mats (Perkin Elmer) via a MicroBeta FilterMate-96 Harvester (Perkin Elmer). Incorporated radioactivity was measured by liquid scintillation counting on a 2450 MicroBeta plate counter (Perkin-Elmer). Each assay was performed in triplicate. The proliferative response was expressed as a stimulation index (SI) calculated by dividing the mean cpm (counts per minute) of anti-CD3 stimulated T cells by the mean cpm of unstimulated (media only) cells.

Cytokine secretion assays

PBMCs (2 X 10^6/ml) were stimulated with 1µg/ml plate-bound purified mouse anti-human CD3 antibody (Life Technologies), or 10 µg/ml lipopolysaccharide (LPS) (Sigma-Aldrich) in flat-bottomed 96-well plates. Supernatants from LPS plates were harvested and frozen after a 4-hour incubation, and supernatants from anti-CD3 plates were harvested and frozen after 48 hours. IFN-γ and IL-2 secretion from anti-CD3 stimulated PBMCs, and TNF-α and IL-6 secretion from LPS stimulated PBMCs were measured using the Human ELISA MAX™ Deluxe (Biolegend, San Diego, CA) as per manufacturer instructions. Each assay was performed in triplicate.

Flow cytometric analyses

PBMCs were washed twice in PBS at 4°C. Fc receptors on PBMCs were blocked by incubation with 1µg purified mouse anti-human CD16 (Biolegend) per 1×10^6 cells for 15min at 4°C. PBMCs were stained with

fluorescence-labeled antibodies (1 µg per 1×10^6 cells) to the following cell surface markers: CD3e, CD4, CD8a, CD69, CD25, CD11c, CD14, HLA-DR. Antibody isotype controls included: mouse IgG_{2a} and mouse IgM. All antibodies except CD16 were purchased from BD Biosciences. Following incubation with the conjugated antibodies for 30 min at 4° C, cells were washed twice in PBS and then fixed in cytofix (BD Biosciences) for flow cytometric analyses. Lymphoid and myeloid cells were gated on forward vs. side scatter and a total of 25,000 events were analyzed on a FC500 Benchtop Cytometer (Beckman Coulter, Pasadena, CA). Flow cytometric analyses were plotted and analyzed using FlowJo 7.6 (Tree Star, Ashland, OR).

3.3.7. Statistical analyses

Spearman's rank correlation was used to determine the relationship between IL-2 and IFN-γ secretion from T cells and T cell proliferation. Differences in IFN-γ secretion from anti-CD3 stimulated T cells between participants with and without self-reported cold or flu episodes in the past month were determined using unpaired t-test. Differences in IL-6 secretion from LPS stimulated PBMCs between participants with and without self-reported cold or flu episodes in the past month were determined using Wilcoxon-Mann-Whitney test. Correlations between LPS-induced IL-6 secretion and anti-CD3 induced IFN-γ secretion and total number of days with cold or flu symptoms was determined via Spearman's rank correlation.

For all linear, logistic, and Poisson regression models, potential confounders (listed in Table 3.2 and 3.3) were selected based on previous published reports demonstrating a relationship between the variable of interest (e.g. BMI, vitamin D, zinc, etc.) and immune outcomes (128, 129,

132, 140-156). Confounding variables varied in different models, and were selected in each model based on their ability to change the slope of the regression line for the predictor. Briefly, a full model was fitted with either effector function or incidence and severity of cold or flu infection as the dependent variable and activation marker expression and all potential confounding factors mentioned above as the independent variables (predictors). Then, the potential confounding factor showing the smallest contribution to the model was removed, and the percent change of β for predictor in the current model relative to β in the full model was calculated. If the change was within ±10%, this potential confounding factor was deleted from the model. The potential confounding factors were removed from the model one by one based on the 10% change criteria until all the variables remaining in the model were significant at $p \leq 0.05$ level or the percent change of β was beyond ±10% range. Automated backward and stepwise elimination were also performed to determine which variables remained in the final model. Similar findings were obtained using all three model-building techniques.

Statistical significance was accepted at the $p \leq 0.05$ level. All data were analyzed using SAS (Statistical Analysis System, Version 9.4, Cary, NC). Graphs were plotted using GraphPad Prism 5 (La Jolla, CA).

3.4. Results

3.4.1. Participant characteristics.

Anthropometric measurements, blood pressure, biochemical characteristics, physical activity, and dietary intake of participants are presented in Table 2.1. All 36 participants (25 females and 11 males)

completed the study. The participants were healthy, young adults (mean age of 28.3±1.0 years). The average BMI was 24.0±0.4 kg/m^2; 26 (72.2%) participants were normal weight, 9 (25.0%) were overweight, and 1 (2.8%) was obese. Their blood pressure was normal and waist circumference, fasting blood glucose, insulin, lipids and lipoproteins, and CRP levels were within the normal range (Table 3.1). Dietary intake and physical activity was assessed from self-reported 3-day dietary recall records and IPAQ responses, respectively. The median daily physical activity intensity (based on self-reported responses) was estimated to be 3.1 METs (range 2.4-5.2 METs). The average daily total calorie intake of participants calculated from 3-day dietary recall records was estimated to be 2281.0±130.8 kcal. The daily intake of macronutrients, vitamins, minerals and n-3 PUFA, caffeine and alcohol are reported in Table 3.1.

Table 3.1. Demographic characteristics of participants [1].

Characteristics	Values (n=36)
Age (yr)	28.3±1.0
Male, n (%)	11 (30.6%)
Body mass index (kg/m^2)	24.0±0.4
≤24.9	26 (72.2%)
25.0 -29.9	9 (25.0%)
≥30	1 (2.78%)
Waist Circumference (cm)	85.2±1.3
Blood pressure (mm Hg)	
Systolic	106.3±1.6
Diastolic	72.6±1.1
Glucose (mg/dL)	87.4±1.2
Insulin (mg/dL)	5.2±0.7
CRP (mg/L)	2.5±0.9
Lipids and Lipoproteins (mg/dL)	
Total cholesterol (TC)	164.7±4.8
LDL cholesterol	92.6±4.3
HDL cholesterol	54.9±1.9
Triglyceride (TG)	85.8±4.8
Physical activity intensity (METs/d)[2]	3.1 (2.4-5.2)
Dietary intake[2] of	
Total calories (kcal/d)	2281.0±130.8
Carbohydrate (g/d)	281.1±17.7
Protein (g/d)	88.4±6.1
Fat (g/d)	90.2±6.0
Vitamin C (mg/d)	69.5±10.7
Vitamin D (IU/d)	106.1±29.3
Vitamin E (mg/d)	3.1±0.5
Iron (mg/d)	14.3±1.2
Selenium (µg/d)	40.2±4.4
Zinc (mg/d)	6.0±0.6
n-3 PUFA (g/d)	0.6±0.1
Caffeine (mg/d)	71.3±14.6
Alcohol consumption (g/d)	2.2±0.9

[1] Values are presented as mean ± SEM, or Median (range) or n (%) depending on the variable.

[2] Physical activity and dietary intake were assessed from self-reported responses to IPAQ and 3-day dietary recall records, respectively.

3.4.2. T cell proliferation and cytokine secretion.

T cell proliferation (Figure 3.1A), IL-2 (Figure 3.1B) and IFN-γ (Figure 3.1C) secretion in response to 1μg/ml anti-CD3 antibody stimulation were measured to determine the effector function of T cells from participants. The mean T cell proliferation (reported as stimulation index) in response to anti-CD3 antibody was 149.40±19.52, (range 15.04 - 511.80). The mean IL-2 and IFN-γ production from anti-CD3 stimulated T cells was 0.37±0.08 ng/ml (range 0.00-1.45 ng/ml) and 102.70±8.09 ng/ml (range 1.35-169.40 ng/ml), respectively. We observed a large variation in all T cell effector functions (proliferation and cytokine secretion). Therefore, the relationship between T cell proliferation and cytokine secretion was examined to determine if subjects with greater T cell proliferation were those with high IL-2 and IFN-γ production. T cell proliferation was significantly correlated with IL-2 secretion (Figure 3.1D; Spearman r=0.3751, p=0.0264), but not with IFN-γ secretion (Figure 3.1E; Spearman r=0.0689, p=0.6941).

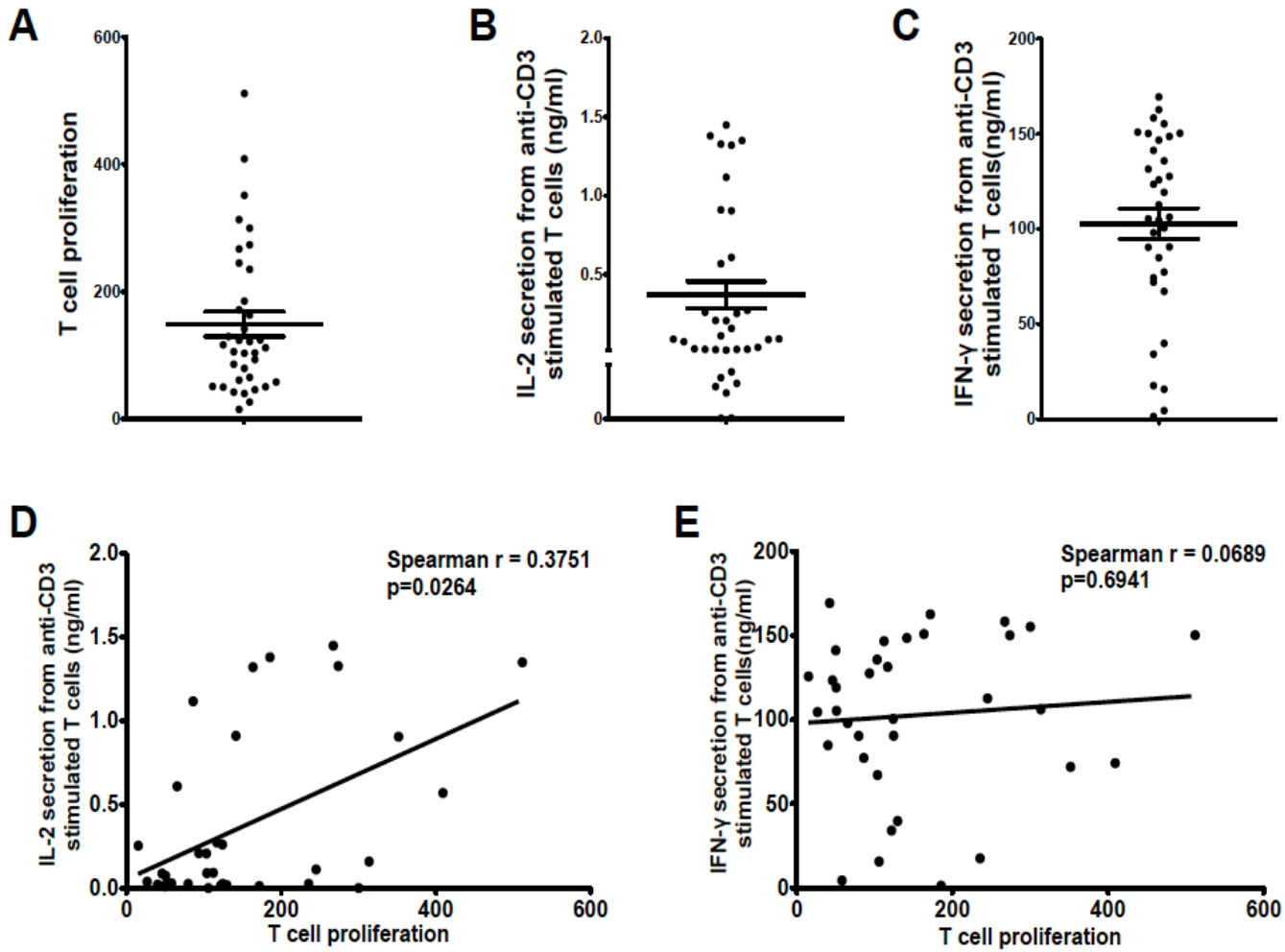


Figure 3.1. Effector function of T cells.
Proliferation of T cells **(A)** in response to anti-CD3 antibody was assessed by tritiated thymidine [^{3}H] incorporation. Stimulation index (SI) was calculated by dividing the mean cpm of stimulated T cells (in response to 1µg/ml anti-CD3) by the mean cpm of unstimulated cells (in media alone). Anti-CD3 stimulated IL-2 **(B)** and IFN-γ **(C)** secretion from T cells was measured by ELISA. Data are presented as mean ± SEM. T cell proliferation was significantly correlated with IL-2 secretion [**(D)**; Spearman r=0.3751, p=0.0264], but not with IFN-γ secretion [**(E)**; Spearman r=0.0689, p=0.6941)].

3.4.3. Activation marker expression on unstimulated T cells.

The percentage of $CD3^+CD69^+$ (Figure 3.2A) and $CD3^+CD25^+$ (Figure 3.2B) cells, and the mean fluorescence intensity (MFI) of CD69 and CD25 on double positive cells (Figure 3.2C and 3.2D, respectively) were quantified to determine the activation status of freshly isolated T cells. Bivariate plots of CD3 vs CD69 expression and CD3 vs CD25 expression are shown from one representative subject in Figure 3.2A and 3.2B inset graphs, respectively. The mean percentage of $CD3^+CD69^+$ T cells in PBMCs was 9.74±1.87% (range 0.03% to 41.92%, Figure 3.2A), and the mean percentage of $CD3^+CD25^+$ T cells in PBMCs was 8.97±1.24% (range 0.92% to 36.39%, Figure 3.2B). The MFI of CD69 on $CD3^+CD69^+$ T cells was 13119±1878 (range 2546-39050), and the average MFI of CD25 on $CD3^+CD25^+$ T cells was 15791±2267 (range 4504-60450). Representative flow histograms of CD69 expression on $CD3^+CD69^+$ cells and CD25 expression on $CD3^+CD25^+$ cells are shown in Figure 3.2C and 3.2D inset graphs, respectively.

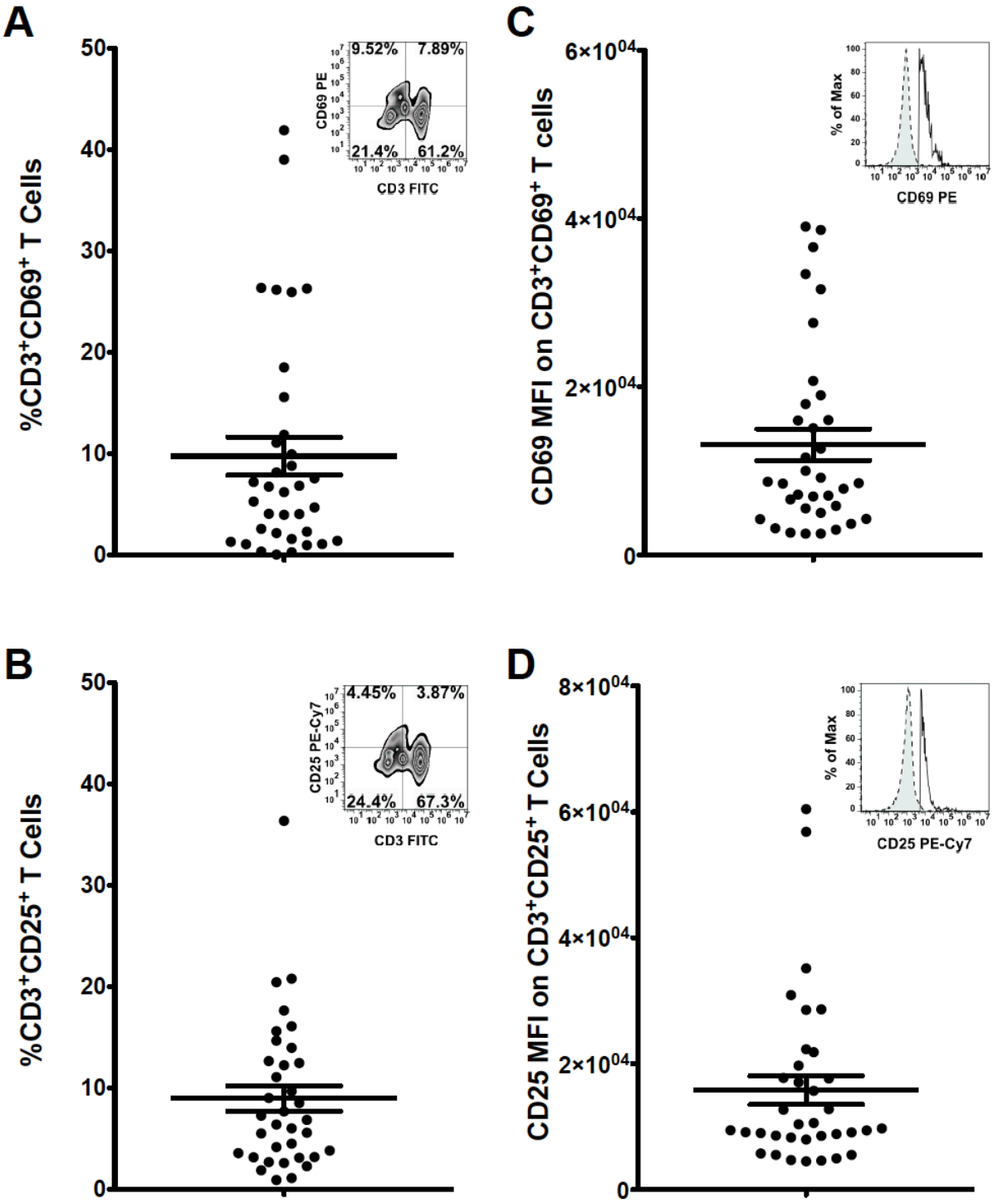


Figure 3.2. Activation marker expression on T cells.
Percentage of $CD3^+CD69^+$ T cells **(A)** and $CD3^+CD25^+$ T cells **(B)** in total PBMCs, and the MFI of CD69 on $CD3^+CD69^+$ T cells **(C)** and the MFI of CD25 on $CD3^+CD25^+$ T cells **(D)** was assessed by flow cytometry. Data are presented as mean ± SEM. Bivariate plots of CD3 vs CD69 expression [**(A)** inset] or CD25 expression [**(B)** inset)] from one representative subject are shown. Insets of **(C, D)** show flow histograms of CD69 MFI on $CD3^+CD69^+$ T cells and CD25 MFI on $CD3^+CD25^+$ cells from one representative subject. Dashed lines represent isotype controls, and solid lines represent experimental samples.

3.4.4. Activation marker expression on unstimulated T cells was associated with T cell proliferation and cytokine secretion.

To determine if activation marker expression (CD69 and CD25 expression) on freshly-isolated, unstimulated T cells and T cell subsets and other host factors (e.g. age, BMI, physical activity and dietary factors) contribute to the variation in effector function (e.g. T cell proliferation and IL-2 and IFN-γ secretion), linear regression analysis was used. Most of the variables in Table 3.1 contributed less than 10% to any of the immune outcomes (T cell proliferation, IL-2 and IFN-γ secretion) when included in the model alone (without activation marker expression), with the exception of age, HDL and vitamin D which contributed 10.3%, 11.0% and 11.3% of the variability in IL-2 secretion (Table 3.2; R^2=10.25%; 10.98% and 11.27%, respectively). Also hs-CRP and vitamin D contributed to 11.6% and 12.1% of the variability in IFN-γ secretion (Table 3.2; R^2 = 11.55% and 12.13%, respectively). However, the MFI of CD69 on $CD3^+CD69^+$ T cells alone accounted for 29% of the variability in T cell proliferation (Table 3.3, model 1, β=0.0058, R^2=29.17%, p=0.0008). When BMI and the MFI of CD69 on unstimulated $CD3^+CD69^+$ T cells were included in the model, 45% of the variability in T cell proliferation was explained (Table 3.3, model 2, β=0.0063, R^2=44.95%, p<0.0001). The MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells was also significantly associated with T cell proliferation. The MFI of CD25 on $CD3^+CD25^+$ T cells alone accounted for 16% of the variability in T cell proliferation (Table 3.3, model 1, β=0.0035, R^2=15.78%, p=0.0181). However, the predicted value of CD25 MFI on unstimulated $CD3^+CD25^+$ T cells was no longer significant when physical activity, daily intake of vitamin D, selenium,

and n-3 PUFA were included in the model (Table 3.3, model 2, β=0.0021, R^2=36.29%, p=0.1490). Similar analyses were conducted to explore the relationship between the MFI of CD69 and CD25 on unstimulated T cell subsets and T cell proliferation. Data are presented in Supplemental Table 3.1 and 3.2. The R^2 of the predictor (activation marker expression) plus the individual confounding variables included in the models described in Table 3.3 were examined to compare the relative contribution of each confounding variable when the predictor was in the model. BMI accounted for 16% of the variability in T cell proliferation when CD69 MFI on $CD3^+CD69^+$ T cells were included in the model (Supplemental Table 3.3; R^2=15.79%). Dietary selenium intake accounted for 10% of the variability in T cell proliferation when CD25 MFI on $CD3^+CD25^+$ T cells was included in the model (Supplemental Table 3.3; R^2=10.30%).

The MFI of CD69 on unstimulated $CD3^+CD69^+$ T cells was also significantly associated with IL-2 secretion from anti-CD3 stimulated T cells. The MFI of CD69 on $CD3^+CD69^+$ T cells accounted for 29% of the variability in IL-2 secretion (Table 3.3, model 1, β=0.0242, R^2=29.02%, p=0.0010). When age, physical activity, daily total calories, vitamin D and iron intake were included in the model as confounding factors, 64% of the variability in IL-2 secretion was explained (Table 3.3, model 2, β=0.0205, R^2=64.19%, p=0.0002). The MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells was also significantly associated with IL-2 secretion. The MFI of CD25 on freshly-isolated, unstimulated $CD3^+CD25^+$ T cells alone accounted for 55% of the variability in IL-2 secretion (Table 3.3, model 1, β=0.0276, R^2=55.29%, p<0.0001). After controlling for age, daily total calories, vitamin D and iron

intake as confounding factors, the MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells and confounding factors accounted for 74% of the variability in IL-2 secretion (Table 3.3, model 2, β=0.0209, R^2=74.00%, p<0.0001). Similar analyses were done to explore the relationship between the MFI of CD69 and CD25 on unstimulated T cell subsets and IL-2 secretion from anti-CD3 stimulated T cells. Data are presented in Supplemental table 3.1 and 3.2. The R^2 of the predictor (activation marker expression) plus the individual confounding variables included in the models described in Table 3.2 were examined to compare the relative contribution of each confounding variable when the predictor was in the model. None of the individual dietary or lifestyle variables contributed more than 10% to variability in IL-2 secretion (Supplemental Table 3.3).

Unlike the relationship between T cell activation marker expression and IL-2 secretion, only the MFI of CD25 on unstimulated $CD3^+CD25^+$ cells was associated with IFN-γ secretion from anti-CD3 stimulated T cells. CD25 MFI on unstimulated $CD3^+CD25^+$ cells alone accounted for 13% of the variability in IFN-γ secretion (Table 3.3, model 1, β=1.2020, R^2=12.91%, p=0.0369). However, the value of CD25 MFI on unstimulated $CD3^+CD25^+$ cells was no longer significant when daily intake of vitamin C, vitamin D, selenium and n-3 PUFA were included in the model as confounding variables (Table 3.3, model 2, β=0.9655, R^2=31.97%, p=0.0958). CD69 MFI on $CD3^+CD69^+$ T cells was not associated with IFN-γ secretion before (Table 3.3, model 1, β=0.9298, R^2=5.28, p=0.1911) and after adjusting for confounding variables (Table 3.3, model 2, β=0.7203, R^2=30.93, p=0.4823). Similar analyses were done to explore the relationship between the MFI of CD69 and CD25 on unstimulated

T cell subsets and IFN-γ secretion from anti-CD3 stimulated T cells. Data are presented in Supplemental table 3.1 and 3.2. Since the MFI of CD69 and CD25 alone on T cells was not associated with IFN-γ secretion, we further examined the relationship between IFN-γ secretion and other potential immunomodulatory factors, including age, BMI, physical activity level, daily intake of total calories, vitamin C, vitamin D, E, selenium, iron, zinc, n-3 PUFA, caffeine, and alcohol consumption to determine which variables significantly impacted IFN-γ secretion. Daily intake of vitamin D was the only variable that was significantly associated inversely with IFN-γ secretion. Vitamin D intake contributed to 12% of the variability in IFN-γ secretion (Table 3.2, β=-88.4027, R^2=12.13, p=0.0470).

Table 3.2. Contributions of individual variables to immune function and cold or flu incidence and severity[1,2,3]

	R^2 (%)					Odds Ratio (95% CI)	95% CI
	SI	IL-2 secretion	IFN-γ secretion	TNF-α secretion	IL-6 secretion	Cold or flu incidence	Number of days with cold or flu
Age	1.38	10.25	2.69	0.01	0.35	1.085 (0.867,1.359)	(-0.105,0.120)
BMI	0.72	0.39	2.70	2.81	0.16	1.085 (0.619,1.901)	(-0.258,0.240)
Insulin	1.77	3.40	3.24	2.27	0.47	0.789 (0.546,1.139)	(-0.040,0.191)
CRP	0.31	3.45	11.55	3.19	1.25	0.908 (0.683,1.208)	(-0.021,0.098)
Serum total cholesterol	6.08	0.07	2.80	0.08	0.17	1.042 (0.989,1.098)	(-0.038,0.002)
Serum HDL	0.00	10.98	0.15	1.36	1.55	0.950 (0.839,1.076)	(-0.042,0.052)
PA	7.28	8.71	0.15	0.13	5.55	1.169 (0.141,9.669)	(-1.362,1.275)
Dietary intake of							
Total calories	0.00	3.33	0.02	16.68	9.89	0.998 (0.994,1.002)	(-0.001,0.001)
Vitamin C	2.97	2.39	1.99	15.48	5.31	1.006 (0.982,1.031)	(-0.009,0.014)
Vitamin D	1.98	11.27	12.13	8.05	4.67	1.002 (0.992,1.012)	(-0.321,0.095)
Vitamin E	9.84	0.03	3.13	0.31	1.19	1.440 (0.894,2.320)	(-0.044,0.013)
Iron	5.53	4.15	4.53	7.63	12.18	1.246 (0.726,2.141)	(-0.171,0.103)
Selenium	2.49	5.07	3.23	0.28	2.93	1.047 (0.964,1.137)	(-0.044,0.013)
Zinc	3.19	2.78	3.35	2.60	4.77	0.809 (0.364,1.799)	(-0.361,0.214)
n-3 PUFA	1.81	0.41	1.44	0.47	5.80	0.013 (0.001,5.176)	(0.029,2.920)
Caffeine	0.04	0.28	0.17	0.02	1.26	1.015 (0.992,1.038)	(-0.024,0.005)
Alcohol consumption	0.55	0.01	1.16	1.87	2.73	1.039 (0.824,1.311)	(-0.085,0.115)

[1]Waist circumference, glucose, SBP and DBP were not included because they were collinear with BMI.
[2]Serum LDL and TG were not included because they were collinear with serum total cholesterol.
[3]Dietary intake of carbohydrates, proteins and fat were not included because they were collinear with total calorie intake.

Table 3.3. Activation marker expression on unstimulated T cells as predictors of T cell effector function[1].

	CD69 MFI on $CD3^+CD69^+$ T cells				CD25 MFI on $CD3^+CD25^+$ T cells			
	β	**R^2 (%)**	**Variables in the model**	**p value**	**β**	**R^2 (%)**	**Variables in the model**	**p value**
Anti-CD3 induced T cell proliferation[1]								
Model 1	0.0058	29.17	CD69 MFI on $CD3^+CD69^+$ T cells	0.0008	0.0035	15.78	CD25 MFI on $CD3^+CD25^+$ T cells	0.0181
Model 2	0.0063	44.95	Model 1 + BMI	<0.0001	0.0021	36.29	Model 1 + PA, vitamin D, selenium, n-3 PUFA	0.1490
Anti-CD3 induced IL-2 secretion from T cells								
Model 1	0.0242	29.02	CD69 MFI on $CD3^+CD69^+$ T cells	0.0010	0.0276	55.29	CD25 MFI on $CD3^+CD25^+$ T cells	<0.0001
Model 2	0.0205	64.19	Model 1 + age, PA, total calories, vitamin D, iron	0.0002	0.0209	74.00	Model 1 + age, PA, total calories, vitamin D, iron	<0.0001
Anti-CD3 induced IFN-ɣ secretion from T cells								
Model 1	0.9298	5.28	CD69 MFI on $CD3^+CD69^+$ T cells	0.1911	1.2020	12.91	CD25 MFI on $CD3^+CD25^+$ T cells	0.0369
Model 2	0.7203	30.93	Model 1 + age, BMI, PA, total calories, vitamin C, D, selenium, zinc, alcohol	0.4823	0.9655	31.97	Model 1 + vitamin C, D, selenium, n-3 PUFA	0.0958

[1] T cell proliferation was evaluated by quantifying tritiated thymidine incorporation following stimulation with anti-CD3 antibodies, and results are reported as a stimulation index. Stimulation index was calculated by dividing the cpm of the anti-CD3 induced T cell proliferation by unstimulated T cells.

3.4.5. Cytokine secretion and HLA-DR expression on DCs and macrophages.

The percentage of DCs ($CD123^+CD11c^+HLA\text{-}DR^+$) and macrophages ($CD14^+HLA\text{-}DR^+$) in PBMCs, and HLA-DR expression on unstimulated DCs and macrophages were both quantified. The percentage of DCs in PBMCs was 4.86±1.12% (range 0.07-14.18%), and the average MFI of HLA-DR on DCs was 94446±10084 (range 23500-255000, data not shown). The percentage of macrophages in PBMCs was 14.38±1.00% (range 3.79-27.22%), and the average MFI of HLA-DR on macrophages was 46817±3528 (range 16100-111000, data not shown). TNF-α and IL-6 secretion from PBMCs in response to 10μg/ml LPS stimulation was also measured. The mean TNF-α and IL-6 production from LPS-stimulated PBMCs was 1.16±0.10 ng/ml (range 0.33-2.39 ng/ml) and 15.01±1.15 ng/ml (range 5.55-25.05 ng/ml), respectively. Of the variables shown in Table 2.1, dietary iron contributed to 12.2% of the variability in IL-6 (Table 2.2; R^2=12.18%) and total calories and vitamin C contributed to 16.7% and 15.5% of the variability in TNF-α secretion (Table 2.2; R^2= 16.68% and 15.48%, respectively). All other variables contributed to less than 10% of the variability in IL-6 and TNF-α secretion. HLA-DR expression on DCs and macrophages was not associated with TNF-α and IL-6 production from LPS stimulated DCs and macrophages (data not shown).

3.4.6. Cytokine secretion was associated with the incidence and severity of cold or flu symptoms.

Participants (n = 34) completed a URTI questionnaire at their clinical visit. According to their self-reported cold or flu incidence, 17 participants had

one or more cold or flu episodes in the past month. The association between activation marker expression (CD69 and CD25); T cell proliferation; T cell cytokine secretion (IL-2 and IFN-γ); HLA-DR expression on antigen presenting cells; and cytokine secretion (TNF-α and IL-6) from LPS stimulated PBMCs, and self-reported cold and flu incidence or severity was examined by logistic and Poisson regression, respectively. No association was observed between activation marker expression, T cell proliferation, HLA-DR expression on antigen presenting cells, and TNF-α secretion from LPS stimulated PBMCs and cold and flu incidence and severity (data not shown). However, IL-6 secretion from LPS stimulated PBMCs was significantly higher in participants with self-reported cold or flu symptoms compared to participants without cold or flu symptoms in the past month (Figure 3.3A, p=0.0471). The average concentration of IL-6 secretion from LPS stimulated PBMCs of participants without self-reported cold or flu episodes was 12.17±1.34 ng/ml, and the concentration of IL-6 secretion from participants with cold or flu episodes was 17.00±1.64 ng/ml. IL-6 secretion was associated with the incidence of cold or flu episodes before (Table 3.4, model 1, β=-0.1471, OR=0.863, 95% CI=(0.749, 0.994), p=0.0414) and after incorporating daily intake of total calories, vitamin C, iron and zinc as confounding variables in the model (Table 3.4, model 2, β=-0.2392, OR=0.787, 95% CI=(0.632, 0.980), p=0.0325). However, IL-6 secretion was not associated with the total number of days with self-reported cold or flu symptoms before (Figure 3.3C, Spearman r=0.2335, p=0.2229; and Table 3.4, model 1, β=0.0252, 95% CI=(-0.042, 0.092), p=0.4609) and after incorporating daily intake of total calories,

vitamin D, iron, and zinc as confounding variables in the model (Table 3.4, model 2, β=0.0801, 95% CI=(-0.018, 0.178), p=0.1105).

In contrast to IL-6 secretion, IFN-γ secretion from anti-CD3 stimulated T cells was significantly lower in participants with self-reported cold or flu symptoms compared to participants without these symptoms in the past month (Figure 3.3B, p=0.0376). The average concentration of IFN-γ secretion from anti-CD3 stimulated T cells of participants without self-reported cold or flu episodes was 119.30±10.45 ng/ml, and the concentration of IFN-γ secretion from participants with cold or flu episodes was 84.19±12.45 ng/ml. IFN-γ secretion was not associated with the self-reported incidence of cold or flu episodes alone (Table 3.4, model 1, β=0.0141, OR=1.014, 95% CI=(0.997, 1.032), p=0.1019), or after incorporating confounding variables (age, BMI, daily intake of vitamin D, iron, zinc, n-3 PUFA, caffeine, and alcohol consumption) in the model (Table 3.4, model 2, β=0.0556, OR=1.057, 95% CI=(0.983, 1.133), p=0.1158). However, IFN-γ secretion was inversely correlated with total number of days with self-reported cold or flu symptoms (Figure 3.3D, Spearman r=-0.4989, p=0.0031). IFN-γ secretion was significantly associated with total number of days with cold or flu symptoms (Table 3.4, model 1, β=-0.0138, 95% CI=(-0.022, -0.006), p=0.0005). After incorporating daily intake of vitamin C, zinc, caffeine, and alcohol consumption as confounding variables in the model, IFN-γ secretion was also significantly associated with total number of days with cold or flu symptoms (Table 3.4, model 2, β=-0.0153, 95% CI=(-0.021, -0.009), p<0.0001). The contributions of the predictor (cytokine secretion) and individual confounding variables in the regression models with cold or flu incidence and severity as

dependent variables are included as Supplemental Table 3.4. Individual variables contributed a small amount to the model (e.g. prediction of cold or flu incidence or total number of days with cold symptoms). However, cytokine secretion better predicted the incidence or severity of cold of flu when dietary factors were included in the models.

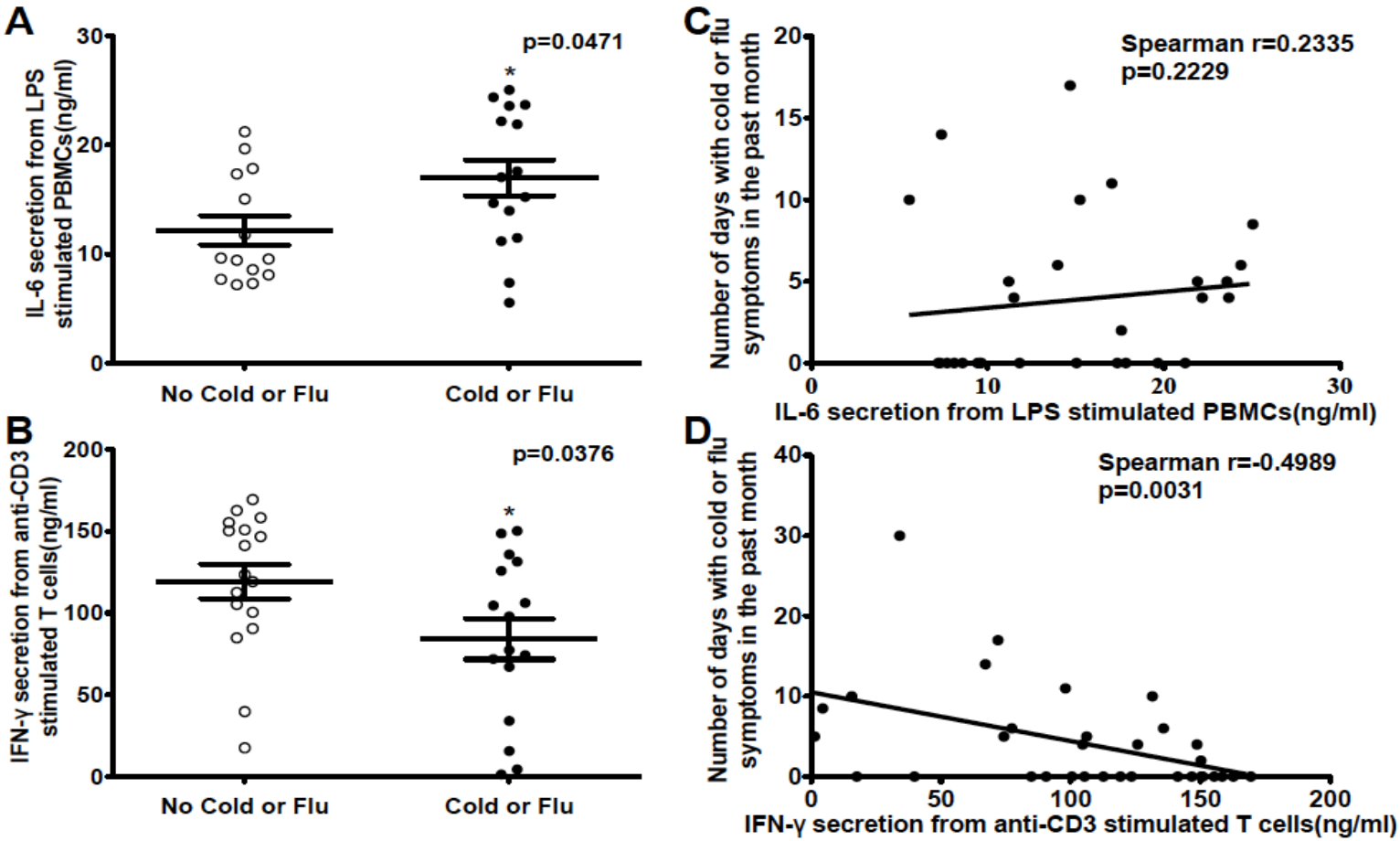


Figure 3.3. LPS-stimulated IL-6 secretion from PBMCs, anti-CD3 stimulated IFN-γ secretion from T cells, and self-reported cold or flu status in human subjects.

IL-6 secretion from LPS-stimulated PBMCs was significantly higher in subjects with self-reported cold or flu symptoms compared to subjects without cold or flu symptoms [**(A)**; Mann-Whitney test, p=0.0471]. IL-6 secretion was not correlated with number of days with cold or flu symptoms [**(C)**; Spearman r=0.2335, p=0.2229]. IFN-γ secretion from anti-CD3 stimulated T cells in subjects with cold or flu symptoms was significantly lower compared to subjects without cold or flu symptoms [**(B)**; unpaired t-test, p=0.0376]. T cell IFN-γ secretion was inversely associated with total number of days with self-reported cold or flu symptoms [**(D)**; Spearman r=-0.4989, p=0.0031]. Asterisk indicates a significant difference from no cold or flu group (p<0.05).

Table 3.4. Cytokine secretion as predictors of self-reported cold or flu incidence and severity[1,2,3].

	LPS induced IL-6 secretion from PBMCs				Anti-CD3 induced IFN-ɣ secretion from T cells			
	β	**Odds ratio (95% CI)**	**Variables in the model**	**p value**	**β**	**Odds ratio (95% CI)**	**Variables in the model**	**p value**
Incidence of cold or flu episode in the past month[1,2]								
Model 1	-0.1471	0.863 (0.749, 0.994)	IL-6 secretion	0.0414	0.0141	1.014 (0.997, 1.032)	IFN-ɣ secretion	0.1019
Model 2	-0.2392	0.787 (0.632, 0.980)	Model 1 + total calories, vitamin C, iron, zinc	0.0325	0.0556	1.057 (0.986, 1.133)	Model 1 + age, BMI, vitamin D, iron, zinc, n-3 PUFA, alcohol, caffeine	0.1158
	β	**95% CI**	**Variables in the model**	**p value**	**β**	**95% CI**	**Variables in the model**	**p value**
Total number of days with cold or flu symptoms in the past month[3]								
Model 1	0.0252	-0.042, 0.092	IL-6 secretion	0.4609	-0.0138	-0.022, -0.006	IFN-ɣ secretion	0.0005
Model 2	0.0801	-0.018, 0.178	Model 1 + total calories, iron, zinc	0.1105	-0.0153	-0.021, -0.009	Model 1 + vitamin C, zinc, alcohol, caffeine	<0.0001

[1] Logistic regression model is fitted to determine the relationship between IL-6 or IFN-ɣ secretion and incidence of cold or flu in the past month.
[2] No cold or flu episode in the past month=0, presence of a cold or flu episode in the past month=1. Probability modeled is No cold or flu episode in the past month.
[3] Poisson regression model is fitted to determine the relationship between IL-6 or IFN-ɣ secretion and total number of days with cold or flu symptoms in the past month.

3.5. Discussion

To our knowledge, this study provides the first documentation that expression of activation markers on freshly-isolated, unstimulated T cells is associated with effector function of activated T cells from healthy human subjects. Flow cytometric analysis of CD69 and CD25 expression on unstimulated T cells and T cell subsets was significantly correlated with anti-CD3 stimulated T cell proliferative response and IL-2 secretion. However, neither CD69 nor CD25 expression was associated with IFN-γ secretion. These data suggest that CD69 and CD25 expression on unstimulated T cells may be a useful predictor of T cell effector function following stimulation. Furthermore, host factors (e.g. age, BMI, physical activity, total calories and select dietary factors) contribute significantly to the relationship between activation marker expression and T cell effector function. Our study is also the first to demonstrate that LPS-stimulated IL-6 secretion from PBMCs and anti-CD3 induced IFN-γ secretion from T cells were significantly associated with self-reported incidence and severity of cold or flu in the past month, respectively. These findings demonstrate the incidence and severity of cold or flu symptoms captured via the URTI questionnaire were accompanied by relevant immunological changes. This relationship was also strengthened by the inclusion of host factors in the model. Collectively, these data suggest lifestyle and dietary factors are important variables that contribute to the immune response and should be included in human clinical trials assessing immune endpoints.

Few clinical studies have examined T cell proliferation, IL-2 and IFN-γ secretion in response to anti-CD3 stimulation in young healthy adults. Results

from our study demonstrate that among this population, T cell effector function varied greatly, with some individuals demonstrating robust T cell effector responses, and others not. To determine if these individuals with high T cell proliferation also had high cytokine secretion, we examined the correlation between T cell proliferation and IL-2 and IFN-γ secretion. T cell proliferation was significantly correlated with IL-2 secretion but not IFN-γ secretion. These results are not surprising given the essentiality of IL-2 for T cell proliferation (157). Previous reports have demonstrated a positive association between IFN-γ secretion and T cell proliferation (158), however, in our population, this was not the case. This may be due to the fact that PBMCs rather than purified T cells were used in the IFN-γ secretion assay. Although T cells are the major sources of IFN-γ secretion, the secretion of IFN-γ by other immune cells (e.g. B cells, nature killer cells) may minimize the association between IFN-γ secretion and T cell proliferation (159). The limited sample size (n=36) in our study may also partly explain the inconsistency between our results and previous studies.

In addition to the heterogeneity in T cell effector responses, we observed that activation marker expression on T cells also varied greatly among subjects. Stimulation of T cells by antigens or mitogens induces upregulation of CD69 within 4 hours, and CD25 between 24 to 48 hours following T cell activation, which in turn triggers downstream signaling pathways that initiates proliferation and differentiation of T cells (157, 160-163). Several previous studies have reported that CD69 or CD25 expression on antigen or mitogen stimulated T cells is up-regulated in parallel with T cell proliferation (determined by [^{3}H] thymidine incorporation) (164-166). However,

these results are not consistent across all studies (164-166). Results vary based on the different types of stimuli (antigen or mitogen) and the length of the assay (164-166). Simms, *et al.* reported that anti-CD3 or staphylococcal enterotoxin (SEB) induced T cell proliferation 72 hour post stimulation is correlated with the percentage of $CD69^+$ T cells 24 hours following stimulation (164). Similar results are observed by Maino and colleagues, who found that the percentage of $CD3^+CD69^+$T cells 4 hours post activation was correlated with PBMC proliferation following stimulation with anti-CD2 receptor antibodies for 72 hours (165). However, there was no correlation between the percentage of $CD69^+$ or $CD25^+$ T cells and T cell proliferation induced by various other stimuli (PHA, SEB, tetanus toxoid or influenza A virus) for longer time periods (72, 120, 168 and 168 hours, respectively) (166). Thus, the nature of mitogenic or antigenic stimuli and the time course of activation influence the relationship between activation marker expression on T cells and proliferative response of T cells. These studies suggest that quantification of CD69 and CD25 expression on stimulated T cells may not be a consistent marker of T cell proliferative capacity because the variability in assay conditions confounds this relationship.

To determine if the activation marker expression on freshly isolated, unstimulated T cells was predictive of T cell proliferation, we used linear regression analyses using the expression of CD69 or CD25 (MFI of CD69 on $CD3^+CD69^+$ or MFI of CD25 on $CD3^+CD25^+$) on unstimulated T cells as the independent variable (predictor) and anti-CD3 induced T cell proliferation as the dependent variable (outcome). The MFI of CD69 on unstimulated $CD3^+CD69^+$ T cell contributed significantly (29.2%) to the variability in T cell

proliferation (Table 3.3). To further explain the heterogeneity in T cell proliferation, we included other host variables in the model that have previously been reported to modulate T cell proliferation such as age, BMI, total calories and select dietary factors (131, 144, 145). The final model included both the MFI of CD69 on $CD3^+CD69^+$ T cells and BMI, and these two variables explained 45% of the variability in T cell proliferation. Our data demonstrate that CD69 MFI on unstimulated $CD3^+CD69^+$ T cells was predictive of T cell proliferation in response to anti-CD3 stimulation in young healthy adults. Numerous studies report a costimulatory role of CD69 in T cell proliferation (157, 160-163, 167). Crosslinking CD69 with antibodies enhances human T cell proliferation induced by phorbol myristate acetate (PMA), phytohaemagglutinin (PHA) or anti-CD3 antibody (160-163, 167). Thus, baseline differences in CD69 expression may contribute to differences in T cell proliferation via varying amounts of co-stimulation. Moreover, we found that the BMI of subjects contributed to the heterogeneity of T cell proliferation, and confounds the association between CD69 expression and T cell proliferation. Therefore, BMI should be quantified and controlled for in studies quantifying T cell proliferation.

The MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells also contributed significantly (16%) to the variability in T cell proliferation (Table 3.3). These results are consistent with previous studies which demonstrate a co-stimulatory role of CD25 in T cell proliferation (157). However, in our study the MFI of CD25 on $CD3^+CD25^+$ T cells was no longer associated with T cell proliferation after incorporating physical activity and daily intake of selenium, vitamin D, and n-3 PUFA as confounding variables in our model (129, 143,

146-150). These data suggest that CD25 MFI may only be marginally predictive of T cell proliferation, but other lifestyle and dietary patterns may contribute significantly to T cell proliferative responses. Similar analyses using activation marker expression on unstimulated $CD4^+$ and $CD8^+$ T cell subsets as predictors find consistent results, which confirm our current observations (Supplemental table 3.1 and 3.2).

Similar linear regression analyses were performed to explore the association between CD69 and CD25 expression on unstimulated T cells and anti-CD3 induced IL-2 secretion from T cells. The MFI of CD69 on unstimulated $CD3^+CD69^+$ T cells contributed significantly (29%) to the variability in IL-2 secretion (Table 3.3). To further explain the heterogeneity in IL-2 secretion from activated T cells, we included several confounding variables in the model that have previously been reported to modulate IL-2 secretion (129, 141, 143, 144, 146, 147, 151-156). The final model included both the MFI of CD69 on $CD3^+CD69^+$ T cells and age, physical activity, and daily intake of total calories, vitamin D and iron. Combined these variables explained 64% of the variability in IL-2 secretion. Our observations are consistent with previous studies, which report that crosslinking CD69 with anti-CD69 antibodies enhance human IL-2 secretion following T cell activation induced by PMA, PHA or anti-CD3 antibody (160-163, 167). However, our findings are novel because we demonstrate that individual lifestyle and dietary variables contribute a small amount to IL-2 secretion. However, these variables significantly improved the predictive relationship between CD69 expression and IL-2 secretion suggesting that host factors are important variables to quantify in clinical studies assessing T cell function.

Similarly, the MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells contributed significantly (55%) to the variability in IL-2 secretion (Table 3.3). In our final model, we controlled for age, physical activity, and daily intake of total calories, vitamin D, and iron, and the MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells and together these variables contributed significantly (74%) to the variability in IL-2 secretion. Our findings are not surprising given the autocrine loop formed between CD25 expression and IL-2 secretion during T cell activation (157). Similar analyses using activation marker expression on unstimulated $CD4^+$ and $CD8^+$ T cell subsets as predictors found consistent results, which confirmed our current observations (Supplemental table 3.1 and 3.2). Our data suggest that the MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells could serve as predictor of IL-2 secretion from T cells in response to anti-CD3 stimulation in young healthy adults. In addition, we found that host factors including age, physical activity, and daily intake of total calories, vitamin D and iron of subjects contributed to the association between the MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells and IL-2 secretion.

Unlike IL-2 secretion, we did not observe an association between anti-CD3 induced IFN-γ secretion from T cells and the MFI of CD69 on unstimulated $CD3^+CD69^+$ T cells either alone, or after controlling for host factors that have previously been reported to modulate T cell IFN-γ secretion (147-150, 155, 156). However, the MFI of CD25 on unstimulated $CD3^+CD25^+$ T cells contributed significantly (13%) to the variability in IFN-γ secretion (Table 3.3). Previous studies demonstrate that the interaction of IL-2 with CD25 (α chain of the IL-2 receptor) induces T cells to secrete IFN-γ (157,

158). These data support the association between MFI of CD25 alone and IFN-γ secretion. However, in our study the association between CD25 MFI on unstimulated $CD3^{+}CD25^{+}$ T cells and IFN-γ secretion was no longer significant after controlling for daily intake of selenium, vitamin C, vitamin D and n-3 PUFA suggesting that these dietary factors significantly influence IFN-γ response in human T cells (147-150, 155, 156). In fact, of the aforementioned dietary variables, vitamin D intake contributed to 12% of the variability in IFN-γ secretion, and daily intake of vitamin D was inversely associated with IFN-γ secretion. This finding is in agreement with previous studies, which report that vitamin D (25-hydroxyvitamin D) and 1,25 dihydroxyvitamin D3, the active form of vitamin D, inhibit IFN-γ secretion from mitogen stimulated T cells in humans and mice (149). Results from our study suggest that activation marker expression (either CD69 or CD25) on freshly-isolated, unstimulated T cells was not strongly predictive of IFN-γ secretion from activated T cells. Moreover, dietary and lifestyle factors (in particular vitamin D status) contributed significantly to the variability in IFN-γ secretion. Thus, the relationship between dietary and lifestyle factors and IFN-γ secretion needs to be examined in a larger study to confirm these findings and to determine the magnitude of the relationship. Similar analyses using activation marker expression on unstimulated $CD4^{+}$ and $CD8^{+}$ T cell subsets as predictors found consistent results (Supplemental table 3.1 and 3.2).

To determine if the incidence and severity of cold or flu symptoms captured via the self-reported URTI questionnaire is accompanied by relevant immunological changes, we used two analytical strategies. First, we performed logistic regression analysis using IL-6 secretion from LPS

stimulated PBMCs as the independent variable (predictor) and incidence of cold or flu episodes as the dependent variable (outcome). Next, we ran Poisson regression analysis using IL-6 secretion from LPS stimulated PBMCs as the independent variable (predictor) and total number of days with cold or flu symptoms as the dependent variable (outcome). From these two approaches, we found that IL-6 secretion was higher in participants with self-reported cold or flu episodes compared to participants without cold or flu episodes, and was significantly associated with cold or flu incidence in the past month (Figure 3.3 and Table 3.4). IL-6 secretion remained significantly associated with incidence of cold or flu after incorporating total calories, vitamin C, iron, and zinc in the model (Table 3.4). However, we did not observe an association between total number of days with cold or flu symptoms and IL-6 secretion alone and with the addition of confounding variables (daily intake of total calories, iron, and zinc). Our findings suggest that IL-6 secretion from LPS stimulated PBMCs was associated with the incidence of cold or flu episodes, but was not related to the severity of cold or flu symptoms captured via self-reported URTI questionnaire. In addition, we found that when dietary factors (total calories, vitamin C, iron, and zinc) were included in the model, the association between IL-6 secretion and the incidence of cold or flu symptoms in young healthy adults was strengthened.

Previous studies report elevated levels of IL-6 during cold or flu infection as a result of ongoing inflammation (168-170). The release of IL-6 during cold or flu is also critical in coordinating the innate and adaptive immune responses for efficient clearance of infection (170). The heterogeneity in IL-6 secretion from PBMCs in response to LPS stimulation

may be attributable to genetic and environmental factors (171, 172). Previous studies demonstrate an association between polymorphisms in the gene encoding IL-6 (G/C polymorphism was detected at position -174) and secreted level of IL-6 following stimulation. In particular, homozygosity of IL-6-174C allele is associated with reduced transcription and secretion of IL-6 in human plasma (173) and with decreased frequency of the common cold (171). Therefore, the heterogeneity in LPS-stimulated IL-6 secretion from PBMCs observed in our study may be due to polymorphisms in the gene encoding IL-6, as well as environmental factors (e.g. BMI, dietary intake and physical activity) which contribute to the difference in IL-6 secretion between participants with and without self-reported cold or flu.

Similar logistic and Poisson regression analyses were also performed to determine if the incidence and severity of cold or flu symptoms captured via the self-reported URTI questionnaire is accompanied by IFN-γ secretion from anti-CD3 stimulated T cells. The incidence of cold or flu was not associated with IFN-γ secretion alone or following inclusion of age, BMI, daily intake of vitamin D, iron, zinc, n-3 PUFA, and caffeine, and alcohol consumption in the model (Table 3.4). However, IFN-γ secretion from anti-CD3 stimulated T cells was significantly associated with total number of days with cold or flu symptoms alone and with the addition of daily intake of vitamin C, zinc, caffeine and alcohol consumption (Table 3.4). Our results suggest that the severity but not the incidence of cold or flu symptoms assessed via the self-reported URTI questionnaire was accompanied by changes in IFN-γ secretion from anti-CD3 stimulated T cells in healthy young adults. We also observed that the association between IFN-γ secretion and severity of cold or flu

symptoms was confounded by daily intake of vitamin C, zinc, caffeine and alcohol consumption, suggesting that these dietary components impact this relationship.

IFN-γ production has been reported to play an important role in the immune response against numerous viral infections (172, 174). Thus, individuals who have lower IFN-γ production may be more susceptible to infection with influenza virus or rhinoviruses causing the common cold. The heterogeneity in IFN-γ secretion following T cell activation that we observed may also be attributable to genetic and environmental factors (171, 172). For example, a single nucleotide polymorphism (SNP) in IFN-γ gene has been studied extensively and is presented as polymorphism +874 A/T. Results from a meta-analysis demonstrate that the variant allele +874 A is correlated with lower levels of IFN-γ production and increased risk of *Mycobacterium tuberculosis* infection (175). It is plausible that individuals carrying the +874A allele may be susceptible to other infections, including influenza infection. Thus, IFN-γ gene polymorphisms may contribute to the differences in IFN-γ secretion between individuals with and without cold or flu episodes and the association between IFN-γ secretion and incidence and severity of cold or flu symptoms in our study. However, Becker *et al,* reported no association between common cold frequency and CD2-induced IFN-γ secretion from T cells in adults 45-65 years old (174). Previous studies demonstrate an age-related reduction in the in synthesis and secretion of IFN-γ from PBMCs (151, 152). Thus, age may also impact the relationship between cold and flu incidence and IFN-γ secretion.

In summary, we demonstrated that expression of CD69 and CD25 expression on freshly isolated, unstimulated T cells was significantly associated with anti-CD3 stimulated T cell proliferation and IL-2 secretion. We also found that IL-6 secretion from LPS stimulated PBMCs was associated with self-reported incidence of cold or flu episodes, and IFN-γ secretion from T cells was associated with self-reported severity of cold or flu symptoms in the past month. Our data suggest that the incidence and severity of cold or flu symptoms captured via the URTI questionnaire was accompanied by relevant immunological changes. In addition, we demonstrated that host-related factors, including age, BMI, physical activity, total calorie intake, and various dietary components contributed to heterogeneity in T cell function and incidence and severity of cold or flu infection. These factors also confounded the association between activation marker expression on T cells and T cell effector function, and the association between innate and adaptive immune response and incidence and severity of cold or flu symptoms. Therefore, quantification of dietary factors in human clinical trials measuring immune function may be crucial to understanding the variability in immune responses among subjects, and to determine the true relationship between an intervention of interest and immune outcomes.

Supplement table 3.1. CD69 and CD25 expression on unstimulated $CD4^+$ T cell as predictors of T cell effector function[1].

	CD69 MFI on $CD3^+CD4^+CD69^+$ T cells				CD25 MFI on $CD3^+CD4^+CD25^+$ T cells			
	β	**R^2 (%)**	**Variables in the model**	**p value**	**β**	**R^2 (%)**	**Variables in the model**	**p value**
Anti-CD3 induced T cell proliferation[1]								
Model 1	0.00584	27.02	CD69 MFI on $CD3^+CD4^+CD69^+$ T cells	0.0014	0.00899	16.67	CD25 MFI on $CD3^+CD4^+CD25^+$ T cells	0.0149
Model 2	0.00681	45.44	Model 1 + BMI	<0.0001	0.00587	35.89	Model 1 + PA, selenium, n-3 PUFA	0.1065
Anti-CD3 induced IL-2 secretion from T cells								
Model 1	0.02571	29.51	CD69 MFI on $CD3^+CD4^+CD69^+$ T cells	0.0009	0.05469	34.94	CD25 MFI on $CD3^+CD4^+CD25^+$ T cells	0.0002
Model 2	0.02011	54.36	Model 1 + age, total calories, vitamin D, zinc, iron	0.0051	0.04065	69.68	Model 1 + age, PA, total calories, vitamin D, zinc, iron	0.0013
Anti-CD3 induced IFN-ɣ secretion from T cells								
Model 1	1.43915	11.36	CD69 MFI on $CD3^+CD4^+CD69^+$ T cells	0.0512	2.68772	10.36	CD25 MFI on $CD3^+CD4^+CD25^+$ T cells	0.0634
Model 2	1.22687	30.84	Model 1 + BMI, vitamin C, D, selenium, n-3 PUFA	0.1385	2.37393	38.13	Model 1 + vitamin D, E, selenium, zinc, iron, n-3 PUFA	0.1273

[1] T cell proliferation was evaluated by quantifying tritiated thymidine incorporation following stimulation with anti-CD3 antibodies, and results are reported as a stimulation index. Stimulation index was calculated by dividing the cpm of the anti-CD3 induced T cell proliferation by unstimulated T cells.

Supplement table 3.2. CD69 and CD25 expression on unstimulated $CD8^+$ T cell as predictors of T cell effector function[1].

	CD69 MFI on $CD3^+CD8^+CD69^+$ T cells				CD25 MFI on $CD3^+CD8^+CD25^+$ T cells			
	β	**R^2 (%)**	**Variables in the model**	**p value**	**β**	**R^2 (%)**	**Variables in the model**	**p value**
Anti-CD3 induced T cell proliferation[1]								
Model 1	0.00604	38.23	CD69 MFI on $CD3^+CD8^+CD69^+$ T cells	<0.0001	0.00145	13.72	CD25 MFI on $CD3^+CD8^+CD25^+$ T cells	0.0285
Model 2	0.00764	61.85	Model 1 + BMI, vitamin C	<0.0001	0.00086	33.57	Model 1 + PA, selenium, n-3 PUFA	0.2023
Anti-CD3 induced IL-2 secretion from T cells								
Model 1	0.02307	31.56	CD69 MFI on $CD3^+CD8^+CD69^+$ T cells	0.0005	0.01196	52.64	CD25 MFI on $CD3^+CD8^+CD25^+$ T cells	<0.0001
Model 2	0.02235	37.72	Model 1+age	0.0006	0.00866	66.63	Model 1 + age, PA, total calories, vitamin D, iron	0.0008
Anti-CD3 induced IFN-ɣ secretion from T cells								
Model 1	0.67363	03.31	CD69 MFI on $CD3^+CD8^+CD69^+$ T cells	0.3034	0.51000	11.76	CD25 MFI on $CD3^+CD8^+CD25^+$ T cells	0.0471
Model 2	0.10972	41.83	Model 1 + age, BMI, PA, total calories, vitamin C, E, selenium, zinc, iron, n-3 PUFA, caffeine, alcohol	0.9145	0.51000	11.76	Model 1	0.0471

[1] T cell proliferation was evaluated by quantifying tritiated thymidine incorporation following stimulation with anti-CD3 antibodies, and results are reported as a stimulation index. Stimulation index was calculated by dividing the cpm of the anti-CD3 induced T cell proliferation by unstimulated T cells.

Supplemental Table 3.3. Individual R^2 (%) of predictor (activation marker expression) and confounding variables in the linear regression models with T cell proliferation, IL-2 secretion and IFN-γ secretion as outcome variables.

	T cell proliferation		IL-2 secretion		IFN-γ secretion	
	CD69 MFI on $CD3^+CD69^+$ T cells	CD25 MFI on $CD3^+CD25^+$ T cells	CD69 MFI on $CD3^+CD69^+$ T cells	CD25 MFI on $CD3^+CD25^+$ T cells	CD69 MFI on $CD3^+CD69^+$ T cells	CD25 MFI on $CD3^+CD25^+$ T cells
Total R^2	44.96	36.29	64.19	74.00	30.94	31.97
Predictor	29.17	15.53	34.72	55.73	1.71	12.91
Age			8.40	4.47	3.68	
BMI	15.79				2.49	
PA		4.41	3.26	5.30	0.84	
Total Calories			7.09	2.62	1.98	
Vitamin C					0.73	2.33
Vitamin D		0.70	5.84	3.48	12.13	10.02
Vitamin E						
Selenium		10.30			1.53	2.92
Zinc					1.07	
Iron			4.88	2.40		
n-3 PUFA		5.35				3.79
Alcohol					4.78	
Caffeine						

Supplemental Table 3.4. Individual contribution of predictor (cytokine secretion) and confounding variables in the regression models with cold/flu incidence and severity as outcome variables.

Dependent variable	Cold or flu incidence (Odds ratio (95% CI))		Cold or flu severity (95% CI)	
	IL-6 secretion	IFN-γ secretion	IL-6 secretion	IFN-γ secretion
Predictor	0.787 (0.632, 0.980)	1.057 (0.986, 1.133)	(-0.018,0.178)	(-0.021, -0.009)
Age		1.232 (0.946, 1.604)		
BMI		0.708 (0.364, 1.378)		
PA				
Total calories	1.001 (0.999, 1.003)		(-0.001, 0.000)	
Vitamin C	0.997 (0.979, 1.015)			(0.004, 0.016)
Vitamin D		1.008 (0.997, 1.020)		
Vitamin E				
Selenium				
Zinc	1.629 (0.862, 3.080)	1.617 (0.805, 3.249)	(-0.455, -0.022)	(-0.317, -0.101)
Iron	0.840 (0.621, 1.138)	0.796 (0.561, 1.129)	(-0.024, 0.185)	
n-3 PUFA		0.083 (0.002, 3.353)		
Alcohol		0.918 (0.776, 1.085)		(-0.013, 0.173)
Caffeine		1.026 (0.996, 1.058)		(-0.021, -0.001)

CHAPTER 4

Consumption of *Bifidobacterium animalis* subsp. *lactis* BB-12 in yogurt reduced expression of TLR-2 on peripheral blood-derived monocytes and pro-inflammatory cytokine secretion in young adults

* The results presented in this chapter were submitted to the *Journal of Nutrition*.

4.1. Abstract

Previous studies have demonstrated that probiotic bacteria modulate immune parameters and inflammatory outcomes; however, observed results vary by genus, species and strain of organism studied. Many studies have evaluated combinations of probiotic bacteria making it difficult to determine the immunomodulatory properties of each organism. There is also evidence that the matrix used to deliver probiotic bacteria may influence the performance and efficacy of probiotic interventions *in vivo*. Therefore, the primary goal of the current study was to evaluate the effect of one species, *Bifidobacterium animalis* subsp. *lactis* BB-12 at a dose of log 10 ± 0.5 CFUs/day on immune responses in a randomized, partially blinded, 4-period crossover, free-living study. A secondary goal was to determine if the immune response to BB-12 differed depending on the delivery matrix of the probiotic bacteria. Healthy adults (n=30) aged 18-40 years old were recruited, and received 4 treatments in a random order: A) yogurt smoothies alone; smoothies with BB-12 added B) before or C) after yogurt fermentation, or D) BB-12 given in capsule form. At baseline and after each 4-week treatment, peripheral blood mononuclear cells (PBMCs) were isolated, and functional and phenotypic marker expression was assessed. BB-12 interacted with peripheral myeloid cells via Toll-like receptor 2 (TLR-2). The percentage of $CD14^{+}HLA\text{-}DR^{+}$ cells in peripheral blood was increased by all yogurt-containing treatments compared to baseline ($p=0.0356$). Participants who consumed yogurt smoothies with BB-12 added post fermentation had significantly lower expression of TLR-2 on $CD14^{+}HLA\text{-}DR^{+}$ cells ($p=0.0186$) and reduction in TNF-α secretion from BB-12 ($p=0.0490$) or LPS ($p=0.0387$)

stimulated PBMCs compared to baseline. These findings not only demonstrate anti-inflammatory properties of BB-12 in healthy adults, but also indicate that the delivery matrix influences the immunomodulatory properties of the probiotic.

The ClinicalTrials.gov identifier: NCT01399996.

4.2. Introduction

There is growing interest in the use of probiotics in the maintenance of both immunological and gastrointestinal health. Numerous studies have examined the influence of probiotic bacteria, either alone or combined, on immune parameters and inflammatory outcomes. Probiotic bacteria in the genera, *Lactobacillus* and *Bifidobacterium* are widely studied (8), and overall the results are mixed. It appears that large strain and species differences exist. In addition variability due to dose and duration of supplementation, characteristics of the study population, and delivery matrix of probiotic have been reported (8). Thus, well-designed clinical trials using a single strain of probiotic on immune and inflammatory outcomes are needed.

Bifidobacterium animalis subsp. *lactis* BB-12 is a widely used probiotic species of *Bifidobacterium* (9). It is popular among food manufacturers due to its ability to remain viable until consumption (9). Moreover, BB-12 is resistant to degradation by gastric acid and bile, and adheres to human intestinal epithelial cells and mucin. These features may allow BB-12 to survive during passage through the gastrointestinal (GI) tract and exert its probiotic effect (9-13). Several studies have investigated the effect of BB-12 consumption in combination with other lactic acid producing bacteria on human systemic innate immunity, in an effort to link probiotic use to a reduction in inflammation-mediated diseases (14-16). The effect of probiotics on innate immunity appears strain specific, and thus the results of these studies vary based on which probiotic bacteria was given in conjunction with BB-12 (14-16).

Studies employing BB-12 alone as the probiotic intervention also yielded mixed results. One *in vitro* study using monocyte-derived dendritic cells (MoDCs) demonstrated that co-culture of BB-12 with MoDCs induced increased IL-12 secretion, low IL-10/IL-12 ratio, and shifted the TNF-α/IL-10 balance, suggesting that BB-12 promotes the induction of strong Th1 response (17). In humans, BB-12 has been used to treat inflammatory diseases of the intestine (e.g., inflammatory bowel disease, irritable bowel syndrome) with some success (18, 19). The mechanisms underlying the beneficial effect of BB-12 in these inflammatory diseases is unknown. However, oral consumption of a similar probiotic strain, *Bifidobacterium animalis* subsp. *lactis* NCC2818 has been shown to reduce colonic inflammation in a murine colitis model via a reduction in the pro-inflammatory markers, cyclooxygenase-2 (COX-2), TNF-α, and IL-6 (29, 116). Therefore, the beneficial effect of BB-12 in inflammatory bowel disease (IBD) patients may be mediated via its role in the modulation of innate immunity, in particular in inflammatory responses. However, in another clinical trial, healthy adults who received BB-12 daily for 3 weeks had no change in serum systemic inflammation markers (hs-CRP and TNF-α) or *in vitro* TNF-α production from *Streptococcus pyogenes* or LPS stimulated PBMCs (20). Therefore, additional studies are needed to clarify the role of BB-12 alone on inflammatory responses in humans.

Probiotics are often considered to act independently of the matrix used to deliver them to the human host (21). Dairy products, especially yogurt, are commonly used to deliver BB-12 (22); however, BB-12 can also be consumed in capsule form (23). While this expands the options for BB-12 consumption, it

is unclear if probiotics delivered in capsule form are as effective in modulating immunity as when delivered in a dairy food (23). Furthermore, BB-12 can be added into yogurt products either prior to or following the yogurt fermentation process, and this varies among manufacturers. Little is known about the timing of the addition of BB-12 to dairy products on the efficacy and functionality of BB-12, even though these differences in manufacturing processes may influence the health benefits of the probiotic organism.

Thus, the primary goal of this study was to evaluate the effect of BB-12 consumption on innate immunity and anti-inflammatory responses in healthy adults. A secondary goal of this study was to determine if the delivery matrix (yogurt smoothie vs capsule), and timing of the addition of probiotic organism to the yogurt smoothie (pre- or post-fermentation) impacted the immunological responses to BB-12 in humans.

4.3. Materials and methods

4.3.1. Participants

Healthy subjects (n=30, 19 women and 11 men) 18-40 years of age were recruited for the study. Exclusion criteria included: BMI greater than 40kg/m^2, smoking and/or use of other tobacco products, blood pressure ≥ 140/90 mm Hg, use of blood pressure or cholesterol lowering medications, history of myocardial infarction, stroke, diabetes mellitus, liver disease, kidney disease and thyroid disease (unless controlled by medication and blood results within the previous six months were provided), lactation, pregnancy or desire to become pregnant during the study, clinical diagnosis of IBD (e.g. Crohn's Disease or ulcerative colitis), excessive alcohol consumption (>14 drinks per week), vegetarianism, lactose intolerance, chronic use of anti-

inflammatory medications (unless able to discontinue), antibiotic use, swallowing disorders or dysphasia to food or pills, and refusal to give blood or plasma during the study. A complete blood count and standard biochemistry panel was obtained at screening to rule out the presence of illness (autoimmune disease, cancer, and immunodeficiency). Blood pressure was measured according to Joint National Committee 7 guidelines (137).

4.3.2. Recruitment and screening

Recruitment strategy used in the study is shown in Figure 4.1. Participants were recruited through advertisements in the local newspaper and university e-mail lists; 203 individuals called or emailed to indicate interest in participating in the study. They were given information about the study, and if interested, were contacted and screened using a series of medical and lifestyle questions by telephone. Of those who were contacted and screened through telephone interview, 136 qualified participants were scheduled for a clinic visit at the Penn State Clinical Research Center (CRC). After written informed consent was provided, participants had their height, weight, waist circumference and blood pressure measured, followed by a fasting blood draw for a complete blood count and health profile (liver and kidney function, and glucose metabolism). BMI was calculated according to body weight and height measured. From the participants who were screened, 36 were eligible to participant in the study. To ensure that there was no effect of treatment order on outcomes, eligible participants (n=36) were randomized to treatment sequences. The randomization scheme was generated by using the website Randomization.com, http://www.randomization.com. However, two participants withdrew at the baseline visit due to a schedule conflict; four

withdrew due to personal reasons. Thirty participants received treatment interventions; however, not all subjects finished all interventions. Two participants were excluded prior to the third and fourth treatment periods, respectively due to pregnancy; one participant withdrew from the study prior to the fourth treatment period due to diagnosis of irritable bowel syndrome; one participant withdrew from the study prior to the second treatment period due to a schedule conflict; one participant withdrew from the study prior to the second treatment period due to discomfort after treatment; and two participants withdrew from the study prior to the first and second treatment periods, respectively due to other personal reasons. All the experiments in this study were performed with approval of the Institutional Review Board of the Pennsylvania State University-University Park campus (University Park, PA).

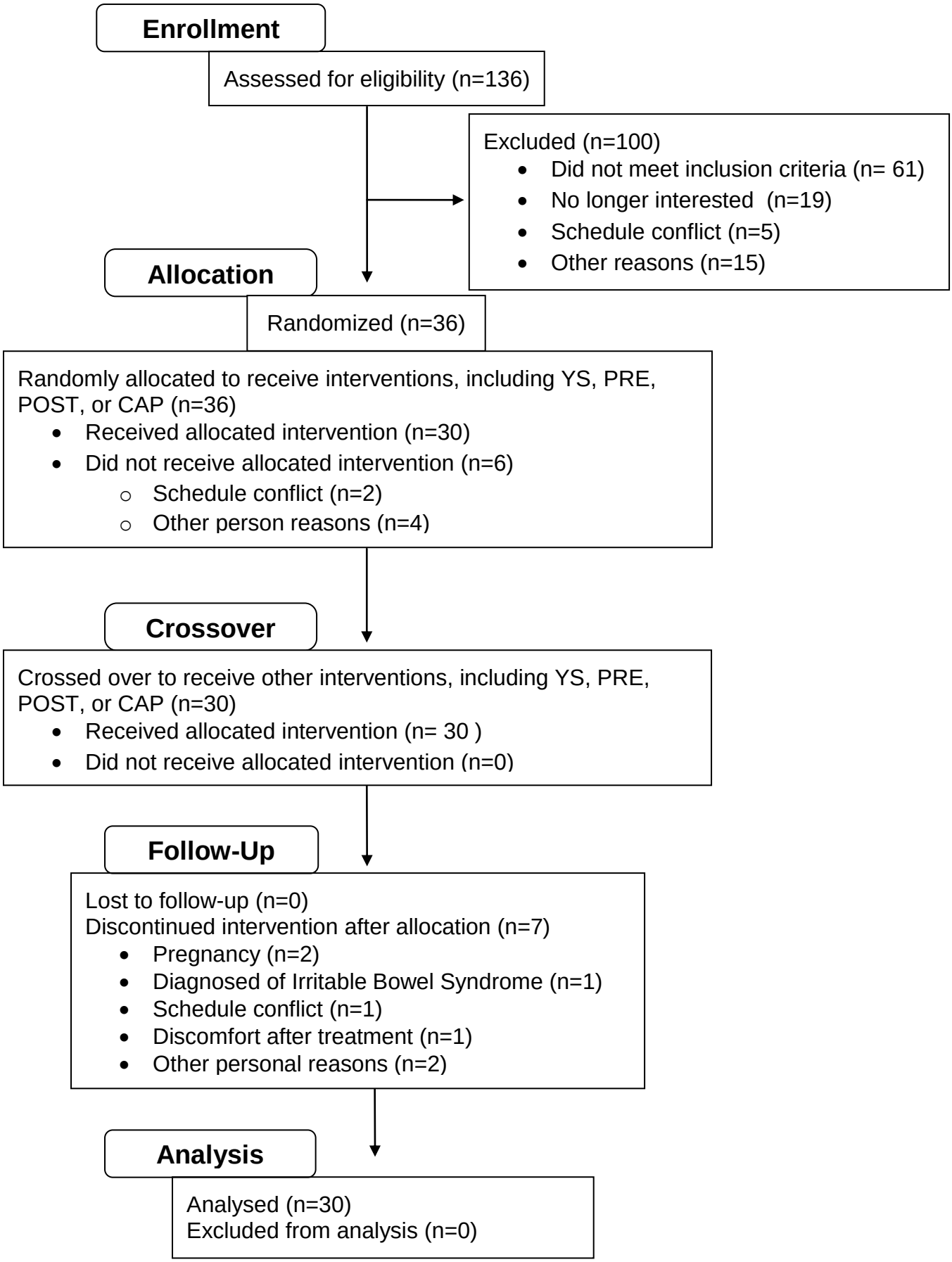


Figure 4.1. Recruitment strategy used in the study.

4.3.3. Design and intervention

The study was a randomized, partially blinded, 4-period crossover free-living study. Prior to the first treatment, participants completed an anthropometric assessment (age, gender, BMI, waist circumference), biochemical measurements (fasting serum glucose, insulin, and hs-CRP), a physical activity questionnaire (self-reported IPAQ), and an immune endpoint assessment for use as baseline values. Participants then began the intervention phase, and received 4 treatments according to their randomized sequence. These treatments included: (A) yogurt smoothies (YS); (B) yogurt smoothies with BB-12 (Chr. Hansen, Milwaukee, WI) added pre-fermentation (PRE); (C) yogurt smoothies with BB-12 added post-fermentation (POST); and (D) one capsule containing BB-12 (CAP). Each treatment period lasted 4 weeks, with a two-week washout period scheduled between treatment periods.

During the yogurt smoothie treatment phases, participants consumed one 8-oz (240 g) serving of yogurt per day. Each smoothie delivered log 10 ± 0.5 CFUs/day. The yogurt smoothie were developed at Pennsylvania State University, and manufactured based on methods described in a previous study (176). Briefly, yogurt mix (milk and dry ingredients) were inoculated with the yogurt starter culture YF-L702 (Chr. Hansen), containing active cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Inoculated yogurt mix was divided into two portions: one portion was further inoculated with BB-12 (PRE); another portion (non-BB-12) was incubated directly. Both portions were allowed to ferment to a pH of 4.6, and a slurry of strawberry, pectin, corn syrup solids, sugar and water was added and

blended into each portion until uniform. The non-BB-12 containing portion of smoothie was divided into two groups. One group was inoculated with BB-12 (POST) while the other remained a BB-12 free product (YS). The smoothie yogurt was then homogenized to produce a drinkable yogurt. The three products were identical in macronutrient composition and differed only by the presence of BB-12 and the timing of BB-12 addition. Viable counts of *B. lactis* were determined by pour-plating on MRS (de Man, Rogosa, and Sharpe)-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate) agar (177) with growth under anaerobic conditions at 37°C for 72 hours.

During the treatment phase involving capsule consumption, participants ingested one capsule per day, which delivered log 10 ± 0.5 CFUs/day. During each 4-week treatment phase, participants were required to avoid consuming any other food or pharmaceutical product containing probiotic bacteria such as commercial yogurt, smoothies, and probiotic bacteria capsules. Participants were asked not to change their habitual diets during the course of the study and to maintain their body weight.

4.3.4. Sample size estimation

The primary immune endpoints used to estimate sample size for the study in Chapter 4 were cytokine secretion (TNF-α and IL-6) from our pilot study and published clinical trials assessing cytokine secretion from LPS-stimulated PBMCs in healthy adults who consumed BB-12 or other *B. lactis* subspecies (20, 61). These endpoints were chosen because previous reports demonstrate modest changes in the magnitude of the cytokine response upon stimulation with various *B. lactis* and high variance. Using the sample size

calculation for crossover study, it was determined that the estimated sample size required to identify a mean difference of 30% in TNF-α and IL-6 secretion from LPS-stimulated PBMCs with α = 0.05 and β = 80% was 19 and 22, respectively. We estimated a high dropout rate (40%) due to the length of the study, and the final sample size was 36. However, 6 participants dropped out of the study before intervention phase, and 30 participants completed at least one allocated interventions. Based on the aforementioned calculations, a sample size of 30 was adequate to observe a treatment effect.

4.3.5. Diet assessment

Dietary intake of the participants was obtained via 24-hour dietary recalls for 3 days, including one weekend day. Briefly, participants were asked to recall their intake of food and beverages during breakfast, lunch, dinner, and snacks in the three days according to detailed instructions provided by trained staff. Portion size of each food item was also provided. Daily intake of total calories, macronutrients, vitamins, minerals, caffeine, and alcohol was analyzed based on the recorded food intake of participants using Food Processor SQL software (ESHA Research, Salem, OR).

4.3.6. Physical activity assessment

Physical activity level of participants was evaluated using the International Physical Activity Questionnaires (IPAQ) questionnaire as previously reported (138). Briefly, the participants recorded the activity they performed during each of the 96 periods of 15 min over a 24-h period for 3 days, including one weekend day. The activities were categorized from 1 to 9 depending on their intensity as previously described (138). Total daily intensity of physical activity was calculated by averaging the approximate

metabolic equivalent of tasks (METs) of activities performed of the categories 3 to 9 over a 24 hour period (96 periods of 15 min).

4.3.7. Blood sample collection and immunological assays

Blood (50 ml) was collected in sterile EDTA (K2)-coated blood tubes (BD Biosciences, San Jose, CA) by trained staff at the CRC on the University Park campus.

Serum markers

Insulin was measured by radioimmunoassay (Quest Diagnostics, Pittsburgh, PA). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose and Lactate Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Serum high-sensitivity C-reactive protein (hs-CRP) was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

Isolation of immune cells

Human blood was diluted 1:2 with phosphate buffer saline (PBS) (Mediatech, Manassas, VA), gently layered on top of lymphocyte separation media (LSM) (Corning, Manassas, VA), and centrifuged at 1600 rpm with low speed and no brake for 30 mins at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected at the plasma/LSM interface; washed twice with complete media RPMI 1640 (Mediatech) containing 10 mM HEPES (Mediatech), 10% heat-inactivated fetal bovine serum (Gemini, West Sacramento, CA), 2 mM L-glutamine (Mediatech), 0.1 mM nonessential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 100U/ml Penicillin/Streptomycin (Mediatech), and 55 μM 2-mercaptoethanol (Life

Technologies, Grand Island, NY) at room temperature; and counted for use in functional and phenotypic analyses.

Cytokine secretion assays

PBMCs (2 X 10^6/ml) were stimulated with 10 µg/ml heat-inactivated BB-12 (Chr. Hansen) or 10 µg/ml lipopolysaccharides (LPS) (Sigma-Aldrich, St. Louis, MO) in flat-bottomed 96-well plates, and supernatants from BB-12 or LPS plates were harvested and frozen after 4 h incubation. TNF-α and IL-6 secretion from BB-12 or LPS stimulated PBMCs were measured using the Human ELISA MAX™ Deluxe (Biolegend, San Diego, CA) as per manufacturer instructions. Each assay was performed in triplicate.

TLR-2 and TLR-4 blocking experiment

The TLR-2 and TLR-4 blocking experiments were conducted at baseline to determine which TLR(s) recognize and interact with surface ligands of BB-12. 8 participants were randomly selected for this experiment. PBMCs (2 X 10^6/ml) were co-cultured with 10 µg/ml anti-TLR-2 blocking antibody (Biolegend), 10 µg/ml anti-TLR-4 blocking antibody (Biolegend), or 10 µg/ml isotype antibody (mouse anti-human IgG_{2a}) in flat-bottomed 96-well plates, respectively. Control PBMCs were not treated with antibody. Following 1 h incubation, cells were stimulated with 10 µg/ml heat-inactivated BB-12 (Chr. Hansen) for another 4 h. Supernatants from plates were harvested and frozen after 4 h incubation, and TNF-α and IL-6 secretion from BB-12 stimulated PBMCs were measured using the Human ELISA MAX™ Deluxe (Biolegend) as per manufacturer instructions. Each assay was performed in triplicate.

Flow cytometric analyses

PBMCs were washed twice in PBS at 4°C. Fc receptors on PBMCs were blocked by incubation with 1 µg purified mouse anti-human CD16 (Biolegend) per 1×10^6 cells for 15 min at 4°C. PBMCs were stained with fluorescence-labeled antibodies (1µg/1×10^6 cells) to the following cell surface markers: CD123, CD11c, CD14, HLA-DR, CD281 (TLR-1), CD282 (TLR-2), CD284 (TLR-4), TLR-5, CD286 (TLR-6). Antibody isotype controls included: mouse IgG_{2a} and mouse IgM. CD281 was purchased from R&D systems (Minneapolis, MN), CD282 and CD284 were purchased from Biolegend, TLR-5 and CD286 were purchased from Thermo Scientific (Waltham, MA) and other antibodies were purchased from BD Biosciences. Following incubation with the conjugated antibodies for 30 min at 4° C, cells were washed twice in PBS and then fixed in cytofix (BD Biosciences) for flow cytometric analyses. Lymphoid and myeloid cells were gated on forward vs. side scatter and a total of 25,000 events were analyzed on a FC500 Benchtop Cytometer (Beckman Coulter, Pasadena, CA). Flow cytometric analyses were plotted and analyzed using Flowjo 7.6 (Tree Star, Ashland, OR).

4.3.8. Statistical analyses

Statistical analyses were performed using SAS (Statistical Analysis System, Version 9.4, Cary, NC). Differences in the percentage of $CD14^+HLA\text{-}DR^+$ cells expressing TLR-1, -2, -4, -5, -6 in PBMCs and the mean fluorescence intensity (MFI) of TLR-1, -2, -4, -5, -6s on $CD14^+HLA\text{-}DR^+$ cells were determined using the Friedman test. In the TLR blocking experiment, differences in TNF-α or IL-6 secretion from BB-12 stimulated PBMCs from subjects on each treatment were determined using the Friedman test, followed by a Dunnett test for multiple comparisons where appropriate.

All immune endpoints in this study were tested for a carryover effect using an exponential decay model (details of model explained in Supplemental Data). No carryover effect of treatments on any immune endpoint was observed.

The mixed models procedure (PROC MIXED) was used to test the effects of treatment on immune outcomes following each treatment period. Outcomes were modeled as repeated measures with unstructured covariance structure. Age, gender, BMI, PA, dietary factors and their interactions with treatment (e.g. age*treatment, gender*treatment, etc.) were included as covariates. Dietary factors (e.g. dietary intake of total calories, vitamin C, E, iron, zinc, and n-3 PUFA) were selected based on their individual contributions to the variation in phenotypic marker expression and function of innate immune cells at baseline (178). The covariates in models varied by different immune outcomes, and were selected based on manual backward selection procedure. Briefly, models were fitted with immune outcomes as dependent variables, and treatment effect and all covariates as independent variables. Covariates were removed from the model one by one based on the significance (assessed by p value of the corresponding covariate in models) of their contribution to the models. Covariates included in the final models contributed significantly to the model. If the interaction was significant but the main effect was not, both the main effect and interaction were included in the model. Participant was designated as a random effect and the treatment effect and covariates were fixed effects. For all outcomes, model selection was based on optimizing fit statistics (evaluated as lowest Bayesian information criterion) and α was set at 0.05 for all tests. Bonferroni correction

was used for post hoc analyses. Means are reported as least squares means ± 95% CI. Statistical significance was accepted at the $p \leq 0.05$ level. Graphs were plotted using GraphPad Prism 5 (La Jolla, CA).

4.4. Results

4.4.1. Participant characteristics.

Anthropometric measurements, blood pressure, biochemical characteristics, and physical activity of participants are presented in Table 4.1. All 30 participants (19 females and 11 males) completed the study. The participants were healthy, young adults (mean age of 28.0±1.2 years). The average BMI was 24.2±0.5kg/m^2; 20 (66.7%) participants were normal weight, 9 (30.0%) were overweight, and 1 (3.3%) was obese. Their blood pressure was normal and waist circumference, fasting blood glucose, insulin, and CRP levels were within the normal range (Table 4.1). Physical activity was assessed from self-reported IPAQ responses. The median daily physical activity intensity (based on self-reported responses) was estimated to be 3.0 METs (range 2.4-5.2 METs). The average daily total calorie intake of participants calculated from 3-day dietary recall records was estimated to be 2350±145 kcal. The daily intake of macronutrients, vitamins, minerals and n-3 PUFA, caffeine and alcohol are reported in Table 4.1.

Table 4.1. Demographic characteristics of participants at baseline [1].

Characteristics	Values (n=30)
Age (yr)	28.0±1.2
Male, n (%)	11 (36.7%)
Body mass index (kg/m^2)	24.2±0.5
≤24.9	20 (66.7%)
25.0 -29.9	9 (30.0%)
≥30	1 (3.3%)
Waist Circumference (cm)	85.1±1.4
Blood pressure (mm Hg)	
Systolic	106.9±1.9
Diastolic	73.2±1.2
Glucose (mg/dL)	87.0±1.3
Insulin (mg/dL)	5.2±0.8
hs-CRP (mg/L)	1.9±0.6
Physical activity (METs)[2]	3.0 (2.4-5.2)
Dietary intake[2] of	
Total calories (kcal/d)	2350±145
Carbohydrate (g/d)	291.1±19.5
Protein (g/d)	91.7±6.8
Fat (g/d)	92.1±6.7
Vitamin C (mg/d)	62.3±11.0
Vitamin D (IU/d)	112.6±34.4
Vitamin E (mg/d)	2.8±0.6
Iron (mg/d)	14.9±1.4
Selenium (µg/d)	37.4±4.7
Zinc (mg/d)	6.2±0.6
n-3 PUFA (g/d)	0.5±0.1
Caffeine (mg/d)	62.3±15.6
Alcohol consumption (g/d)	2.1±1.0

[1] Values are presented as mean ± SEM or n (%) or median (range).
[2] Physical activity and dietary intake were assessed from self-reported responses to IPAQ and 3-day dietary recall records, respectively.

4.4.2. BB-12 interacts with peripheral myeloid cells via TLR-2.

The percentage of $CD14^+HLA\text{-}DR^+$ cells expressing TLR-1, -2, -4, -5, -6 (Fig. 4.2A), and the MFI of TLR-1, -2, -4, -5, -6 on PBMC-derived monocytes (Fig. 4.2B) was quantified to determine the expression pattern of common surface TLRs on human $CD14^+HLA\text{-}DR^+$ cells. Overall, the percentage of $CD14^+HLA\text{-}DR^+$ cells expressing different TLRs and the MFI of TLRs on $CD14^+HLA\text{-}DR^+$ cells significantly differed (Fig. 4.2A; $\chi^2=23.92$, $p<0.0001$ and Fig. 4.2B; $\chi^2=26.30$, $p<0.0001$), with both the percentage of $CD14^+HLA\text{-}DR^+$ PBMC-derived monocytes expressing TLR-2 (Fig. 4.2A), and the MFI of TLR-2 on $CD14^+HLA\text{-}DR^+$ cells (Fig. 4.2B) being highest. Several subjects also had a high number of $CD14^+HLA\text{-}DR^+$ cells expressing TLR-4 (Fig. 4.2A), and the MFI of TLR-4 was elevated in one subject (Fig. 4.2B).

Fresh PBMCs were treated with either anti-TLR-2 blocking antibody, anti-TLR-4 blocking antibody, or the appropriate isotype control antibody (all antibodies added at 10 µg/mL), and stimulated with heat-inactivated BB-12 (10 µg/mL). TNF-α (Fig. 4.2C) and IL-6 (Fig. 4.2D) secretion from PBMCs was assessed to determine via which TLR on human myeloid cells BB-12 may be signaling. PBMCs treated with anti-TLR-2 blocking antibody had significantly reduced TNF-α (Fig. 4.2C; $\chi^2=23.13$, $p<0.0001$) and IL-6 (Fig. 4.2D; $\chi^2=17.67$, $p=0.0005$) secretion in response to BB-12 stimulation as compared to PBMCs treated with isotype control antibody or without treatment. In contrast, blocking TLR-4 signaling did not reduce BB-12-induced TNF-α or IL-6 secretion from PBMCs (Fig. 4.2C and 4.2D, respectively).

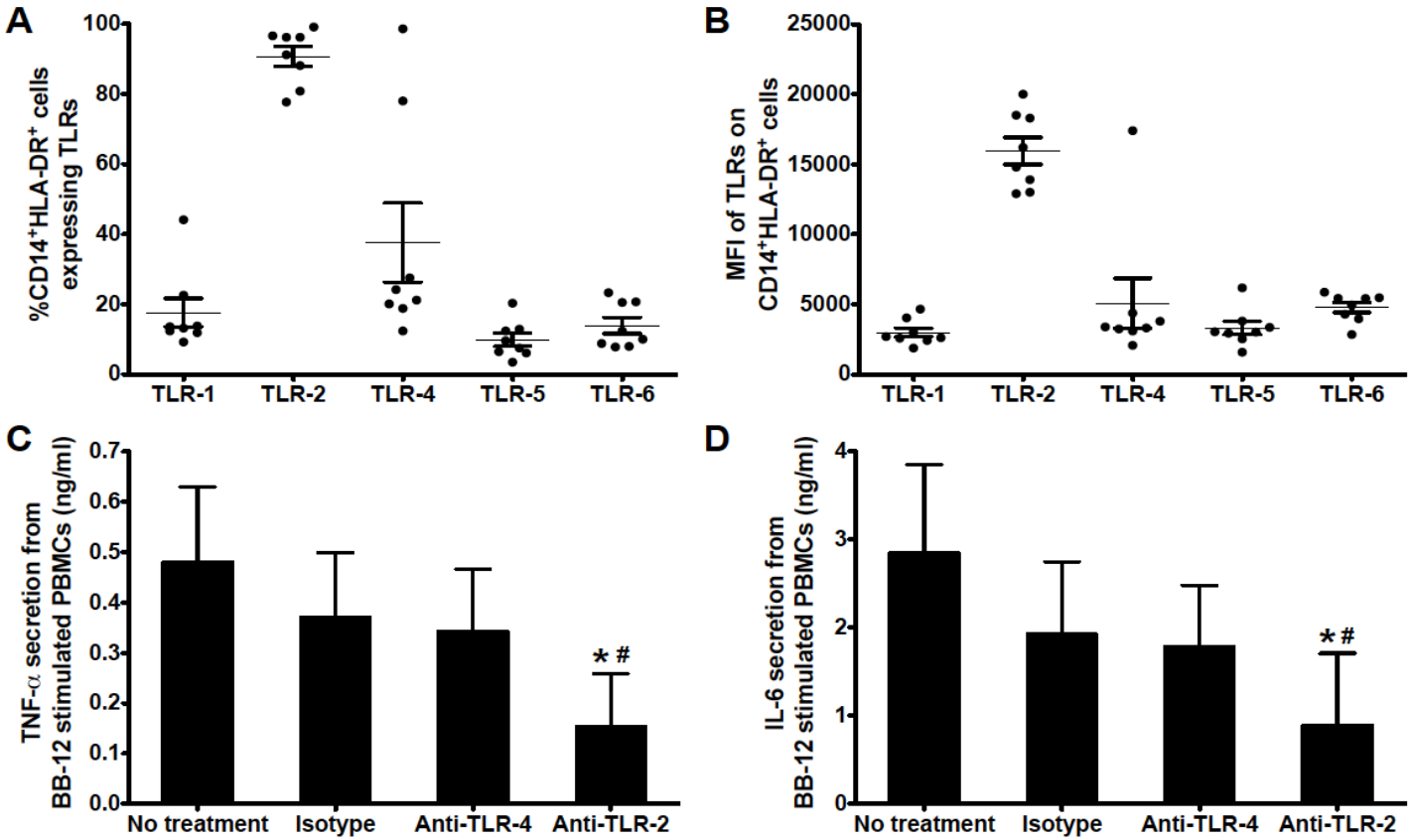


Figure 4.2. TLR expression pattern and functional characteristics.
Percentage of $CD14^+HLA\text{-}DR^+$ cells expressing TLR-1, -2, -4, -5, -6 in PBMCs **(A)**; and the MFI of TLRs on $CD14^+HLA\text{-}DR^+$ cells **(B)** was assessed by flow cytometry. Overall, the percentage of $CD14^+HLA\text{-}DR^+$ cells expressing different TLRs significantly differed [**(A)**; χ^2=23.92, p<0.0001]; and the MFI of TLRs on $CD14^+HLA\text{-}DR^+$ cells also differed [**(B)**; χ^2=26.30, p<0.0001]. PBMCs were treated with anti-TLR-2, anti-TLR-4, or isotype control antibodies (10 µg/mL) for 1 h, and stimulated with heat-inactivated BB-12 for 4 h. TNF-α **(C)** and IL-6 **(D)** secretion from stimulated PBMCs was measured by ELISA. Data are presented as mean ± SEM. Both TNF-α [**(C)**; χ^2=23.13, p<0.0001] and IL-6 [**(D)**; χ^2=17.67, p=0.0005] secretion were significantly lower from PBMCs treated with anti-TLR-2 antibody compared to cells treated with isotype control antibodies or no antibody treatment. Post hoc analyses were conducted using Dunnett's test. Asterisk indicates a significant difference from no treatment group (p<0.05). Pound sign indicates a significant difference from isotype control group (p<0.05).

4.4.3. Consumption of yogurt smoothies with BB-12 added post-fermentation altered the distribution and expression of TLR-2 of $CD14^+HLA\text{-}DR^+$ cells.

The percentage of $CD123^+CD11c^+HLA\text{-}DR^+$ dendritic cells and $CD14^+HLA\text{-}DR^+$ monocytes among total PBMCs and the expression and MFI of TLR-2 on $CD14^+HLA\text{-}DR^+$ monocytes was quantified by flow cytometry. Overall, the percentage of $CD123^+CD11c^+HLA\text{-}DR^+$ cells in peripheral blood differed between baseline and treatments, but when individual comparison were made, no individual treatment was significantly different from baseline (Fig.4.3A; $F_{(4,91)}=2.92$, $p=0.0254$). The percentage of $CD14^+HLA\text{-}DR^+$ cells in peripheral blood increased in participants following consumption of yogurt smoothies alone and with BB-12 added both pre- and post-fermentation compared to baseline (Fig. 4.3B; $F_{(4,97)}=2.69$, $p=0.0356$). However, the increase in the percentage of $CD14^+HLA\text{-}DR^+$ cells in peripheral blood following consumption of all yogurt-containing treatments occurred only in male participants (Supplemental Fig.4.1). The percentage of $CD14^+HLA\text{-}DR^+$ cells expressing TLR-2 was significantly lower in participants following all four treatments compared to baseline, and was lowest in participants following the consumption of the yogurt smoothies with BB-12 added post-fermentation among all treatment groups (Fig. 4.3C; $F_{(4,76)}=3.16$, $p=0.0187$). Similarly, the MFI of TLR-2 on $CD14^+HLA\text{-}DR^+$ cells was significantly lower in subjects following consumption of the yogurt smoothies with BB-12 added post-fermentation compared to baseline (Fig. 4.3D; $F_{(4,74)}=3.17$, $p=0.0186$).

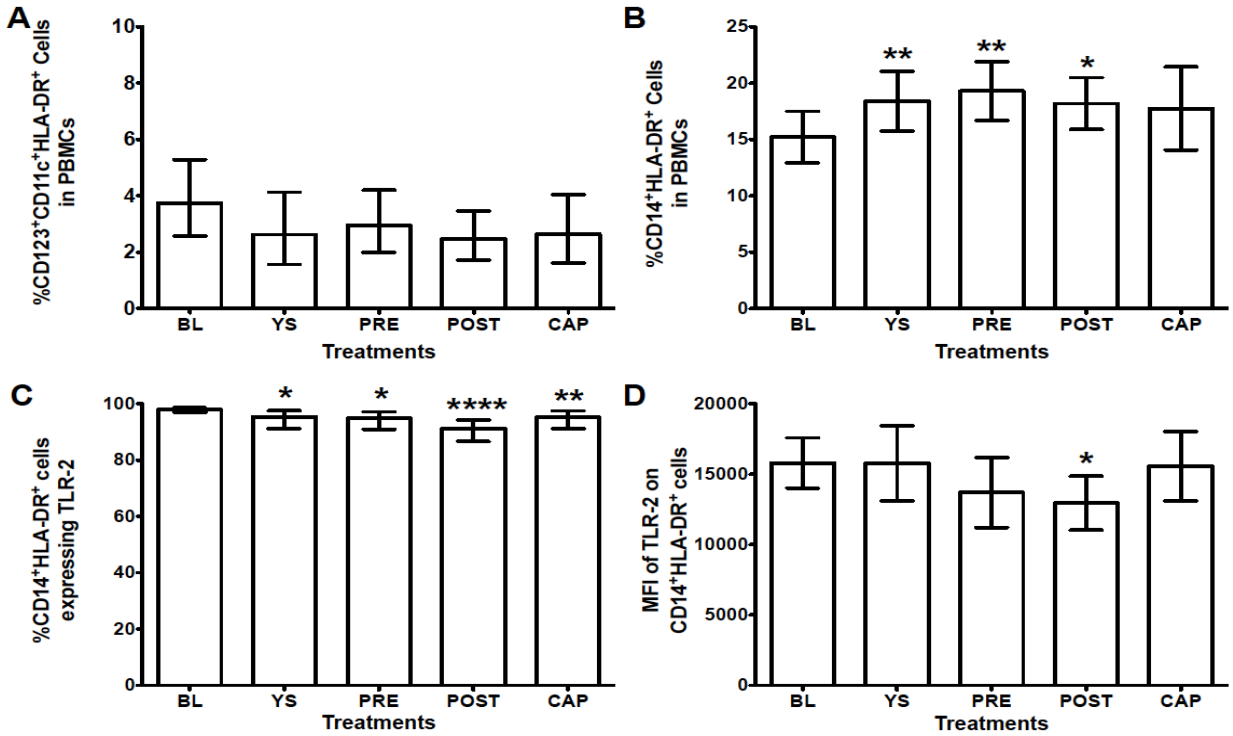


Figure 4.3. Distribution of myeloid cells in peripheral blood, and TLR-2 expression on $CD14^+HLA\text{-}DR^+$ cells in participants following each treatment.

The percentage of $CD123^+CD11c^+HLA\text{-}DR^+$ in peripheral blood was different between baseline and treatments, but post hoc analysis using Bonferonni's correction observed no difference following any treatment compared to baseline [**(A)**; $F_{(4,91)}=2.92$, $p=0.0254$; covariates in model: gender, PA, treatment*gender, treatment*PA]. The percentage of $CD14^+HLA\text{-}DR^+$ cells in peripheral blood was higher in all yogurt-containing treatments compared to baseline [**(B)**; $F_{(4,97)}=2.69$, $p=0.0356$; covariates in model: gender, treatment*gender]. The percentage of $CD14^+HLA\text{-}DR^+$ cells expressing TLR-2 was lower following all treatments, and was lowest in participants following the consumption of the yogurt smoothie with BB-12 added post-fermentation among all treatment groups [**(C)**; $F_{(4,76)}=3.16$, $p=0.0187$; covariates in model: PA, treatment*PA]. The MFI of TLR-2 on $CD14^+HLA\text{-}DR^+$ cells was also significantly lower in participants who consumed yogurt smoothie with BB-12 added post-fermentation compared to baseline [**(D)**; $F_{(4,74)}=3.17$, $p=0.0186$; covariates in model: gender, PA, vitamin C, and treatment*vitamin C]. Data are presented as least squares means ± 95% CI. Post hoc analyses were conducted using Bonferroni correction for multiple comparisons. Asterisk indicates a significant difference from baseline (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

4.4.4. Consumption of BB-12 delivered in a yogurt smoothie significantly reduced TNF-α secretion from BB-12 or LPS stimulated PBMCs.

TNF-α (Fig. 4.4A) and IL-6 (Fig. 4.4B) secretion from PBMCs stimulated with heat-inactivated BB-12 (10 µg/mL) were measured at baseline and following each treatment. PBMCs from participants who received yogurt smoothies with BB-12 added post-fermentation had significantly lower TNF-α (Fig. 4.4A; $F_{(4,92)}$=2.48, p=0.0490) but not IL-6 (Fig. 4.4B; $F_{(4,95)}$=0.44, p=0.78) secretion compared to baseline in response to *in vitro* BB-12 challenge.

To determine if BB-12 consumption could also alter TNF-α and IL-6 secretion following a robust inflammatory stimulus, PBMCs were stimulated with LPS (10 µg/mL). Participants who consumed yogurt smoothies with BB-12 added post fermentation had significantly lower TNF-α secretion from LPS-stimulated PBMCs compared to baseline (Fig. 4.4C; $F_{(4,88)}$=2.64, p=0.0387). Participants did not alter LPS-induced IL-6 secretion following any treatment (Fig. 4.4D; $F_{(4,100)}$=0.93, p=0.45).

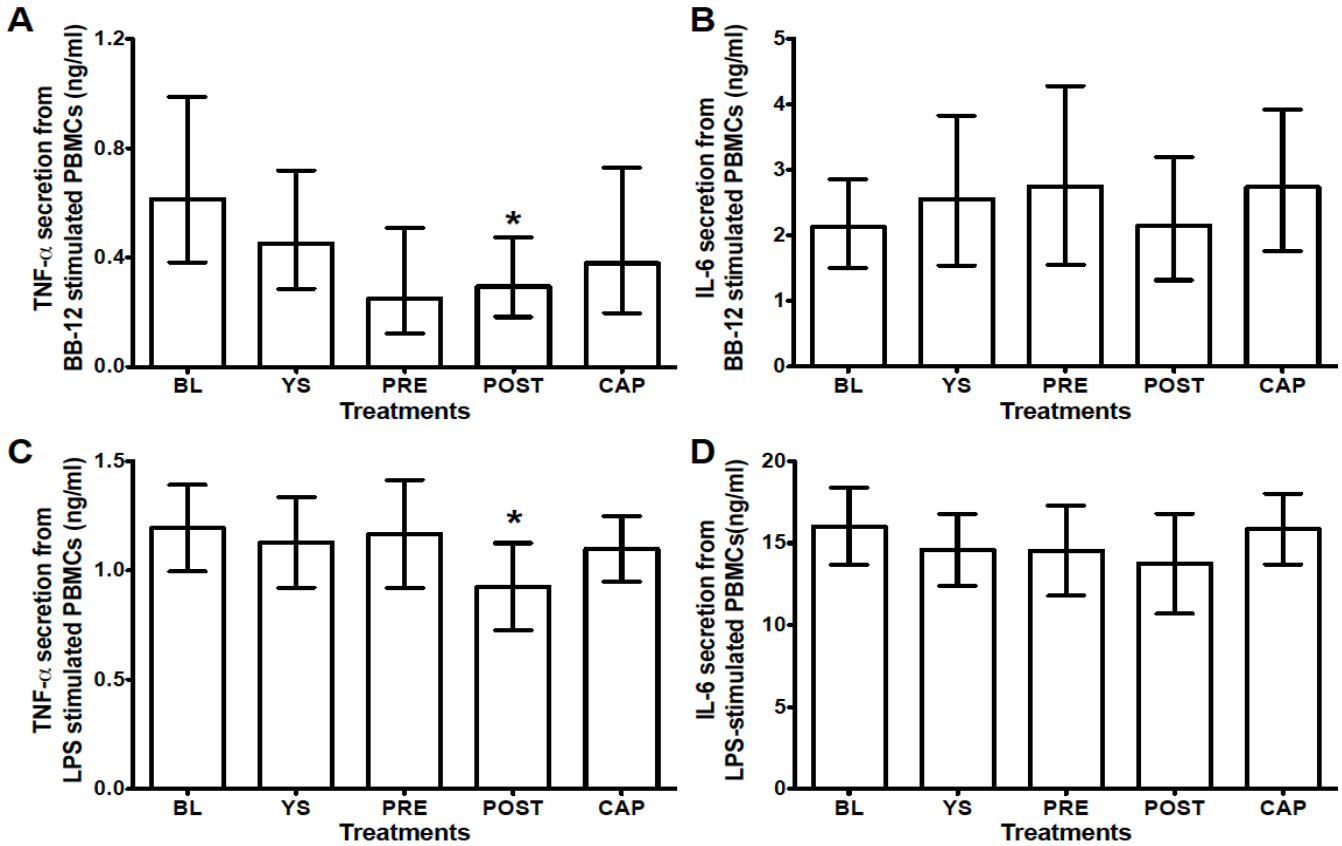


Figure 4.4. TNF-α and IL-6 secretion from PBMCs stimulated with heat-inactivated BB-12 or LPS following each treatment. Participants who consumed yogurt smoothie with BB-12 added post-fermentation had significantly lower TNF-α secretion from PBMCs stimulated with heat-inactivated BB-12 compared to baseline [**(A)**; $F_{(4,92)}$=2.48, p=0.0490; covariates in model: zinc and treatment*zinc]. TNF-α secretion from PBMCs stimulated with LPS was also significantly lower in participants who consumed yogurt smoothie with BB-12 added post-fermentation compared to baseline [**(C)**; $F_{(4,88)}$=2.64, p=0.0387; covariates in model: gender, total calories, treatment*gender, treatment*total calories]. There was no difference in IL-6 secretion from myeloid cells stimulated with BB-12 [**(B)**; $F_{(4,95)}$=0.44, p=0.78; covariates in model: gender, PA, and zinc] or LPS [**(D)**; $F_{(4,100)}$=0.93, p=0.45; covariates in model: gender] in participants following treatments compared to baseline. Data are presented as least squares means ± 95% CI. Post hoc analyses were conducted using Bonferroni correction for multiple comparisons. Asterisk indicates a significant difference from baseline (*p<0.05, ***P<0.001).

4.5. Discussion

To our knowledge, the current study provides the first documentation that consumption of *Bifidobacterium animalis* subsp. *lactis* (BB-12) delivered in a yogurt smoothie significantly reduced TNF-α secretion from PBMCs stimulated with heat-inactivated BB-12 or LPS in young healthy adults. These data suggest an anti-inflammatory effect of BB-12 consumption when administered in yogurt in healthy adults. Our study is also the first to report that BB-12 interacted with peripheral blood-derived myeloid cells via TLR-2. The reduction in cytokine secretion observed in PBMCs collected from participants following consumption of BB-12 delivered in a yogurt smoothie may be mediated via decreased TLR-2 expression on peripheral blood-derived monocytes. These findings demonstrate that daily consumption of BB-12 in a yogurt smoothie could impact immune responses in peripheral blood and specifically, that BB-12 consumption could reduce pro-inflammatory cytokine production by PBMCs. Lastly, results from this study demonstrate that the delivery matrix of BB-12 (e.g. yogurt vs. capsule) influenced the effect of BB-12 on the immune response, as BB-12 delivered in a yogurt smoothie significantly reduced inflammatory cytokine production from BB-12 or LPS stimulated PBMCs whereas BB-12 consumed as a capsule did not.

Similar to pathogenic bacteria, the recognition and interaction of probiotic bacteria with myeloid cells and intestinal epithelial cells (IECs) are mediated via TLRs. It is currently unknown which TLR(s) on human myeloid cells recognize and interact with BB-12. In the current study, we demonstrated that TLR-2 was the most abundant TLR on $CD14^{+}HLA\text{-}DR^{+}$ cells. Furthermore, our in *vitro* TLR-blocking experiments demonstrated that

BB-12-induced TNF-α and IL-6 secretion was mediated via the interaction of BB-12 with TLR-2 but not TLR-4. A previous study in rats demonstrated that IL-6 gene expression is undetectable in IECs from TLR2$^{-/-}$ germ-free rats fed with BB-12 compared to wild type controls, indicating that the cross-talk between BB-12 and rodent IECs is mediated via TLR-2 (179). Our results in human PBMCs demonstrate that BB-12 interacted with TLR-2, thus suggesting that BB-12 binds to TLR-2 in both rats and humans.

To gain an understanding of the role of probiotic consumption on the distribution and activation status of peripheral blood-derived myeloid cells, we quantified the percent of CD123^{+}CD11c^{+}HLA-DR^{+} and CD14^{+}HLA-DR^{+} cells in PBMCs following each treatment period. We found no significant treatment effect on the distribution of CD123^{+}CD11c^{+}HLA-DR^{+} DCs. However, the percentage of CD14^{+}HLA-DR^{+} cells was increased in participants following consumption of all yogurt-containing treatments (smoothies alone and smoothies with BB-12 added both pre- and post-fermentation) compared with baseline, suggesting that yogurt or a component of yogurt may increase the frequency of peripheral blood-derived monocytes. Interestingly, the increase in the percentage of CD14^{+}HLA-DR^{+} cells following consumption of all yogurt-containing treatments occurred only in male participants, indicating a gender difference in responsiveness of CD14^{+}HLA-DR^{+} cells to yogurt or yogurt components. In addition, we quantified the percentage of CD14^{+}HLA-DR^{+} cells that expressed TLR-2 and the MFI of TLR-2 on CD14^{+}HLA-DR^{+} cells following each treatment. Despite the increase in the percentage of CD14^{+}HLA-DR^{+} cells in peripheral blood in men following consumption of all the yogurt-containing treatments, we found a significant reduction in the

percentage of $CD14^+HLA\text{-}DR^+$ cells that expressed TLR-2 following all treatments among all participants. Furthermore, the greatest reduction in the percentage of TLR-2 expressing $CD14^+HLA\text{-}DR^+$ cells occurred following consumption of the yogurt smoothies with BB-12 added post-fermentation. A significant reduction in the surface expression of TLR-2 on $CD14^+HLA\text{-}DR^+$ cells was also observed in all participants following consumption of the yogurt smoothie with BB-12 added post-fermentation.

To determine if the reduction in the percent of TLR-2 expressing $CD14^+HLA\text{-}DR^+$ cells and the expression of TLR-2 on these cells following consumption of BB-12 added post-fermentation to the yogurt smoothies had any physiological consequences, we assessed TNF-α and IL-6 secretion from BB-12 stimulated PBMCs collected at the end of each treatment. We found that consumption of BB-12 added post-fermentation to a yogurt smoothie significantly reduced BB-12 induced *in vitro* TNF-α secretion from PBMCs. Next we determined if the consumption of BB-12 reduced pro-inflammatory cytokine secretion in response to a known, robust, inflammatory stimulus, LPS. We observed that consumption of the yogurt smoothies with BB-12 added post-fermentation significantly reduced *in vitro* TNF-α secretion from PBMCs in response to LPS stimulation compared to baseline, demonstrating the anti-inflammatory effect of BB-12 delivered in a yogurt smoothie. We did not observe a similar effect on BB-12 or LPS induced TNF-α secretion following consumption of BB-12 in capsule form, suggesting that the matrix in which BB-12 was administered influenced the effect of BB-12 on the immune response.

Yogurt is rich in various nutrients, including peptides, free amino acids, free fatty acids, folic acid, and calcium, which can support the growth, survival and activity of BB-12 until consumption (180). Following consumption, the acidic environment and buffering system provided by yogurt may confer a survival advantage to BB-12 while traveling through the human GI tract. The nutrient composition and buffering capacity of yogurt may also directly influence the efficacy of BB-12 by modifying the probiotic effect of BB-12 in the intestine (22, 125, 126). However, these effects may be dependent on the timing of the probiotic bacteria to the yogurt cultures, as we did not observe any effect of yogurt smoothies on innate immunity when BB-12 was added pre-fermentation. The yogurt fermentation process may reduce the efficacy and probiotic activity of BB-12 or may be altering the metabolites produced by BB-12, which may influence the immune response. Furthermore, our results suggest that the probiotic effect of BB-12 may be influenced by the timing of its addition during the yogurt manufacturing process and warrants further study.

TLR-2 signaling has been shown to induce the production of pro-inflammatory cytokines from macrophages (181). Our data suggest that decreased TLR-2 expression on peripheral blood derived monocytes may lessen the interaction between BB-12 and these cells, and contribute to the reduction in pro-inflammatory cytokines production in response to BB-12. Previous studies report that lactic acid producing probiotics (e.g. *Lactobacillus casei*) can inhibit the nuclear factor-κB (NF-κB) pathway via stabilizing the I-κB α subunit in human IECs (182-184). The NF-κB pathway is downstream of TLR-2 activation, and is crucial in initiating gene expression and production of

pro-inflammatory cytokines in human macrophages (181). Thus, additional studies are needed to investigate if the BB-12 induced down-regulation of TLR-2 expression on $CD14^{+}HLA\text{-}DR^{+}$ cells can lead to subsequent blockage of NF-κB activity and reduction in pro-inflammatory cytokines.

Additionally, the probiotic-induced reduction in TNF-α secretion from LPS-stimulated myeloid cells observed in our study may also be mediated via a probiotic-induced inhibition of NF-κB activity. Pre-culture of RAW264.7 macrophages with *Bifidobacterium adolescentis* decreases mRNA levels of TNF-α in response to LPS stimulation (185). The reduction in TNF-α mRNA is accompanied by a reduced phosphorylation *of* I-κB α subunit of NF-kB and increased gene expression of suppressor of cytokine signaling (SOCS) proteins 1 and 3, which are negative regulators of NF-κB (185). Similarly, Riedel and colleagues reported anti-inflammatory effects of 8 *Bifidobacterium* species (including *Bifidobacterium lactis NCC362*) on HT-29 cells by inhibition of NF-κB activation and subsequent down-regulation of TNF-α gene expression in response to LPS stimulation (186). Overall, the inhibition of NF-κB activity may explain the anti-inflammatory effect of BB-12 delivered in yogurt smoothie in our participants. Future studies are needed to investigate the effect of BB-12 on LPS-stimulated NF-κB pathways in human myeloid cells.

In addition to alteration in innate immunity, other *in vitro* studies also shed light on the anti-inflammatory effect of BB-12. In the intestinal lumen, BB-12 has been shown to interact with IECs and inhibit pathogenic microorganisms by various mechanisms. BB-12 can produce various metabolites, including organic acid (acetate and lactate) and hydrogen

peroxide, which can directly inhibit the growth of pathogenic microorganisms (9, 187). Pathogenic bacteria or their surface ligands (such as LPS) can enter intestinal epithelium by passing between IECs, disseminate throughout the blood stream from the gut, and induce systemic inflammation (188). Commane *et al.* demonstrated that BB-12 increases trans-epithelial electric resistance of Caco-2 cells, indicating the ability of BB-12 to enhance the strength of tight junction proteins between IECs (189). Improvement in tight junction strength between IECs prevents the pathogens or LPS from entering intestinal epithelium and spread in the blood stream, and reduces the incidence in systemic inflammation. BB-12 has also been shown to adhere to human intestinal mucin and IECs better than common pathogenic bacteria, including *Clostridium difficile, Listeria monocytogenes,* and thus may compete for adhesion sites with pathogenic bacteria and may prevent the adhesion and interactions of these pathogenic bacteria to IECs (190). Moreover, BB-12 has been shown to influence the composition of gut microbiota, which may interact with intestinal epithelial cells and/or immune cells and potentially result in the attenuation or prevention of gut and/or systemic inflammation. A clinical study reported that preterm infants fed with formula supplemented with 4.9×10^9 cfu of BB-12 per day for 21 days modifies their gut microbiota by increasing the number of *Bifidobacteria* and decreasing the number of harmful bacteria, including *Enterobacteriaceae* and Clostridia (191). Similarly, Ahmed et al found that consumption of as low as 6.5×10^7 cfu/day of *Bifidobacterium. lactis* NH019 for 4 weeks in older adults increases the cell count of *Bifidobacteria*, *Lactobacilli*, and *Enterococci*, and deceases the count of *Enterobacteriaceae* in feces (192). Similar changes in gut microbiota were

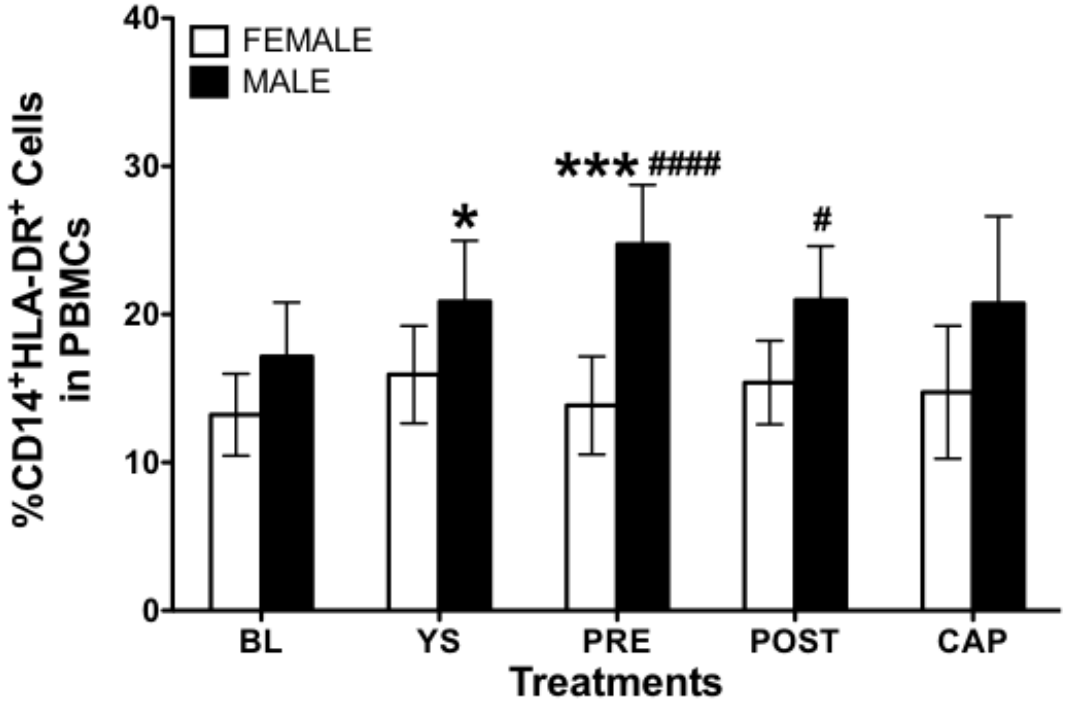
also observed in adult and aged rats fed with BB-12 in conjunction with *Lactobacillus delbrueckii* subsp. *rhamnosus* GG and the prebiotic inulin for 21 days (193). Combined, the anti-inflammatory effect of BB-12 delivered in yogurt smoothie may be linked the ability of BB-12 to alter microbial balance by increasing the ratio of beneficial to pathogenic microorganisms.

In summary, we demonstrated that consumption of BB-12 delivered in a yogurt smoothie significantly reduced pro-inflammatory cytokine (TNF-α) secretion from peripheral myeloid cells stimulated with heat-inactivated BB-12 or LPS in young healthy adults, suggesting an anti-inflammatory effect of BB-12. We also found that BB-12 interacted with peripheral myeloid cells via TLR-2, and the reduction in TNF-α secretion in participants who consumed yogurt smoothies containing BB-12 was related to the decreased TLR-2 expression on peripheral blood derived monocytes. These findings are not only the first to demonstrate anti-inflammatory properties of BB-12, but also indicate that the matrix of BB-12, and the timing of its addition to yogurt in relation to the fermentation process influenced the immunological effects of BB-12. Future studies will focus on understanding the mechanisms underlying the anti-inflammatory effect of BB-12 and determine if the current findings are applicable in clinical conditions of chronic inflammation.

Supplemental Statistical Analyses

An exponential decay model was constructed to determine if there was a carryover effect of BB-12 among different treatments. We assumed the probiotic treatment effect decayed exponentially *in vivo* after participants stopped receiving a given treatment. Following each treatment period, the total probiotic effect was calculated by adding the effect of treatment administered during the current treatment period ($\alpha_{current}$), to the residual effect of the treatment administered during the previous treatment period ($\alpha_{previous}$). The probiotic effect at the end of the first treatment period was only the effect of the treatment administered during period one. A dummy variable "decay proportion" (c) was created and defined as the percentage of residual effect of the treatment administered during the previous treatment period. The proportion of previous treatment that had already decayed during washout and current treatment period was 1-c. The equation describing the exponential decay model was $y=\beta_0+\beta_1*x_1+\beta_2*x_2+\beta_3*x_3+\beta_4*x_4+\alpha_{current}+c*\alpha_{previous}+\varepsilon$. In the model, y was the immune endpoint of our study; x_1-x_4 were covariates, including age, gender, BMI, and PA of participants; β_0 was the intercept of the regression line; β_1 to β_4 were slope of regression line for covariates x_1-x_4, respectively. The model was fitted with c equal to 0, 10%, 20%, and up to 90%, and R^2 was recorded with each choice of c. The percent change in R^2 when c = 10%, 20% up to 90% relative to the R^2 when c = 0 was calculated. If the percentage change between the two was less than 10%, we surmised that R^2 when c = 10%, 20% up to 90% was not different from R^2 when c = 0, indicating that the model when c=0 was the closest to the true model. When c=0, 100% of the previous treatment effect has decayed, and

we concluded that there were no carryover effect from the previous treatment. All immune endpoints in this study were tested for a carryover effect using the exponential decay model, and no carryover effect of treatments on any immune endpoint was observed.



Supplemental Figure 4.1. Distribution of $CD14^+HLA\text{-}DR^+$ cells in peripheral blood in male and female participants following each treatment.

The treatment effect on the percentage of $CD14^+HLA\text{-}DR^+$ cells differed by gender ($F_{(4,97)}=2.69$, $p_{treatment*gender}=0.0366$). The increase in the percentage of $CD14^+HLA\text{-}DR^+$ cells following consumption of all yogurt-containing treatments occurred only in male participants. Data are presented as least squares means ± 95% CI. Post hoc analyses were conducted using Bonferroni correction for multiple comparisons. Asterisk indicates a significant difference from male participants at baseline (*$p<0.05$, ***$p<0.001$). Pond indicates a significant difference from female participants at the same treatment period ($^{\#}p<0.05$, $^{\#\#\#\#}p<0.001$).

CHAPTER 5

Consumption of *Bifidobacterium animalis* subsp. *lactis* BB-12 impacts upper respiratory tract infection and the function of T cells and NK cells in young adults

* The results presented in this chapter are currently in preparation for submission to the *Journal of Nutrition*.

5.1. Abstract

Evidence suggests that consumption of *Bifidobacterium animalis* subsp. *Lactis* BB-12, alone or combined with other probiotics, can modulate immunity and reduce upper respiratory tract infection (URTI) in humans. However, no study has investigated the effect of consumption of BB-12 on T cell and NK cell function concurrently with the self-reported URTI questionnaire data to determine if BB-12 induces alterations in immune response that may underlie its impact URTI-related outcomes. There is also evidence that delivery matrix of BB-12 may influence its probiotic effect *in vivo*. The primary goal of the current study was to evaluate the effect of BB-12 at a dose of log 10 ± 0.5 CFUs/day on NK and T cell function in conjunction with self-reported cold or flu incidence and severity in a randomized, partially blinded, 4-period crossover, free-living study. A sub-aim was to determine if the immune response to BB-12 differed depending on the delivery matrix. Healthy adults (n=30) aged 18-40 years old were recruited, and received 4 treatments for 4 weeks in a random order: A) yogurt smoothies alone; smoothies with BB-12 added B) before or C) after yogurt fermentation, or D) BB-12 capsule. At baseline and after each treatment, peripheral blood mononuclear cells (PBMCs) were isolated, and functional and phenotypic marker expression was assessed. Incidence and severity of cold or flu infection in the past month was assessed using established self-reported URTI questionnaire. Participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form had elevated IL-2 secretion and NK cell cytotoxicity, concurrently with fewer number of days with URTI. However, consumption of yogurt smoothies

with BB-12 added post-fermentation did not change T cell and NK cell function; and did not alter severity of URTI. These findings demonstrate that timing of BB-12 addition to yogurt smoothies in relation to the fermentation process influenced the impact of BB-12 on the activity of T cells and NK cells and cold or flu severity in young healthy adults.

5.2. Introduction

The consumption of probiotics is associated with numerous health benefits, including modulating immune function, and attenuating the risk and/or severity of infections. *Lactobacillus* and *Bifidobacterium* are lactic acid producing probiotic strains that have been commonly used and extensively studied for their impact on immune parameters and infectious outcomes (8). Large strain and species differences exist in the capacity of probiotic bacteria to impact the immune response and infection-related health outcomes (8). Moreover, variability due to dose of probiotics, duration of supplementation, characteristics of the study population, and delivery matrix of probiotics have been reported (8). Thus, well-designed clinical trials using a single strain of probiotic bacteria on immune and infectious disease outcomes are needed.

Bifidobacterium animalis subsp. *lactis* BB-12 is a widely used probiotic species of *Bifidobacterium*, and is favored over many other probiotic strains among food manufacturers due to numerous production advantages and reported health benefits (9-13). BB-12, used alone or in combination with other probiotic bacteria or ingredients, has been shown to reduce upper respiratory tract infections (URTI) in children and adults (117-120). Oral consumption of powder containing BB-12 and *Lactobacillus Rhamnosus* GG (LGG) for 12 weeks significantly shortens the self-reported URTI duration by two days and reduces the URTI severity score by 34% in healthy college students (117). Another clinical trial observed that infants fed BB-12 and LGG supplemented formula for 12 months have a lower incidence of acute infections and fewer recurrent respiratory infections in the first year of life in comparison to infants receiving placebo (118). Additionally, consumption of

yogurt supplemented with BB-12 and the prebiotic, inulin, for 16 weeks significantly reduces the number of days with URTI symptoms in healthy toddlers (119). Similarly, infants that received BB-12 using a slow-release tablet in a pacifier from 2 to 8 months of age have fewer respiratory infections compared to infants receiving placebo (120). Overall, these data demonstrated that BB-12 supplementation may reduce susceptibility to URTI. However, the mechanisms underlying the beneficial effect of BB-12 on URTI risk and severity is unknown.

The immune response of the host is an important component of the pathogenesis of URTI (121). The cellular immune responses to viruses that induce URTI are mediated by T cells and NK cells, which limit the severity of infection by exerting cytotoxic activity or inducing apoptosis to viral-infected cells (122-124). Several studies have investigated the effect of BB-12 consumption in combination with other lactic acid producing bacteria on adaptive immune responses in humans, in an effort to link probiotic use to immune changes that may underlie the relationship between probiotic consumption and URTI risk and/or severity (14, 16). Due to strain and species differences in the immunomodulatory capacity of probiotics, the results of these studies vary based on which probiotic bacteria were given in conjunction with BB-12 (14, 16). However, studies employing BB-12 alone as the probiotic intervention also yield mixed results, suggesting that dose, duration or mode of delivery may impact immune responses to BB-12 consumption (15, 20). No study to date has investigated the effect of consumption of BB-12 alone on T cell and NK cell function concurrently with self-reported URTI questionnaire data to determine if BB-12 induced

alteration in immune responses are associated with its impact on URTI. Therefore, additional studies are needed to clarify the role of BB-12 on NK and T cell function and URTI-related outcomes in humans.

BB-12 is primarily consumed in dairy product, especially yogurt; however, it can also be consumed in capsule form as a dietary supplement (22, 23). While the use of multiple delivery matrices expands the options for BB-12 consumption, it is unknown if the delivery matrix influences the immunomodulatory effect of BB-12. Furthermore, BB-12 can be added into yogurt either prior to or following the yogurt fermentation process, which varies among manufacturers. Little is known about the timing of the addition of BB-12 to yogurt on the efficacy and functionality of BB-12, and if this alters immune responses and URTI risk and severity.

Thus, the primary goal of this study was to evaluate the effect of BB-12 consumption on NK and T cell distribution and function concurrently with self-reported cold or flu incidence and severity in healthy adults. A secondary goal of this study was to determine if the delivery matrix (yogurt smoothies vs capsule), and timing of the addition of BB-12 to the yogurt smoothies (pre- or post-fermentation) impacted the immunological responses and infection-related outcomes to BB-12 in healthy adults.

5.3. Materials and methods

5.3.1. Participants

Healthy subjects (n=30, 19 women and 11 men) 18-40 years of age were recruited for the study. Exclusion criteria included: BMI greater than 40kg/m^2, smoking and/or use of other tobacco products, blood pressure ≥ 140/90 mm Hg, use of blood pressure or cholesterol lowering medications,

history of myocardial infarction, stroke, diabetes mellitus, liver disease, kidney disease and thyroid disease (unless controlled by medication and blood results within the previous six months were provided), lactation, pregnancy or desire to become pregnant during the study, clinical diagnosis of IBD (e.g. Crohn's Disease or ulcerative colitis), excessive alcohol consumption (>14 drinks per week), vegetarianism, lactose intolerance, chronic use of anti-inflammatory medications (unless able to discontinue), antibiotic use, swallowing disorders or dysphasia to food or pills, and refusal to give blood or plasma during the study. A complete blood count and standard biochemistry panel was obtained at screening to rule out the presence of illness (autoimmune disease, cancer, and immunodeficiency). Blood pressure was measured according to Joint National Committee 7 guidelines (137).

5.3.2. Recruitment and screening

Recruitment strategy used in the study is shown in Figure 5.1. Participants were recruited through advertisements in the local newspaper and university e-mail lists; 203 individuals called or emailed to indicate interest in participating in the study. They were given information about the study, and if interested, were contacted and screened using a series of medical and lifestyle questions by telephone. Of those who were contacted and screened through telephone interview, 136 qualified participants were scheduled for a clinic visit at the Penn State Clinical Research Center (CRC). After written informed consent was provided, participants had their height, weight, waist circumference and blood pressure measured, followed by a fasting blood draw for a complete blood count and health profile (liver and kidney function, and glucose metabolism). BMI was calculated according to

body weight and height measured. From the participants who were screened, 36 were eligible to participant in the study. To ensure that there was no effect of treatment order on outcomes, eligible participants (n=36) were randomized to treatment sequences. The randomization scheme was generated by using the website Randomization.com, http://www.randomization.com. However, two participants withdrew at the baseline visit due to a schedule conflict; four withdrew due to personal reasons. Thirty participants received treatment interventions; however, not all subjects finished all interventions. Two participants were excluded prior to the third and fourth treatment periods, respectively due to pregnancy; one participant withdrew from the study prior to the fourth treatment period due to diagnosis of irritable bowel syndrome; one participant withdrew from the study prior to the second treatment period due to a schedule conflict; one participant withdrew from the study prior to the second treatment period due to discomfort after treatment; and two participants withdrew from the study prior to the first and second treatment periods, respectively due to other personal reasons. All the experiments in this study were performed with approval of the Institutional Review Board of the Pennsylvania State University-University Park campus (University Park, PA).

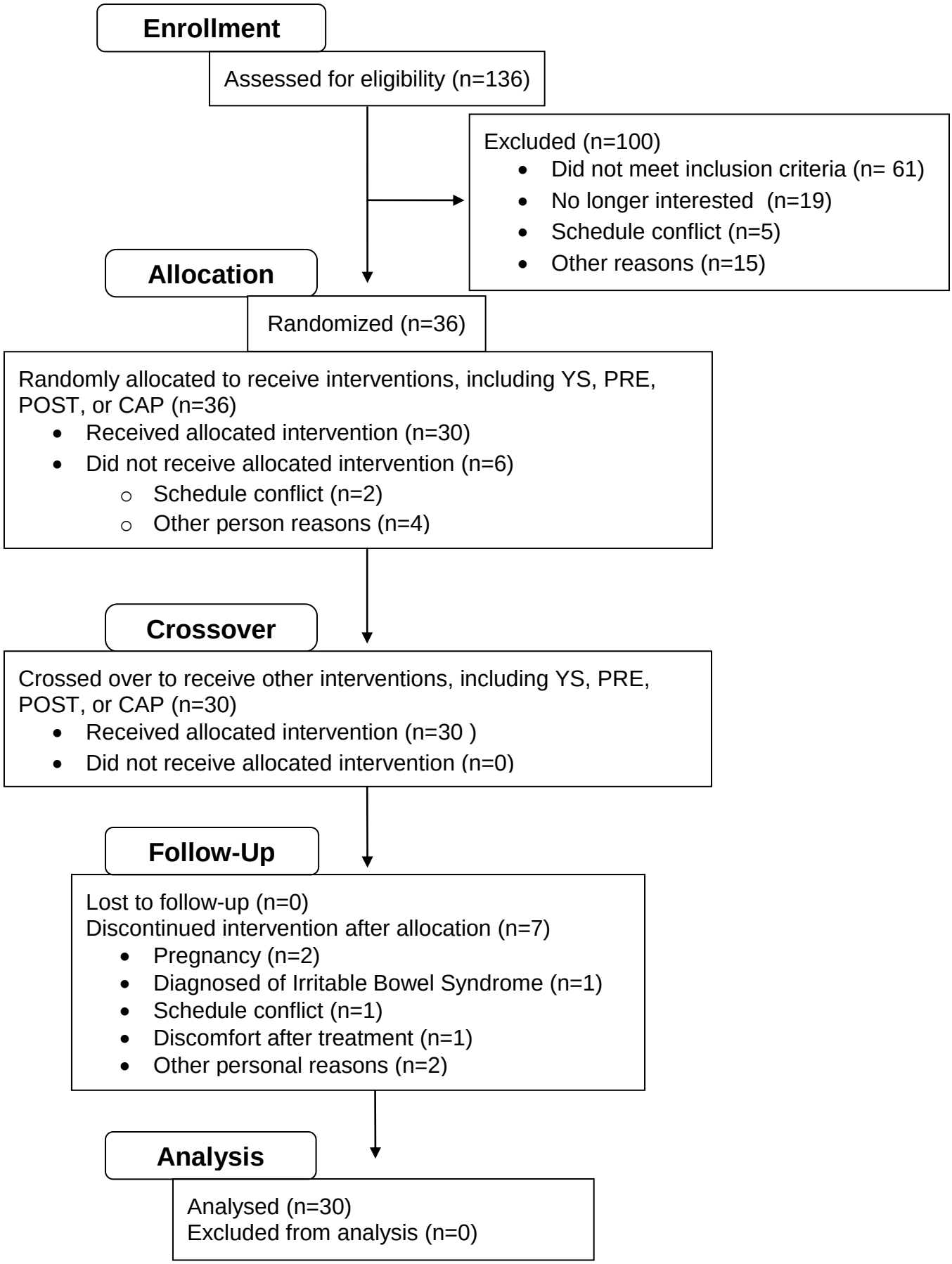


Figure 5.1. Recruitment strategy used in the study.

5.3.3. Design and intervention

The study was a randomized, partially-blinded, 4-period crossover free-living study. Prior to the first treatment, participants completed an anthropometric assessment (age, gender, BMI, waist circumference), biochemical measurements (fasting serum glucose, insulin, and hs-CRP), a 3-day dietary recall record, a physical activity questionnaire (self-reported IPAQ), an URTI questionnaire and an immune endpoint assessment for use as baseline values. Participants then began the intervention phase, and received 4 treatments according to their randomized sequence. These treatments included: (A) yogurt smoothies (YS, without added BB-12); (B) yogurt smoothies with BB-12 (Chr. Hansen, Milwaukee, WI) added pre-fermentation (PRE); (C) yogurt smoothies with BB-12 added post-fermentation (POST); and (D) one capsule containing BB-12 (CAP). Each treatment period lasted 4 weeks, with a two-week washout period scheduled between treatment periods.

During the yogurt smoothies treatment phases, participants consumed one 8-oz (240 g) serving of yogurt per day. Each smoothie delivered log 10 ± 0.5 CFUs/day. The yogurt smoothies were developed at Pennsylvania State University, and manufactured based on methods described in a previous study (176). Briefly, yogurt mix (milk and dry ingredients) were inoculated with the yogurt starter culture YF-L702 (Chr. Hansen), containing active cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Inoculated yogurt mix was divided into two portions: one portion was further inoculated with BB-12 (PRE); another portion (non-BB-12) was incubated directly. Both portions were allowed to ferment to a pH of 4.6, and a slurry of

strawberry, pectin, corn syrup solids, sugar and water was added and blended into each portion until uniform. The non-BB-12 containing portion of smoothie was divided into two groups. One group was inoculated with BB-12 (POST) while the other remained a BB-12 free product (YS). The smoothie yogurt was then homogenized to produce a drinkable yogurt. The three products were identical in macronutrient composition and differed only by the presence of BB-12 and the timing of BB-12 addition. Viable counts of *B. lactis* were determined by pour-plating on MRS (de Man, Rogosa, and Sharpe)-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate) agar (177) with growth under anaerobic conditions at 37°C for 72 hours.

During the treatment phase involving capsule consumption, participants ingested one capsule per day, which delivered log 10 ± 0.5 CFUs/day. During each 4-week treatment phase, participants were required to avoid consuming any other food or pharmaceutical product containing probiotic bacteria such as commercial yogurt, smoothies, and probiotic bacteria capsules. Participants were asked not to change their habitual diets during the course of the study and to maintain their body weight.

5.3.4. Sample size estimation

The primary immune endpoint used to estimate sample size for the study in Chapter 5 was self-reported total number of days with cold or flu symptoms from our pilot study and published clinical trials assessing the effect of BB-12 consumption on cold or flu severity in healthy adults (117). Using the sample size calculation for crossover study, it was determined that the estimated sample size required to identify a mean difference of 60% in

total number of days with cold or flu symptoms with α = 0.05 and β = 80% was 39. The dropout rate in our pilot study was 15%. If we estimate a dropout rate of 15%, the final sample size was 52. However, only 36 participants were recruited in the study, and 30 participants completed at least one allocated interventions. Thus, the assessment of the effect of BB-12 consumption on total number of days with cold or flu symptoms was underpowered.

5.3.5. Diet assessment

Dietary intake of the participants was obtained via 24-hour dietary recalls for 3 days, including one weekend day. Briefly, participants were asked to recall their intake of food and beverages during breakfast, lunch, dinner, and snacks in three days according to detailed instructions provided by trained staff. Portion size of each food item was also provided. Daily intake of total calories, macronutrients, vitamins, and minerals was analyzed based on the recorded food intake of participants using Food Processor SQL software (ESHA Research, Salem, OR).

5.3.6. Physical activity assessment

Physical activity level of participants was evaluated using the International Physical Activity Questionnaires (IPAQ) questionnaire as previously reported (138). Briefly, the participants recorded the activity they performed during each of the 96 periods of 15 min over a 24-h period for 3 days, including one weekend day. The activities were categorized from 1 to 9 depending on their intensity as previously described (138). Total daily intensity of physical activity was calculated by averaging the approximate metabolic equivalent of tasks (METs) of activities performed of the categories 3 to 9 over a 24 hour period (96 periods of 15 min).

5.3.7. Upper respiratory tract infection questionnaire

Participants completed a self-administered URTI questionnaire, which was developed from established, frequently used instruments (146) on the incidence (whether or not have experienced cold or flu episodes, with symptoms including a sore throat, runny or stuffy nose, coughing sneezing, fever, headache, general aches and pains, fatigue and discomfort); severity (total number of days with cold or flu symptoms) of cold or flu symptoms; number of days in bed and/or absent from work due to cold or flu; and sick score (ranging from 1-10 from not very severe to severe) due to cold or flu infection over the past month. Participants were instructed to recall the occurrence of cold or flu in the last month, and advised on questionnaire completion.

5.3.8. Blood sample collection and immunological assays

Blood (50 ml) was collected in sterile EDTA (K2)-coated blood tubes (BD Biosciences, San Jose, CA) by trained staff at the CRC on the University Park campus.

Serum markers

Insulin was measured by radioimmunoassay (Quest Diagnostics). Glucose was determined by an immobilized enzyme biosensor using the YSI 2300 STAT Plus Glucose and Lactate Analyzer (Yellow Springs Instruments). Serum high-sensitivity C-reactive protein (hs-CRP) was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

Isolation of immune cells

Human blood was diluted 1:2 with phosphate buffer saline (PBS) (Mediatech, Manassas, VA), gently layered on top of lymphocyte separation

media (LSM) (Corning, Manassas, VA), and centrifuged at 1600 rpm with low speed and no brake for 30 mins at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected at the plasma/LSM interface; washed twice with complete media RPMI 1640 (Mediatech) containing 10 mM HEPES (Mediatech), 10% heat-inactivated fetal bovine serum (Gemini, West Sacramento, CA), 2 mM L-glutamine (Mediatech), 0.1 mM nonessential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 100U/ml Penicillin/Streptomycin (Mediatech), and 55 µM 2-mercaptoethanol (Life Technologies, Grand Island, NY) at room temperature; and counted for use in functional and phenotypic analyses.

Lymphocyte proliferation assay

PBMCs (2 X 10^6/ml) were incubated with 0 or 1µg/ml plate-bound purified mouse anti-human CD3 antibody (Life Technologies) in flat-bottomed 96-well plates. After 54 hours in culture, the cells were pulsed with [^{3}H] thymidine (1 µCi/well; Perkin Elmer, Waltham, MA) and harvested 18 hours later. Following incubation, cells were harvested onto glass fiber filter mats (Perkin Elmer) via a MicroBeta FilterMate-96 Harvester (Perkin Elmer). Incorporated radioactivity was measured by liquid scintillation counting on a 2450 MicroBeta plate counter (Perkin-Elmer). Each assay was performed in triplicate. The proliferative response was expressed as a stimulation index (SI) calculated by dividing the mean cpm (counts per minute) of anti-CD3 stimulated T cells by the mean cpm of unstimulated (media only) cells.

Cytokine secretion assays

PBMCs (2 X 10^6/ml) were stimulated with 1µg/ml plate-bound purified mouse anti-human CD3 antibody (Life Technologies) in flat-bottomed 96-well

plates. Supernatants from anti-CD3 plates were harvested and frozen after 48 hours. IFN-γ, TNF-α and IL-2 secretion from anti-CD3 stimulated PBMCs were measured using the Human ELISA MAX™ Deluxe (Biolegend, San Diego, CA) as per manufacturer instructions. Each assay was performed in triplicate.

Flow cytometric analyses

PBMCs were washed twice in PBS at 4°C. Fc receptors on PBMCs were blocked by incubation with 1 µg purified mouse anti-human CD16 (Biolegend) per 1×10^6 cells for 15 min at 4°C. PBMCs were stained with fluorescence-labeled antibodies (1µg/1×10^6 cells) to the following cell surface markers: CD3, CD4, CD8, CD69, CD25, CD56, CD14, CD123, CD11c, HLA-DR. Antibody isotype controls included: mouse IgG_{2a} and mouse IgM. All antibodies were purchased from BD Biosciences. Following incubation with the conjugated antibodies for 30 min at 4° C, cells were washed twice in PBS and then fixed in cytofix (BD Biosciences) for flow cytometric analyses. Lymphoid and myeloid cells were gated on forward vs. side scatter and a total of 25,000 events were analyzed on a FC500 Benchtop Cytometer (Beckman Coulter, Pasadena, CA). Flow cytometric analyses were plotted and analyzed using Flowjo 7.6 (Tree Star, Ashland, OR).

Cytotoxicity assays

Natural killer cell cytotoxicity (NKCC) assay was assessed in standard 4-hour chromium release assay as previously described (194), using 200:1 effector to target (E:T) ratio. NKCC experiments were performed using PBMCs as effector cells and ^{51}Cr-labeled K562 as target cells. Adjusted NKCC measured NKCC on a per cell basis, and was calculated based on the

percentage of NK cells ($CD56^{+}CD3^{-}$) in the peripheral blood as quantified via flow cytometry. All experiments were performed in triplicate.

5.3.9. Gene expression

Gene expression of FoxP3, TGF-β, IL-10, and IL-12 in PBMCs was assessed in a subset of participants (n=13). Total RNA of PBMCs was extracted and genomic DNA contamination was removed by using RNeasy Mini Kit (Qiagen, Valencia, CA) as per manufacturer instructions. Total RNA was quantified by using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), and reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies). Real-time qPCR was performed by using Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies). Quantification of gene expression was performed in 96-well plates by adding 20 ng of cDNA with 2X TaqMan PCR Master Mix (Life Technologies) and TaqMan Gene Expression primers (Life Technologies). Supplemental Table 5.1 describes the 5 target genes and primers. The amplification protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Cycle threshold (Ct) values of the genes and endogenous control gene (GAPDH) were used with the ΔΔCt method to determine relative levels of gene expression. Relative quantification or fold change in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{target} - Ct_{endogenous}) - (Ct_{reference} - Ct_{endogenous})$, with GAPDH as endogenous control and baseline value as reference control.

5.3.10. Statistical analyses

Statistical analyses were performed using SAS (Statistical Analysis System, Version 9.4, Cary, NC). Differences in the fold change in gene

expression of FOXP3, TGF-β, IL-10, and IL-12 among treatment were determined using the Friedman test, followed by a Dunnett test for multiple comparisons where appropriate.

All immune endpoints in this study were tested for a carryover effect using an exponential decay model (details of model explained in Supplemental Data). No carryover effect of treatments on any immune endpoint was observed.

The mixed models procedure (PROC MIXED) was used to test the effects of treatment on immune outcomes, including percent change from baseline following each treatment period and raw values at baseline and following treatments. Outcomes were modeled as repeated measures with unstructured covariance. Age, gender, BMI, PA, dietary factors and their interactions with treatment (e.g. age*treatment, gender*treatment, etc.) were included as covariates. Dietary factors (e.g. dietary intake of total calories, vitamin C, D, E, iron, zinc, and n-3 PUFA) were selected based on their individual contributions to the variation in immune cell distribution, phenotypic marker expression and function of T cells and NK cells at baseline (178). The covariates in models varied by different immune outcomes, and were selected based on manual backward selection procedure. Briefly, models were fitted with immune outcomes as dependent variables, and treatment effect and all covariates as independent variables. Covariates were removed from the model one by one based on the significance (assessed by p value of the corresponding covariate in models) of their contribution to the models. Covariates included in the final models contributed significantly to the model. If the interaction was significant but the main effect was not, both the main

effect and interaction were included in the model. Participant was designated as a random effect and the treatment effect and covariates were fixed effects. For all outcomes, model selection was based on optimizing fit statistics (evaluated as lowest Bayesian information criterion) and α was set at 0.05 for all tests. Bonferroni correction was used for post hoc analyses. Means are reported as least squares means ± 95% CI.

Logistic regression (PROC LOGISTIC) was used to test the effects of treatment on self-reported cold or flu incidence following each treatment period. Poisson regression (PROC GENMOD) was used to test the effects of treatment on self-reported number of cold or flu episodes, total number of days with cold or flu symptoms, number of days in bed and/or absent from work due to cold or flu infection, and sick score due to cold or flu infection following each treatment period. Outcomes were modeled as repeated measures. Covariates and selection procedure of covariates were the same as described above. Means are reported as least squares means ± 95% CI. Statistical significance was accepted at the $p \leq 0.05$ level. Graphs were plotted using GraphPad Prism 5 (La Jolla, CA).

5.4. Results

5.4.1. Participant characteristics.

Anthropometric measurements, physical activity intensity and dietary intake of participants are presented in Table 5.1. All 30 participants (19 females and 11 males) completed the study. The participants were healthy, young adults (mean age of 28.0±1.2 years). The average BMI was 24.2±0.5kg/m^2; 20 (66.7%) participants were normal weight, 9 (30.0%) were overweight, and 1 (3.3%) was obese. Their average waist circumference was

85.1±1.4 cm. Their blood pressure was normal and fasting blood glucose, insulin, and CRP levels were within the normal range (data not shown). Physical activity (PA) was assessed from self-reported IPAQ responses. The median daily physical activity intensity (based on self-reported responses) was estimated to be 3.0 METs (range 2.4-5.2 METs). The average daily total calorie intake of participants calculated from 3-day dietary recall records was estimated to be 2350±145 kcal. The daily intake of macronutrients, vitamins, minerals and n-3 PUFA are reported in Table 5.1.

Table 5.1. Demographic characteristics of participants at baseline [1].

Characteristics	Values (n=30)
Age (yr)	28.0±1.2
Male, n (%)	11 (36.7%)
Body mass index (kg/m^2)	24.2±0.5
≤24.9	20 (66.7%)
25.0 -29.9	9 (30.0%)
≥30	1 (3.3%)
Waist Circumference (cm)	85.1±1.4
Physical activity (METs)[2]	3.0 (2.4-5.2)
Dietary intake[2] of	
Total calories (kcal/d)	2350±145
Carbohydrate (g/d)	291.1±19.5
Protein (g/d)	91.7±6.8
Fat (g/d)	92.1±6.7
Vitamin C (mg/d)	62.3±11.0
Vitamin D (IU/d)	112.6±34.4
Vitamin E (mg/d)	2.8±0.6
Iron (mg/d)	14.9±1.4
Selenium (µg/d)	37.4±4.7
Zinc (mg/d)	6.2±0.6
n-3 PUFA (g/d)	0.5±0.1

[1] Values are presented as mean ± SEM or n (%) or median (range).
[2] Physical activity and dietary intake were assessed from self-reported responses to IPAQ and 3-day dietary recall records, respectively.

5.4.2. Consumption of BB-12 did not alter the distribution of T cells and subsets.

The percentage of total ($CD3^+$), helper ($CD3^+CD4^+$) and cytotoxic ($CD3^+CD8^+$) T cells in peripheral blood at baseline and following each treatment was quantified by flow cytometry. None of the treatments altered the percentage of total ($CD3^+$; $F_{(4,101)}=0.31$, $p=0.87$), helper ($CD3^+CD4^+$; $F_{(4,101)}=0.21$, $p=0.93$) and cytotoxic ($CD3^+CD8^+$; $F_{(4,101)}=0.53$, $p=0.71$) T cells in peripheral blood in comparison to baseline (data not shown). The ratio of $CD4^+$:$CD8^+$ (helper: cytotoxic) T cells was also not different between baseline and treatments ($F_{(4,99)}=0.76$, $p=0.55$; data not shown). Expression of activation markers CD69 and CD25 on total $CD3^+$ T cells, $CD4^+$ and $CD8^+$, subsets at baseline and following each treatment was also assessed by flow cytometry. No consistent treatment effects were observed on activation marker expression (data not shown).

5.4.3. T cell proliferation and cytokine secretion were not altered in participants who consumed yogurt smoothies with BB-12 added post-fermentation

The percent change from baseline in T cell proliferation (Fig. 5.2A); and secretion of IFN-γ (Fig. 5.2B), TNF-α (Fig. 5.2C), and IL-2 (Fig. 5.2D) in response to 1 μg/ml anti-CD3 antibody stimulation following each treatment was measured to determine if BB-12 consumption alters the effector function of T cells. The percent change in T cell proliferation was not different among treatments (Fig. 5.2A; $F_{(3,72)}=2.10$, $p=0.11$). The percent change from baseline in anti-CD3 induced IFN-γ secretion (Fig. 5.2B; $F_{(3,72)}=0.39$, $p=0.76$) and TNF-α secretion from T cells did not differ among treatments (Fig. 5.2C;

$F_{(3,72)}$=1.89, p=0.14). However, subjects who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation and BB-12 in capsule form all had elevated TNF-α secretion from T cells in response to anti-CD3 stimulation. However, participants who consumed yogurt smoothies with BB-12 added post-fermentation had reduced TNF-α secretion. The percent change from baseline in IL-2 secretion from anti-CD3 stimulated T cells was marginally different among treatments (Fig. 5.2D; $F_{(3,72)}$=2.50, p=0.0665). Similar to the pattern of TNF-α secretion in response to treatment, participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation and BB-12 in capsule form all had increased IL-2 secretion; whereas participants who consumed yogurt smoothies with BB-12 added post-fermentation did not increase IL-2 secretion from anti-CD3 stimulated T cells.

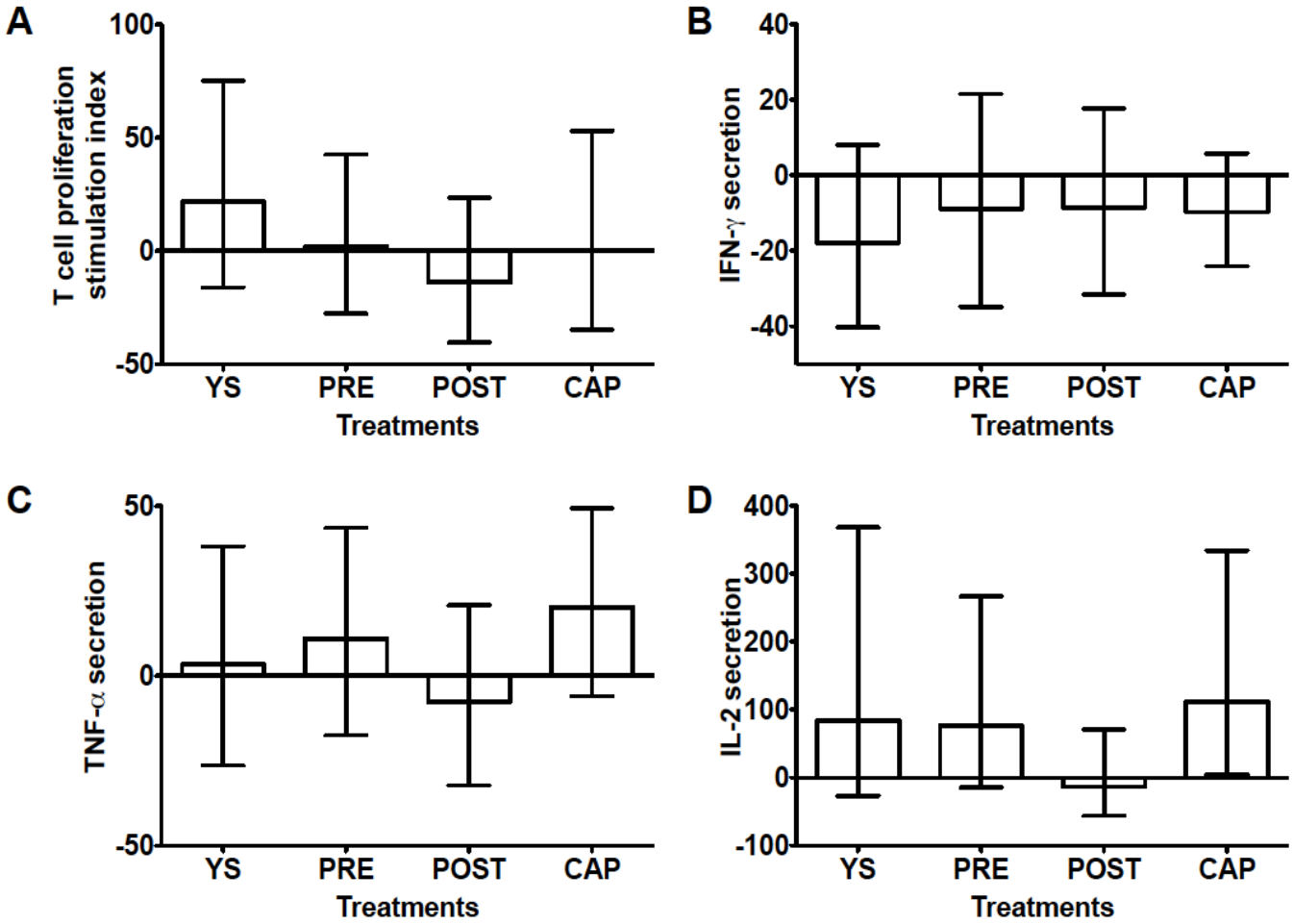


Figure 5.2. T cell proliferation and cytokine secretion in participants following each treatment.
The percent change from baseline in anti-CD3 induced T cell proliferation [**(A)**; $F_{(3,72)}$=2.10, p=0.11], and IFN-γ [**(B)**; $F_{(3,72)}$=0.39, p=0.76] and TNF-α [**(C)**; $F_{(3,72)}$=1.89, p=0.14] secretion did not differ among treatments in participants. The percent change from baseline in IL-2 secretion from anti-CD3 stimulated T cells was marginally different among treatments [**(D)**; $F_{(3,72)}$=2.50, p=0.0665]. Data are presented as least squares means ± 95% CI.

5.4.4. Distribution of NK cells in peripheral blood and NKCC varied by treatment group.

The distribution of NK cells ($CD56^{+}CD3^{-}$) in peripheral blood (percent change from baseline; Fig. 5.3A) and NKCC (Fig. 5.3B) were measured following each treatment. Participants who consumed yogurt smoothies with BB-12 added pre- and post-fermentation had a greater reduction in the percentage of NK cells in peripheral blood than subjects who consumed yogurt alone, or BB-12 in capsule form (Fig. 5.3A; $F_{(3,64)}=4.82$, $p=0.0044$). The change in NKCC was marginally different among treatments (200:1 E:T ratio; $F_{(3,55)}=2.37$, $p=0.08$; data not shown). Participants who consumed yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; or BB-12 capsule had increased cytotoxic function of NK cells. In contrast, participants who consumed yogurt smoothies with BB-12 added post-fermentation had no change in NK cell cytotoxicity (data not shown). Since a significant change in the distribution of NK cells was observed (Fig. 5.3A), the NKCC was adjusted based on the percentage of NK cells in PBMCs to determine the effect of treatment on NKCC on a per cell basis. Participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation, or BB-12 in capsule form had elevated NKCC; whereas, subjects who consumed yogurt smoothies with BB-12 added post-fermentation had no increase in NKCC (Fig. 5.3B; $F_{(3,52)}=4.23$, $p=0.0094$).

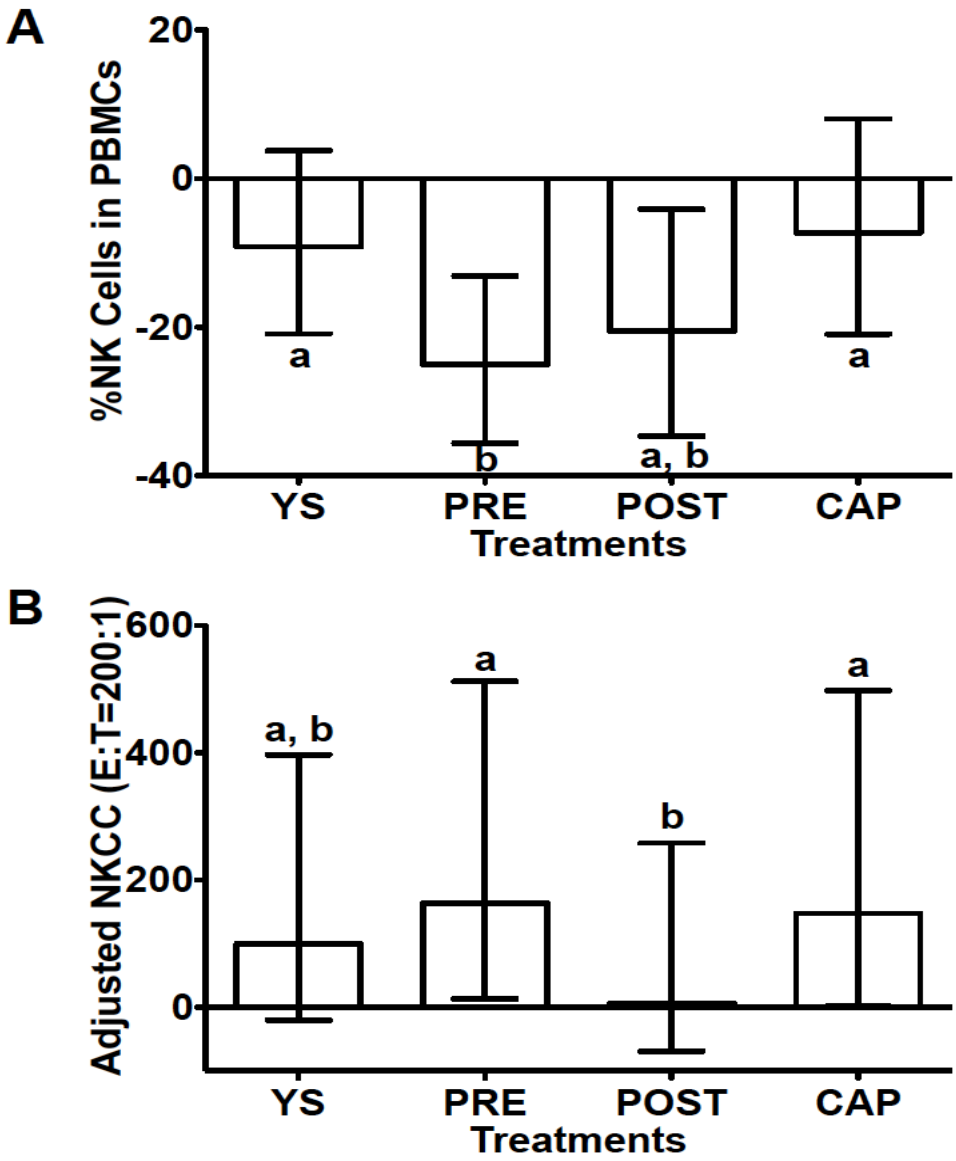


Figure 5.3. Distribution of NK cells in peripheral blood and NKCC in participants following each treatment.
The percent change from baseline in NK cell distribution in peripheral blood following yogurt smoothies with BB-12 added pre-fermentation was significantly different from yogurt smoothie alone and BB-12 capsule [**(A)**; $F_{(3,64)}$=4.82, p=0.0044; covariates in model: gender, zinc, treatment*zinc]. The percent change from baseline in adjusted NKCC at 200:1 E:T ratio was significantly different among treatments [**(B)**; $F_{(3,52)}$=4.23, p=0.0094; covariate in model: BMI, PA, treatment*BMI, treatment*PA]. Data are presented as least squares means ± 95% CI. Post hoc analyses were conducted using Bonferroni correction for multiple comparisons. Different lowercase letters within endpoints indicate significant differences between treatments.

5.4.5. Reduction in HLA-DR expression on antigen-presenting cells (APCs) may contribute to the treatment effect of BB-12 on T cell and NK cell function.

To determine the potential mechanisms underlying the differences in immune outcomes assessed in subject who consumed yogurt smoothies with BB-12 added post-fermentation, we assessed the fold change from baseline in mRNA expression of the transcription factor FoxP3 (Fig. 5.4A); the regulatory cytokines TGF-β (Fig. 5.4B), IL-10 (Fig. 5.4C), and IL-12 (Fig. 5.4D); and the surface expression of HLA-DR on dendritic cells (DCs, $CD123^{+}CD11c^{+}HLA\text{-}DR^{+}$; Fig. 5.4E) and peripheral blood-derived monocytes ($CD14^{+}HLA\text{-}DR^{+}$; Fig. 5.4F) following each treatment. None of the treatments altered gene expression of FoxP3 (Fig. 5.4A; χ^2=1.15, p=0.76), TGF-β (Fig. 5.4B; χ^2=0.70, p=0.87), IL-10 (Fig. 5.4C; χ^2=2.68, p=0.44), and IL-12 (Fig. 5.4D; χ^2=1.43, p=0.70) in participants.

The MFI of HLA-DR on DCs (percent change from baseline) was not different among treatments (Fig. 5.4E; $F_{(3,72)}$=0.49, p=0.69). However, participants who consumed yogurt smoothies with BB-12 added pre-fermentation or BB-12 capsule had elevated MFI of HLA-DR on DCs. In contrast, participants who consumed yogurt smoothies alone or with BB-12 added post-fermentation had no change in HLA-DR expression on DCs. The MFI of HLA-DR on peripheral blood-derived monocytes (percent change from baseline) was significantly lower in participants who consumed yogurt smoothies with BB-12 added post fermentation compared to other three treatments (Fig. 5.4F; $F_{(3,72)}$=2.84, p=0.0440).

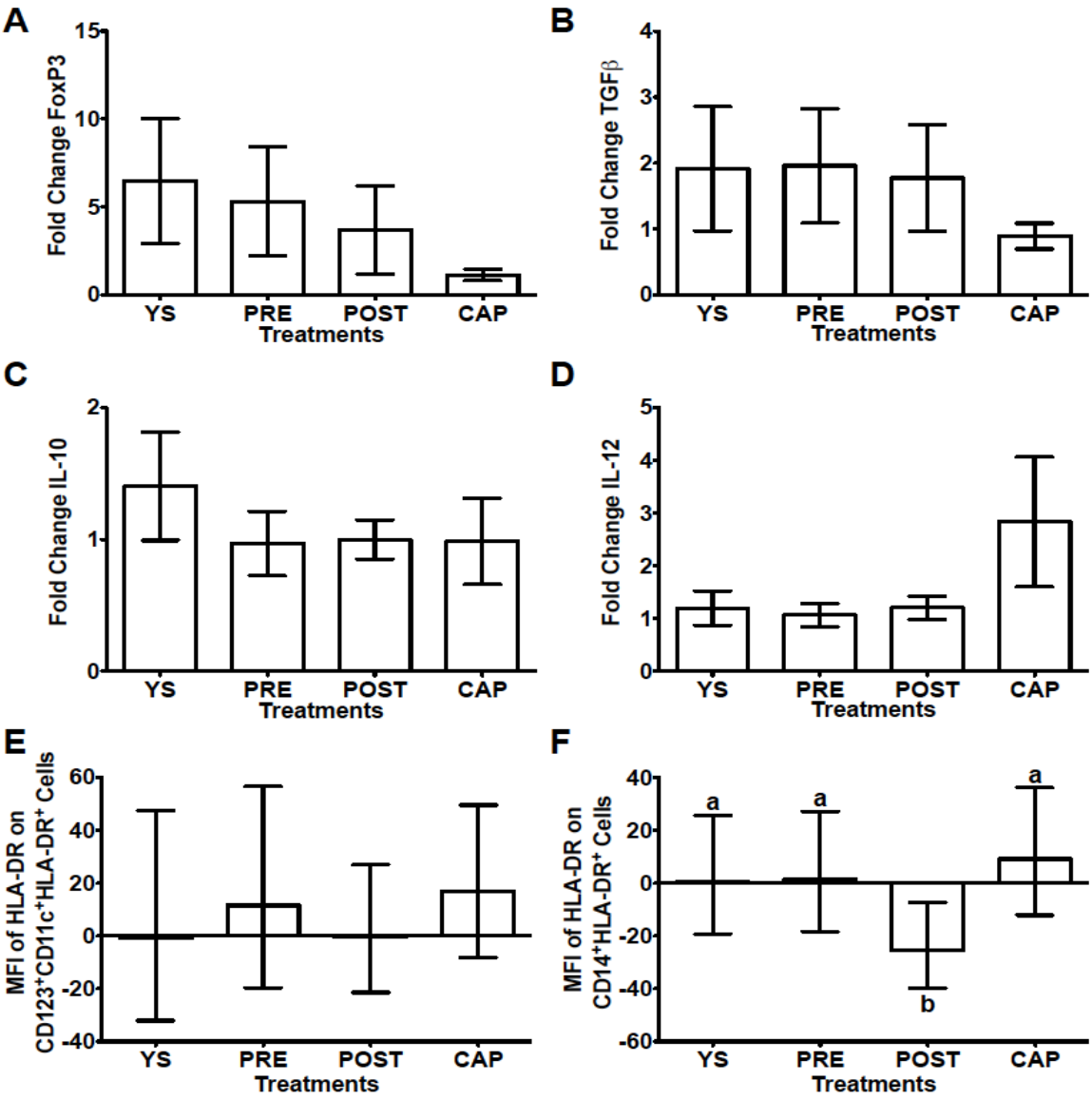


Figure 5.4. Gene expression of regulatory mediators in PBMCs, and HLA-DR expression on DCs and peripheral blood-derived monocytes in participants following each treatment.

None of the treatment altered the fold change in gene expression of FOXP3 [**(A)**; χ^2=1.15, p=0.76], TGF-β [**(B)**; χ^2=0.70, p=0.87], IL-10 [**(C)**; χ^2=2.68, p=0.44], and IL-12 [**(D)**; χ^2=1.43, p=0.70] in our participants. The percent change from baseline in MFI of HLA-DR on DCs was not different among treatments [**(E)**; $F_{(3,72)}$=0.49, p=0.69; covariate in model: gender]. The percent change from baseline in MFI of HLA-DR on peripheral blood-derived monocytes was significantly lower in participants who consumed yogurt smoothies with BB-12 added post fermentation compared to other treatments [**(F)**; $F_{(3,72)}$=2.84, p=0.0440; covariate in model: age]. Data in Fig. 5A-D are presented as mean±SEM, and data in Fig. 5E and F are presented as least squares means ± 95% CI. Post hoc analyses were conducted using Bonferroni correction for multiple comparisons. Different lowercase letters within endpoints indicate significant differences between treatments.

5.4.6. All treatments except yogurt smoothies with BB-12 added post-fermentation reduced self-reported cold or flu severity in participants.

To determine if the alteration in T and NK cell function, or the change in NK cell distribution following treatments had any clinical consequences, we assessed the incidence of cold or flu infection; number of cold or flu episodes; total number of days with cold or flu symptoms; number of days in bed and/or absent from work due to cold or flu; and sick score due to cold or flu infection over the past month based on self-reported URTI questionnaire. None of the treatments altered the incidence of cold or flu infection (Table 5.2; Wald χ^2=6.40, p=0.17); number of cold or flu episodes (Table 5.2; χ^2=5.59, p=0.23); number of days in bed and/or absent from work (Table 5.2; χ^2=3.88, p=0.42); and sick score (Table 5.2; χ^2=8.92, p=0.06) due to cold or flu infection in participants. However, consumption of yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; and BB-12 capsule (but not participants who consumed yogurt smoothies with BB-12 added post-fermentation) significantly decreased the self-reported total number of days with cold or flu symptoms by approximately 2-3 days (Table 5.2; χ^2=9.45, p=0.0509).

Table 5.2. Difference in self-reported cold or flu incidence and severity in the past month between BL and treatments[1,2,3].

	BL (N=30)	YS (N=26)	PRE (N=26)	POST (N=26)	CAP (N=27)	
	n (%)	n (%)	n (%)	n (%)	n (%)	p value[1]
Incidence of cold or flu						0.1709
No	15 (50.0)	20 (76.9)	20 (76.9)	17 (65.4)	16 (59.3)	
Yes	15 (50.0)	6 (23.1)	6 (23.1)	9 (34.6)	11 (40.7)	
	mean (95% CI)	mean (95% CI)	mean (95% CI)	mean (95% CI)	mean (95% CI)	p value[2]
Number of cold or flu episodes[3]	0.85 (0.53,1.36)	0.37 (0.17,0.82)	0.34 (0.15,0.75)	0.54 (0.30,0.99)	0.49 (0.30,0.81)	0.2316
Number of days with cold or flu symptoms[3]	3.87 (2.42,6.19)	1.51 (0.74,3.06)*	1.20 (0.55,2.60)**	2.37 (1.31,4.29)	1.96 (1.14,3.36)*	0.0509
Number of days in bed or away from work due to cold or flu	0.90 (0.34,2.36)	0.50 (0.10,2.58)	0.08 (0.01,0.53)	0.08 (0.02,0.29)	0.33 (0.12,0.93)	0.4220
Sick score due to cold or flu	3.03 (2.01,4.58)	1.04 (0.48,2.26)	0.92 (0.40,2.10)	1.31 (0.71,2.40)	1.93 (1.12,3.31)	0.0632

[1] p values for overall difference between BL and treatments based on Logistic regression.
[2] p values for overall difference between BL and treatments based on Poisson regression.
[3] covariates include gender.
*represents p<0.05 compared to BL; **represents p<0.01 compared to BL.

5.5. Discussion

To our knowledge, our study provides the first comprehensive documentation of the effect of *Bifidobacterium animalis* subsp. *lactis* (BB-12) consumption, delivered in yogurt smoothies or capsule, on the function of T cells and NK cells concurrently with self-reported cold or flu incidence and severity in young adults. We observed that participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form had an elevation in IL-2 secretion and NKCC. In contrast, participants who consumed yogurt smoothies with BB-12 added post-fermentation had no increase in IL-2 production from stimulated T cells or NKCC. These findings suggest that the timing of the addition of BB-12 to the yogurt smoothies impacts the immunomodulatory properties of BB-12. We also observed that consumption of yogurt smoothies with BB-12 added post-fermentation decreased the expression of HLA-DR on peripheral blood-derived monocytes. This may contribute to the difference in cytokine secretion observed in this treatment group. Moreover, subjects who consumed yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; or BB-12 capsule had significantly fewer number of days with cold or flu symptoms. However, subjects who consumed yogurt smoothies with BB-12 added post-fermentation had no reduction in the number of days with cold or flu symptoms. These data demonstrate that changes in URTI severity are correlated with both NK cell and T cell functional outcomes. Furthermore, these data demonstrate that timing of the addition of BB-12 to the yogurt mixture significantly impacts the efficacy of BB-12 to modulate immune responses and URTI severity.

To gain a better understanding of the role of BB-12 consumption on T cell function in healthy adults, we quantified the proliferative capacity and cytokine secretion (IFN-γ, TNF-α, and IL-2) of T cells in response to anti-CD3 stimulation. We observed that subjects who consumed yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; and BB-12 in capsule form had a elevation in IL-2 secretion from T cells; whereas the consumption of yogurt smoothies with BB-12 added post-fermentation did not result in an increase in IL-2 secretion. Although we observed no statistically significant differences in T cell proliferation, IFN-γ or TNF-α secretion, a pattern emerged in TNF-α secretion that was similar to the pattern of IL-2 secretion among treatments. TNF-α and IL-2 secretion in subjects who consumed yogurt smoothies with BB-12 added post-fermentation was unchanged in response to the intervention. This suggests that this treatment (consumption of yogurt smoothies with BB-12 added post-fermentation) results in physiological changes that are unique, as exposure to all other treatment groups (including yogurt alone) resulted in an elevation of IL-2 and TNF-α.

The elevation in T cell IL-2 secretion observed in participants who consumed yogurt smoothies alone may be due to immunomodulatory effect of bacteria-free components of yogurt (195). The yogurt fermentation process can increase the bioavailability of minerals in yogurt (195). One study found that long-term yogurt consumption is associated with increased concentrations of ionized calcium in serum; and calcium has been shown to enhance IL-2 secretion from T cells (195). Thus, consumption of yogurt smoothies alone may increase IL-2 secretion via improving the bioavailability

of calcium. The elevation in IL-2 secretion following consumption of yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form may be due to an effect of BB-12 on T cell function. Although the elevation in IL-2 secretion observed in these three treatments may be due to various individual treatment effects, a potential placebo effect may also explain the consistent changes in IL-2 secretion by three different treatments. Recent evidence demonstrates that placebo effects may influence human immune response via expectations, behavior modifications and learning processes associated with the placebo response (196).

Consumption of yogurt smoothies with BB-12 added post-fermentation did not increase *in vitro* TNF-α and IL-2 secretion from T cells, suggesting that BB-12 may lose the probiotic effect and/or its ability to modulate immunity after being added post yogurt fermentation. Consuming yogurt smoothies with BB-12 added post-fermentation may induce signaling pathways in T cells that differ from other three treatments. In the same study population, we also found that consumption of yogurt smoothies with BB-12 added post-fermentation significantly reduces heat-inactivated BB-12 or LPS-induced TNF-α secretion from peripheral myeloid cells. This reduction in TNF-α secretion in participants who consumed yogurt smoothies with BB-12 added post-fermentation was associated with lower expression of Toll-like receptor 2 (TLR-2) on peripheral blood-derived monocytes [Meng, *et al*, submitted manuscript]. Transcription factor nuclear factor-κB (NF-κB) is downstream of TLR-2 in myeloid cells and is crucial in initiating gene expression of pro-inflammatory cytokines, including TNF-α. Several *in vitro* studies reported that lactic acid producing probiotics can inhibit the activity of NF-κB, and

concurrently reduce TNF-α mRNA expression in human intestinal epithelial cells, or RAW264.7 macrophages in response to probiotic bacteria or LPS stimulation (182, 185, 186). In T cells, NF-κB can be activated by protein kinase Cθ and IKKβ, which are induced by the combination of T cell receptor (TCR) and co-stimulatory signaling (197). Once activated, NF-κB plays an important role in T cell development and function, including T cell proliferation and cytokine production (197). Moreover, NF-κB activity is crucial for Th1 responses, including secretion of TNF-α (197). Therefore, changes in NF-kB signaling may be induced in both monocytes and T cells following consumption of yogurt smoothies with BB-12 added post-fermentation, reducing both monocyte and T cell responses to stimulation. Moreover, TCR and co-stimulatory signaling-induced NF-κB activation results in translocation of NF-kB into the nucleus, and binding of NF-kB to the proximal promoter of human IL-2 and IL-2 receptor genes, resulting in increased IL-2 secretion and expression of the IL-2 receptor (198-200). IL-2 in turn stimulates T cell responses in an autocrine fashion, and regulates cellular events including activation of the NF-κB pathway (198-200). Thus, consumption of yogurt smoothies with BB-12 added post-fermentation may result in blunted NF-κB signaling and a subsequent reduction in IL-2 secretion, which further reduces NF-κB signaling. Future studies are needed to investigate the effect of BB-12 on NF-κB pathways in human T cells, and determine if the inhibition of NF-κB activity may explain the reduction in T cell TNF-α and IL-2 secretion following consumption of BB-12 delivered in yogurt smoothies post-fermentation in our participants.

To determine the role of consumption of BB-12 on NK cells, we quantified the percentage of NK cells in peripheral blood and the cytotoxic function of NK cells. All treatments reduced the percentage of NK cells in peripheral blood, but consumption of yogurt smoothies with BB-12 added pre- and post-fermentation resulted in the greatest reduction. When the NK function was assessed, subjects who consumed yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; and BB-12 in capsule form had an elevation in NK cell cytotoxicity. However, since we observed a significant reduction in the percent of circulating NK cells in subjects who consumed yogurt smoothies with BB-12 added both pre- and post-fermentation, we adjusted the NK cell cytotoxicity based on the percent of NK cells in the sample to determine if any of the treatments altered NK cell function on a per cell basis. After adjusting for the percent of NK cells in the sample, we observed that consumption of yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; and BB-12 in capsule form resulted in an elevation of NK cell cytotoxicity; whereas consumption of yogurt smoothies with BB-12 added post-fermentation had no effect on NK cell function. Similar to T cell IL-2 cytokine secretion, the consistent elevations in NK cell cytotoxicity following consumption of yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; and BB-12 capsule may be due to a placebo effect of being a participant in a clinical trial. Moreover, IL-2 is an essential growth factor for NK cells, and has been shown to enhance NK cell cytotoxic activity (198). Therefore, increased IL-2 secretion may also contribute to the elevation in NK cell cytotoxicity in the three treatment groups. In contrast, NK cell cytotoxicity was not affected by consumption of yogurt

smoothies with BB-12 added post-fermentation, which may be attributable to lack of effect of this treatment on IL-2 secretion from T cells. Overall, our results demonstrated an inhibitory effect of BB-12 delivered in yogurt smoothies post-fermentation on distribution and function of NK cells, and also suggest that the timing of addition of BB-12 to the yogurt may influence the impact of BB-12 on NK cells.

Three different biological mechanisms may underlie the treatment differences observed in T cell and NK cell function. First, BB-12 may influence the responses of T cells and NK cells in the periphery indirectly by modulating gut microbiota composition. A number of clinical studies demonstrate that oral consumption of BB-12, alone or combined with other probiotic bacteria or bioactive compounds, influences the composition of gut microbiota, which may interact with intestinal epithelial cells and/or immune cells. Mohan *et al* reported that daily supplementation with infant formula containing 4.9×10^9 cfu of BB-12 for 21 days increases the number of *Bifidobacteria* and decreases the number of harmful bacteria, including *Enterobacteriaceae* and Clostridia in the intestine of preterm infants (191). Clinical studies with healthy adults found similar results. Alander *et al* reported that healthy adults who consumed 3×10^{10} cfu/d of BB-12 alone or in combination with 4 grams of galacto-oligosaccharide significantly increase the number of *Bifidobacteria* in feces (201). Another clinical study found that oral consumption of fermented milk supplemented with 10^9 to 4×10^{10} cfu/d of BB-12;1×10^9 cfu/d of *Lactobacillus acidophilus* LA5; and green tea extract for 4 weeks modifies gut bacteria composition by increasing the count of *Bifidobacteria* and *Lactobacilli* and decreasing the count of *Enterococci* in healthy adults in comparison to

baseline (202). Similarly, Matto *et al* observed that consumption of a total number of 1 x 10^{11} cfu/d of BB-12, *Lactobacillus acidophilus* NCFB 1748, and *Lactobacillus paracasei* subsp. *paracasei* F19 for 2 weeks increases the total number of *Bifidobacteria* and *Lactobacilli* in feces in healthy adults (203). Similar changes in gut microbiota were also observed in adult and aged rats fed with BB-12 in conjunction with LGG and the prebiotic inulin for 21 days (193). The mechanisms underlying how changes in microbiota composition may impact T cell and NK cell function are still unclear. However, gut microbiota may induce cytokine secretion by intestinal epithelial cells, regulate gut-brain axis, and produce various metabolites (e.g. short chain fatty acids), which may mediate the relationship between microbiota and immune function (204).

The second mechanism by which BB-12 consumption may be altering immune cell function via a change in the level of regulatory mediators, including transcription factor of regulatory T cells (Treg) FoxP3 and regulatory cytokines TGF-β, IL-10, and IL-12, all of which have been shown to influence activity of T cells and NK cells (205-208). Tregs are part of the adaptive immune system that maintain self-tolerance and contribute to homeostasis of immune responses by shutting down the activities of immune cells, including T cells and NK cells, after their successful clearance of invading pathogens (205). FoxP3 is a transcription factor that regulates the development and suppressive function of Tregs (205). TGF-β can be secreted by many immune cells, and has been well-known for its role in controlling proliferation and differentiation of lymphocytes (206). TGF-β has been shown to induce expression of FoxP3 in Tregs, and mediates the suppressive capacity of

Tregs (206). Moreover, TGF-β is also able to block the activity of T cells and NK cells, including T cell proliferation and cytokine secretion, NK cell cytotoxicity and IFN-γ secretion (206). IL-10 is an anti-inflammatory cytokine that regulates immune response during infection or inflammatory process (207). In particular, IL-10 can inhibit the activity of T helper 1 cells, NK cells, and macrophages (207). In contrast, IL-12 contributes to inflammatory responses by inducing the production of pro-inflammatory cytokines from T cells and NK cells, and the differentiation of T cells towards a T helper 1 phenotype (208). We observed that participants who consumed yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; and BB-12 in capsule form had an elevation in T cell and NK cell function; whereas consumption of yogurt smoothies with BB-12 added post-fermentation did not increase responses of T cells and NK cells. Thus, we hypothesized that consumption of yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation, and BB-12 capsule decrease gene expression of FoxP3, TGF-β, and IL-10, and increase gene expression of IL-12. In contrast, we hypothesized that yogurt smoothies with BB-12 added post-fermentation increase gene expression of FoxP3, TGF-β, and IL-10, and decrease gene expression of IL-12. In order to test this hypothesis, we assessed the fold change from baseline in mRNA expression of FoxP3, TGF-β, IL-10, and IL-12 in PBMCs following each treatment period by using real-time qPCR. We observed that none of the treatments altered the expression of these regulatory mediators. The effect of BB-12 consumption alone on gene expression of FoxP3 and TGF-β in PBMCs has not been reported previously. However, consumption of yogurt containing 10^{10} cfu/d of BB-12

combined with two *Lactobacilli* strains (with and without low calorie diet) for 8 weeks increases gene expression of FoxP3 and TGF-β in PBMCs from overweight and obese adults (41). Previous studies report no effect of BB-12 consumption on protein levels of IL-10 and IL-12, which correlated with lack of effect in gene expression of IL-10 and IL-12 in PBMCs observed in our study. One clinical study reported that serum levels of IL-10 and secretion of IL-12 from influenza A.H3N2 stimulated PBMCs does not change in healthy adults who consumed a milk drink containing BB-12 for 3 weeks (20). Another study found no change in serum concentrations of IL-10 in healthy adults that consumed 10^9 cfu/d of BB-12 for 6 weeks (15). Moreover, IL-10 secretion from PHA-stimulated PBMCs was not altered in healthy adults following consuming BB-12 capsules at doses of 10^8-10^{11} cfu/d for 3 weeks (14). Overall, our results suggest that BB-12 induced alterations in function of T cells and NK cells were not due to changes in Treg function or the production regulatory cytokines.

Lastly, HLA-DR, which is the surface major histocompatibility complex II (MHCII) on DCs and macrophages, is part of “signal I” of T cell activation. HLA-DR can present foreign antigen peptides to the T cell receptor inducing subsequent T cell activation. We quantified the expression of HLA-DR on DCs and peripheral blood-derived monocytes at baseline and following each treatment period to determine if BB-12 consumption altered T cell cytokine secretion via changing HLA-DR expression on APCs. We found that HLA-DR expression on peripheral blood-derived monocytes was significantly lower in participants who consumed yogurt smoothies with BB-12 added post-fermentation compared to other three treatments. Our results suggest that the

reduction in anti-CD3 induced secretion of TNF-α and IL-2 in participants who consumed yogurt smoothies with BB-12 added post-fermentation may be due to the reduction in HLA-DR expression on peripheral blood-derived monocytes. In addition, activation of TLR-2 signaling induces a cascade of intracellular events, which can contribute to a greater expression of HLA-DR on APCs (209). Our previous study found that consumption of yogurt smoothies with BB-12 added post-fermentation reduces expression of TLR-2 on peripheral blood-derived monocytes, which may contribute to the decreased HLA-DR expression on these cells. In addition to "signal I", expression of co-stimulatory molecules, including CD80 (B7.1) and CD86 (B7.2), on APCs is also essential in activation and function of T cells. Upregulation of TLR-2 expression has also been shown to increase the expression of CD80 and CD86 on APCs (209). Thus, consumption of yogurt smoothies with BB-12 added post-fermentation reduces expression of TLR-2 on peripheral blood-derived monocytes, which may contribute to lower expression of HLA-DR and co-stimulatory molecule expression on peripheral blood-derived monocytes, and result in reduction in T cell cytokine secretion.

To determine if the alterations in function of T cells and NK cells have clinical relevance, we assessed self-reported outcomes associated with cold of flu incidence and severity in the past month of our participants. We found that participants who received yogurt smoothies alone; yogurt smoothies with BB-12 added post-fermentation; and BB-12 capsule had significantly fewer total number of days with cold or flu symptoms. In contrast, we did not see a protective effect of yogurt smoothies with BB-12 added post-fermentation on URTI symptoms. The beneficial effect of BB-12 alone has also been observed

in infants in previous studies. Healthy children 12-48 months of age significantly reduce number of days with URTI symptoms following consuming yogurt supplemented with BB-12 and prebiotic inulin once daily for 16 weeks (119). Similarly, infants that received BB-12 by using a slow-release tablet from 2 to 8 months old have fewer respiratory infections compared to infants received placebo (120). We also observed a protective effect of yogurt smoothies alone on self-reported cold or flu infection in our participants, indicating a potential placebo effect on cold or flu outcomes. Participants may have increased expectations for health and behavioral modifications towards healthy lifestyle when they participate in clinical interventions, and these factors may contribute to a reduction in severity of cold or flu infection observed in the yogurt only treatment group. We did not observe a similar effect of BB-12 added in yogurt smoothies post-fermentation on severity of cold of flu infection in participants, indicating that the beneficial effect of yogurt smoothies alone is counteracted after BB-12 is added to the yogurt smoothies post-fermentation. The lack of response in the post-fermentation group may be due to the lack of effect of this treatment on the function of T cells and NK cells. Overall, we observed that consumption of yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation; and BB-12 in capsule form significantly reduced severity of cold or flu infection, concurrently had elevated T cell and NK cell function in our participants. In contrast, participants who consumed yogurt smoothies with BB-12 added post-fermentation did not alter the severity of cold or flu infection, or T cell and NK cell activities. Our results suggest that BB-12 induced alterations in severity of cold or flu infection are associated with changes in T cell and NK cell function.

Overall, we observed beneficial effects of yogurt smoothies alone, yogurt smoothies with BB-12 added post-fermentation, and BB-12 capsule on T cells and NK cell function and severity of cold or flu infection. Yogurt has numerous health claims, including immunomodulatory properties, due to its rich in various nutrients (e.g. peptides, free amino acids, free fatty acids, folic acid, and calcium) and bioactive compounds (e.g. casein) (180). Yogurt can also support the growth, survival and activity of BB-12 until consumption, and provide adequate nutrients, acidic environment and buffering system to support the survival of BB-12 while passing through the human GI tract (125, 126, 180). Thus, yogurt can influence the efficacy of BB-12 by modifying the probiotic effect of BB-12 in the intestine (22, 125, 126). However, these beneficial effects may be dependent on the timing of addition of the BB-12 to the yogurt cultures, as observed no effect of BB-12 delivered in yogurt smoothies post-fermentation on T cells and NK cell function and cold or flu infection. BB-12 may produce different metabolites when it is added before or after the yogurt fermentation process, which may influence the efficacy and probiotic activity of BB-12 and may be altering the immune response and infectious outcomes. Furthermore, our results suggest that the probiotic effect of BB-12 may be influenced by the timing of its addition during the yogurt manufacturing process and warrants further study.

In summary, we demonstrated that participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form had an elevation in IL-2 and TNF-α secretion from T cells and elevated NK cell cytotoxicity. In contrast, consumption of BB-12 delivered in yogurt smoothies post-fermentation had no change in secretion of TNF-α

and IL-2 in response to anti-CD3 stimulation and no change in NKCC. We also found that HLA-DR expression on blood-derived monocytes was reduced following receiving yogurt smoothies with BB-12 added post-fermentation, which may contribute to the lack of effect on T cell cytokine secretion and NKCC. Lastly, we observed that consumption of yogurt smoothies alone; yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form significantly reduced self-reported total number of days with cold or flu symptoms in young healthy adults. However, consumption of yogurt smoothies with BB-12 added post-fermentation did not alter the severity of cold or flu infection. The URTI severity scores correspond to the aforementioned changes in NK and T cell function among treatment groups. Furthermore, these findings indicate that the timing of its addition to yogurt in relation to the fermentation process influenced the impact of BB-12 on the activity of T cells and NK cells and cold or flu severity in young healthy adults. Future studies will focus on understanding the mechanisms underlying BB-12 induced immunomodulation, and its relationship with infection-related outcomes.

Supplemental Statistical Analyses

An exponential decay model was constructed to determine if there was a carryover effect of BB-12 among different treatments. We assumed the probiotic treatment effect decayed exponentially *in vivo* after participants stopped receiving a given treatment. Following each treatment period, the total probiotic effect was calculated by adding the effect of treatment administered during the current treatment period ($\alpha_{current}$), to the residual effect of the treatment administered during the previous treatment period ($\alpha_{previous}$). The probiotic effect at the end of the first treatment period was only the effect of the treatment administered during period one. A dummy variable "decay proportion" (c) was created and defined as the percentage of residual effect of the treatment administered during the previous treatment period. The proportion of previous treatment that had already decayed during washout and current treatment period was 1-c. The equation describing the exponential decay model was $y=\beta_0+\beta_1*x_1+\beta_2*x_2+\beta_3*x_3+\beta_4*x_4+\alpha_{current}+c*\alpha_{previous}+\varepsilon$. In the model, y was the immune endpoint of our study; x_1-x_4 were covariates, including age, gender, BMI, and PA of participants; β_0 was the intercept of the regression line; β_1 to β_4 were slope of regression line for covariates x_1-x_4, respectively. The model was fitted with c equal to 0, 10%, 20%, and up to 90%, and R^2 was recorded with each choice of c. The percent change in R^2 when c = 10%, 20% up to 90% relative to the R^2 when c = 0 was calculated. If the percentage change between the two was less than 10%, we surmised that R^2 when c = 10%, 20% up to 90% was not different from R^2 when c = 0, indicating that the model when c=0 was the closest to the true model. When c=0, 100% of the previous treatment effect has decayed, and

we concluded that there were no carryover effect from the previous treatment. All immune endpoints in this study were tested for a carryover effect using the exponential decay model, and no carryover effect of treatments on any immune endpoint was observed.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

The studies in this dissertation were designed to explore the immunomodulatory property of BB-12, delivered in yogurt smoothies (pre- or post-fermentation) or in capsule form, in young healthy adults. Specifically, these studies investigated the effect of BB-12 consumption on the distribution, phenotypic marker expression and pro-inflammatory cytokine secretion from peripheral blood derived monocytes and dendritic cells. These studies also investigated the effect of BB-12 consumption on the distribution and function of T cells and NK cells and impact on self-reported incidence and severity of cold or flu infection in humans. Prior to analyzing the data from this clinical trial, we investigated what dietary and lifestyle variables contributed to the inter-subject variability in immune responses. This chapter briefly summarizes the main findings and identifies future directions for research.

The goals of study 1 were to determine which endogenous and exogenous host factors contribute to the heterogeneity in innate and adaptive immune responses and cold or flu status among healthy participants; and if these host factors also confound the relationship between phenotypic marker expression, immune function, and cold or flu status by using baseline data of our clinical trial. These questions are important because the heterogeneity in both innate and adaptive immune responses that we observed among healthy adults results in large inter-subject variability, and makes it challenging to evaluate the effect of dietary interventions, like probiotic consumption on immune function

(131, 133). This included anti-CD3 induced T cell proliferation and secretion of IL-2 and IFN-γ, and LPS stimulated TNF-α and IL-6 secretion from PBMCs. We also quantified dietary intake (via 3-day dietary recall records), physical activity level (via IPAQ questionnaire), and the incidence and severity of cold or flu symptoms (via self-reported upper respiratory tract infection [URTI] questionnaires) in our participants.

We demonstrated that host-related factors (including age, BMI, physical activity, and daily intake of total calories and various dietary components) contributed significantly to heterogeneity in T cell effector function, and cold or flu status, and confounded the associations between activation marker expression on T cells and T cell effector function. These host factors also contributed significantly to the associations between innate and adaptive immune response and incidence and severity of cold or flu infection. We also found that individual lifestyle and dietary variables contribute a small amount to T cell effector function and cold or flu status. However, combinations of these variables significantly improved the predictive relationship between activation marker expression and T cell proliferation and IL-2 secretion, and also strengthened the associations between IL-6 and IFN-γ secretion and the incidence and severity of cold or flu symptoms, respectively. These results suggest that lifestyle and dietary factors are important variables that collectively contribute to T cell responses and cold or flu status, and should be included in human clinical trials that assess immune endpoints. This analysis using immune data in our baseline sample has enabled us to understand the variability in human immune outcomes and has been insightful

about how to analyze the treatment effects of BB-12 consumption on immune outcomes in our clinical trial.

Study 2 tested the hypothesis that consumption of BB-12 alone increased phenotypic marker expression (e.g. TLR-2) on peripheral blood derived monocytes, and secretion of pro-inflammatory cytokines (TNF-α and IL-6) in peripheral myeloid cells in response to *in vitro* challenge with heat-inactivated BB-12 or LPS in young healthy adults. We also proposed that BB-12 delivered in yogurt smoothies (either pre- or post- fermentation) had greater impact on innate immune responses than BB-12 in capsule form. Our results did not support our original hypothesis. Instead, we found that consumption of yogurt smoothies with BB-12 added post-fermentation significantly reduced the secretion of the inflammatory cytokine, TNF-α, from PBMCs in response to *in vitro* BB-12 and LPS stimulation. These finding are not only the first to demonstrate the anti-inflammatory property of BB-12, but also suggests that the delivery matrix of BB-12 and the timing of addition of BB-12 to the yogurt fermentation process influences the immunomodulatory effect of BB-12. *Bifidobacterium animalis* subsp. *lactis* NCC2818 reduces colonic inflammation in a murine colitis model via a reduction in the pro-inflammatory markers, cyclooxygenase-2 (COX-2), TNF-α, and IL-6 (29, 116), further confirming an anti-inflammatory effect of *B. lactis*. In humans, BB-12 has been used to treat inflammatory diseases of the intestine (e.g., inflammatory bowel disease, irritable bowel syndrome) with some success (18, 19). Therefore, the beneficial effect of BB-12 in patients with intestinal

inflammatory disease may be mediated via its role in reducing production of pro-inflammatory cytokines.

Our study also found that BB-12 binds to the cell surface receptor, toll-like receptor 2 (TLR-2) on human peripheral blood-derived monocytes. A previous study demonstrated that the cross-talk between BB-12 and rat intestinal epithelial cells (IECs) is mediated via TLR-2 (179). Our finding in human PBMCs is novel, and demonstrates that BB-12 binds to TLR-2 in both rats and humans. This contributes to our understanding of how BB-12 influences immune function. Moreover, consumption of yogurt smoothies with BB-12 added post-fermentation for 4 weeks significantly decreased TLR-2 expression on peripheral blood-derived monocytes. This reduction may lessen the interaction between BB-12 and these cells, and contribute to the reduction in TNF-α secretion observed in BB-12 or LPS stimulated PBMCs collected from participants following consumption of BB-12 delivered in yogurt smoothies post-fermentation.

TLR-2 can signal through nuclear factor-κB (NF-κB) pathway, which is crucial in gene expression and production of pro-inflammatory cytokines in human macrophages (181). Previous studies reported that lactic acid producing probiotics inhibit the NF-κB pathway via stabilizing the I-κB α subunit in human IECs *in vitro* (182-184), indicating that the reduction in TNF-α secretion from BB-12-stimulated PBMCs observed in our study may be mediated via BB-12-induced inhibition of NF-κB activity. In addition, pre-culture of RAW264.7 macrophages or human IECs with *Bifidobacterium species* decreases mRNA levels of TNF-α in response to LPS stimulation, and concurrently reduces activity *of* NF-kB and/or

increases gene expression of negative regulators of NF-κB (185, 186). These data suggest that the BB-12 induced reduction in TNF-α secretion from LPS-stimulated PBMCs observed in our study may also be mediated via BB-12-induced inhibition of NF-κB activity. Future studies are needed to investigate if the BB-12 induced down-regulation of TLR-2 expression on human peripheral blood-derived monocytes can lead to subsequent blockage of BB-12 or LPS-induced NF-κB activity and concurrent reduction in production of pro-inflammatory cytokines.

Our findings in study 2 have significant clinical relevance. Future studies should focus on examining if consumption of yogurt smoothies with BB-12 added post-fermentation can be used to reduce inflammatory signals via changes in NF-kB activation in clinical conditions of chronic inflammation, including obesity, cardiovascular disease or other inflammatory diseases. Additionally, clinical studies are needed to examine the effect of BB-12 consumption on serum markers of chronic inflammation, including serum high-sensitive CRP, pro-inflammatory cytokines TNF-α and IL-6, and LPS-binding protein (marker of subclinical endotoxemia) (210).

Study 3 tested the hypothesis that consumption of BB-12 alone increased T cell proliferation and cytokine (IFN-γ, TNF-α, and IL-2) secretion, NK cell cytotoxicity, and reduced URTI incidence and severity in young healthy adults. We also proposed that BB-12 delivered in yogurt smoothies (either pre- or post-fermentation) had greater effect on these immune responses and infection-related outcomes than BB-12 in capsule form. Our hypothesis was partially

supported by our results, however some of our findings were unexpected. We found that participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form had an elevation in TNF-α and a significant increase in IL-2 secretion in response to *in vitro* anti-CD3 stimulation. We also found that these three treatments increased NK cell cytotoxicity. In contrast, consumption of yogurt smoothies with BB-12 added post-fermentation did not increase TNF-α and IL-2 secretion or NK cell cytotoxic activity. These findings suggest that the timing of addition of BB-12 to the yogurt fermentation process impacts the modulation of T cell and NK cell function by BB-12. We also found that consumption of yogurt smoothies with BB-12 added post-fermentation significantly reduced the expression of HLA-DR on peripheral blood-derived monocytes compared to other three treatments, and this reduction may contribute to the lack of effect of BB-12 delivered in yogurt smoothies post-fermentation on T cell cytokine secretion in participants. Expression of HLA-DR on peripheral blood derived monocytes can be regulated via TLR-2 signaling (209). In study 2, we found that consumption of yogurt smoothies with BB-12 added post-fermentation reduced expression of TLR-2 on peripheral blood-derived monocytes, which may contribute to the decreased HLA-DR expression on these cells.

We also found that alterations in T cell and NK cell function are associated with the severity of URTI. Consumption of yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation, and BB-12 capsule reduced self-reported total number of days with cold or flu symptoms (by 2-3 days) in young

healthy adults, concurrently with elevations in T cell and NK cell function. However, consumption of yogurt smoothies with BB-12 added post-fermentation did not alter the severity of cold or flu infection. The lack of a beneficial effect of consuming yogurt smoothies with BB-12 added post-fermentation on cold or flu severity may be due to the lack of effect of this treatment on T cell and NK cell function. To further explore if alterations in URTI severity is mediated by BB-12 induced changes in T cell and NK cell function, future studies could utilize a murine influenza model to test this hypothesis. Mice could be given oral administration of yogurt alone; yogurt with BB-12 added pre- or post-fermentation; or BB-12 pellet, and receive an intranasal challenge with influenza virus. Survival rate, influenza virus titer in the lungs, and immune responses (including NK cell cytotoxicity and influenza virus-specific T cell proliferation and cytokine secretion) could be measured to determine if yogurt alone, yogurt with BB-12 added pre-fermentation and BB-12 pellet reduces survival rate and virus titer by enhancing NK cell and T cell activity.

In both study 2 and 3, we found that consumption yogurt smoothies with BB-12 added post-fermentation reduced TNF-α secretion from either BB-12 or LPS stimulated peripheral myeloid cells or anti-CD3 stimulated T cells. Thus, BB-12 when added to yogurt post-fermentation may induce specific signaling pathways that regulate TNF-α secretion in myeloid cells and T cells. In study 2, we reported that the reduction in TNF-α secretion from BB-12 or LPS stimulated myeloid cells may be associated with a reduction in expression of TLR-2 on peripheral blood-derived monocytes in participants who consumed yogurt

smoothie with BB-12 added post-fermentation. Data from previous studies suggest that the NF-κB pathway may mediate this association (182-186). T cell receptor and co-stimulatory signaling-induced upregulation of the NF-κB pathway also plays an important role in TNF-α secretion from T cells (197). Therefore, future studies are needed to investigate the effect of BB-12 on NF-κB pathways in human T cells, and determine if the inhibition of NF-κB activity may explain the reduction in TNF-α secretion following consumption of yogurt smoothies with BB-12 added post-fermentation.

In addition, two other mechanisms may be contributing to the distinct changes in immunity and infection outcomes that we observed when BB-12 added to yogurt post-fermentation. We assessed the viability of BB-12 in yogurt smoothies in both pre- and post-fermentation group before consumption, and did not observe significant difference (data not shown). However, it is not known if different delivery matrices alter the viability of BB-12 in the intestine following consumption. When BB-12 was added before the fermentation process, the stress and harsh conditions may improve the ability of BB-12 to adapt to acidic environment (211), which may allow BB-12 to survive better during passing through GI tract. In contrast, BB-12 may not experience similar adaptations when added post-fermentation. Thus, future studies could evaluate the viability of BB-12 following consumption by investigating the levels of BB-12 DNA or recovery of live BB-12 in human fecal samples. This will enable us to determine if the lack of effect on T cell and NK cell function by yogurt smoothies with BB-12 added post-fermentation is due to low BB-12 viability in the intestine of participants.

Moreover, BB-12 may induce immunological responses via exposure to bacterial cell wall components. The major components of BB-12 cell walls include peptidoglycans (PGN) and lipoteichoic acid (LTA), which have immunostimulatory properties (74, 212). Large differences in the composition of cell wall components exist from one bacterial species to another in both probiotic and pathogenic bacteria (74, 212, 213). These differences may be recognized or sensed by TLRs on immune cells, and thus lead to different immunological consequences (74, 212, 213). One animal study reported that colonizing wild-type C57BL/6 mice with *B. breve* UCC2003 in the presence or absence of exopolysaccharide (EPS) expression on cell wall of the strain generated different B cell responses (214). However, no data exists on the cell wall composition of BB-12 and immunomodulatory effects of these components. The effect of delivery matrix on cell wall components of BB-12 is also not clear. Therefore, in future studies PBMCs can be challenged with cell wall extracts from BB-12 isolated from different matrices to explore if the matrix of BB-12 alters its cell wall composition and immunomodulatory properties.

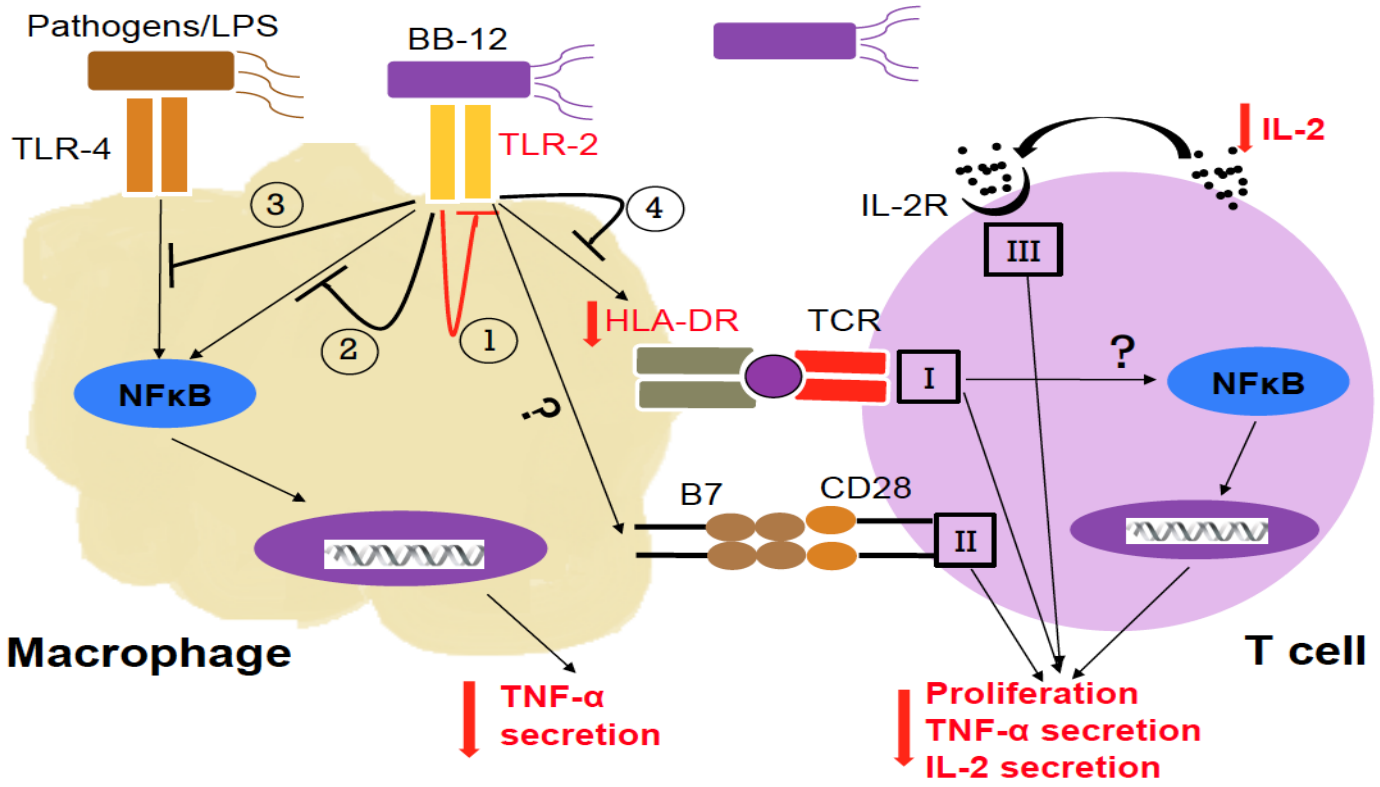


Figure 6.1. Mechanisms underlying BB-12 induced alteration in innate and adaptive immune responses.
1) Consumption of BB-12 delivered in yogurt smoothie post-fermentation reduces TLR-2 expression on human peripheral blood-derived monocytes. 2) BB-12 may inhibit the activity of NFκB downstream of TLR-2. 3) BB-12 may inhibit the activity of NFκB downstream of TLR-4. 4) Consumption of BB-12 delivered in yogurt smoothie post-fermentation reduces HLA-DR expression on human peripheral blood-derived monocytes. I) Signal I of T cell activation: interaction between T cell receptor and HLA-DR. Consumption of BB-12 delivered in yogurt smoothie post-fermentation may reduce Signal I via reduction in HLA-DR expression on peripheral blood-derived monocytes. II) Signal II of T cell activation: interaction between CD28 and co-stimulatory molecules (B7.1 and B7.2). Consumption of BB-12 delivered in yogurt smoothie post-fermentation may reduce Signal II via reduction in expression of co-stimulatory molecules on peripheral blood-derived monocytes. III) Signal III of T cell activation: interaction between IL-2 and IL-2 receptor. Consumption of BB-12 delivered in yogurt smoothie post-fermentation may reduce Signal III via reduction in both Signal I and II, and inhibition of NFκB downstream of Signal I and II.

Results from both study 2 and 3 demonstrate that oral consumption of BB-12 altered function of peripheral immune cells in participants. Although the cross-talk between ingested BB-12 and the systemic immune system is unclear, several hypotheses linking BB-12 and the gut-associated lymphoid tissues (GALT) can be generated based on previous studies (Figure 6.2). Following consumption, BB-12 travels through the GI tract and arrives in the intestinal lumen, where it may interact with GALT. DCs in the lamina propria may sample BB-12 by passing the dendrites between IECs. BB-12 may enter intestinal epithelium by transcytosis via microfold cells (M cells), gain access to the Peyer's patches, and interact with DCs or macrophages in the Peyer's patches. BB-12 antigens may also translocate between IECs, and interact with DCs or macrophages in the lamina propria. In the Peyer's patches, antigen-loaded myeloid cells may present BB-12 antigens to T cells, and may induce the activation of T cells. Activated T cells may then enter mesenteric lymph nodes (MLNs) via draining lymph, and join the blood circulation or lymphatic systems. Antigen-loaded myeloid cells in the Peyer's patches or lamina propria may gain direct access to MLNs via draining lymph. In the MLNs, myeloid cells can either present BB-12 antigens to T cells, or they may disseminate throughout the peripheral immune system via bloodstream or lymphatic systems. We then can capture and detect myeloid cells that have interacted with ingested BB-12 in the peripheral blood. Future studies are needed to explore the interactions between ingested BB-12 and immune cells in GALT using fluorescence-labeled BB-12 in

animal models to better understand how BB-12 influences systemic immune function.

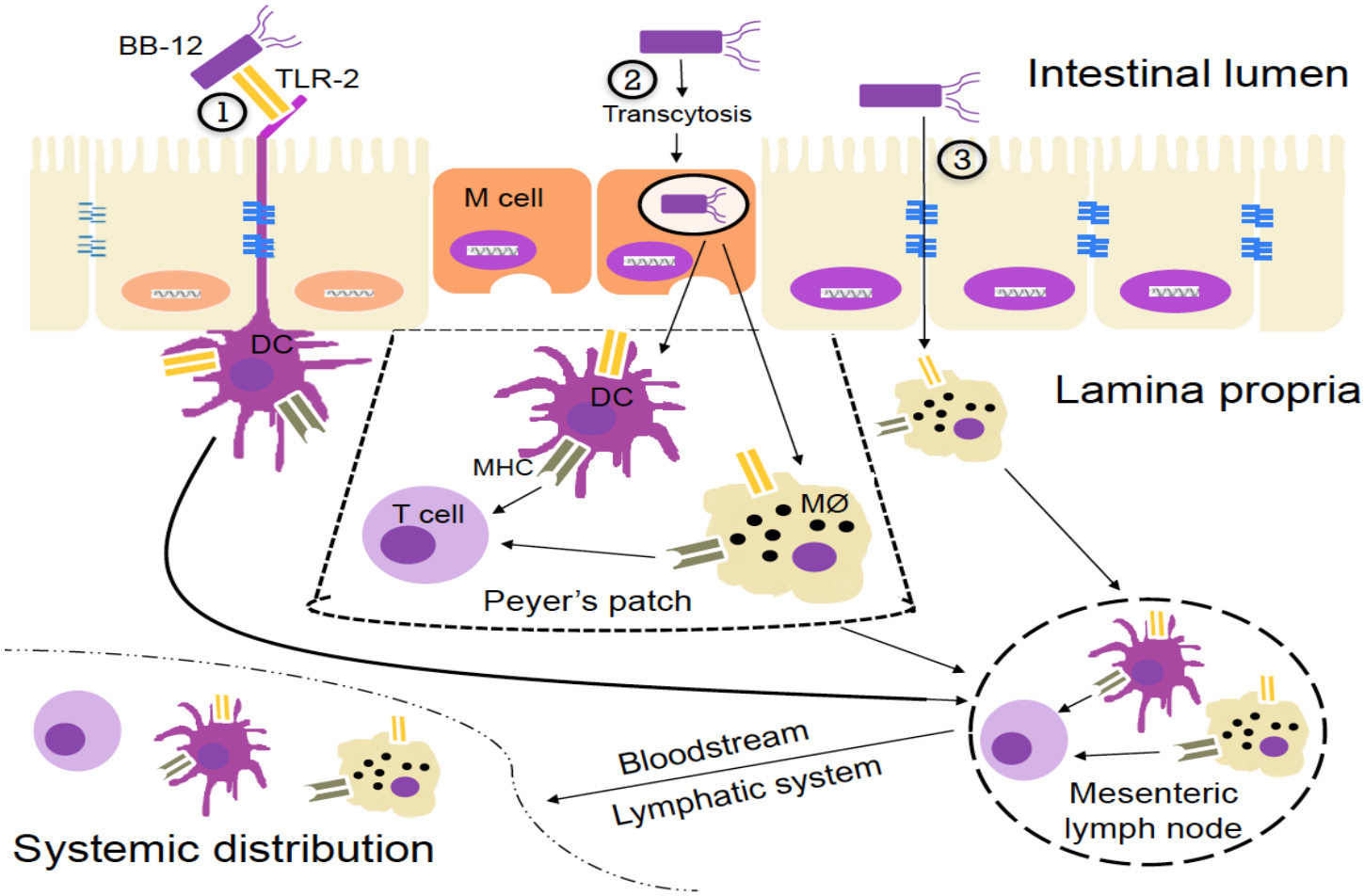


Figure 6.2. Mechanisms underlying how BB-12 influences peripheral immune cells.
BB-12 may influence systemic immune function via its interaction with gut-associated lymphoid tissues (GALT). Following consumption, BB-12 travels through GI tract and arrives in the intestinal lumen, where it may interact with GALT. 1) DCs in the lamina propria may sample BB-12 by passing the dendrites between IECs. 2) BB-12 may enter intestinal epithelium by transcytosis via microfold cells (M cells), gain access to the Peyer's patches, and interact with DCs or macrophages in the Peyer's patches. 3) BB-12 antigens may translocate between IECs, and interact with DCs or macrophages in the lamina propria.

Collectively, both study 2 and 3 demonstrated an anti-inflammatory property of BB-12 via reducing pro-inflammatory cytokine (TNF-α) secretion from peripheral myeloid cells and T cells in healthy adults. In addition, other *in vivo* and *in vitro* studies also shed light on the anti-inflammatory effect of BB-12 (Figure 6.3). In the intestinal lumen, BB-12 has been shown to interact with IECs and inhibit pathogenic microorganisms by various mechanisms, including production of pathogen-killing metabolites (9, 187); improvement in tight junction strength between IECs (189); competition with pathogenic bacteria for adhesion sites to human intestinal mucin and IECs (190); and/or modulation of gut microbiota composition by increasing the ratio of beneficial to pathogenic microorganisms (191-193). However, no clinical trial has examined if the delivery matrix and timing of addition of BB-12 in yogurt fermentation process alters these mechanisms *in vivo* in humans.

To explore the mechanisms underlying the anti-inflammatory effect of yogurt smoothies with BB-12 added post-fermentation, future studies could assess if BB-12, added to yogurt smoothies post-fermentation produces more metabolites (e.g. acetate, lactate, butyrate) that may inhibit pathogens than a BB-12 added to yogurt smoothies pre-fermentation and BB-12 in capsule form. These metabolites could be measured in *in vitro* cell culture supernatants or in fecal samples of human subjects via metabolomics (215). Serum levels of metabolites could also be measured to determine if BB-12 metabolites gain direct access to peripheral blood and interact with PBMCs; and if their levels are elevated in serum when BB-12 is added to yogurt post-fermentation. Additionally,

it would be interesting to determine if BB-12 and its delivery matrix altered the ability of IECs to produce antimicrobial peptides, such as β-defensin, *in vitro* and *in vivo*. BB-12 could be co-cultured with human IEC cell lines, and levels of antimicrobial peptides in culture supernatants could be quantified. Antimicrobial peptides in fecal samples of human subjects following consumption of BB-12 delivered in different matrices could also be quantified.

Futures studies could also determine if BB-12 delivered in yogurt smoothies added post-fermentation improve gut barrier function in humans by using the ratio of lactulose to mannitol excretion as a marker for gut permeability (216, 217). The ability of BB-12 to adhere to mucin and IECs has been reported previously (190); however, it is not clear if BB-12 delivered in different matrices alters this feature. Thus, *in vitro* co-culture of BB-12 in different delivery matrices (pre- or post-fermentation, and capsule) with human IEC cell lines or mucin isolated from human fecal samples could be evaluated to determine the adhesion capacity of BB-12.

Lastly, we need to assess the effect of BB-12 delivered in different matrices on gut microbiota composition, and determine if BB-12 delivered in yogurt smoothies post-fermentation can alter the ratio of beneficial to harmful bacteria in human intestine. Specifically, the number of beneficial bacteria, including *Bifidobacterium, Lactobacillus,* and *Enterococci*, and harmful bacteria, including *Enterobacteriaceae* and *Clostridia*, can be examined in human fecal samples.

Combined, the results from our clinical trial demonstrate that consumption of 10^9-10^{10} cfu/d of BB-12 for 4 weeks was effective in modulating both innate and adaptive immune function in young adults. Specifically, we disproved our original hypothesis that consumption of BB-12 would increase secretion of pro-inflammatory cytokines (TNF-α and IL-6) from heat-inactivated BB-12 or lipopolysacchride (LPS) stimulated PBMCs, and alter expression of HLA-DR and Toll-like receptor 2 on peripheral blood-derived monocytes. Instead, we found that BB-12 interacted with peripheral myeloid cells via TLR-2. Participants who consumed yogurt smoothies with BB-12 added post fermentation had significantly lower expression of TLR-2 on $CD14^+HLA\text{-}DR^+$ cells and reduction in TNF-α secretion from BB-12 or LPS stimulated PBMCs compared to baseline. We also supported our second hypothesis that BB-12 would increase T cell and NK cell effector function, and concurrently reduce incidence and severity of cold or flu infection. Specifically, we found that participants who consumed yogurt smoothies alone, yogurt smoothies with BB-12 added pre-fermentation or BB-12 in capsule form had elevated IL-2 secretion and NK cell cytotoxicity, concurrently with fewer number of days with URTI. However, consumption of yogurt smoothies with BB-12 added post-fermentation did not change T cell and NK cell function; and did not alter severity of URTI. Thus, the results from this clinical trial also demonstrate that the delivery matrix of BB-12 (e.g. yogurt smoothies vs. capsule) and timing of BB-12 addition to yogurt influenced the effect of BB-12 on immune response, and had impact on inflammation and infection-related

outcomes. Thus, these variables need to be considered, and controlled for in subsequent studies using BB-12.

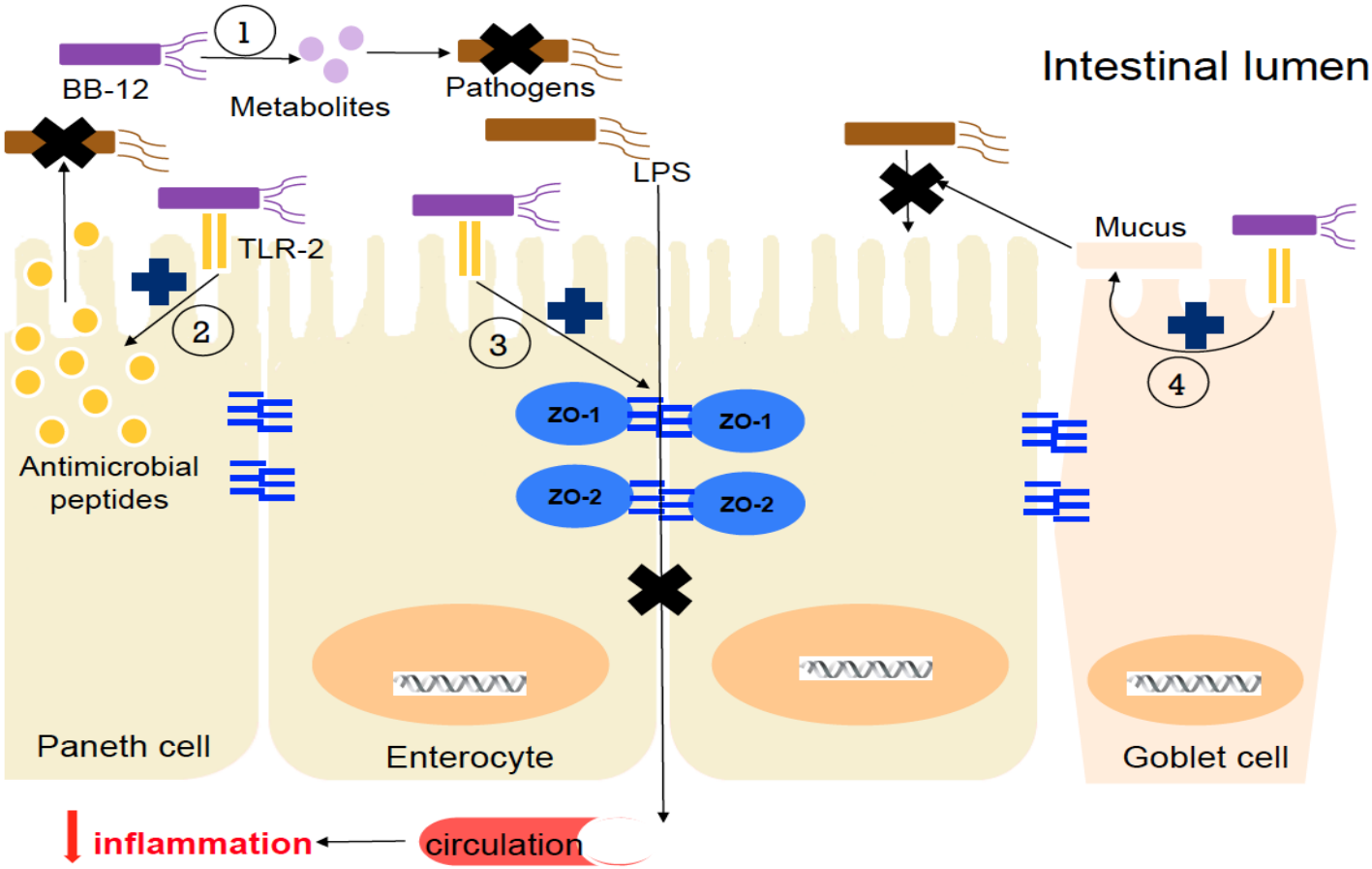


Figure 6.3. Mechanisms underlying the anti-inflammatory effect of BB-12.
In the intestinal lumen, BB-12 has been shown to interact with IECs and inhibit pathogenic microorganisms by various mechanisms. 1) BB-12 can product metabolites, such as acetate and hydrogen peroxide, and directly inhibit the growths of pathogenic microorganisms; 2) BB-12 may stimulate Paneth cells to produce antimicrobial peptides, which can kill pathogens; 3) BB-12 can improve tight junction strength between IECs, and prevents LPS from passing through between IECs; 4) BB-12 can adhere to intestinal mucin in a greater rate than pathogenic bacteria, and prevents the adhesion or interaction of pathogenic bacteria with IECs.

APPENDIX

Sample size estimation

Sample size calculation for crossover study**:** $n = \frac{\sigma_d^2 (Z_{1-\alpha/2} + Z_{1-\beta})^2}{\Delta^2}$

With a significance of α = 0.05 and power β = 0.20, $(Z_{1-\alpha/2} + Z_{1-\beta})^2 = (1.96+0.84)^2 = 7.84$

σ_d^2 = within subject variance for BB-12 group (yogurt smoothie with BB-12 added post-fermentation) and control group (baseline).

Δ= magnitude of the difference in endpoints between BB-12 group and control group.

Sample size estimation for Chapter 4

The primary immune endpoints used to estimate sample size for the study in Chapter 4 were cytokine secretion (TNF-α and IL-6) from our pilot study and published clinical trials assessing cytokine secretion from LPS-stimulated PBMCs in healthy adults who consumed BB-12 or other *B. lactis* subspecies (20, 61). These endpoints were chosen because previous reports demonstrate modest changes in the magnitude of the cytokine response upon stimulation with various *B. lactis* and high variance. Using the sample size calculation for crossover study, it was determined that the estimated sample size required to identify a mean difference of 30% in TNF-α and IL-6 secretion from LPS-stimulated PBMCs with α = 0.05 and β = 80% was 19 and 22, respectively. We estimated a high dropout rate (40%) due to the length of the study, and the final sample size was 36. However, 6 participants dropped out of the study before intervention phase, and 30 participants completed at least one allocated interventions. Based on the aforementioned calculations, a sample size of 30 was adequate to observe a treatment effect.

Detailed calculations:

1. Sample size calculation based on TNF-α secretion from LPS-stimulated PBMCs measured in healthy adults.

   Three randomized clinical trials (including our study) have investigated the effect of *B. lactis* (BB-12 or *B. lactis* CUL34) on TNF-α secretion from LPS-stimulated PBMCs in healthy adults (20, 61). Combined, 20-30% change was observed in these studies. Thus, we expect to see a 30% reduction in TNF-α secretion from LPS-stimulated PBMCs in healthy adults who consume yogurt smoothies with BB-12 added post-fermentation.

   Δ=30% * mean TNF-α secretion at baseline =30% * 1.22 (ng/ml)=0.366 (ng/ml)

   $\sigma^2=\sigma_{BL}^2+\sigma_{POST}^2=0.16+0.16=0.32$

   $n=7.84*0.32/(0.366)^2=18.7\approx19$

2. Sample size calculation based on IL-6 secretion from LPS-stimulated PBMCs measured in healthy adults.

   Three randomized clinical trials (including our study) have investigated the effect of *B. lactis* (BB-12 or *B. lactis* CUL34) on IL-6 secretion from LPS-stimulated PBMCs in healthy adults (20, 61). Combined, 0-60% change was observed in these studies. We choose the mean of percent reduction observed in previous studies, and expect to see a 30% reduction in IL-6 secretion from LPS-stimulated PBMCs in healthy adults who consume yogurt smoothies with BB-12 added post-fermentation.

   Δ=30% * mean IL-6 secretion at baseline =30% * 15.26 (ng/ml)=4.578 (ng/ml)

$\sigma^2=\sigma_{BL}^2+\sigma_{POST}^2=18.09+37.78=56.87$

$n=7.84*56.87/(4.578)^2=21.3\approx22$

Sample size calculation for Chapter 5

The primary immune endpoint used to estimate sample size for the study in Chapter 5 was self-reported total number of days with cold or flu symptoms from our pilot study and published clinical trials assessing the effect of BB-12 consumption on cold or flu severity in healthy adults (117). Using the sample size calculation for crossover study, it was determined that the estimated sample size required to identify a mean difference of 60% in total number of days with cold or flu symptoms with $\alpha = 0.05$ and $\beta = 80\%$ was 39. The dropout rate in our pilot study was 15%. If we estimate a dropout rate of 15%, the final sample size was 52. However, only 36 participants were recruited in the study, and 30 participants completed at least one allocated interventions. Thus, the assessment of the effect of BB-12 consumption on total number of days with cold or flu symptoms was underpowered.

Detailed calculations:

3. Sample size calculation based on self-reported total number of days with cold or flu symptoms in healthy adults.

   Two randomized clinical trials (including our study) have investigated the effect of BB-12 on total number of days with cold or flu symptoms in healthy adults (117). Combined, 40-70% reduction was observed in these studies. We expect to see a 60% reduction in total number of days with cold or flu symptoms in healthy adults who consume yogurt smoothies with BB-12 added post-fermentation.

Δ=60% * mean of total number of days with cold or flu symptoms=60% * 3.87 =2.268

$\sigma^2=\sigma_{BL}^2+\sigma_{POST}^2=13.07+12.03=25.1$

$n=7.84*25.1/(2.268)^2=38.3\approx39$

REFERENCES

1. Madsen K. Probiotics and the immune response. J Clin Gastroenterol 2006;40(3):232-4. doi: http://www.ncbi.nlm.nih.gov/pubmed/16633128.
2. Gill HS, Rutherfurd KJ, Cross ML, Gopal PK. Enhancement of immunity in the elderly by dietary supplementation with the probiotic Bifidobacterium lactis HN019. Am J Clin Nutr 2001;74(6):833-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/11722966.
3. Jain S, Yadav H, Sinha PR. Stimulation of innate immunity by oral administration of dahi containing probiotic Lactobacillus casei in mice. J Med Food 2008;11(4):652-6. doi: 10.1089/jmf.2006.0132.
4. Chiang BL, Sheih YH, Wang LH, Liao CK, Gill HS. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (Bifidobacterium lactis HN019): optimization and definition of cellular immune responses. Eur J Clin Nutr 2000;54(11):849-55. doi: http://www.ncbi.nlm.nih.gov/pubmed/11114680.
5. Lee YN, Youn HN, Kwon JH, Lee DH, Park JK, Yuk SS, Erdene-Ochir TO, Kim KT, Lee JB, Park SY, et al. Sublingual administration of Lactobacillus rhamnosus affects respiratory immune responses and facilitates protection against influenza virus infection in mice. Antiviral Res 2013;98(2):284-90. doi: 10.1016/j.antiviral.2013.03.013.
6. Ghadimi D, de Vrese M, Heller KJ, Schrezenmeir J. Lactic acid bacteria enhance autophagic ability of mononuclear phagocytes by increasing Th1 autophagy-promoting cytokine (IFN-gamma) and nitric oxide (NO) levels and reducing Th2 autophagy-restraining cytokines (IL-4 and IL-13) in response to Mycobacterium tuberculosis antigen. Int Immunopharmacol 2010;10(6):694-706. doi: 10.1016/j.intimp.2010.03.014.
7. Olivares M, Diaz-Ropero MP, Sierra S, Lara-Villoslada F, Fonolla J, Navas M, Rodriguez JM, Xaus J. Oral intake of Lactobacillus fermentum CECT5716 enhances the effects of influenza vaccination. Nutrition 2007;23(3):254-60. doi: 10.1016/j.nut.2007.01.004.
8. Lomax AR, Calder PC. Probiotics, immune function, infection and inflammation: a review of the evidence from studies conducted in humans. Curr Pharm Des 2009;15(13):1428-518. doi: http://www.ncbi.nlm.nih.gov/pubmed/19442167.
9. Jungersen M, Wind A, Johansen E, Christensen J, Stuer-Lauridsen B, Eskesen D. The Science behind the Probiotic Strain Bifidobacterium animalis subsp. lactis BB-12®. Microorganisms 2014;2(2):92-110. doi: 10.3390/microorganisms2020092.
10. Vernazza CL, Gibson GR, Rastall RA. Carbohydrate preference, acid tolerance and bile tolerance in five strains of Bifidobacterium. J Appl Microbiol 2006;100(4):846-53. doi: 10.1111/j.1365-2672.2006.02832.x.
11. Vinderola CG, Reinheimer JA. Lactic acid starter and probiotic bacteria: a comparative “in vitro” study of probiotic characteristics and biological barrier resistance. Food Research International 2003;36(9-10):895-904. doi: 10.1016/s0963-9969(03)00098-x.
12. He F, Ouwehan AC, Hashimoto H, Isolauri E, Benno Y, Salminen S. Adhesion of Bifidobacterium spp. to human intestinal mucus. Microbiol

Immunol 2001;45(3):259-62. doi: http://www.ncbi.nlm.nih.gov/pubmed/11345536.
13. Laparra JM, Sanz Y. Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. Lett Appl Microbiol 2009;49(6):695-701. doi: 10.1111/j.1472-765X.2009.02729.x.
14. Christensen HR, Larsen CN, Kaestel P, Rosholm LB, Sternberg C, Michaelsen KF, Frokiaer H. Immunomodulating potential of supplementation with probiotics: a dose-response study in healthy young adults. FEMS Immunol Med Microbiol 2006;47(3):380-90. doi: 10.1111/j.1574-695X.2006.00109.x.
15. Rizzardini G, Eskesen D, Calder PC, Capetti A, Jespersen L, Clerici M. Evaluation of the immune benefits of two probiotic strains Bifidobacterium animalis ssp. lactis, BB-12(R) and Lactobacillus paracasei ssp. paracasei, L. casei 431(R) in an influenza vaccination model: a randomised, double-blind, placebo-controlled study. Br J Nutr 2012;107(6):876-84. doi: 10.1017/S000711451100420X.
16. Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci 1995;78(3):491-7. doi: 10.3168/jds.S0022-0302(95)76659-0.
17. Lopez P, Gueimonde M, Margolles A, Suarez A. Distinct Bifidobacterium strains drive different immune responses in vitro. Int J Food Microbiol 2010;138(1-2):157-65. doi: 10.1016/j.ijfoodmicro.2009.12.023.
18. Kajander K, Myllyluoma E, Rajilic-Stojanovic M, Kyronpalo S, Rasmussen M, Jarvenpaa S, Zoetendal EG, de Vos WM, Vapaatalo H, Korpela R. Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. Aliment Pharmacol Ther 2008;27(1):48-57. doi: 10.1111/j.1365-2036.2007.03542.x.
19. Bin-Nun A, Bromiker R, Wilschanski M, Kaplan M, Rudensky B, Caplan M, Hammerman C. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. J Pediatr 2005;147(2):192-6. doi: 10.1016/j.jpeds.2005.03.054.
20. Kekkonen RA, Lummela N, Karjalainen H, Latvala S, Tynkkynen S, Jarvenpaa S, Kautiainen H, Julkunen I, Vapaatalo H, Korpela R. Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. World J Gastroenterol 2008;14(13):2029-36. doi: http://www.ncbi.nlm.nih.gov/pubmed/18395902.
21. Isolauri E, Juntunen M, Rautanen T, Sillanaukee P, Koivula T. A human Lactobacillus strain (Lactobacillus casei sp strain GG) promotes recovery from acute diarrhea in children. Pediatrics 1991;88(1):90-7. doi: http://www.ncbi.nlm.nih.gov/pubmed/1905394.
22. Sanders ME, Marco ML. Food formats for effective delivery of probiotics. Annu Rev Food Sci Technol 2010;1:65-85. doi: 10.1146/annurev.food.080708.100743.
23. Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JA. Functional foods and nondairy probiotic food development: trends, concepts, and products. Comprehensive Reviews in Food Science and Food Safety 2010;9(3):292-302.

24. Yang HY, Liu SL, Ibrahim SA, Zhao L, Jiang JL, Sun WF, Ren FZ. Oral administration of live Bifidobacterium substrains isolated from healthy centenarians enhanced immune function in BALB/c mice. Nutr Res 2009;29(4):281-9. doi: 10.1016/j.nutres.2009.03.010.
25. group FWw. Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Córdoba, Argentina (October 1–4, 2001) 2001.
26. Reuter G. The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol 2001;2(2):43-53. doi: http://www.ncbi.nlm.nih.gov/pubmed/11721280.
27. Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EM, Sartor RB, Sherman PM, Mayer EA. An update on the use and investigation of probiotics in health and disease. Gut 2013;62(5):787-96. doi: 10.1136/gutjnl-2012-302504.
28. Fedorak R, Demeria D. Probiotic bacteria in the prevention and the treatment of inflammatory bowel disease. Gastroenterol Clin North Am 2012;41(4):821-42. doi: 10.1016/j.gtc.2012.08.003.
29. Nanau RM, Neuman MG. Nutritional and probiotic supplementation in colitis models. Dig Dis Sci 2012;57(11):2786-810. doi: 10.1007/s10620-012-2284-3.
30. Ng SC, Hart AL, Kamm MA, Stagg AJ, Knight SC. Mechanisms of action of probiotics: recent advances. Inflamm Bowel Dis 2009;15(2):300-10. doi: 10.1002/ibd.20602.
31. Bassaganya-Riera J, Viladomiu M, Pedragosa M, De Simone C, Hontecillas R. Immunoregulatory mechanisms underlying prevention of colitis-associated colorectal cancer by probiotic bacteria. PLoS One 2012;7(4):e34676. doi: 10.1371/journal.pone.0034676.
32. Zhang LL, Chen X, Zheng PY, Luo Y, Lu GF, Liu ZQ, Huang H, Yang PC. Oral Bifidobacterium modulates intestinal immune inflammation in mice with food allergy. J Gastroenterol Hepatol 2010;25(5):928-34. doi: 10.1111/j.1440-1746.2009.06193.x.
33. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. J Gastroenterol 2009;44(1):26-46. doi: 10.1007/s00535-008-2296-0.
34. Roessler A, Friedrich U, Vogelsang H, Bauer A, Kaatz M, Hipler UC, Schmidt I, Jahreis G. The immune system in healthy adults and patients with atopic dermatitis seems to be affected differently by a probiotic intervention. Clin Exp Allergy 2008;38(1):93-102. doi: 10.1111/j.1365-2222.2007.02876.x.
35. Gianotti L, Morelli L, Galbiati F, Rocchetti S, Coppola S, Beneduce A, Gilardini C, Zonenschain D, Nespoli A, Braga M. A randomized double-blind trial on perioperative administration of probiotics in colorectal cancer patients. World J Gastroenterol 2010;16(2):167-75. doi: http://www.ncbi.nlm.nih.gov/pubmed/20066735.
36. Zhang JW, Du P, Gao J, Yang BR, Fang WJ, Ying CM. Preoperative probiotics decrease postoperative infectious complications of colorectal cancer. Am J Med Sci 2012;343(3):199-205. doi: 10.1097/MAJ.0b013e31823aace6.
37. Roller M, Clune Y, Collins K, Rechkemmer G, Watzl B. Consumption of prebiotic inulin enriched with oligofructose in combination with the probiotics

Lactobacillus rhamnosus and Bifidobacterium lactis has minor effects on selected immune parameters in polypectomised and colon cancer patients. Br J Nutr 2007;97(4):676-84. doi: 10.1017/S0007114507450292.
38. Schunter M, Chu H, Hayes TL, McConnell D, Crawford SS, Luciw PA, Bengmark S, Asmuth DM, Brown J, Bevins CL, et al. Randomized pilot trial of a synbiotic dietary supplement in chronic HIV-1 infection. BMC Complement Altern Med 2012;12:84. doi: 10.1186/1472-6882-12-84.
39. Koyama T, Kirjavainen PV, Fisher C, Anukam K, Summers K, Hekmat S, Reid G. Development and pilot evaluation of a novel probiotic mixture for the management of seasonal allergic rhinitis. Can J Microbiol 2010;56(9):730-8. doi: 10.1139/w10-061.
40. Shadnoush M, Shaker Hosseini R, Mehrabi Y, Delpisheh A, Alipoor E, Faghfoori Z, Mohammadpour N, Zaringhalam Moghadam J. Probiotic yogurt Affects Pro- and Anti-inflammatory Factors in Patients with Inflammatory Bowel Disease. Iran J Pharm Res 2013;12(4):929-36. doi: http://www.ncbi.nlm.nih.gov/pubmed/24523774.
41. Zarrati M, Shidfar F, Nourijelyani K, Mofid V, Hossein zadeh-Attar MJ, Bidad K, Najafi F, Gheflati Z, Chamari M, Salehi E. Lactobacillus acidophilus La5, Bifidobacterium BB12, and Lactobacillus casei DN001 modulate gene expression of subset specific transcription factors and cytokines in peripheral blood mononuclear cells of obese and overweight people. Biofactors 2013;39(6):633-43. doi: 10.1002/biof.1128.
42. Klein A, Friedrich U, Vogelsang H, Jahreis G. Lactobacillus acidophilus 74-2 and Bifidobacterium animalis subsp lactis DGCC 420 modulate unspecific cellular immune response in healthy adults. Eur J Clin Nutr 2008;62(5):584-93. doi: 10.1038/sj.ejcn.1602761.
43. Mendonca FH, Santos SS, Faria Ida S, Goncalves e Silva CR, Jorge AO, Leao MV. Effects of probiotic bacteria on Candida presence and IgA anti-Candida in the oral cavity of elderly. Braz Dent J 2012;23(5):534-8. doi: http://www.ncbi.nlm.nih.gov/pubmed/23306230.
44. Campeotto F, Suau A, Kapel N, Magne F, Viallon V, Ferraris L, Waligora-Dupriet AJ, Soulaines P, Leroux B, Kalach N, et al. A fermented formula in pre-term infants: clinical tolerance, gut microbiota, down-regulation of faecal calprotectin and up-regulation of faecal secretory IgA. Br J Nutr 2011;105(12):1843-51. doi: 10.1017/S0007114510005702.
45. Rautava S, Arvilommi H, Isolauri E. Specific probiotics in enhancing maturation of IgA responses in formula-fed infants. Pediatr Res 2006;60(2):221-4. doi: 10.1203/01.pdr.0000228317.72933.db.
46. Leyer GJ, Li S, Mubasher ME, Reifer C, Ouwehand AC. Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics 2009;124(2):e172-9. doi: 10.1542/peds.2008-2666.
47. Trois L, Cardoso EM, Miura E. Use of probiotics in HIV-infected children: a randomized double-blind controlled study. J Trop Pediatr 2008;54(1):19-24. doi: 10.1093/tropej/fmm066.
48. Yang YJ, Sheu BS. Probiotics-containing yogurts suppress Helicobacter pylori load and modify immune response and intestinal microbiota in the Helicobacter pylori-infected children. Helicobacter 2012;17(4):297-304. doi: 10.1111/j.1523-5378.2012.00941.x.

49. Amit-Romach E, Uni Z, Reifen R. Multistep mechanism of probiotic bacterium, the effect on innate immune system. Mol Nutr Food Res 2010;54(2):277-84. doi: 10.1002/mnfr.200800591.
50. Lollo PC, Cruz AG, Morato PN, Moura CS, Carvalho-Silva LB, Oliveira CA, Faria JA, Amaya-Farfan J. Probiotic cheese attenuates exercise-induced immune suppression in Wistar rats. J Dairy Sci 2012;95(7):3549-58. doi: 10.3168/jds.2011-5124.
51. Roller M, Pietro Femia A, Caderni G, Rechkemmer G, Watzl B. Intestinal immunity of rats with colon cancer is modulated by oligofructose-enriched inulin combined with Lactobacillus rhamnosus and Bifidobacterium lactis. Br J Nutr 2004;92(6):931-8. doi: http://www.ncbi.nlm.nih.gov/pubmed/15613255.
52. Cilieborg MS, Thymann T, Siggers R, Boye M, Bering SB, Jensen BB, Sangild PT. The incidence of necrotizing enterocolitis is increased following probiotic administration to preterm pigs. J Nutr 2011;141(2):223-30. doi: 10.3945/jn.110.128561.
53. Vlasova AN, Chattha KS, Kandasamy S, Liu Z, Esseili M, Shao L, Rajashekara G, Saif LJ. Lactobacilli and bifidobacteria promote immune homeostasis by modulating innate immune responses to human rotavirus in neonatal gnotobiotic pigs. PLoS One 2013;8(10):e76962. doi: 10.1371/journal.pone.0076962.
54. Chattha KS, Vlasova AN, Kandasamy S, Esseili MA, Siegismund C, Rajashekara G, Saif LJ. Probiotics and colostrum/milk differentially affect neonatal humoral immune responses to oral rotavirus vaccine. Vaccine 2013;31(15):1916-23. doi: 10.1016/j.vaccine.2013.02.020.
55. Pestka JJ, Ha CL, Warner RW, Lee JH, Ustunol Z. Effects of ingestion of yogurts containing Bifidobacterium and Lactobacillus acidophilus on spleen and Peyer's patch lymphocyte populations in the mouse. J Food Prot 2001;64(3):392-5. doi: http://www.ncbi.nlm.nih.gov/pubmed/11252486.
56. Roller M, Rechkemmer G, Watzl B. Prebiotic inulin enriched with oligofructose in combination with the probiotics Lactobacillus rhamnosus and Bifidobacterium lactis modulates intestinal immune functions in rats. J Nutr 2004;134(1):153-6. doi: http://www.ncbi.nlm.nih.gov/pubmed/14704309.
57. Tejada-Simon MV, Lee JH, Ustunol Z, Pestka JJ. Ingestion of yogurt containing Lactobacillus acidophilus and Bifidobacterium to potentiate immunoglobulin A responses to cholera toxin in mice. J Dairy Sci 1999;82(4):649-60. doi: 10.3168/jds.S0022-0302(99)75281-1.
58. Tejada-Simon MV, Pestka JJ. Proinflammatory cytokine and nitric oxide induction in murine macrophages by cell wall and cytoplasmic extracts of lactic acid bacteria. J Food Prot 1999;62(12):1435-44. doi: http://www.ncbi.nlm.nih.gov/pubmed/10606148.
59. Kaushal D, Kansal VK. Age-related decline in macrophage and lymphocyte functions in mice and its alleviation by treatment with probiotic Dahi containing Lactobacillus acidophilus and Bifidobacterium bifidum. J Dairy Res 2011;78(4):404-11. doi: 10.1017/S0022029911000537.
60. de Vrese M, Winkler P, Rautenberg P, Harder T, Noah C, Laue C, Ott S, Hampe J, Schreiber S, Heller K, et al. Effect of Lactobacillus gasseri PA 16/8, Bifidobacterium longum SP 07/3, B. bifidum MF 20/5 on common cold episodes: a double blind, randomized, controlled trial. Clin Nutr 2005;24(4):481-91. doi: 10.1016/j.clnu.2005.02.006.

61. Hepburn NJ, Garaiova I, Williams EA, Michael DR, Plummer S. Probiotic supplement consumption alters cytokine production from peripheral blood mononuclear cells: a preliminary study using healthy individuals. Benef Microbes 2013;4(4):313-7. doi: 10.3920/BM2013.0012.
62. Bassaganya-Riera J, Viladomiu M, Pedragosa M, De Simone C, Carbo A, Shaykhutdinov R, Jobin C, Arthur JC, Corl BA, Vogel H, et al. Probiotic bacteria produce conjugated linoleic acid locally in the gut that targets macrophage PPAR gamma to suppress colitis. PLoS One 2012;7(2):e31238. doi: 10.1371/journal.pone.0031238.
63. Mariman R, Kremer B, van Erk M, Lagerweij T, Koning F, Nagelkerken L. Gene expression profiling identifies mechanisms of protection to recurrent trinitrobenzene sulfonic acid colitis mediated by probiotics. Inflamm Bowel Dis 2012;18(8):1424-33. doi: 10.1002/ibd.22849.
64. Fitzpatrick LR, Hertzog KL, Quatse AL, Koltun WA, Small JS, Vrana K. Effects of the probiotic formulation VSL#3 on colitis in weanling rats. J Pediatr Gastroenterol Nutr 2007;44(5):561-70. doi: 10.1097/MPG.0b013e31803bda51.
65. Reiff C, Delday M, Rucklidge G, Reid M, Duncan G, Wohlgemuth S, Hormannsperger G, Loh G, Blaut M, Collie-Duguid E, et al. Balancing inflammatory, lipid, and xenobiotic signaling pathways by VSL#3, a biotherapeutic agent, in the treatment of inflammatory bowel disease. Inflamm Bowel Dis 2009;15(11):1721-36. doi: 10.1002/ibd.20999.
66. Wang X, O'Gorman MR, Bu HF, Koti V, Zuo XL, Tan XD. Probiotic preparation VSL#3 alters the distribution and phenotypes of dendritic cells within the intestinal mucosa in C57BL/10J mice. J Nutr 2009;139(8):1595-602. doi: 10.3945/jn.109.109934.
67. Mastrangeli G, Corinti S, Butteroni C, Afferni C, Bonura A, Boirivant M, Colombo P, Di Felice G. Effects of live and inactivated VSL#3 probiotic preparations in the modulation of in vitro and in vivo allergen-induced Th2 responses. Int Arch Allergy Immunol 2009;150(2):133-43. doi: 10.1159/000218116.
68. Calcinaro F, Dionisi S, Marinaro M, Candeloro P, Bonato V, Marzotti S, Corneli RB, Ferretti E, Gulino A, Grasso F, et al. Oral probiotic administration induces interleukin-10 production and prevents spontaneous autoimmune diabetes in the non-obese diabetic mouse. Diabetologia 2005;48(8):1565-75. doi: 10.1007/s00125-005-1831-2.
69. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boirivant M. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol 2005;174(6):3237-46. doi: http://www.ncbi.nlm.nih.gov/pubmed/15749854.
70. Schiavi E, Barletta B, Butteroni C, Corinti S, Boirivant M, Di Felice G. Oral therapeutic administration of a probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy. Allergy 2011;66(4):499-508. doi: 10.1111/j.1398-9995.2010.02501.x.
71. Salim SY, Young PY, Lukowski CM, Madsen KL, Sis B, Churchill TA, Khadaroo RG. VSL#3 probiotics provide protection against acute intestinal ischaemia/reperfusion injury. Benef Microbes 2013;4(4):357-65. doi: 10.3920/BM2013.0026.
72. Barletta B, Rossi G, Schiavi E, Butteroni C, Corinti S, Boirivant M, Di Felice G. Probiotic VSL#3-induced TGF-beta ameliorates food allergy

inflammation in a mouse model of peanut sensitization through the induction of regulatory T cells in the gut mucosa. Mol Nutr Food Res 2013;57(12):2233-44. doi: 10.1002/mnfr.201300028.
73. Pronio A, Montesani C, Butteroni C, Vecchione S, Mumolo G, Vestri A, Vitolo D, Boirivant M. Probiotic administration in patients with ileal pouch-anal anastomosis for ulcerative colitis is associated with expansion of mucosal regulatory cells. Inflamm Bowel Dis 2008;14(5):662-8. doi: 10.1002/ibd.20369.
74. Ashraf R, Shah NP. Immune system stimulation by probiotic microorganisms. Crit Rev Food Sci Nutr 2014;54(7):938-56. doi: 10.1080/10408398.2011.619671.
75. Christensen HR, Frokiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 2002;168(1):171-8. doi: http://www.ncbi.nlm.nih.gov/pubmed/11751960.
76. Xiao JZ, Kondo S, Yanagisawa N, Takahashi N, Odamaki T, Iwabuchi N, Miyaji K, Iwatsuki K, Togashi H, Enomoto K, et al. Probiotics in the treatment of Japanese cedar pollinosis: a double-blind placebo-controlled trial. Clin Exp Allergy 2006;36(11):1425-35. doi: 10.1111/j.1365-2222.2006.02575.x.
77. Schiffrin EJ, Brassart D, Servin AL, Rochat F, Donnet-Hughes A. Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. Am J Clin Nutr 1997;66(2):515S-20S. doi: http://www.ncbi.nlm.nih.gov/pubmed/9250141.
78. Shu Q, Lin H, Rutherfurd KJ, Fenwick SG, Prasad J, Gopal PK, Gill HS. Dietary Bifidobacterium lactis (HN019) enhances resistance to oral Salmonella typhimurium infection in mice. Microbiol Immunol 2000;44(4):213-22. doi: http://www.ncbi.nlm.nih.gov/pubmed/10832963.
79. Kim N, Kunisawa J, Kweon MN, Eog Ji G, Kiyono H. Oral feeding of Bifidobacterium bifidum (BGN4) prevents CD4(+) CD45RB(high) T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. Clin Immunol 2007;123(1):30-9. doi: 10.1016/j.clim.2006.11.005.
80. MacSharry J, O'Mahony C, Shalaby KH, Sheil B, Karmouty-Quintana H, Shanahan F, Martin JG. Immunomodulatory effects of feeding with Bifidobacterium longum on allergen-induced lung inflammation in the mouse. Pulm Pharmacol Ther 2012;25(4):325-34. doi: 10.1016/j.pupt.2012.05.011.
81. Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, Jaudszus A, Ahrens B, Groneberg DA, Wahn U, Hamelmann E. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. Clin Exp Allergy 2007;37(4):498-505. doi: 10.1111/j.1365-2222.2006.02629.x.
82. Ezendam J, de Klerk A, Gremmer ER, van Loveren H. Effects of Bifidobacterium animalis administered during lactation on allergic and autoimmune responses in rodents. Clin Exp Immunol 2008;154(3):424-31. doi: 10.1111/j.1365-2249.2008.03788.x.
83. Shu Q, Qu F, Gill HS. Probiotic treatment using Bifidobacterium lactis HN019 reduces weanling diarrhea associated with rotavirus and Escherichia coli infection in a piglet model. J Pediatr Gastroenterol Nutr 2001;33(2):171-7. doi: http://www.ncbi.nlm.nih.gov/pubmed/11568519.

84. Finamore A, Roselli M, Britti MS, Merendino N, Mengheri E. Lactobacillus rhamnosus GG and Bifidobacterium animalis MB5 induce intestinal but not systemic antigen-specific hyporesponsiveness in ovalbumin-immunized rats. J Nutr 2012;142(2):375-81. doi: 10.3945/jn.111.148924.
85. Zhou JS, Gill HS. Immunostimulatory probiotic Lactobacillus rhamnosus HN001 and Bifidobacterium lactis HN019 do not induce pathological inflammation in mouse model of experimental autoimmune thyroiditis. Int J Food Microbiol 2005;103(1):97-104. doi: 10.1016/j.ijfoodmicro.2004.11.031.
86. O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, et al. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology 2005;128(3):541-51. doi: http://www.ncbi.nlm.nih.gov/pubmed/15765388.
87. Peng S, Lin JY, Lin MY. Antiallergic effect of milk fermented with lactic acid bacteria in a murine animal model. J Agric Food Chem 2007;55(13):5092-6. doi: 10.1021/jf062869s.
88. Schabussova I, Hufnagl K, Wild C, Nutten S, Zuercher AW, Mercenier A, Wiedermann U. Distinctive anti-allergy properties of two probiotic bacterial strains in a mouse model of allergic poly-sensitization. Vaccine 2011;29(10):1981-90. doi: 10.1016/j.vaccine.2010.12.101.
89. van der Kleij H, O'Mahony C, Shanahan F, O'Mahony L, Bienenstock J. Protective effects of Lactobacillus rhamnosus [corrected] and Bifidobacterium infantis in murine models for colitis do not involve the vagus nerve. Am J Physiol Regul Integr Comp Physiol 2008;295(4):R1131-7. doi: 10.1152/ajpregu.90434.2008.
90. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic Bifidobacteria infantis: An assessment of potential antidepressant properties in the rat. J Psychiatr Res 2008;43(2):164-74. doi: 10.1016/j.jpsychires.2008.03.009.
91. Silva AM, Barbosa FH, Duarte R, Vieira LQ, Arantes RM, Nicoli JR. Effect of Bifidobacterium longum ingestion on experimental salmonellosis in mice. J Appl Microbiol 2004;97(1):29-37. doi: 10.1111/j.1365-2672.2004.02265.x.
92. Tejada-Simon MV, Ustunol Z, Pestka JJ. Ex vivo effects of lactobacilli, streptococci, and bifidobacteria ingestion on cytokine and nitric oxide production in a murine model. J Food Prot 1999;62(2):162-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/10030636.
93. Singh A, Hacini-Rachinel F, Gosoniu ML, Bourdeau T, Holvoet S, Doucet-Ladeveze R, Beaumont M, Mercenier A, Nutten S. Immune-modulatory effect of probiotic Bifidobacterium lactis NCC2818 in individuals suffering from seasonal allergic rhinitis to grass pollen: an exploratory, randomized, placebo-controlled clinical trial. Eur J Clin Nutr 2013;67(2):161-7. doi: 10.1038/ejcn.2012.197.
94. Perdigon G, Locascio M, Medici M, Pesce de Ruiz Holgado A, Oliver G. Interaction of bifidobacteria with the gut and their influence in the immune function. Biocell 2003;27(1):1-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/12847910.
95. Park JH, Um JI, Lee BJ, Goh JS, Park SY, Kim WS, Kim PH. Encapsulated Bifidobacterium bifidum potentiates intestinal IgA production.

Cell Immunol 2002;219(1):22-7. doi: http://www.ncbi.nlm.nih.gov/pubmed/12473264.
96. Paineau D, Carcano D, Leyer G, Darquy S, Alyanakian MA, Simoneau G, Bergmann JF, Brassart D, Bornet F, Ouwehand AC. Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. FEMS Immunol Med Microbiol 2008;53(1):107-13. doi: 10.1111/j.1574-695X.2008.00413.x.
97. Holscher HD, Czerkies LA, Cekola P, Litov R, Benbow M, Santema S, Alexander DD, Perez V, Sun S, Saavedra JM, et al. Bifidobacterium lactis Bb12 enhances intestinal antibody response in formula-fed infants: a randomized, double-blind, controlled trial. JPEN J Parenter Enteral Nutr 2012;36(1 Suppl):106S-17S. doi: 10.1177/0148607111430817.
98. Yang H, Liu A, Zhang M, Ibrahim SA, Pang Z, Leng X, Ren F. Oral administration of live Bifidobacterium substrains isolated from centenarians enhances intestinal function in mice. Curr Microbiol 2009;59(4):439-45. doi: 10.1007/s00284-009-9457-0.
99. Shu Q, Gill HS. A dietary probiotic (Bifidobacterium lactis HN019) reduces the severity of Escherichia coli O157:H7 infection in mice. Med Microbiol Immunol 2001;189(3):147-52. doi: http://www.ncbi.nlm.nih.gov/pubmed/11388612.
100. Hiramatsu Y, Hosono A, Konno T, Nakanishi Y, Muto M, Suyama A, Hachimura S, Sato R, Takahashi K, Kaminogawa S. Orally administered Bifidobacterium triggers immune responses following capture by CD11c(+) cells in Peyer's patches and cecal patches. Cytotechnology 2011;63(3):307-17. doi: 10.1007/s10616-011-9349-6.
101. Yasui H, Shida K, Matsuzaki T, Yokokura T. Immunomodulatory function of lactic acid bacteria. Antonie Van Leeuwenhoek 1999;76(1-4):383-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/10532394.
102. Lewis MC, Patel DV, Fowler J, Duncker S, Zuercher AW, Mercenier A, Bailey M. Dietary supplementation with Bifidobacterium lactis NCC2818 from weaning reduces local immunoglobulin production in lymphoid-associated tissues but increases systemic antibodies in healthy neonates. Br J Nutr 2013;110(7):1243-52. doi: 10.1017/S0007114513000251.
103. Zhang L, Su P, Henriksson A, O'Rourke J, Mitchell H. Investigation of the immunomodulatory effects of Lactobacillus casei and Bifidobacterium lactis on Helicobacter pylori infection. Helicobacter 2008;13(3):183-90. doi: 10.1111/j.1523-5378.2008.00595.x.
104. Kim H, Kwack K, Kim DY, Ji GE. Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model. FEMS Immunol Med Microbiol 2005;45(2):259-67. doi: 10.1016/j.femsim.2005.05.005.
105. Gibson RA, Barclay D, Marshall H, Moulin J, Maire JC, Makrides M. Safety of supplementing infant formula with long-chain polyunsaturated fatty acids and Bifidobacterium lactis in term infants: a randomised controlled trial. Br J Nutr 2009;101(11):1706-13. doi: 10.1017/S0007114508084080.
106. Macfarlane S, Cleary S, Bahrami B, Reynolds N, Macfarlane GT. Synbiotic consumption changes the metabolism and composition of the gut microbiota in older people and modifies inflammatory processes: a randomised, double-blind, placebo-controlled crossover study. Aliment Pharmacol Ther 2013;38(7):804-16. doi: 10.1111/apt.12453.

107. Arunachalam K, Gill HS, Chandra RK. Enhancement of natural immune function by dietary consumption of Bifidobacterium lactis (HN019). Eur J Clin Nutr 2000;54(3):263-7. doi: http://www.ncbi.nlm.nih.gov/pubmed/10713750.
108. Sheil B, MacSharry J, O'Callaghan L, O'Riordan A, Waters A, Morgan J, Collins JK, O'Mahony L, Shanahan F. Role of interleukin (IL-10) in probiotic-mediated immune modulation: an assessment in wild-type and IL-10 knock-out mice. Clin Exp Immunol 2006;144(2):273-80. doi: 10.1111/j.1365-2249.2006.03051.x.
109. Groeger D, O'Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, Shanahan F, Quigley EM. Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. Gut Microbes 2013;4(4):325-39. doi: 10.4161/gmic.25487.
110. Foo NP, Ou Yang H, Chiu HH, Chan HY, Liao CC, Yu CK, Wang YJ. Probiotics prevent the development of 1,2-dimethylhydrazine (DMH)-induced colonic tumorigenesis through suppressed colonic mucosa cellular proliferation and increased stimulation of macrophages. J Agric Food Chem 2011;59(24):13337-45. doi: 10.1021/jf203444d.
111. Garrigues C, Johansen E, Pedersen MB. Complete genome sequence of Bifidobacterium animalis subsp. lactis BB-12, a widely consumed probiotic strain. J Bacteriol 2010;192(9):2467-8. doi: 10.1128/JB.00109-10.
112. Barrangou R, Briczinski EP, Traeger LL, Loquasto JR, Richards M, Horvath P, Coute-Monvoisin AC, Leyer G, Rendulic S, Steele JL, et al. Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04. Journal of Bacteriology 2009;191(13):4144-51. doi: JB.00155-09 [pii]
10.1128/JB.00155-09.
113. Leyer GJ, Li SG, Mubasher ME, Reifer C, Ouwehand AC. Probiotic Effects on Cold and Influenza-Like Symptom Incidence and Duration in Children. Pediatrics 2009;124(2):E172-E9. doi: <Go to ISI>://000268377000056.
114. Sanders ME. Summary of probiotic activities of *Bifidobacterium lactis* HN019. Journal of Clinical Gastroenterology 2006;40(9):776-83.
115. Marteau P, Cuillerier E, Meance S, Gerhardt MF, Myara A, Bouvier M, Bouley C, Tondu F, Bommelaer G, Grimaud JC. *Bifidobacterium animalis* strain DN-173 010 shortens the colonic transit time in healthy women: A double-blind, randomized, controlled study. Alimentary Pharmacology and Therapeutics 2002;16:587-93.
116. Philippe D, Favre L, Foata F, Adolfsson O, Perruisseau-Carrier G, Vidal K, Reuteler G, Dayer-Schneider J, Mueller C, Blum S. Bifidobacterium lactis attenuates onset of inflammation in a murine model of colitis. World J Gastroenterol 2011;17(4):459-69. doi: 10.3748/wjg.v17.i4.459.
117. Smith TJ, Rigassio-Radler D, Denmark R, Haley T, Touger-Decker R. Effect of Lactobacillus rhamnosus LGG(R) and Bifidobacterium animalis ssp. lactis BB-12(R) on health-related quality of life in college students affected by upper respiratory infections. Br J Nutr 2013;109(11):1999-2007. doi: 10.1017/S0007114512004138.
118. Rautava S, Salminen S, Isolauri E. Specific probiotics in reducing the risk of acute infections in infancy--a randomised, double-blind, placebo-

controlled study. Br J Nutr 2009;101(11):1722-6. doi: 10.1017/S0007114508116282.
119. Ringel-Kulka T, Kotch JB, Jensen ET, Savage E, Weber DJ. Randomized, Double-Blind, Placebo-Controlled Study of Synbiotic Yogurt Effect on the Health of Children. J Pediatr 2015. doi: 10.1016/j.jpeds.2015.02.038.
120. Taipale T, Pienihakkinen K, Isolauri E, Larsen C, Brockmann E, Alanen P, Jokela J, Soderling E. Bifidobacterium animalis subsp. lactis BB-12 in reducing the risk of infections in infancy. Br J Nutr 2011;105(3):409-16. doi: 10.1017/S0007114510003685.
121. Turner RB. Epidemiology, pathogenesis, and treatment of the common cold. Ann Allergy Asthma Immunol 1997;78(6):531-9; quiz 9-40. doi: 10.1016/S1081-1206(10)63213-9.
122. Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, Lambkin-Williams R, Gilbert A, Oxford J, Nicholas B, et al. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. Nat Med 2012;18(2):274-80. doi: 10.1038/nm.2612.
123. Hillaire ML, van Trierum SE, Kreijtz JH, Bodewes R, Geelhoed-Mieras MM, Nieuwkoop NJ, Fouchier RA, Kuiken T, Osterhaus AD, Rimmelzwaan GF. Cross-protective immunity against influenza pH1N1 2009 viruses induced by seasonal influenza A (H3N2) virus is mediated by virus-specific T-cells. J Gen Virol 2011;92(Pt 10):2339-49. doi: 10.1099/vir.0.033076-0.
124. Schultz-Cherry S. Role of NK cells in influenza infection. Curr Top Microbiol Immunol 2015;386:109-20. doi: 10.1007/82_2014_403.
125. Sachdeva A, Nagpal J. Effect of fermented milk-based probiotic preparations on Helicobacter pylori eradication: a systematic review and meta-analysis of randomized-controlled trials. Eur J Gastroenterol Hepatol 2009;21(1):45-53. doi: 10.1097/MEG.0b013e32830d0eff.
126. Urbanska AM, Bhathena J, Martoni C, Prakash S. Estimation of the potential antitumor activity of microencapsulated Lactobacillus acidophilus yogurt formulation in the attenuation of tumorigenesis in Apc(Min/+) mice. Dig Dis Sci 2009;54(2):264-73. doi: 10.1007/s10620-008-0363-2.
127. Calder PC, Kew S. The immune system: a target for functional foods? Br J Nutr 2002;88 Suppl 2:S165-77. doi: 10.1079/BJN2002682.
128. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. Nat Immunol 2004;5(2):133-9. doi: 10.1038/ni1033.
129. Pedersen BK, Hoffman-Goetz L. Exercise and the immune system: regulation, integration, and adaptation. Physiol Rev 2000;80(3):1055-81. doi: http://www.ncbi.nlm.nih.gov/pubmed/10893431.
130. Schuurs AH, Verheul HA. Effects of gender and sex steroids on the immune response. J Steroid Biochem 1990;35(2):157-72. doi: http://www.ncbi.nlm.nih.gov/pubmed/2407902.
131. Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, Samartin S, Sanderson IR, Van Loo J, Vas Dias FW, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br J Nutr 2005;94(3):452-81. doi: http://www.ncbi.nlm.nih.gov/pubmed/16176618.

132. MacGregor RR. Alcohol and immune defense. JAMA 1986;256(11):1474-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/3747066.
133. Yaqoob P, Newsholme EA, Calder PC. Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells. Cytokine 1999;11(8):600-5. doi: 10.1006/cyto.1998.0471.
134. Cieslak TJ, Frost G, Klentrou P. Effects of physical activity, body fat, and salivary cortisol on mucosal immunity in children. J Appl Physiol (1985) 2003;95(6):2315-20. doi: 10.1152/japplphysiol.00400.2003.
135. Klentrou P, Cieslak T, MacNeil M, Vintinner A, Plyley M. Effect of moderate exercise on salivary immunoglobulin A and infection risk in humans. Eur J Appl Physiol 2002;87(2):153-8. doi: 10.1007/s00421-002-0609-1.
136. Gleeson M, Bishop N, Oliveira M, McCauley T, Tauler P, Muhamad AS. Respiratory infection risk in athletes: association with antigen-stimulated IL-10 production and salivary IgA secretion. Scand J Med Sci Sports 2012;22(3):410-7. doi: 10.1111/j.1600-0838.2010.01272.x.
137. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr., et al. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. JAMA 2003;289(19):2560-72. doi: 10.1001/jama.289.19.2560.
138. Bouchard C, Tremblay A, Leblanc C, Lortie G, Savard R, Theriault G. A method to assess energy expenditure in children and adults. Am J Clin Nutr 1983;37(3):461-7. doi: http://www.ncbi.nlm.nih.gov/pubmed/6829488.
139. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18(6):499-502. doi: http://www.ncbi.nlm.nih.gov/pubmed/4337382.
140. Calder PC, Jackson AA. Undernutrition, infection and immune function. Nutr Res Rev 2000;13(1):3-29. doi: 10.1079/095442200108728981.
141. Nieman DC. Exercise, upper respiratory tract infection, and the immune system. Med Sci Sports Exerc 1994;26(2):128-39. doi: http://www.ncbi.nlm.nih.gov/pubmed/8164529.
142. Cox AJ, Gleeson M, Pyne DB, Callister R, Hopkins WG, Fricker PA. Clinical and laboratory evaluation of upper respiratory symptoms in elite athletes. Clin J Sport Med 2008;18(5):438-45. doi: 10.1097/JSM.0b013e318181e501.
143. Nieman DC, Nehlsen-Cannarella SL, Markoff PA, Balk-Lamberton AJ, Yang H, Chritton DB, Lee JW, Arabatzis K. The effects of moderate exercise training on natural killer cells and acute upper respiratory tract infections. Int J Sports Med 1990;11(6):467-73. doi: 10.1055/s-2007-1024839.
144. Scrimshaw NS, SanGiovanni JP. Synergism of nutrition, infection, and immunity: an overview. Am J Clin Nutr 1997;66(2):464S-77S. doi: http://www.ncbi.nlm.nih.gov/pubmed/9250134.
145. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol 2004;25(1):4-7. doi: http://www.ncbi.nlm.nih.gov/pubmed/14698276.
146. Chubak J, McTiernan A, Sorensen B, Wener MH, Yasui Y, Velasquez M, Wood B, Rajan KB, Wetmore CM, Potter JD, et al. Moderate-intensity exercise reduces the incidence of colds among postmenopausal women. Am J Med 2006;119(11):937-42. doi: 10.1016/j.amjmed.2006.06.033.

147. Maggini S, Wintergerst ES, Beveridge S, Hornig DH. Selected vitamins and trace elements support immune function by strengthening epithelial barriers and cellular and humoral immune responses. Br J Nutr 2007;98 Suppl 1:S29-35. doi: 10.1017/S0007114507832971.
148. Ferencik M, Ebringer L. Modulatory effects of selenium and zinc on the immune system. Folia Microbiol (Praha) 2003;48(3):417-26. doi: http://www.ncbi.nlm.nih.gov/pubmed/12879758.
149. Cantorna MT, Waddell A. The vitamin D receptor turns off chronically activated T cells. Ann N Y Acad Sci 2014;1317:70-5. doi: 10.1111/nyas.12408.
150. Calder PC. n-3 fatty acids, inflammation and immunity: new mechanisms to explain old actions. Proc Nutr Soc 2013;72(3):326-36. doi: 10.1017/S0029665113001031.
151. Abb J, Abb H, Deinhardt F. Age-related decline of human interferon alpha and interferon gamma production. Blut 1984;48(5):285-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/6326901.
152. Kovaiou RD, Herndler-Brandstetter D, Grubeck-Loebenstein B. Age-related changes in immunity: implications for vaccination in the elderly. Expert Rev Mol Med 2007;9(3):1-17. doi: 10.1017/S1462399407000221.
153. Hance KW, Rogers CJ, Hursting SD, Greiner JW. Combination of physical activity, nutrition, or other metabolic factors and vaccine response. Front Biosci 2007;12:4997-5029. doi: http://www.ncbi.nlm.nih.gov/pubmed/17569626.
154. Rogers CJ, Berrigan D, Zaharoff DA, Hance KW, Patel AC, Perkins SN, Schlom J, Greiner JW, Hursting SD. Energy restriction and exercise differentially enhance components of systemic and mucosal immunity in mice. J Nutr 2008;138(1):115-22. doi: http://www.ncbi.nlm.nih.gov/pubmed/18156413.
155. Weiss G. Iron and immunity: a double-edged sword. Eur J Clin Invest 2002;32 Suppl 1:70-8. doi: http://www.ncbi.nlm.nih.gov/pubmed/11886435.
156. Sun YY, Ma AG, Yang F, Zhang FZ, Luo YB, Jiang DC, Han XX, Liang H. A combination of iron and retinol supplementation benefits iron status, IL-2 level and lymphocyte proliferation in anemic pregnant women. Asia Pac J Clin Nutr 2010;19(4):513-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/21147712.
157. Kuziel WA, Greene WC. Interleukin-2 and the IL-2 receptor: new insights into structure and function. J Invest Dermatol 1990;94(6 Suppl):27S-32S. doi: http://www.ncbi.nlm.nih.gov/pubmed/1693645.
158. Kasahara T, Hooks JJ, Dougherty SF, Oppenheim JJ. Interleukin 2-mediated immune interferon (IFN-gamma) production by human T cells and T cell subsets. J Immunol 1983;130(4):1784-9. doi: http://www.ncbi.nlm.nih.gov/pubmed/6403613.
159. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 2004;75(2):163-89. doi: 10.1189/jlb.0603252.
160. Cebrian M, Yague E, Rincon M, Lopez-Botet M, de Landazuri MO, Sanchez-Madrid F. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. J Exp Med 1988;168(5):1621-37. doi: http://www.ncbi.nlm.nih.gov/pubmed/2903209.
161. Nakamura S, Sung SS, Bjorndahl JM, Fu SM. Human T cell activation. IV. T cell activation and proliferation via the early activation antigen EA 1. J

Exp Med 1989;169(3):677-89. doi: http://www.ncbi.nlm.nih.gov/pubmed/2466940.
162. Risso A, Cosulich ME, Rubartelli A, Mazza MR, Bargellesi A. MLR3 molecule is an activation antigen shared by human B, T lymphocytes and T cell precursors. Eur J Immunol 1989;19(2):323-8. doi: 10.1002/eji.1830190216.
163. Testi R, Phillips JH, Lanier LL. T cell activation via Leu-23 (CD69). J Immunol 1989;143(4):1123-8. doi: http://www.ncbi.nlm.nih.gov/pubmed/2501389.
164. Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. Clin Diagn Lab Immunol 1996;3(3):301-4. doi: http://www.ncbi.nlm.nih.gov/pubmed/8705673.
165. Maino VC, Suni MA, Ruitenberg JJ. Rapid flow cytometric method for measuring lymphocyte subset activation. Cytometry 1995;20(2):127-33. doi: 10.1002/cyto.990200205.
166. Caruso A, Licenziati S, Corulli M, Canaris AD, De Francesco MA, Fiorentini S, Peroni L, Fallacara F, Dima F, Balsari A, et al. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. Cytometry 1997;27(1):71-6. doi: http://www.ncbi.nlm.nih.gov/pubmed/9000587.
167. Alari-Pahissa E, Notario L, Lorente E, Vega-Ramos J, Justel A, Lopez D, Villadangos JA, Lauzurica P. CD69 does not affect the extent of T cell priming. PLoS One 2012;7(10):e48593. doi: 10.1371/journal.pone.0048593.
168. Kishimoto T. IL-6: from its discovery to clinical applications. Int Immunol 2010;22(5):347-52. doi: 10.1093/intimm/dxq030.
169. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011;1813(5):878-88. doi: 10.1016/j.bbamcr.2011.01.034.
170. Hussell T, Goulding J. Structured regulation of inflammation during respiratory viral infection. Lancet Infect Dis 2010;10(5):360-6. doi: 10.1016/S1473-3099(10)70067-0.
171. Nieters A, Brems S, Becker N. Cross-sectional study on cytokine polymorphisms, cytokine production after T-cell stimulation and clinical parameters in a random sample of a German population. Hum Genet 2001;108(3):241-8. doi: http://www.ncbi.nlm.nih.gov/pubmed/11354638.
172. Ramshaw IA, Ramsay AJ, Karupiah G, Rolph MS, Mahalingam S, Ruby JC. Cytokines and immunity to viral infections. Immunol Rev 1997;159:119-35. doi: http://www.ncbi.nlm.nih.gov/pubmed/9416507.
173. Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo P. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. J Clin Invest 1998;102(7):1369-76. doi: 10.1172/JCI2629.
174. Becker N, Krause G, Rensch K, Meuer SC. Epidemiologic investigation of serum levels of the soluble forms of CD25, CD54 and CD58, and T cell responsiveness after stimulation via the CD2-dependent pathway in a random sample of the general population. Immunobiology 1996;195(1):47-60. doi: 10.1016/S0171-2985(96)80005-2.

175. de Albuquerque AC, Rocha LQ, de Morais Batista AH, Teixeira AB, Dos Santos DB, Nogueira NA. Association of polymorphism +874 A/T of interferon-gamma and susceptibility to the development of tuberculosis: meta-analysis. Eur J Clin Microbiol Infect Dis 2012;31(11):2887-95. doi: 10.1007/s10096-012-1660-4.
176. Merenstein D, Gonzalez J, Young AG, Roberts RF, Sanders ME, Petterson S. Study to investigate the potential of probiotics in children attending school. Eur J Clin Nutr 2011;65(4):447-53. doi: 10.1038/ejcn.2010.290.
177. Laroia S, Martin JH. Methods for enumerating and propagating bifidobacteria. Cultured dairy products journal 1991;26(2):32-4.
178. Meng H, Lee Y, Ba Z, Fleming JA, Furumoto EJ, Roberts RF, Kris-Etherton PM, Rogers CJ. In vitro production of IL-6 and IFN-γ is influenced by dietary variables and predicts upper respiratory tract infection incidence and severity respectively in young adults. Front Immunol 2015;6(94). doi: doi: 10.3389/fimmu.2015.00094.
179. Ruiz PA, Hoffmann M, Szcesny S, Blaut M, Haller D. Innate mechanisms for Bifidobacterium lactis to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats. Immunology 2005;115(4):441-50. doi: 10.1111/j.1365-2567.2005.02176.x.
180. Shahani KM, Chandan RC. Nutritional and healthful aspects of cultured and culture-containing dairy foods. J Dairy Sci 1979;62(10):1685-94. doi: 10.3168/jds.S0022-0302(79)83481-5.
181. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001;1(2):135-45. doi: 10.1038/35100529.
182. Tien MT, Girardin SE, Regnault B, Le Bourhis L, Dillies MA, Coppee JY, Bourdet-Sicard R, Sansonetti PJ, Pedron T. Anti-inflammatory effect of Lactobacillus casei on Shigella-infected human intestinal epithelial cells. J Immunol 2006;176(2):1228-37. doi: http://www.ncbi.nlm.nih.gov/pubmed/16394013.
183. Nakamura YK, Omaye ST. Metabolic diseases and pro- and prebiotics: Mechanistic insights. Nutr Metab (Lond) 2012;9(1):60. doi: 10.1186/1743-7075-9-60.
184. Amaral FA, Sachs D, Costa VV, Fagundes CT, Cisalpino D, Cunha TM, Ferreira SH, Cunha FQ, Silva TA, Nicoli JR, et al. Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 2008;105(6):2193-7. doi: 10.1073/pnas.0711891105.
185. Okada Y, Tsuzuki Y, Hokari R, Komoto S, Kurihara C, Kawaguchi A, Nagao S, Miura S. Anti-inflammatory effects of the genus Bifidobacterium on macrophages by modification of phospho-I kappaB and SOCS gene expression. Int J Exp Pathol 2009;90(2):131-40. doi: 10.1111/j.1365-2613.2008.00632.x.
186. Riedel CU, Foata F, Philippe D, Adolfsson O, Eikmanns BJ, Blum S. Anti-inflammatory effects of bifidobacteria by inhibition of LPS-induced NF-kappaB activation. World J Gastroenterol 2006;12(23):3729-35. doi: http://www.ncbi.nlm.nih.gov/pubmed/16773690.
187. Martins FS, Silva AA, Vieira AT, Barbosa FH, Arantes RM, Teixeira MM, Nicoli JR. Comparative study of Bifidobacterium animalis, Escherichia

coli, Lactobacillus casei and Saccharomyces boulardii probiotic properties. Arch Microbiol 2009;191(8):623-30. doi: 10.1007/s00203-009-0491-x.
188. Andreasen AS, Krabbe KS, Krogh-Madsen R, Taudorf S, Pedersen BK, Moller K. Human endotoxemia as a model of systemic inflammation. Curr Med Chem 2008;15(17):1697-705. doi: http://www.ncbi.nlm.nih.gov/pubmed/18673219.
189. Commane DM, Shortt CT, Silvi S, Cresci A, Hughes RM, Rowland IR. Effects of fermentation products of pro- and prebiotics on trans-epithelial electrical resistance in an in vitro model of the colon. Nutr Cancer 2005;51(1):102-9. doi: 10.1207/s15327914nc5101_14.
190. Collado MC, Meriluoto J, Salminen S. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Lett Appl Microbiol 2007;45(4):454-60. doi: 10.1111/j.1472-765X.2007.02212.x.
191. Mohan R, Koebnick C, Schildt J, Schmidt S, Mueller M, Possner M, Radke M, Blaut M. Effects of Bifidobacterium lactis Bb12 supplementation on intestinal microbiota of preterm infants: a double-blind, placebo-controlled, randomized study. J Clin Microbiol 2006;44(11):4025-31. doi: 10.1128/JCM.00767-06.
192. Ahmed M, Prasad J, Gill H, Stevenson L, Gopal P. Impact of consumption of different levels of Bifidobacterium lactis HN019 on the intestinal microflora of elderly human subjects. J Nutr Health Aging 2007;11(1):26-31. doi: http://www.ncbi.nlm.nih.gov/pubmed/17315077.
193. Lesniewska V, Rowland I, Cani PD, Neyrinck AM, Delzenne NM, Naughton PJ. Effect on components of the intestinal microflora and plasma neuropeptide levels of feeding Lactobacillus delbrueckii, Bifidobacterium lactis, and inulin to adult and elderly rats. Appl Environ Microbiol 2006;72(10):6533-8. doi: 10.1128/AEM.00915-06.
194. Trinchieri G. Biology of natural killer cells. Adv Immunol 1989;47:187-376. doi: http://www.ncbi.nlm.nih.gov/pubmed/2683611.
195. Meydani SN, Ha WK. Immunologic effects of yogurt. Am J Clin Nutr 2000;71(4):861-72. doi: http://www.ncbi.nlm.nih.gov/pubmed/10731490.
196. Albring A, Wendt L, Benson S, Witzke O, Kribben A, Engler H, Schedlowski M. Placebo effects on the immune response in humans: the role of learning and expectation. PLoS One 2012;7(11):e49477. doi: 10.1371/journal.pone.0049477.
197. Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol 2002;2(10):725-34. doi: 10.1038/nri910.
198. Gaffen SL, Liu KD. Overview of interleukin-2 function, production and clinical applications. Cytokine 2004;28(3):109-23. doi: 10.1016/j.cyto.2004.06.010.
199. Paul S, Schaefer BC. A new look at T cell receptor signaling to nuclear factor-kappaB. Trends Immunol 2013;34(6):269-81. doi: 10.1016/j.it.2013.02.002.
200. Hemar A, Cereghini S, Cornet V, Blank V, Israel A, Greene WC, Dautry-Varsat A. Kappa B binding proteins are constitutively expressed in an IL-2 autocrine human T cell line. J Immunol 1991;146(7):2409-16. doi: http://www.ncbi.nlm.nih.gov/pubmed/2005404.
201. Alander M, Mättö J, Kneifel W, Johansson M, Kögler B, Crittenden R, Mattila-Sandholm T, Saarela M. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence

of Bifidobacterium lactis Bb-12 in the gastrointestinal tract. International Dairy Journal 2001;11(10):817-25.
202. Savard P, Lamarche B, Paradis ME, Thiboutot H, Laurin E, Roy D. Impact of Bifidobacterium animalis subsp. lactis BB-12 and, Lactobacillus acidophilus LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. Int J Food Microbiol 2011;149(1):50-7. doi: 10.1016/j.ijfoodmicro.2010.12.026.
203. Mättö J, Fondén R, Tolvanen T, von Wright A, Vilpponen-Salmela T, Satokari R, Saarela M. Intestinal survival and persistence of probiotic Lactobacillus and Bifidobacterium strains administered in triple-strain yoghurt. International dairy journal 2006;16(10):1174-80.
204. Hemarajata P, Versalovic J. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. Therap Adv Gastroenterol 2013;6(1):39-51. doi: 10.1177/1756283X12459294.
205. Pedroza-Pacheco I, Madrigal A, Saudemont A. Interaction between natural killer cells and regulatory T cells: perspectives for immunotherapy. Cell Mol Immunol 2013;10(3):222-9. doi: 10.1038/cmi.2013.2.
206. Yang L, Pang Y, Moses HL. TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. Trends Immunol 2010;31(6):220-7. doi: 10.1016/j.it.2010.04.002.
207. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol 2008;180(9):5771-7. doi: http://www.ncbi.nlm.nih.gov/pubmed/18424693.
208. Xu M, Mizoguchi I, Morishima N, Chiba Y, Mizuguchi J, Yoshimoto T. Regulation of antitumor immune responses by the IL-12 family cytokines, IL-12, IL-23, and IL-27. Clin Dev Immunol 2010;2010. doi: 10.1155/2010/832454.
209. Hassan GS, Mourad W. An unexpected role for MHC class II. Nat Immunol 2011;12(5):375-6. doi: 10.1038/ni.2023.
210. Romani J, Caixas A, Escote X, Carrascosa JM, Ribera M, Rigla M, Vendrell J, Luelmo J. Lipopolysaccharide-binding protein is increased in patients with psoriasis with metabolic syndrome, and correlates with C-reactive protein. Clin Exp Dermatol 2013;38(1):81-4. doi: 10.1111/ced.12007.
211. Settachaimongkon S, van Valenberg HJ, Winata V, Wang X, Nout MJ, van Hooijdonk TC, Zwietering MH, Smid EJ. Effect of sublethal preculturing on the survival of probiotics and metabolite formation in set-yoghurt. Food Microbiol 2015;49:104-15. doi: 10.1016/j.fm.2015.01.011.
212. Taverniti V, Guglielmetti S. The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: proposal of paraprobiotic concept). Genes Nutr 2011;6(3):261-74. doi: 10.1007/s12263-011-0218-x.
213. Lebeer S, Vanderleyden J, De Keersmaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 2010;8(3):171-84. doi: 10.1038/nrmicro2297.
214. Fanning S, Hall LJ, Cronin M, Zomer A, MacSharry J, Goulding D, Motherway MO, Shanahan F, Nally K, Dougan G, et al. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Natl Acad Sci U S A 2012;109(6):2108-13. doi: 10.1073/pnas.1115621109.

215. Mozzi F, Ortiz ME, Bleckwedel J, De Vuyst L, Pescuma M. Metabolomics as a tool for the comprehensive understanding of fermented and functional foods with lactic acid bacteria. Food Research International 2013;54(1):1152-61.
216. Leber B, Tripolt NJ, Blattl D, Eder M, Wascher TC, Pieber TR, Stauber R, Sourij H, Oettl K, Stadlbauer V. The influence of probiotic supplementation on gut permeability in patients with metabolic syndrome: an open label, randomized pilot study. Eur J Clin Nutr 2012;66(10):1110-5. doi: 10.1038/ejcn.2012.103.
217. Teixeira TF, Souza NC, Chiarello PG, Franceschini SC, Bressan J, Ferreira CL, Peluzio Mdo C. Intestinal permeability parameters in obese patients are correlated with metabolic syndrome risk factors. Clin Nutr 2012;31(5):735-40. doi: 10.1016/j.clnu.2012.02.009.

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PUBLICATIONS

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Meng, H., Lee, Y., Fleming, J., Roberts, R.F., Kris-Etherton, P., Ba, Z., Furumoto, E.J., Rogers, C.J. In vitro production of IL-6 and IFN-ɣ predict upper respiratory tract infection incidence and severity in young adults. *Front. Immunol*. 04 March 2015. doi: 10.3389/fimmu.2015.00094.

Zhang, M., Zou, X., Lin, X., Bian, J., **Meng, H**., & Liu, D. (2015). Effect of Excessive Potassium Iodide on Rat Aorta Endothelial Cells. *Biological trace element research*, 1-9.

Turbitt, W.J., Black, A.J., Collins, S.C, **Meng, H**., Xu, H., Washington, S., Aliaga, C., El-Bayoumy, K., Manni, A., Rogers, C.J., Fish oil differentially alters T cell function and tumor growth and induces the emergence of $Gr1^+/CD11b^+$ cells in two models of mammary carcinogenesis. *Nutrition & Cancer.* (Accepted)

Meng, H., Ba, Z., Lee, Y., Peng, J., Lin, J., Roberts, R.F., Kris-Etherton, P., Fleming, J., Furumoto, E.J., Rogers, C.J. Consumption of yogurt smoothie with *Bifidobacterium animalis* subsp. *lactis* BB-12 added post-fermentation reduced expression of TLR-2 on peripheral blood-derived monocytes and pro-inflammatory cytokine secretion in young adults (*submitted to the Journal of Nutrition*).

Meng, H., Lee, Y., Ba, Z., Peng, J., Lin, J., Boyer, A.S., Roberts, R.F., Kris-Etherton, P., Fleming, J., Furumoto, E.J., Rogers, C.J. Consumption of *Bifidobacterium animalis* subsp. *lactis* BB-12 impacts upper respiratory tract infection and the function of T cells and NK cells in young adults (for *submission to the Journal of Nutrition*).

AWARDS AND HONORS

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