Modification of the animal immune system by feeding probiotics

Mohamed Abdulraheem Osman

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Modification of the animal immune system by feeding probiotics

by

Mohamed Abdulraheem Osman

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Ames, Iowa

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Probiotics, Prebiotics, and Microbiota: Historical Background

1.1.1 Probiotics

Probiotics are defined as “live microorganisms, which when administered in adequate amounts to the host confer health benefits (WHO, 2001)”. The term probiotics originates from the Greek language “pro-bios” means “for life” (Schrezenmeir and de Verse, 2001).

Use of probiotics to treat humans ailments emerged early in human history. In the era before Jesus Christ, Pilinus, a Roman historian, described the use of fermented milk to treat digestive system inflammations (Caramaia and Silvi, 2011). Genesis 18:1-8 in a Persian version of the Old Testament stated that “Abraham owed his longevity to the consumption of the sour milk” (Schrezenmeir and de Verse, 2001). Ancient Indian, Egyptian, and Sumerian civilizations believed that fermented milk can cure infection with Tuberculosis. Thus, they used fermented milk as “a broad-spectrum therapeutic” to rid blood of pathogens and toxins and even as “sleep aid” that prevents insomnia and induces hypnosis (Caramaia and Silvi, 2011; Malago et al., 2011).

The recent history of probiotics, however, started in the early 1900s. Metchnikoff (1845-1916) made the elegant observation that the shepherds of Caucasus and Bulgaria had longer average life than the Americans (87 vs. 48 years) (Malago et al., 2011; Caramaia and Silvi, 2011). Metchnikoff attributed shepherds’ longevity to the consumption of fermented milk, a valuable source of antiputrefactive microorganisms. He believed that certain colonic-inhabitant microbiota putrefy foods resulting in the production of toxic metabolites in the gut (Malago et al., 2011; Caramaia and Silvi, 2011).
In 1906, the French Society “Le Fermente” produced the first nutraceutical probiotics-enriched yoghurt named “Lactobacilline” that contained *Lactobacillus delbruekii* subspecies *bulgaricus* and was intended as a therapy for children’s diarrhea (Malago et al, 2009).

The therapeutic value of Lactobacilline as a treatment for children’s diarrhea gained more credit after Metchnikoff published his book “The Prolongation of Life: Optimistic Studies” in 1907 in which he emphasized the health benefit of some members of the digestive system microbiota. The rationale for manufacturing Lactobacilline is based on the assumption that the type of diet consumed determines the microbial composition of the digestive system microbiota. Thus, the digestive system microbiota can be transformed in a noteworthy manner to replace the harmful bacterial species by adopting specific dietary measures (Metchnikoff, 1908; Caramia and Silvi, 2011).

![Streptococcus thermophilus and Lactobacillus bulgaricus from yogurt matrix at scanning electron microscope. Adopted from M. Benevelli-Dept. “Scienze degli alimenti”, Bologna University, Italy). With permission number 2754390218981 from Springer on 9/22/2011.](image)
Complete transformation of human fecal microbiota was efficiently achieved by daily feeding of 500-1000 mL of *Lactobacillus acidophilus* milk (Cheplin and Rettger, 1920b). Of special interest to note, attempts to implant *Bacillus bulgaricus* in the gut of rodents and humans did not succeed. These results lend themselves to the conclusion that only bacteria of intestinal origin can be implanted in the human intestine (Cheplin and Rettger, 1920a and b).

To date, one popular mechanism via which *Lactobacillus acidophilus* is believed to alter the bacterial constituents of the intestinal microbiota is through production of lactic acid and hydrogen peroxide that kill other nonlactic acid-producing/utilizing commensals and pathogens (Attasi and Servin, 2010). Earlier reports by Cheplin and Rettger (1920a, and b), however, indicated that, in humans and rodents, dominance of the *Lactobacillus acidophilus* in the fecal microbiota was not necessarily accompanied by alteration of the hydrogen ion concentration.

### 1.1.1.1 Invention of the term “probiotics”

It was not until 1965 when Lilly and Stillwell used the term *probiotics for the first time* to describe a factor produced by the protozoan *Colpidium campylum* and stimulates growth of *Tetrahymena pyrofomis*. In their report, Lilly and Stillwell (1965) contrasted the stimulatory and growth-promoting effect of “probiotics” factor to the growth-inhibitory effect of the antibiotics.

By the advent of 1989, Fuller revised the definition of the term probiotics to depict “the live microbial additives that exert beneficial effect on the host by improving its intestinal microbial balance”. It was this definition coupled with the early perception of the
significance of transformation of the intestinal microbiota that ignited more interest in elucidating the role of the intestinal microbiota in promoting health or inciting diseases.

1.1.2 Prebiotics and their use to transform digestive system microbiota

As early as the beginning of the 20th century, interest in transforming the gut microbiota was increased (Cheplin and Rettger, 1920). The daily addition of 1 g of either lactose or dextrin to a basal diet of albino rats composed of bread and meat induced a transformation of the intestinal microbiota within about 4 days, and enhanced the development of *Lactobacillus acidophilus* to displace almost 50% of most common bacterial species that constitute the intestinal microbiota (Cheplin and Rettger, 1920). Interestingly, feeding more than 2 grams daily of lactose stimulated the proliferation of *Bifidobacterium bifidum* to such extent that it dominated the intestinal microbiota and displaced even *L. acidophilus* (Cheplin and Rettger, 1920a).

1.1.3 Microbiota

Our knowledge of the precise microbiota role in shaping the gut morphology remains to be fully unraveled. The current molecular biology techniques are appreciably enhancing our understanding of their mechanism of action and therapeutic potential for animal and human digestive system ailments (Patel and Lin, 2010).

Because probiotics are defined as live microorganisms that when administered in adequate amount induce health benefit to the host (FAO/WHO, 2001), distinct commensals inhabiting the digestive tract can be considered probiotics as well (walker, 2008).

After birth, the sterile fetal gut starts acquiring an enormously varied commensal microbiota. Eventually, commensal bacteria proliferate to outnumber the host cells by tenfold (Hooper et al., 2001; Bakhed and ley, 2005). The established symbiotic relationship
between the commensal microbiota and host furnishes the microbiota with a nutrient-rich and temperature-favorable niche. In return, the host commensal microbiota facilitates nutrient assimilation (Bakhed et al., 2005), protection against injury (Hooper et al., 2001), intestinal homeostasis (Rakoff-Nahoum et al., 2004), and development of GALT immunity (Rhee et al., 2004; Spencer et al., 2009). Density of microbiota increases from $10^3$ cells·g$^{-1}$ in the stomach and duodenum to $10^8$ cells·g$^{-1}$ in the jejunum and ileum. The greatest density of microbiota, however, occurs in the colon where it reaches $10^{11}$ cells·g$^{-1}$ and contains highly diverse species (Neish, 2009).

The preliminary acquisition of commensal microbiota is dependent on parturition methodology. Initially, the fetus acquires microbiota from species dominate the maternal vagina and colon, such as *Enterobacteriae*, *Enterococci* and *Staphylococci* (Patel and Lin, 2010). Subsequent milk feeding provides newborns with commensal microbiota inhabiting the mammary gland and its skin. After weaning, newborn gut microbiota is further altered and expanded in accordance with the diet consumed (Patel and Lin, 2010).

1.1.3.1 Microbiota dysbiosis thwarts host resistance to diseases

By the mid of 1950s, Finger and Wood (1955) and Miller et al (1957) pioneered studies to explore the effects of the antibiotic-induced perturbation and thus dysbiosis of the intestinal microbiota on susceptibility of mice to acquire *Salmonella enteritidis* infection. These pioneering studies grew out of the curiosity about the secondary bacterial infections that are associated with antibiotic administration.

Secondary bacterial infections that are associated with antibiotic therapy are mostly caused by avirulent species, such as *Pseudomonas* or yeast-like fungi and occasionally are initiated by virulent *Staphylococci* (Miller et al, 1957). Because these microorganisms are
normal inhabitants of the digestive tract, their contribution to infection during antibiotic treatment raised concern about the role that the digestive tract microbiota plays in disease prevention (Miller et al., 1957).

Intriguingly, whereas $1 \times 10^1$ CFU of streptomycin-resistant *Salmonella enteritidis* were sufficient to infect more than 50% of the streptomycin-treated mice, as much as $1 \times 10^6$ CFU of *Salmonella* were required to induce *Salmonella* infection in the non-streptomycin-treated control mice (Miler et al., 1957).

![Image](image_url)

**Figure 2.** Modification of the microbiota and its effect on intestinal immunobiology. Effect of antibiotic administration on composition and function of digestive system microbiota. Adopted from Preidis and Versalovic (2009) with permission from Elsevier on 6/9/11

Of significance, fecal culture from the streptomycin-treated mice showed the disappearance of the Gram-negative bacilli (Coliforms, *Proteus, Aerobacterium aerogenes*, and *Bacteroides*) from the intestinal microbiota. In contrast, the Gram-positive bacterial populations were not altered by streptomycin treatment (Miler et al., 1957; Bohnoff et al., 1964).
The reestablishment of the aforementioned gram-negative bacilli in the mice intestine, however, did not restore the intact resistance to *Salmonella* infection. In contrast, feces from control mice inoculated into the streptomycin-treated mice were able to restore resistance to *Salmonella*.

Further investigations of the fecal matter characteristics of normal mice led the authors to suggest that streptomycin-sensitive, unculturable, anaerobic strains contribute the resistance to *Salmonella enteritidis* infection in mice (Miller et al., 1957) through yet-to-be elucidated mechanisms.

Later, Bohnoff and colleagues (1964) elegantly unraveled the inhibitory mechanism via which these Gram-negative anaerobes inhibit the growth of *Salmonella enteritidis* infection in mice. They showed that these anaerobes produce butyric, acetic, and lactic acids that directly antagonize the growth of pathogenic bacteria.

Following streptomycin oral administration, the pH in colon increased and the concentrations of the short-chain fatty acids (SCFA) decreased. These alterations were accompanied by a concurrent exacerbated susceptibility to *Salmonella enteritidis* infection. On day three after streptomycin administration, SCFA concentrations and pH were restored to normal values; however, the elevated lactic acid concentration abolished the ability of the SCFA to inhibit the growth of *Salmonella*, favoring increased susceptibility of mice to infection (Bohnoff et al., 1964).

Findings of Bohnoff and colleagues (1964) suggested that in addition to these anaerobes, lactic acid-utilizing species were eliminated by the streptomycin administration. Note worthy, the intestinal microbiota is composed of highly intricate populations of archea, fungi, and bacteria that exceed $10^{13}$ microorganisms (Bailey et al., 2010; Sekirov et al., 2010). It is
now well established that these different bacterial species that constitute the digestive system microbiota are critical for the development of the gut-associated lymphoid tissues (GALT) and their microbiome contribution to metabolizing macronutrients exceeds that of the human genome (Gill et al., 2006).

It has been suggested that the co-evolution of the digestive system and microbiota determines brain development and programming and dictates the type of motor control and anxiety-related behavior (Heijtz et al., 2011). In support of this hypothesis, specific pathogen-free mice colonized with normal gut flora displayed decreased motor activity and increased anxiety in comparison with germ-free mice (Heijtz et al., 2011).

Besides contributing to gut inflammation, dysbiosis induces central nervous system disorders as well (Bercik et al., 2011). To induce dysbiosis, specific pathogen-free Balb/c mice treated or not with sub-diaphragmatic vagotomy or chemical sympathectomy and germ-free mice were administered with antibiotic mixture in the drinking water for one week (Bercik et al., 2011). The oral antibiotic mixture temporarily altered their microbiota composition (Figure 2) and enhanced their exploratory attitude and increased expression of the brain-derived nuclear factor (BDNF) in the hippocampus.

In contrast, colonization of the germ-free Balb/c mice with microbiota from NIH Swiss mice enhanced their exploratory activities and the concentrations of BDNF. On the other hand, colonization of germ-free NIH Swiss mice with microbiota from Balb/c retarded the exploratory activities by NIH Swiss mice (Bercik et al., 2011).

Taken together, the findings derive considerable evidence that dysbiosis contribute to induction of certain behavioral pattern through modulating brain biochemistry. Interestingly, dysbiosis-induced modulation of brain biochemistry was not associated with intestinal
inflammation, digestive system neurotransmitters function, or induction of sympathetic nervous system stimuli (Bercik et al., 2011).

1.1.4 Taxonomy and significance of *Mycobacterium avium* subspecies *paratuberculosis*, the causative agent of Johne’s disease

The murine and bovine microbiome compromises major phyla of *Firmicutes* and *Bacteroides* that make up about 90-98% of the total microbiota. The remaining portion of the microbiota (2-10%) is made up from the phyla of *Actinobacteria*, *Proteobacteria*, *Cyanobacteria*, and *Verrucomicrobia* (Ley et al., 2005; Bailey et al., 2010).

The phylum Actinobacterium is known to comprise the order Actinomycetales that contains the family Mycobacteriaceae, the genus *Mycobacterium*, the species *avium*, and the subspecies *paratuberculosis*. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is recognized as the etiologic agent of Johne’s disease (JD), a major disease of ruminants. Since 1985, JD continues to have significant welfare and economic impact (Ott et al., 1999; NAHMS, 2008) on livestock worldwide. JD is characterized by slowly developing chronic granulomatous enterocolitis and digestive system lymphadenitis and lymphangitis (Dennis et al., 2008). Collectively, these inflammatory conditions induce significant mucosal injury and protein malabsorption (Johnson et al., 2011), chronic intermittent diarrhea (Clarke and Little, 1996), and progressive weight loss (Radostits et al., 1994).

To date, there is no effective chemotherapeutic treatment for JD. Following infection, mycobacteria becomes dormant and resistant to chemotherapeutic agents (Deb et al., 2009). In humans, MAP is prevalent in blood and tissues of 40% of healthy individuals and in 80% of individuals affected with Crohn’s disease (Bull et al., 2003). Recent investigations have shown that probiotic microorganisms have the potential to enhance the host innate immunity
and increase expression of genes associated with host defense against pathogens as well as genes that control energy homeostasis (Nerstedt et al., 2007; Ibnou-Zekri et al. 2003; Wei et al., 2007).

In these studies, we investigated the immunological mechanisms of prevention of JD progression in a mouse model and explored efficacy of probiotics to transform the intricate microbiome of a dairy cow rumen and its subsequent effect on nutrient digestibility, energy harvest, and immunity.

**DISSERTATION ORGANIZATION**

This dissertation is presented as four complete papers with abstracts, introductions, materials and methods, results, discussions, conclusions, acknowledgements, and references for submission to the Journal of Immunobiology and Journal of Dairy Science. The first article is entitled “Tolerance of specific pathogen-free Balb/c mice to varying concentrations of *Lactobacillus acidophilus* strain NP51”. The second article is entitled “Probiotic *Lactobacillus acidophilus* strain NP51 curtails progression of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in Blab/c mice”. The third article is entitled “*Lactobacillus acidophilus* strain NP51 restricts progression of *Mycobacterium avium* subspecies *paratuberculosis* through activating the CD8α⁺ immune cell-mediated immunity.” The fourth paper is entitled “Transformation of lactating dairy cow digestive system microbiome by feeding Bovamine®: Effects on productivity and immune responses).

The dissertation has been prepared from original research conducted to partly fulfill the requirements for a Doctor of Philosophy degree from the Department of Animal Science and the Interdepartmental Program of Immunobiology at Iowa State University, Ames, IA. The
novel findings of this research indicate that probiotic feeding of *Lactobacillus acidophilus* strain NP51 to Balb/c mice infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of JD in ruminant animals, conclusively prevented the development of Johne’s disease.

Also, feeding probiotic Bovamine® (*Lactobacillus acidophilus* strain NP51 plus *Probionibacterium freudenreichii* strain NP24) to dairy cattle transformed their digestive system microbiome and improved ruminal feed digestibility. Because the probiotics NP51 and NP24 are safe commensal microorganisms that their feeding to dairy cows does not entail condemnation of milk and meat, their inclusion in the dairy cattle diet will enhance the profitability to the dairy farmers around the globe. A literature review and associated references list precede the articles, general discussion, and recommendations for future research. The acknowledge section concludes the dissertation.
CHAPTER 2. LITERATURE REVIEW

2.1 Probiotics

Probiotics have numerous beneficial effects on animal and human health including modification of the aberrant immune responses through balancing of Th1 and Th2 responses (Moreau et al., 1988) and preventing of intestinal disorders via production of antimicrobial peptides, competitive exclusion of enteric pathogens, and enhancement of gut barrier function (Boesten and de Vos, 2008). Probiotics also decrease risk of cancer by lowering $\beta$-glucuronidase and carcinogens concentrations in the intestine (Bezirtzoglou and Stavropoulou, 2011).

The co-evolution of the microbiota with the mammalian host digestive system has ensured tolerance to the multitude of the bacterial species that comprise the microbiota (Gill et al., 2006). Nevertheless, certain members of microbiota can acquire virulence ability and proliferate causing anomalous autoimmune and intestinal disorders (Willing et al., 2010). Such conditions are predominantly accompanied with microbiota dysbiosis (Willing et al., 2010; Tarasova et al., 2010). Probiotics administration also has potential to normalize the dysbiosis and resolve the aberrant autoimmune response (Tarasova et al., 2010).

2.1.1 Characteristics of probiotics

Scientists from the Food and Agriculture Organization (FAO) and World Health Organization (WHO) defined probiotics as being “live microorganisms which, when administered in a adequate amounts, confer health benefits on the host” (FAO/WHO, 2001).

It is widely agreed that substances produced by probiotic microorganisms, however, are not probiotics themselves (Guarner et al., 2010). Thus, the conserved and unique
extracellular molecules on the outer microbial envelope, such as peptidoglycans, cell wall associated polysaccharides, and lipoteichoic acid that are known as pathogen-associated molecular patterns (PAMPs) are not per se probiotics (Guarner et al., 2010).

Because also the heat-killed and gamma-irradiated probiotics are widely used and have shown potential to positively alter the host immune responses, it appears that the definition set forth by (Fuller, 1989; FAO/WHO, 2001) is relatively invalid. To include the effect of heat-killed probiotics, Salminen and colleagues (1999) revised the definition of probiotics to read as “probiotics are microbial cell preparations or components of microbial cells that have beneficial effects on the health and well-being of the host.”

The World Gastroenterology Organization Practice Guidelines on Probiotics and Prebiotics (2008) defined potential probiotic bacteria as microorganisms that are:

1. Normal inhabitant (intrinsic) of the human or animal gastrointestinal tract.
2. Resistant to antibiotics because of genomic DNA mutations.
3. Able to resist the acidic gastric and pancreatic juices, intestinal enzymes, and bile salts and colonize the digestive tract when administered at a minimal dose of $10^7$ CFU·day$^{-1}$. Laboratory procedures used to evaluate potential probiotics that are able to survive transiting and colonizing the gastrointestinal tract include exposing the bacteria at $10^9$ CFU·mL$^{-1}$ to 3 g·L$^{-1}$ pepsin in sterile phosphate buffered saline (PBS) at pH 2.0 (adjusted with HCL) followed by exposure to pancreatin at 1 g·L$^{-1}$. To test the ability of potential probiotics to survive the toxic action of bile, the bacteria are exposed to 0.5% (w/v) Ox-gall in sterile PBS at 37°C. Following incubation, bacterial cells are withdrawn at different time points and viability is determined by
plate count on de Man, Rogosa, and Sharp (MRS) media (Collado and Sanz, 2007; Pan et al., 2010).

4. Able to adhere to the enterocytes via interaction of the surface proteins on the probiotics plasma membrane with the mucosal cells. This interaction coupled with probiotics secreted proteins should cause immune response.

5. Able to modify advantageously the intestinal metabolic function to improve the animal and human health and antagonize pathogens proliferation by secreting bactriocin and enhance secretion of defensin peptides.

6. Safe immunologically to animals and humans.

7. Stable properties during storage time.

8. Cost-effective when administered in adequate dose.

2.1.1.1 Resistance to gastric juice

The harsh acidic environment of the stomach and the bile salts secreted into the duodenum are substantial impediments to the survival of ingested bacteria. Potential probiotic microorganisms must be able to transit through the gastric juice of the stomach (Hendriksson et al., 1999; Nagata et al., 2009) prior to reaching the small and large intestines.

Marteau et al (1993) demonstrated that microbial colonization of the digestive system was enhanced by gastrectomy. Similarly, the use of the proton pump inhibitors decreased the stomach acidity and induced microbial colonization of the stomach. To isolate and determine probiotic ability of Lactobacilli isolated from human ileum, Dunne et al (2001) mixed gastric juice obtained from healthy individuals with MRS media. Subsequently, the modified MRS media was inoculated with the Lactobacilli of interest to determine its ability to survive the acidic environment of the stomach.
The carbon source for probiotics also can affect transit of probiotics through the stomach. Incubation of *Lactobacillus plantarum* at simulated gastric media that either contained glucose or fructose as a carbon source was examined (Nagata et al., 2009). Viability of *L. plantarum* remained high at pH 3.5, regardless of the carbon source. When the pH was decreased to 3.0, the viability of *L. plantarum* incubated in the fructose-supplemented medium decreased after two hours, whereas incubation in the glucose-supplemented medium did not affect the *L. plantarum* growth. Noteworthy, decreasing the pH to 2.5 decreased *L. plantarum* viability, regardless of the carbon source within an hour of incubation (Nagata et al., 2009).

These findings clearly demonstrated the effect of the carbon source prevalent in the gastrointestinal tract on modulating the resistance of the probiotics to the gastric acidity. Thus, when formulating synbiotics, prior assessment of the effect of prebiotics and the prevalent carbohydrate in the prebiotic or the diet on the viability of the probiotics is warranted.

Indeed, ability of probiotics to resist the high acidity of stomach can be enhanced. Collado and Sanz (2007) incubated six acid-sensitive strains belonging to the species *Bifidobacterium longum* and *Bifidobacterium catenulatum* at pH 2.0 for extended periods of time. Following the acid incubation, the metabolic and phenotypic profiles of these strains were significantly enhanced. It was found that the acid-incubated strains showed better ability to grow in the presence of bile salt (1-3%) and NaCl (6-10%). Also, they developed higher resistance to elevated temperatures of 60-70°C for 10 min compared with the parental strains.

Additionally, the acid-incubated strains exhibited higher fermentative ability and enhanced enzymatic activities. Intriguingly, the acid-incubated strains also demonstrated
elevated susceptibility to variety of antibiotics compared with the nonacid-incubated parental strains (Collado and Sanz, 2007).

2.1.1.2 Resistance to pancreatic juice

Resistance to pancreatic secretion is essential for probiotics to colonize the intestinal tract. To examine the ability of probiotic Lactobacilli and Bifidobacteria strains to resist action of pancreatic juice, Del Piano and coworkers (2008) measured the viability of lactobacilli inoculated in media supplemented with human pancreatic juice obtained from subjects during an endoscopic cholangiopancreatography procedure or with standard artificial pancreatic juice. After one hour of contact with the artificial or human pancreatic secretion, the viability of lactobacilli strains was decreased by 19.7 and 28.6%, respectively. Similarly, the viability of Bifidobacteria strains was decreased by 42.2 and 44.4%, respectively (Del Piano et al., 2008). Thus, high tolerance of probiotics to standard artificial pancreatic juice (1.0 g/L pancreatin) is essential to screening potential probiotics.

2.1.1.3 Resistance to bile

Bile tolerance is a critical attribute that determines the ability of bacteria to survive in the small intestine, and, consequently, tolerance to bile is considered a critical criterion in selecting probiotics (FAO/WHO, 2001; Hamon et al., 2011). Hamon and colleagues (2011) used comparative proteomic analyses to examine differences in protein expression by L. plantarum 299 V (bile-resistant), L. plantarum LC 804 (relatively bile-resistant), and L. plantarum LC 56 (bile-sensitive) when challenged by bile salts. Proteins that dictate the resistance of L. plantarum to bile salt were characterized as being a two glutathione reductases that are involved in protection against the bile salts-induced oxidative damage, a cyclopropane-fatty-acyl-phospholipid synthase required for cell membrane integrity, a bile
salt hydrolase, an ATP-Bound Cassette (ABC) transporter, and a F$_0$F$_1$-ATP synthase that pumps out intracellular bile-related stress molecules (Hamon et al., 2011).

### 2.1.2 Mechanisms of probiotic modulation of immune system

#### 2.1.2.1 Secretion of soluble bioactive proteins

Probiotics synthesize its extracellular proteins and transport it unfolded to the plasma membrane via the Sec channel (Sutcliffe and Harrington, 2002, von Heijne, 1989). Alternatively, probiotics may secret its extracellular proteins folded through specific ABC transporters (Jongbloed et al., 2006).

Screening of *Lactobacillus reuteri* DSM 20016 cell wall revealed that this probiotic bacterium express about 52 novel genes encoding extracellular proteins that are critical for host/microbial interactions, transport of molecules, and regulation of sensor molecules. Also, some of these genes encoded for enzymes, conserved hypothetical proteins, or unconserved hypothetical proteins (Wall et al., 2003).

Soluble extracellular proteins secreted by probiotics include the pentapeptide (CHWPR) that is secreted by *Bifidobacterium animalis* subspecies *lactis* strain BB-12 and has been implicated with promoting interleukin (IL)-6 and c-myc genes expression in the HL-60 cell line (Mitsuma et al., 2008). Whereas IL-6 is critical for switching the innate immunity into adaptive immunity (Jones, 2005), c-myc expression is linked to cell proliferation and its over-expression is associated with cancer development (Musgrove et al., 2008).

Also, *Bifidobacterium longum* subspecies *longum* strain NCC2705 has been shown to produce the extracellular elastase-like protease inhibitor (serpine) (Ivanove et al., 2006). In this regard, serpine can deactivate the neutrophils elastase that is released by activated neutrophils during inflammation of the mucosa. Neutrophils elastase is known to cause
extracellular matrix destruction and tissue damage (Chua and Laurent, 2006). Because numerous biological processes that take place inside the inflammasome and the apoptosome are initiated by proteolytic events it likely that Bifidobacterial serpine plays critical immune inhibitory function in the gut of the mammalian host (Ivanov et al., 2006).

Similarly, *B. infantis* has been shown to secret a bioactive soluble protein that increases transepithelial resistance and enhanced tight junction by increasing zonula-occludin-1 expression (decrease intestinal leakage) in human T84 epithelial cell line cocultered with tumor necrosis factor (TNF)-α and interferon (IFN)-γ (Ewaschuck et al., 2008). These immune modulations were associated with increased expression of the extracellular-regulated kinase (ERK) and decreased p38 mitogen-activated protein kinase (MAPK). Also, oral administration *Bifidobacterium*-conditioned media to IL-10-deficient mice ameliorated their intestinal inflammation and decreased colon permeability *in vivo* (Ewaschuck et al., 2008). Several yet-to-be characterized and identified bioactive proteins have been shown to be probiotic products are secreted primarily by *Bifidobacteria* and *Lactobacilli* play critical immuno-modulatory roles.

Also, a protein product of a *Bifidobacteria breve* C50 has been shown to induced DC maturation and increased expression of B7-1 and B7-2 via signaling through p38MAPK and phosphatidyleinositol 3-kinase (PI3K) pathway (Hoarau et al., 2008). Inhibition of ERK and glucose synthase kinase (GSK)-1 did not abolish *B. breve* C50 protein effect on DC maturation (Hoarau et al., 2008). Also, C50 protein-stimulated DC secreted high concentration of IL-10 and low concentration of IL-12 suggesting a tolerogenic phenotype. Secretion of IL-10 was completely abrogated by inhibiting p38MAPK and to a lesser extent
by inhibiting ERK and PI3K inhibitor (Hoarau et al., 2008). In contrast, inhibition of GSK-1 and p38 MAPK completely abolished IL-12 secretion by C50 protein-stimulated DC.

Similarly, work by Konstantinov et al (2008) illustrated that *Lactobacillus acidophilus* strain NCFM surface layer A protein (SlpA) binds DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) receptor and program DC to secret copious amount of IL-10 and low amount of IL-12p70. Overall, probiotics interaction with DC appears to destine DC to the tolerogenic phenotype.

Probiotics secretory bioactive proteins have been implicated with triggering immune modifications in the mammalian host, such as induction of effector molecules (chemokines, and cytokines), antimicrobial peptides bactriocin and defensins). In addition, probiotics secretory bioactive proteins have been shown to increase the mucus secretion, enhance the tight junctions by increasing occluding and zonula-1 (Ewaschuck et al., 2008), and promote the innate immunity via up-regulating DC maturation and survival (Hoarau et al., 2008; Konstantinov et al., 2008).

To induce an immune response, probiotic microorganisms must ‘‘speak’’ to the immune cells that are endowed with conserved patterns recognition receptors (PRRs) sensitive to PAMPs, such as DNA, metabolites, and cell wall components.

Examination of the intestinal epithelial cells responses to cocultered adherent probiotics demonstrated the ability of probiotics to regulate the secretion of numerous anti- and proinflammatory cytokines (Haller et al., 2001; Ruiz et al., 2005).

### 2.1.2.2 Regulation of NFkB and MAPK pathway.

It has been shown that *Bifidobacterium lactis*, after colonizing the intestinal tract of germ-free rats, transiently phosphorylates and activate the Re1A subunit of NF-κB and the p38
MAPK in the intestinal epithelial cells (Ruiz et al., 2005). As a result, IL-6 gene expression was significantly increased.

On the contrary, *Bacteroides vulgates* administration to germ-free rats, though phosphorylated NF-κB subunit Re1A, it was failed to phosphorylate MAPK p38 and, thus, did not induce IL-6 gene expression (Ruiz et al., 2005). Inhibition of MAPK pathway in intestinal epithelial cell line impaired the *Bifidobacterium lactis* induction of IL-6 gene expression in vitro (Ruiz et al., 2005).

To unravel the mechanism of the *Bifidobacterium lactis*-intestinal epithelial cell (*IEC*) cross-talk, Ruiz and colleagues (2005) fed *Bifidobacterium lactis* to toll-like receptor (*TLR*)-2 knockout mice. TLR-2 deficiency abrogated the ability of *Bifidobacterium lactis* to promote IL-6 gene expression.

IL-6 signaling is essential to resolving the innate immunity and promoting the acquired immunity (Jones, 2005). Transition from innate to acquired immunity is pivotal to resolution of all inflammatory conditions, and disruption of this immunological switch may potentially promote the onset of chronic inflammatory disorders or autoimmune diseases (Hoebe et al., 2004).

### 2.1.2.3 Induction of the innate immunity.

To investigate the IECs and DC immune responses to probiotic and pathogenic bacteria, O’Hara et al (2006) incubated HT-29 human IECs with *Bifidobacterium infantis* 35624, *Lactobacillus salivarius* UCC118, or *Salmonella typhimurium* UK1 for different times.

Alternatively, IECs were cocultered with the probiotics *Bifidobacterium infantis* 35624, *Lactobacillus salivarius* UCC118 for 2 h before being exposed to *S. typhimurium* or flagellin. Examination of the inflammatory genes expression revealed that exposure of IECs to *S.*
*typhimurium* increased expression of 36 proinflammatory genes of the 847 genes assayed, including NF-κB and CXCL-8. In contrast, exposing the IECs to the commensal bacteria did not alter any gene expression of the 847 genes examined.

Also, the baseline and flagellin-induced secretion CXCL-8 by IECs was decreased by the exposure to *B. infantis* and *L. salivarius*. Though the treatment with the probiotics did not prevent adhesion of *S. typhimurium* to the HT-29 cell line, it did not increase MUC3 or E-cadherin gene expression. Conversely, IL-10 and tumor necrosis factor (TNF)-α secretion by DC were augmented. This immune response suggests that whereas immune responses of HT-29 IECs remained unaltered when exposed to probiotic bacteria, probiotics bacteria mediate host responses to flagellin and pathogenic bacteria via activating intestinal innate immunity cells, such as DC (O’Hara et al., 2006).

### 2.1.2.4 Induction of immune tolerance.

It is now well established that the enormous number of intestinal microbiota share a plethora of PAMPs that are recognized by the innate immunity PRRs (Kim et al., 2008; Ryu et al., 2009). Curiously enough, the PRRs of the innate immunity are able to distinguish between pathogenic and probiotic PAMPs and maintain homeostasis of normal microbiota (Shi and Walker, 2004).

Ryu et al (2009) examined the immunogenic pattern of the lipoteichoic acid (LTA), an immunogenic protein on the cell wall of pathogenic, nonpathogenic, and probiotic Gram-positive bacteria. Ultra pure LTA from *Staphylococcus aureus* (pathogenic) and *Bacillus subtilis* (non-pathogenic) activated TLR-2, downstream myeloid differentiation primary response gen 88 (MyD88), NF-κB, and the transcription factor activator protein-1 (AP-1) of
the RAW 264.7 murine macrophage cell line and stimulated their production of NO and TNF-α (Figure 5) (Ryu et al., 2009; Kim et al., 2008).

Figure 1. The effect of heat-killed bacteria on NO production by the RAW 264.7 macrophage cells. RAW 264.7 at $1 \times 10^5$ cells/mL were stimulated with heat-killed Gram-positive bacteria *L. plantarum*, *B. subtilis*, or *S. aureus* at $0$, $10^5$, $10^6$, $10^7$, $10^8$ CFU/mL for 48 h. Nitrite accumulation was determined as an indicator of NO production in the cultured media. Values are mean ± S.D. of three replicates for each group. (*) indicates a significant difference compared with the control group at $P$-value ≤ 0.05. Adopted from (Ryu et al, 2009).

On the contrary, ultra pure LTA from *Lactobacillus plantarum* (probiotic) failed to activate TLR-2 as verified by the decreased NFkB luciferase activity (Figure 2; Kim et al., 2008). Also, ultra pure LTA from *L. plantarum* did not induce production of NO (Figure 1). This immunogenic pattern was further verified by showing that stimulation of the RAW
264.7 with the heat-killed *S. aureus* and *B. subtilis* triggered significant production of NO and TNF-α in a dose-dependent manner. In contrast, the stimulation of RAW 264.7 with the heat-killed *L. plantarum* induced only little NO and TNF-α production (Figure 1; Ryu et al, 2009).

![Graph](image)

**Figure 2.** Effect of liptoteichoic acid (LTA) from *Lactobacillus plantarum* and *Staph aureus* on the luciferase activity of U937 macrophage cell line. U937 macrophages cell line were transiently cotransfected with *L. plantarum* NF-κB-luciferase and *L. plantarum* RL-SV40. Transfectants were prestimulated with or without 100 µg/ml of *L. plantarum* LTA for 20 h, and then restimulated with 50 µg/ml of either *L. plantarum* LTA or *S. aureus* LTA for 18 h. The cells were lysed, and the luciferase activity was measured. Data are normalized for Renilla activity. (**) indicates $P \leq 0.01$ compared with *S. aureus* LTA alone. Adopted from Kim et al (2008).

Also, the abrogation of TLR-2 on RAW 264.7 by NaOH or pretreating RAW 264.7 with
L. plantarum LTA abolished the ability of LTA from S. aureus to induce NO and TNF-α. Thus, it appears that TLRs are able to “finger print” various bacterial PAMPs and orchestrate a very unique immune response characterized by tolerance to probiotics and intolerance to pathogenic bacteria.

### 2.1.3 Health-promoting effects of probiotics

It is increasingly been accepted that the mammalian digestive tracts are intricate symbiotic systems of multispecies microorganisms (Forssten et al., 2011) that bestow numerous health benefits to the mammalian host.

#### 2.1.3.1 Antidyslipidemic effect

The interesting findings by Mann and Spoerry (1974) that serum cholesterol concentrations have decreased dramatically in male African Maasai warriors following consumption of large amounts of fermented milk that contained Lactobacillus strain drew great attention to the cholesterol-lowering effect of the probiotic lactobacilli. In a recent report, Pan et al (2010) examined the cholesterol-lowering ability of Lactobacillus fermentum strain SM-7 isolated from fermented milk. They inoculated the 1% (v/v) SM-7 strain into the MRS-cholesterol broths supplemented with 0, 1, 2, 3, or 4 g·L⁻¹ Ox-gall (bile salt) at 37°C for 24 hours. The supernatant, washing buffer, and the pelleted bacterial cells were then collected and measured for their cholesterol content. Cholesterol was lowered significantly when SM-7 was incubated in a concentration of 3 g·L⁻¹ Ox-gall. Elevating the ox-gall concentration to 4 g·L⁻¹ slightly decreased the cholesterol-lowering ability of SM-7, indicating that bile salts at 4 g·L⁻¹ are likely to inhibit growth of SM-7.

Elegantly, Pan and coworkers (2010) examined the in vivo cholesterol-lowering effect of SM-7 in mice. Mice were fed daily for four weeks a high-fat diet that contained
10 g cholesterol, 100 g lard, 50 g yolk and 2 g ox-gall·kg\(^{-1}\) diet. Simultaneously, SM-7 was gavaged to mice daily for four weeks. They revealed that gavaging the SM-7 to mice decreased substantially the fasting serum total cholesterol and triacylglycerols by 21.2 and 9.8%, respectively, lower than that of control mice fed the high fat diet only. Importantly, serum LDL-cholesterol was decreased significantly by 17.8% lower than that of the control. Overall, AI values showed a significant decrease whereas the anti-atherogenic index (AAI) values were increased in the group of mice fed the SM-7.

The mechanism whereby probiotics alleviate hypercholesteremia is poorly understood. Some bacteria, however, can assimilate cholesterol \textit{in vitro}. Decreased cholesterol absorption by the intestine has been pointed out as one mechanism that is triggered by probiotics (Anderson et al., 1995).

Microbiomics and metabolomic studies in humanized microbiome mouse model (germ-free mouse colonized with human baby flora) have been conducted to explore the transgenomic and metabolic effects of \textit{L. paracasei} or \textit{L. rhamnosus} in mice (Marin et al., 2008). Using multivariate compartmental analysis, it was shown that inclusion of the probiotic Lactobacilli in the mice microbiome decreased concentrations of LDL and VLDL and increased TAG and phosphorylcholin (PC) concentrations in plasma (Martin et al., 2008). Also, bile acids excretion in feces was decreased (Martin et al., 2008). Previously, it has been shown that increased uptake of bile acids by \textit{Lactobacilli} decreases their excretion (Kurdi et al., 2000). Alternatively, probiotics feeding alters the assimilation of bile acids and lowers cholesterol and plasma lipid concentrations (Nguyen et al., 2007). The probiotic-induced cholesterol-lowering effects is mediated, at least in part, through down-regulation of the Niemann-Pick C1-like 1 and the liver X receptors.
2.1.3.2 Antiallergic effect

Allergic rhinitis is a widely prevalent disease that affects about 500 million people around the world (Bousquet et al., 2008). Effect of short-term oral administration of \textit{Lactobacillus paracasei} strain ST11 fed to patients with pollen grain-induced allergic rhinitis on their nasal congestion test and immune responses was conducted (Wassenberg et al., 2011). Subjects were randomized to either take placebo or ST11 fermented milk for four weeks. All subjects were intranasally nebulized with 0.2 mL of six pollen grains cocktail.

Remarkably, ST11 consumption decreased the nasal congestion and pruritus. In addition, secretion of IL-5, IL-8, and IL-10 by peripheral blood mononuclear cells decreased significantly compared with that of the placebo-fed group. Also, consumption of the ST11 fermented milk lowered significantly the allergen specific IgG\(_4\). Elegantly, findings of Wassenberg et al (2011) suggest strongly that probiotics consumption down regulates important immune pathways that are active in the pathogenesis of allergic rhinitis.

2.1.3.3 Intestinal diseases-preventive effect

The GALT starts developing in utero during the fetal stage and becomes able to trigger sensible immune response against pathogenic insults at birth (Insoft et al., 1996). For the GALT, however, to be fully protective and able to trigger adequate immune response, exposure to commensal and probiotics microbiota is a prerequisite (Shi et al., 2004).

Stimulation of the GALT with microbiota enables the GALT to synthesize and secrete the polymeric IgA that neutralizes viruses, bacteria, and toxins. Also, microbiota-induced stimulation of the GALT balances the aberrant Th-1 and Th-2 to Th-3 ratios. A balanced T helper cells ratio is purported for oral tolerance induction and prevention of allergic and chronic bowel disorders (Moreau et al., 1988).
2.1.3.4 Antioxidative stress effect

Though Lactobacilli are sensitive to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) damage, *Lactobacillus sakei* strain YSI8 expresses a novel heme-dependent catalase that degrades H\textsubscript{2}O\textsubscript{2}. In contrast, *Lactobacillus rhamnosus* lacks the ability to express catalase, but it is a valuable probiotic starter cultures that is extensively used for fermenting meat products. An and coworkers (2010) examined the heterologous expression of *L. sakei* catalase gene *katA* in *L. rhamnosus* on its resistance to oxidative stress. The recombinant *L. rhamnosus* strain AS 1.2466 exhibited catalase activity and degraded H\textsubscript{2}O\textsubscript{2} efficiently. In addition, the survival rate of *L. rhamnosus* strain AS 1.2466 was increased 10,000- and 600-fold when exposed to H\textsubscript{2}O\textsubscript{2} at the exponential and stationary phases, respectively.

2.1.3.5 Pain-soothing effect

Chronic inflammatory bowel disease (IBS) significantly increases gut permeability causing chronic intestinal pain. Chronic intestinal pain constitutes a leading symptom for referral to a specialist (Francavilla et al., 2010). *Lactobacillus GG* was tested for its efficacy to relieve recurrent abdominal pain in children. Feeding *Lactobacillus GG* for 8 weeks in a randomized, double-blind, placebo-controlled trial to children with inflammatory bowel disease decreased significantly the severity and frequency of abdominal pain (Francavilla et al., 2010). The GG pain-reducing effect persisted to the end of the eight-week follow-up period and was associated with a significant decrease of the gut permeability.

Probiotic feeding has been found to abolish pain response by decreasing dorsal root ganglion single unit activity and prevented the pressure-dependent bradycardia induced by distending the colorectum of Sprague-Dawley rats (Kamiya et al., 2005). *Lactobacillus*
Paracasei administration to rat pups prevented stress-induced visceral pain and maintained gut permeability (Eutamene et al., 2007). Molecular investigation revealed that Lactobacillus farciminis feeding suppresses expression of Fos, a marker of general neuron activity in the central nervous system indicating that L. farciminis downregulate the spinal nociceptive neurons sensitized by stress (Ait-Belgnaoui et al., 2009).

In conclusion, antibiotics are clinically used to delete or inhibit growth of an overgrown member(s) of the microbiota. Deleterious side effects of antibiotic use include the evolvement of antibiotic-resistance genes. Horizontal transfer of these genes to other susceptible microbiota members could induce state of global antibiotic resistance (Nakano et al., 2011).

In contrast, the use of probiotics in animal feed re-introduces a deleted beneficial microbiota component or enhances its growth by inhibiting growth of competitors. The use of prebiotics in the feed can provides necessary nutrients required to enhance the growth of that microbiota component. Thus, the prebiotics and probiotics action is additive and provides a means to introduce beneficial alterations in the microbiome to enhance the health of animals (Martin et al., 2008; Preidis and Versalovic, 2009).

2.1.4 Adverse side effect of probiotics

Probiotics could potentially have side effects in vulnerable humans, such as induction of microbial infection, aberrant immune stimulation, toxic metabolites production, and transfer of antibiotic resistance genes (Mateau and Seksik, 2004). Reported cases of bacterial infection caused by probiotics use are very scarce and occurred primarily in individuals with critical medical conditions (Mateau and Seksik, 2004).
Recently a high death rate in a Dutch clinical trial carried out by researchers in Utrecht University has raised much concern (Vogel, 2008; Tavernier and Paye, 2011). Acute pancreatitis is known to affect patients with alcoholism and is induced by acute overgrowth and infection by commensal microbiota that is refractory to antibiotic therapy. It is characterized by acute abdominal pain associated with elevated plasma lipase by more than three-fold the physiological value (Vogel, 2008; Tavernier and Paye, 2011).

The Dutch Acute Pancreatitis Study Group fed about 100 patients diagnosed with acute pancreatitis fed them a combination of probiotics. Another 100 patients were fed the placebo. In fact, far more patients died in the probiotics group relative to those in the control group. The death rate was 16 and 6% in the probiotic and the placebo groups, respectively (Vogel, 2008). An intriguing cause of death was multiple organ failure in the probiotics group, a side effect that triggered by the probiotic treatment (Vogel, 2008).

2.2 Mechanisms of microbiota modulation of immune system

2.2.1 Induction of the GALT development

In germ-free animals, GALT is ill developed. Thus, polymeric immunoglobulin (Ig) A secretion is retarded causing inadequate oral tolerance to commensals and food antigens (Rhee et al., 2004; Shi and Walker, 2004). To verify whether commensals microbiota are required for GALT development, Rhee and colleagues (2004) introduced commensal bacteria into rabbits in which the appendices were rendered germ-free and identified intestinal commensal species that promote GALT development.

The combination of *Bacteroides fragilis* and *Bacillus subtilis* predominantly promoted GALT development and increased the frequency of the somatic hypermutations in the B cell V(D)J genes of Ig. Adequate somatic hypermutations is required to ensure development and
diversity of the IgM+ B cells repertoire. In humans, GALT has been shown to accommodate molecular tools that diversify the B cell repertoire by somatic hypermutation, class switch recombination, and receptor editing (Figures 2A and 3). Thus, a well-developed GALT is pivotal to enhancing the diversity of mature B cells and their clonal progeny of plasma cells to secret integrated range of highly diverse polyclonal antibodies (Spencer et al., 2009).

It was evident that the GALT-promoting effect of *Bacteroides fragilis* and *Bacillus subtilis* was synergistic and equally required. Intriguingly enough, neither species alone predominantly mediated GALT induction. Intriguingly, only commensals were required for GALT induction, and introduction of *Escherichia coli, Clostridium subterminale, Bacillus fragilis or Staphylococcus epidermidis* did not mediate GALT induction (Rhee et al., 2004).

To reveal the molecular mechanisms via which commensals microbiota mediate GALT induction, Rhee and coworkers (2004) introduced stress-response mutant *B. subtilis* into the germ free-appendix rabbits. It was found that *Spo0A* (gene that promotes postexponential phase sporulation) and biofilm formation by *B. subtilis* is required to signal through *YqxM* (gene required for extracellular matrix development essential for biofilm formation) to induce GALT development (Rhee et al., 2004; Hera et al., 2008). Similarly, ligation of rabbit appendices at birth to prevent microbial colonization arrested lymphoid follicle development (Perey and Good, 1968). When the ligated appendix was reconnected with the intestinal lumen, the lymphoid follicle development was restored indicative of a developed GALT (Figure 3).
Figure 3. Paraffin-embedded sections of human GALT stained with hematoxylin and eosin. Panel A. Payer’s patch with prominent germinal center and intimate association with the follicle-associated epithelium (FAE). Panel B. Colonic lymphoid germinal center (LGC) with small or absent germinal center and a narrower FAE zone. Unlike the Payer’s patches that are exclusively in the mucosa, LGC cross the *muscularis mucosa*. Panel C. mucosal-associated lymphoid tissue (MALT) with a large germinal center in stomach. Normal stomach does not contain lymphoid tissue, but MALT can be acquired in response to infection with *Helicobacter pylori*. Adopted from Spencer et al., 2009.

2.2.2 Modulation of the antigen-regulated immune response

The symbiotic relationship with commensal microbiota and elimination of invading bacterial pathogens by the mammalian host is essential for survival of the mammal host. Recognition of microbes by the host immune system is brought about by detection of the highly conserved PAMPs by the host PRRs, such as the TLRs and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors; NLRs) (Akira et al., 2006; Inhora et al., 2005).

Membrane-bound TLRs recognize bacterial ligands at the cell surface or inside the phagolysosomes (Akira et al., 2006). NLRs, on the other hand, induce innate immune
responses via recognizing PAMPs inside the cytosol (Ting and Davis, 2005; Inohara et al., 2005). Two NLR family members, NOD-1 and NOD-2, sense bacterial muramyl dipeptide (MDP), a conserved sequence of all peptidoglycan (PG).

This PAMPs-PRRs “cross-talk”, however, is intriguingly dependent on the microenvironment (Figure 4) (Koropatnick et al., 2004). For example, the tracheal cytotoxin (TCT), a component of the bacterial cell wall-associated PG, has been shown to cause extensive tissue damage, unique lesions to gonorrhea and whooping cough infections. Though components of PG and lipopolysaccharides (LPS) are typically associated with disease pathogenesis, the symbioant *Vibrio fischeri* also released TCT which act in synergy.
with LPS to induce tissue development of its host squid *Euprymna scolopes* (Koropatnick et al., 2004).

It is evident, therefore, that the host interpretation of PAMPs-PRRs is dependent on the predominant microenvironment and differences in host interpretations to PAMPs-PRRs interaction results in disease development or symbiosis establishment (Figure 4; koropatnick et al., 2004).

2.2.3 Regulation of the PRRs signaling by microbiota prevents GIT inflammation.

2.2.3.1 Regulation of TLRs signaling

Of importance to note, ligands for the PRRs known as TLRs are not unique to pathogenic bacteria, and are also expressed by commensal microbiota (Rakoff-Nahoum et al., 2004). It has been shown that under normal steady state microbiota is recognized by TLRs, and that this recognition is pivotal for the IECs homeostasis (Rakoff-Nahoum et al., 2004; Rhee et al., 2004).

Compared to stimulation with pathogens, stimulation of TLRs by microbiota is required for developing protection against intestinal injury and ensuing deaths. To address the role of TLRs family in the intestinal inflammation, Rakoff-Nahoum and coworkers (2004) administered 2% (w/v) dextran sodium sulfate (DSS) in drinking water to mice deficient of MyD88 (Figure 5). MyD88 is an adaptor molecule critical for all TLRs, IL-1, and IL-18 receptors signaling (Takeda et al., 2003). DSS was also administered to WT- and TLR-2- and TLR-4-deficient mice. Curiously enough, MyD88-deficient mice developed sustained GIT inflammation and high mortality whereas all WT mice survived (Figures 5 and 6). These findings indicated the critical role of commensal microbiota signaling via TLR in maintaining intestinal epithelial homeostasis and preventing intestinal inflammation.

Similar intestinal injuries and subsequent mortalities were seen in the TLR-2- and TLR-4-deficient mice administered DSS. These interesting findings revealed the critical role of the TLR family in maintaining IEC homeostasis to protect host from intestinal injury and drew the attention to the TLR family as therapeutic targets for the intestinal diseases (Rakoff-Nahoum et al., 2004).
2.2.3.2 Regulation of nucleotide-binding oligomerization domain (NOD)-1 and 2 signaling

The NOD family of cytoplasmic PRRs is involved in the innate recognition of the microbiota PAMPs, the muramyl dipeptide (MDP) (Girardin et al., 2003; Akira et al., 2006). MDP is a conserved structure in all type of PG (Girardin et al., 2003; Inohara et al., 2003). Upon recognition of MDP, NOD-1 and 2 mediate gene expression through a pathway involves NFκB transcription factor, the MAPK, and the adaptor molecule receptor interacting protein (RIP) 1 signaling (Girardin et al., 2000; Hsu et al., 2007).

Concordant with that of mutant TLRs (Rakoff-Nahoum et al., 2004), mutant NOD-1 and 2 have been implicated in the etiology of the mucosal injury and Crohn’s disease (CD) pathogenesis. To reveal the role of NOD family in the recognition of the commensal microbiota, Radkoff-Nahoum et al (2005) administered MyD88+/−, RIP-1−/− mice with DSS.
Unlike MyD88−/− mice, RIP-1−/− mice where completely resistant to intestinal injury suggesting that the protective role of commensal is mediated via TLRs, not RIP-1.

Abundant evidence exists that signaling of PG of microbiota through NOD-1 on epithelial cells is critical for induction of the intestinal lymphoid tissue and β-defensin in mice (Bouskra et al., 2008). However, subsequent signaling of microbiota through the TLR is essential for maturation of intestinal lymphoid tissue into GALT with large B cell cluster. Mice with deficient intestinal lymphoid follicle exhibit significant dysbiosis (Bouskra et al., 2008).

To elucidate mechanistically the cytoprotective role of commensal microbiota that is mediated through TLR signaling, production of IL-6, TNF-α, and keratinocyte-derived chemokines (KC)-1 was quantified in colonocytes from WT and MyD88−/− mice (Rakoff-Nahoum et al., 2004). It was found that colonocytes of WT mice produce IL-6, TNF-α, and KC-1 at baseline, and production of IL-6 and KC-1 was substantially elevated after DSS administration. On the contrary, colonocytes of MyD88−/− mice release significantly low concentrations of these cytoprotective cytokines, and production of these cytokines was entirely abrogated after DSS administration (Rakoff-Nahoum et al., 2004).

It was evident, then, that commensal microbiota prevents intestinal injury via signaling through TLR and the downstream MyD88 to MAPK to translocate NFκB to the nucleus. Subsequent molecular events result in the production of the cytoprotective cytokines IL-6, KC-1, and TNF-α.

Besides promoting adaptive immunity (Ovlisen et al., 2009), a wide range of cytoprotective role has been ascribed to microbiota-regulated IL-6, KC-1, and TNF-α in numerous types of cell, such as alveolar epithelial cells (Kida et al, 2005), endothelial cells
(Liu et al., 2010), intestinal epithelial cells (Pagnini et al., 2010), and hepatocytes (Wruck et al., 2011). Also, IL-6 has been shown to have antioxidative stress functions (Wruck et al., 2011).

2.2.4 Induction of secretory IgA

Additionally, PRRs on DC allow DC to efficiently sample intestinal microbiota and even retain viable microbial cells for several days. Retention of viable microbiota by DCs enables the selective induction of a microbiota-specific sIgA that restrains microbiota translocation (Macpherson and Uhr, 2004) and ensures tolerance of commensal microbiota for symbiotic purposes. Depending on the pathogenicity of the engulfed bacteria, DC can trigger a pro- or anti-inflammatory immune response (Mazzoni and Segal, 2004; Smits et al., 2005).

2.3 Prebiotics

2.3.1 Prebiotics fermentation

The 2008 annual meeting of the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined prebiotics as being “dietary prebiotics are selectively fermented ingredients that result in specific changes in the consumption and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health”. Whereas a variety of molecules can be prebiotics, however, mainly dietary fibers and oligosaccharide are considered to be so (Courbeyre et al., 2011). Prebiotics, also along with the polyunsaturated fatty acids and phytochemicals constitute a wide proportion of the bioactive food ingredients known as nutraceuticals (Laparra and Sanz, 2010).

Prebiotics differ from probiotics in their mode of action. Prebiotics serve as “food” for specific members of the microbiota that exist within the microenvironment of the digestive
system to induce benefits to the host (ISAPP, 2008). The guidelines of the ISAPP (2008) and others (Roberfroid, 2007) classify food ingredients as prebiotics based on the followings:

1. Non-digestible carbohydrates those are resistant to mammalian gastric and intestinal enzymes.
2. Fermented chiefly by the digestive system microbiota.
3. Selectively stimulates activity and/or growth of beneficial members of the digestive system microbiota.
4. Heat- and desiccation-stable and can be stored at room temperature.

Prebiotics include non-digestible carbohydrates fibers, such as galacto- and fructooligosaccharides (GOS and FOS, respectively), and xylooligosaccharides (Arslanoglu et al., 2007; Shadid et al., 2007; Seifery and Watzl, 2008). GOS are obtained from fructose after it is treated with $\beta$-galactosidase, whereas inulin and FOS are produced by enzymatic digestion of chicory (Macfarlane et al., 2006).

Inulin is a fructose polymer composed of 10 to 60 fructose residues linked $\beta$ (2-1). Partial hydrolysis of inulin produces FOS that are composed of 3 to 7 residues of fructose. Inulin and FOS are natural components of wheat, asparagus, and onion (Gibson, 1994; Macfarlane et al., 2006). FOS slow the gastric emptying rate, causing a decrease in the glycemic index of the diet and thus improve glucose and lipid metabolism of the host (Russo et al., 2010).

Prebiotics selectively stimulates growth of certain beneficial genera of the microbiome (Cummings and Macfarlane, 2001). FOS (fructose polymers) are fermented selectively by Lactobacilli and Bifidobacteria at a high rate (Gibson et al., 2005; Falony et al., 2009). It has been shown that fructose released during fermentation of FOS by Bifidobacteria is utilized as a source of carbon by local inhabitant bacteria (Falony et al., 2006). Anaerostipes caccae
DSM 14662 do not ferment FOS but survive on fructose released by *Bifidobacteria* during FOS fermentation. On the other hand, *Roseburia intestinalis* DSM 14610 degrades FOS merely in the presence of acetate produced by *Bifidobacterium*.

FOS can be fermented by other bacterial genera, such as *Bacteroides* and *Clostridia*. But because their absorption of FOS is inadequate, they grow slower than *Bifidobacteria* (Van der Meulen et al., 2006: Falony et al., 2009).

### 2.3.2 Immune mechanisms of prebiotics

Prebiotics can have direct antimicrobial effects by acting as a decoy receptor that directly bind pathogens or their toxins (Futher et al., 2010). Milk oligosaccharides have been shown to bind *Clostridium difficile* exotoxins by serving as a decoy receptor for inactivation of the exotoxins (El-Hawiet et al., 2011). Alternatively, GOS have bacterial receptor homolog that can compete with pathogenic bacteria on binding to sites on the enterocytes apical membrane. This prevents pathogens from binding to enterocytes and colonizing the gut an initial step in bacterial diseases pathogenesis (Searle et al., 2010; Quintero et al., 2011) (Figure 5B).
Prebiotics fed to constipated rats reversed the constipation-induced decrease of superoxide dismutase and the increase in malondialdehyde, an oxidative stress marker (Li et al., 2011). Also, the prebiotic feeding resolved the histological damage induced by constipation and increased production of secretory IgA (Li et al., 2011).

The dust mite *Dermatophagoides farina* is a known etiologic agent of respiratory allergy. FOS supplement to diet of mice affected with *D. farina*-induced allergic bronchitis resolved the allergic and inflammatory symptoms as evident by the significant decrease in the allergen-specific IgG1 in the serum (Yasuda et al., 2011). Also, supplemental FOS tended to attenuate expression of eotaxin and IL-5 in the lungs of affected mice. The histopathological examination of the mucosa lining of the bronchi indicated a decrease in goblet cells hyperplasia and attenuation of the eosinophilic inflammation (Yasuda et al., 2011). The molecular interaction that underlies this immune response is yet to be precisely elucidated.
One possible mechanism of this immune response is thought to be via FOS-triggered alteration in microbiota (Yasuda et al., 2011).

Effect of prebiotic preparations of acidic oligosaccharides, FOS, and GOS that mimic human milk oligosaccharides were tested for their immuno-modulatory effects in a mouse model of influenza virus vaccine (Vos et al., 2007). Acidic oligosaccharides enhanced the influenza vaccine-specific delayed-type hypersensitivity (DTH) response and attenuated Th2 responses in vitro. Feeding combination of three prebiotics to mice increased population of Bifidobacteria and Lactobacillus in their feces and adjuvanted their Th1 response to influenza vaccine (Vos et al., 2007). Of interest, at day 9 of feeding the prebiotic oligosaccharides, the cecal Lactobacilli numbers correlated with the DTH suggesting that prebiotic mediate its vaccine adjuvant effect by modifying the microbiome (Vos et al., 2010).

Scientists from Max Rubner Institute in Germany suggest that prebiotics can directly mediate an antiinflammatory response independent of the gut microbiota (Zenhom et al., 2011). To examine whether activation of the peptidoglycan recognition protein-3 (P GlyRP3) is involved in the oligosaccharides-induced modulation of the GALT immunity, Zenhom and colleagues (2011) treated Caco-2 cells with oligosaccharides, α-sialyllactose, or FOS. It was found that secretion of IL-12 was significantly decreased by Caco-2 cells. This finding was further supported by showing that gene expression of IL-12p35, IL-8, and TNF-α was simultaneously repressed (Zenhom, 2011). Because gene expression and translocation to the nucleus was inhibited, the authors concluded that these anti-inflammatory actions were mediated via a pathway involves NFκB (Zenhom et al., 2011).

Further characterization of the molecular mechanism to understand the anti-inflammatory action of these prebiotics indicated that the prebiotics treatment of Caco-2 cell induced
expression of PGlyRP3 in a time- and a dose-dependent manner and induced peroxisomes proliferator-activated receptor (PPAR)-γ. Targeting of PGlyRP3 with siRNA abrogated the prebiotics-mediated anti-inflammatory action. Also, co-culturing of the Caco-2 cells with the GW9662, a PPAR-γ antagonist, suppressed expression of PGlyRP3 by the prebiotics and prevented subsequent induction of the anti-inflammatory response. These findings implied that prebiotics provoke the anti-inflammatory response via increasing expression of PPAR-γ that induces PGlyRP3 expression (Zenhom et al., 2011).

2.3.3 Health-promoting effects of prebiotics

2.3.3.1 Anti-diabetic effect

Developmental plasticity allows the fetus to sense and respond to the biochemical milieu induced by the nutritional status of dam during gestation and lactation. Fetal responses to unbalanced nutrition of the dam are characterized by altered gene expressions that can destine the neonate to acquiring anomalous metabolism (Gniuli et al., 2008). To examine such effect, Maurer and Reimer (2011) fed high-prebiotic fiber, high-protein, or regular chow diets to Wistar rat dams during gestation and lactation and monitored subsequent effects on their progeny metabolism profile. Weanling rat pups from all treatment groups were fed the regular rodent chow staring on d 21 postpartum. Feeding the high-prebiotics fiber diet decreased plasma glucose and amylin and increased glucagon-like peptide (GLP-1) on day 28 postpartum (Gniuli et al., 2011). Also, feeding the high-prebiotic fiber to gestating and lactating Wistar dams increased gene expression of GLUT2 and the sodium-dependent glucose/galactose transporter in their progeny on day 21 postpartum (Gniuli et al., 2011).

The decrease of amylin concentrations in plasma of neonate rat pups of dams fed the high-prebiotic fiber diet suggests significant health implication. Amylin has been shown to
generate $\text{H}_2\text{O}_2$ that oxidatively damages the pancreatic $\beta$-cells and plays role in the pathogenesis of the type II diabetes (Masad et al., 2007). GLP-1 is an anorectic incretin that decreases the energy intake by increasing satiety signal (Verhoef et al., 2011). Also, when FOS were given at 16 g/d to human subjects, their energy intake was significantly decreased by 11% and the area under the curve for GLP-1 was significantly increased over that of the placebo treatment on day 13 of the study (Verhoef et al., 2011).

Prebiotics have the potential to improve markers of diabetes type II in rats. The diabetes type II rat model Goto-Kakizaki (GK) rats were fed prebiotic resistant starch at 30% of their diet for 10 weeks. Rats were subsequently mated and after the pregnancy and lactation periods dams were euthanized. Feeding the prebiotic resistant starch increased $\beta$-cell mass and the insulin content of the pancreas. In addition, plasma GLP-1 and cecal SCFA concentration were increased. Also, the butyrate-producing bacterial population in the cecum was greatly enhanced (Shen et al., 2011). The anti-diabetic effect of the prebiotic seemed to be mediated through a microbiome shift that involves an increase of Bifidobacteria-associated GLP-1 (Shen et al., 2011; Neyrinck et al., 2011).

On the other hand, the neonate pups of GK rats fed the prebiotic fiber diet demonstrated normal growth rate and their fasting plasma glucose significantly improved (Shen et al., 2011). The neonate GK rats developed pancreatic $\beta$-cells aplasia by week three postpartum (Tourrel et al., 2002) that underlies the hyperglycemia characteristic of type II diabetes. Treating GK neonate with 400 $\mu$g/kg GLP-1 daily during the pre-diabetic stage (first week after birth) replenished $\beta$-cell mass in their pancreas and improved their glucose tolerance test results at adulthood (Tourrel et al., 2002).
The findings of (Gniuli et al., 2011; Verhoef et al., 2011; Shen et al., 2011) highlight the efficacy of prebiotics as likely therapy for type II diabetes and as preventative for the pregnancy-associated type II diabetes. The findings also indicate that some prebiotics mediate their immune effect through microbiota modulation-induced GLP-1.

2.3.3.2 Antidyslipidemic effect

Glucan prebiotics, such as chitin-glucans (CG) from fungus has been shown to resolve the dysbiosis-induced obesity (Neyrick et al., 2011) and improve lipid and glucose metabolism. Supplementation of C57b16/J mice fed high-fat diets with 10% CG (w/w) increased the population of the clostridial cluster 14a that comprises *Roseburia* species, which was significantly decreased following high-fat diet feeding in C57b16 mice.

Of note, CG inclusion in the high-fat diet significantly prevented the weight gain by the high-fat diet and decreased fasting blood glucose and glucose intolerance. Also, CG feeding ameliorated hepatic triacylglycerols (TAG) and plasma cholesterol regardless of the energy density of the diet (Neyrick et al., 2011). Of importance to note, CG mediated its health-enhancing effect independent of GLP-1, whose expression was not altered by feeding the CG.

2.3.3.3 Antiinflammatory effect

Prebiotic feeding can ameliorate gut inflammation in obese diabetic mice through modulating gut microbiota to improve the epithelial tight junction (Cani et al., 2009). Obese diabetic mice exhibit greater intestinal permeability that lead to endotoxemia, a leading cause in the pathogenesis of metabolic disorders (Cani et al., 2008).

To explore the effect of feeding prebiotics to obese diabetic C57BL/6 mice on their intestinal permeability and inflammation, mice were fed either the prebiotic FOS or non-
prebiotic carbohydrates (control) and, subsequently, treated with GLP-2 agonist or antagonist (Cani et al., 2009). Prebiotic treatment increased expression of the tight junction proteins occludin and zonula-occludin-1 and increased GLP-2 expression. On the other hand, the prebiotic treatment decreased mRNA expression of TLR-4, monocytes chemo-attractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1, and CD68 (markers of macrophage infiltration), TNF-α and plasminogen activator inhibitor (PAI-1) (inflammation markers), and iNOS and NADPH oxidase (oxidative stress markers). Also plasma LPS was significantly decreased. Indeed, these anti-inflammatory effects of the prebiotic oligofructose were completely abrogated by GLP-2 antagonist administration in the prebiotic-treated mice. Conversely, administration of the GLP-2 agonist restored the anti-inflammatory action of the prebiotics (Cani et al., 2009).

Subsequent denaturing gradient gel electrophoresis (DGGE) on the microbiome of the cecal microbiome undertaken to characterize the role of gut microbiota revealed a prebiotic-induced increased of the total bacteria, Bifidobacteria, and Lactobacilli counts (Cani et al., 2009).

2.3.3.4 Intestinal disease-preventive effect

Prebiotics have shown efficacy in treating infant diarrhea (Passariello et al., 2011). In a single-blind, prospective, controlled study, children age 3-36 months affected with diarrhea were assigned to receive either a hypotonic rehydrant (59 males and 59 females) or a hypotonic rehydrant added with zinc sulfate and prebiotics (60 males and females) (Passariello et al., 2011). After 72 h of administering either treatment regimen, the diarrhea resolution rate was 50 and 72.9% in the control and prebiotic treatment group, respectively. Palatability of the rehydrant-added prebiotics was higher and the average intake during the
first 24 h was 50 mL/kg bw. In the control group, the average intake during the first 24 h was 22 mL/kg (Passariello et al., 2011).

Of economic importance, parents of infants in the prebiotics group missed only 0.39 working days, whereas parents of infants in the control group missed 1.45 working days. Previously, it has been shown that addition of zinc sulfate to standard rehydrant, however, decreases duration of diarrhea and amount of stool (Bhatnagar et al., 2004). Findings of Passariello and colleagues (2011) would be of more value if a third treatment group that was given the standard hypotonic rehydrant plus zinc sulfate alone were examined simultaneously.

2.3.3.5 Anticancer effect

Prebiotics have been shown to lower potential for developing colon cancer (Hijova et al., 2011). Fecal $\beta$-glucuronidase activity in individuals with colon cancer is 12.1-fold higher relative to healthy controls (Kim and Jin, 2001). To explore the $\beta$-glucuronidase-lowering effect of prebiotics and thus prevention of colon cancer, Hijova and colleagues (2011) fed rats the high-fat diet (10% fat; control) or the high-fat diet supplemented with 2% of fat with probiotics in combination with or without the bioactive food Hyppocastani extractum siccum at 2% or Lini oleum virginalae at 1% for two weeks. Subsequently, to induce $\beta$-glucuronidase, rats were injected with 20 mg/kg bw dimethylhydrazine intraperitonealy twice in two weeks.

Prebiotics alone or in combination with hyppocastani extractum siccum or Lini oleum virginalae significantly decreased the secretion of bile acids and production of the bacterial $\beta$-glucuronidase. Also, the prebiotic feeding decreased total plasma cholesterol and TAG and increased the production of the SCFA (Hijova et al., 2011).
mRNA of osteopontin (OPN; anti-apoptotic for colon cancer cells) and cyclooxygenase (COX)-2 were shown to be increased substantially in \textit{ex vivo} colon cancer tissues relative to those of the adjacent healthy colon tissues (Jahns et al., 2011). Addition of butyrate to the culture media decreased expression of OPN and COX-2 in all healthy and colon cancer tissues. Also, in colon cancer tissues, OPN mRNA expression was decreased by 50\%, whereas that of COX-2 was decreased by 35\% relative to the control after addition of the butyrate (Jahns et al., 2011).

2.4. Johne’s disease

Johne’s disease (JD; paratuberculosis) is the infection with \textit{avium} subspecies \textit{paratuberculosis} (MAP) in wild and domesticated ruminant animals. Whereas subclinical JD is characterized by decreased milk production, clinical JD is causes diarrhea and wasting in infected animals.

2.4.1. Etiology of Johne’s disease

MAP is the etiologic bacterium that causes JD in ruminant animals worldwide (Harris and Barletta, 2001; Olsen et al., 2002). MAP is a mycobactin J-dependent, acid-fast, slow-grower mycobacterial species that multiplies inside intestinal macrophages of the host (Momotani et al., 1988; Sweeney, 1996). The taxonomic classification of MAP indicates its kinship to the Suborder Corynebacterineae, order Actinomycetales, and phylum actinobacteria under the Domain Bacteria.

2.4.2. Transmission and epizootology of Johne’s disease

Johne’s disease is a chronic granulomatous enterocolitis, local adenitis, and lymphangitis and is manifested as an intermittent or profuse watery diarrhea accompanied with progressive emaciation that concludes by death of the affected animal. Once described in 1885, by John
and Forthingham, JD was an episodic disease that posed insignificant economical loss. Economical impact of JD, however, has intensified by increasing animal densities in the modern animal farming industry (Hutchinson, 1996).

Ability of MAP to form a biofilm (Wu et al., 2009) makes MAP ubiquitously prevalent in the domesticated animal (Pickup et al., 2006) and wildlife environment (Davidson et al., 2004), enhances its resistance to eradication by currently adopted control strategies, and also increases its pathogenicity.

As early as 1984, culturing ileocecal lymph nodes from cull cows indicated that JD is prevalent in about 2.9% of all cull cows in the US (Merkal et al., 1987). On herd level, however, 34% of dairy herds in Wisconsin have been found infected with JD (Collins et al., 1994). Wells and Wagner (2000) screened sera from about 79% of the US dairy herds in different geographical locations for antibodies to MAP by using ELISA assay. Results demonstrated that 3.4% of cows and 21.6% of dairy herds were infected with MAP.

Less preventative measures, such as delayed time of separation of calf from dam and nature of hygienic measures adopted including disinfecting teats and udder before collecting colostrum from dam, were highly associated with increasing prevalence of JD among herds (Wells and Wagner, 2000).

In another different approach, ELISA tests designed to detect MAP-specific IgG1 coupled with PCR to detect MAP IS900 genomic DNA in pooled bulk tank milk were used by Wilson and colleagues (2010) to examine prevalence of MAP in milk from dairies in Utah area. MAP was detected at least once in about 39% of dairy herds tested. The result highlights the fact that JD is steadily spreading among dairy cattle despite the raising awareness about the disease and the stringent control strategies being applied.
2.4.3. Morphology of *Mycobacterium avium* subspecies *paratuberculosis*

MAP average about 1.0 µm in length ranging from 0.5 to 1.5 µm. MAP possesses a relatively thick waxy cell wall that is composed of 60% lipid. This thick lipid layer permits MAP to gain the characteristics of resistance to decolorization by the acidic Ziehl-Neelsen stain (acid fastness), increased tolerance to disinfectants, such as chlorine (Whan et al. 2006), strong negative charge and hydrophobicity (Boster et al., 2009), resistance to pasteurization, (Grant et al. 1998), and ability to spread in bio-aerosols (Eisenberg et al., 2011).

MAP cell wall is composed of an internal PG layer surrounded by an intermediate bilayer of long-chain mycolic acid arabinoglactan complex and an external layer of peptidoglycolipids composed mainly of lipomannan (LM) and lipoarabinomannan (LAM), 19-kDa lipoprotein, mycolyl-arabinoglactan peptidoglycan complex (Brennan and Nishina, 1995), and trehalose 6,6’-dimycolate (Welsh et al, 2008).

LM, the precursor of LAM, is composed of a mannan core immobilized to the cell wall by a phosphatidylinositol molecule. Arabinosylation of the mannan core of LM at its free side by an arabinan domain derives LAM. The arabinan domain is a highly branched arabinofuranosyl side chains that make up the basic cell wall component of both pathogenic and nonpathogenic mycobacteria (Chatterjee and Khoo, 2000).

The arabinan domain can be capped by various motifs (Briken et al., 2004). Pathogenic mycobacteria, such as MAP, possess a arabinan domain that is capped by an oligomannose cap (Man-LAM). In contrast, the arabinan domain of non-pathogenic *Mycobacterium smegmatis* is capped by phospho-inositol (PI-LAM) and that of *Mycobacterium chelonae* is uncapped (Ara-LAM) (Chatterjee and Khoo, 1998; Briken et al., 2004).
Molecular pathogenesis studies of MAP suggest that recognition of MAP by the innate immunity surveillance patterns and the ensuing immune response outcome is dictated primarily by the presence or absence of the arabinan-capping motifs (Schlesinger, 1993; Maeda et al., 2002). Whereas AraLAM is proinflammatory, ManLAM is anti-inflammatory and allows MAP to evade the innate immunity detection through invasion of macrophage using the mannose receptor as a safe portal of entry (Schorey and Cooper, 2003).

2.4.4 Signaling of *Mycobacterium avium* subspecies *paratuberculosis* surface proteins

Upon phagocytosis of MAP and other mycobacteria, PI-LAM and Man-LAM become integrated into the cell membrane and localized to the membrane lipid rafts of the macrophage anchored by its glycosylphosphatidylinositol anchor (Welin et al., 2008). Membrane rafts are cholesterol-rich bilayer that constitutes the cell signaling molecules aggregates (Pike, 2006).

Ara-LAM is expressed constitutively by nonpathogenic bacteria and demonstrates high affinity to binding CD14, a co-receptor associated with TLR-4 and the lymphocyte antigen-96 (MD-2), that binds LPS and other PAMPs (Kitchens, 2000). Signaling of Ara-LAM via TLR-4 results in secretion of chemokines by macrophages and induction of TNF-α, IL-1α, IL-1β, and IL-6 (Juffermans et al., 2000).

Alternatively, Man-LAM localization to the membrane lipid rafts suppressed the translocation of the lysosomal marker CD63 to the phagosome of human macrophage cell line hMDMs suggesting that Man-LAM inhibits phagosome maturation (Welin et al., 2008). Inhibition of phagosome maturation by Man-LAM did not seem to involve disruption of TLR-2 and 4 signaling nor activation of p38 MAPK (Welin et al., 2008).
In the bovine animal, the paradigm of immune responses triggered against MAP infection shows that Th1 responses targeted to MAP were the first to be activated and dominate during the subclinical stage (Stabel, 1996; Stabel, 2006). Indeed, the main Th1 cytokine involved is IFN-γ (Stabel, 1996) and single nucleotide polymorphism (SNP) in IFNGR that encodes the IFN-γ receptor is associated with JD in dairy cattle (Pant et al., 2011). As the disease proceeds to the clinical stage, a switch to Th2 responses becomes dominant (Stabel, 2006) with increased IL-10 secretion. Subsequent histopathological characterization of the disease uncovered that the subclinical stage in context of Th1 involves paucibacillary (tuberculoid) granulomatous reaction that sieges the MAP dispersion in tissues and causes significant destruction to the pathogen (Kurade et al., 2004). In contrast, the multibacillary form of JD involves humoral immunity development with increase of MAP-specific antibodies production.

In the ovine animal, the classical immune response shift has been noted by Kurade et al (2004). A JD transitional state characterized by upregulation of both Th1 and Th2 responses was seen in lambs (Kurade et al., 2004). These two forms of ovine JD are characterized by distinct molecular patterns dictated by differential chemokine expression, leading to the classical immune cell infiltration unique to each of the two JD forms (Smeed et al., 2010). Detection of different forms of JD, however, requires the concurrent use of the ELISA testing and the immune proliferation assay (Kurade et al., 2004).

Recently, the dogma of the classical switch of JD from the cell-mediated immunity in the early stage to the humoral immunity at later stage has been questioned (Begg et al., 2011). Simultaneous MAP-specific IFN-γ and antibody responses have been detected in the early
stage of JD in sheep (Robinson et al., 2009; Begg et al., 2011) and even as early as two weeks post-infection in calves (Water et al., 2003).

These findings indicated the fact that probably immune responses against MAP are likely more intricate than previously thought (Stabel, 2006; Begg et al., 2011). Because about 50% of all infected sheep exhibited concurrent cell-mediated and humoral immunity, Begg and colleagues (2011) suggested that rather than a classical shift to the multibacillary form, the immune system of a JD-affected animal becomes malfunctional resulting in initial permanent loss of the cell-mediated immunity (Begg et al., 2011).

Given the ability of MAP to subvert the initial strong cell-mediate immunity (characterized by IFN-γ secretion leading to the paucibacillary granulomas formation) and to induce the non-protective humoral immunity (characterized by increased IL-10 secretion and the lepromatous granulomas development), we would question the efficacy of developing JD vaccines that trigger Th1 responses. It is well established that ruminant animal can overcome natural MAP infection and development of JD (Kurade et al., 2004). Stress, however, can aid MAP to break the host immune siege and disseminates (Chiodini et al., 1984) causing the fatal multibacillary form of JD.

2.4.5 Metabolism in Mycobacterium avium subspecies paratuberculosis

Once inside the mammalian host cell, MAP has the privilege of a wide array of nutrients available for them to metabolize (Barclay and Wheeler, 1989). MAP chiefly depends on glucose for harvesting energy (Wheeler, 1984). Metabolizing glucose in the glycolysis and pentose phosphate pathway generates a plethora of metabolic intermediates that can be utilized for building of macromolecules (Wheeler, 1984).
Acetyl-CoA and oxaloacetate will be condensed in the tricarboxylic acid cycle to generate citrate and reducing equivalents (NADH and FADH$_2$) (Wheeler, 1984). Whereas citrate can be a building block for the synthesis of mycolic acid, a principal component of MAP cell wall, the reducing equivalent can be utilized by the oxidative phosphorylation to generate ATP (Bloom, 1994).

Because lipids are available to MAP cells in vivo in excess of carbohydrate, lipid synthesis enzymes will be feedback-inhibited (Wheeler et al., 1990; Wheeler and Ratledge, 1992). Host-derived fatty acids by MAP acyl-hydrolase phospholipase (Wheeler and Ratledge, 1992) will be elongated and used by MAP to synthesize its own cell wall components (Wheeler et al., 1990). β-oxidation of host-derived fatty acids can take place inside the MAP cells to produce additional ATP and acetyl-CoA that accelerate the tricarboxylic acid cycle to aid proliferation of MAP (Barclay and Wheeler, 1989).

Host cell-available amino acids are assimilated by MAP cell to synthesize enzymes for protein synthesis. Also, the host preformed purines, pyrimidines, and nucleotides are imported inside the MAP cell and are utilized for building MAP nucleic acids (Wheeler, 1987).

MAP, however, is iron-requiring mycobacteria that fail to grow in artificial media deprived of mycobactin J, an iron-chelating substance (Bloom, 1994). Proliferation of MAP in macrophage cultured in vitro was inhibited by addition of iron-chelating substances (Fernandez et al., 2010). Microarray genomic comparison unraveled genetic divergence between two the MAP strains; S MAP that causes ovine JD and C MAP that induces bovine JD (Marsh et al., 2006). The genetic divergence involves three substantial genomic deletions in 24 open reading frames in
the S MAP (Marsh et al., 2006) that seem to underlay the distinct microbiological variance of each strain.

When iron is replete, S MAP express iron-dependent regulator SIder that suppresses gene expression of the putative bacterioferritin (BfrA) and hypothetical protein (MAP2073c), which is indicative of a defective iron storage mechanism (Janagama et al., 2010). In contrast, C MAP upregulates iron storage gene BfrA, virulence-associated antigen-85 and Esx-5, and ribosomal proteins and inhibits aconitase and succinate dehydrogenase to spare iron for cell division and proliferation (Janagama et al., 2010).

As yet, some aspects of MAP metabolism are poorly characterized (Bloom, 1994). For illustration, MAP is a facultative aerobe that may require O₂ to grow and harvest energy in the oxidative phosphorylation pathway. Because the mammalian host holds O₂ tightly bound to hemoglobin, myoglobin, and cytochromes, means by which MAP acquire O₂ await full elucidation (Bloom, 1994).

The extremely slow growth of MAP (one division each 18 h), as an example of mycobacteria, compared to that of E. coli (one division each 20 min) has been a topic of research. Largely, the thick waxy cell wall has been implicated as being a less permeable barrier to hydrophilic nutrients. Uptake of hydrophobic nutrients by MAP is accomplished through porin channels that penetrate the thick waxy lipid bilayer (Trias et al., 1992; Trias and Benz, 1994). In addition, the nucleic acid synthesis by mycobacteria and their sensitivity to inhibition by rifampicin has been hypothesized to be the rate-limiting step in the growth of the slow-growing mycobacteria (Woodley et al., 1972).
2.4.6 Economic impact of Johne’s disease

Infection with JD significantly decreases milk production and thereby causes economic loss to dairy producers (Smith et al., 2009). Cows that test positive for MAP through ELISA serum testing and fecal culture produce about 1.0 Kg per day less 4% fat-corrected milk than herdmates that test negative for MAP (Aly et al., 2010). Detection of MAP at greater than 30 CFU/g feces in a dairy cow fecal culture was associated with a decrease in milk production that amounted to about 4.0 Kg milk per day less than milk production of a JD-free cow (Smith et al., 2009).

Infection with MAP decreases milk quantity and alters its quality (Gonda et al., 2007). Deterioration of milk quantity was severer in cows that tested positive by fecal culture relative to cows that test positive by serum ELISA. Holstein cows that were infected with JD produced about 304 Kg less milk, 11.5 Kg less fat, and 9.5 Kg less protein per lactation relative to JD uninfected cows (Gonda et al., 2007). Despite its deep effect on milk production, JD, however, does not seem to affect the udder health or increase milk somatic cell counts (Hendrick et al., 2005; Gonda et al., 2007).

Additionally, MAP form feces of JD-infected cow may contaminate meat during slaughtering decreasing its quality (Alonso-Hearn et al., 2009; Mutharia et al., 2010). Despite that recent investigations have shown that viable MAP can be inactivated in lamb meat homogenate cooked to recommend standards (Whittington et al., 2010) prevalence of MAP in meat would exacerbate the economic burden to farmers because of JD. The findings also highlights the need for developing and maintaining more stringent biosafety measures to ensure fitness of meat intended for human consumption.
The USDA National Animal Health Monitoring System's (NAHMS) 1996 national dairy study indicated that JD is prevalent in about 21.6% of all dairy herd tested. Also, JD-infected herds lost US$ 100 per cow in comparison with JD-free dairy herds as a result of decreased milk production and increased culling and replacement of infected cows (Ott et al., 1999).

When percentage of culled cows because of JD reached 10% of the herd, economic losses amounted to US$ 200 per cow. Also, milk production was decreased by 700 kg per cow in dairy herds that experienced high prevalence of JD. Overall, the increased cow mortality and premature culling coupled with the decreased productivity because of JD, the national dairy industry loses about US$ 250 million per year (Ott et al., 1999).

Because the prevalence of JD has increased from 21.6% in 1999 (Ott et al., 1999) to about 68% in 2007 (NAHMS, 2007), the annual economic losses because of JD is currently greater than US$ 250 million.

2.4.7 Prevention and control measures of Johne’s disease

Prevention and control strategies in play to eliminate MAP infection and the spread of JD among dairy animals include adopting sound husbandry practices to eliminate exposure of young animals to the disease. Prevention strategies also call for continuous testing of young heifers to detect and cull infected animals. The JD prevention program also necessitates raising awareness about JD among dairy and beef farmers and urges the development of an effective vaccine (Kennedy and Benedictus, 2001). The control JD, however, has been difficult because of lack of an effective vaccine.

2.4.7.1 Husbandry and hygiene practices

MAP is ubiquitously present in the environment and in the untreated water from farm runoff Whan et al., 2005). In water, MAP can be ingested by the free-living naturally
occurring protozoa, such as *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* (Whan et al., 2006). Ingested MAP can multiply inside the protozoa, and their number can increase by 1-1.5 log₁₀ within three weeks of ingestion. Interestingly, ingestion of MAP by protozoa enhances resistance of MAP to killing by chlorine (Whan et al., 2006).

MAP also is carried in settled dust inside dairy farms where the herd is heavily infected with JD. Cleaning barns of MAP “super shedder” dairy cows with cold, high-pressure water and disinfectants followed by depopulating the barns for two weeks effectively decreased detection of viable MAP from about 86% of the samples to zero (Eisenberg et al., 2010). Repopulating barns with MAP-shedding cows resulted in wide spread of viable MAP in the settled dust inside the farm and in the surroundings, suggesting spread of MAP in the bio-aerosols (Eisenberg et al., 2011).

Minimizing the exposure of calves to adult feces has proven to be the most effective measure for eliminating JD in dairy herds (Merce et al., 2011). A compliance with this procedure is the removal of neonate calves from their dams during the first 12 hours after birth. Also, replacement heifers should be housed separately from the rest of milking herd until they are one year old. Segregation of calves before weaning, however, had no effect on transmission of JD.

Milk and colostrum from JD-infected dams can transmit JD to neonate calves (Streeter et al., 1995; Nielsen et al., 2008). Significant transmission of MAP infection, however, occurs through consumption of MAP-contaminated colostrum rather than of milk (Nielsen et al., 2008).

Indeed, feeding neonate calves pasteurized colostrum from JD-uninfected dams alleviates JD incidence in calves during the first year of life (Stabel, 2008). Of noteworthy, a JD-
preventative effect of the pasteurized colostrum was demonstrated in neonate calves born to dams naturally infected with MAP. The high thermal resistance of MAP, however, could enable a few number of MAP to survive pasteurization (Grant et al., 2001) and seed an infection.

Similarly, feeding plasma-derived colostrum replacer to neonate Holstein calves born in JD-infected herds prevented significant transmission of MAP infection relative to calves fed raw colostrum (Pithua et al., 2009). Calves fed the plasma-derived colostrum seemed less likely to become JD-infected ($R^2 = 0.55$) based on the serological and bacteriological testing modalities used (Pithua et al., 2009).

In addition to peripartal infection, congenital infection with JD has been reported (Koets, 2006) and about 9% of neonatal calves could acquire MAP infection when JD prevails in 40% of the herd (Whittington and Windsor, 2009). Adult cattle are also susceptible, and yearling cattle can acquire JD after intensive grazing of a MAP-contaminated pasture (Fecteau et al., 2010).

The use of new technology, such as the radiofrequency power to thermally disinfect wastewater from dairy farms and kill livestock pathogens, such as MAP, *E. coli* 0157:H7, and *Salmonella* species, has proven effective (Lagunas-Solar et al., 2005). Bacterial pathogens were heat-killed completely by raising the wastewater temperature to 65$^\circ$ C for less than one minute.

Milk bulk tank somatic cell counts (**BTSCC**) indicates milk quality and are used to price milk. Current SCC standards are more rigorous requiring that BTSCC be no greater than 400,000 (Reneau, 2001). To lower BTSCC, dairy farmers use milk from cows with high SCC to feed calves (Babtista et al., 2008).
Appreciable correlation between detection of MAP antibodies in milk and high SCC could not be established after screening 1,733 dairy cows milk in Danish dairy herds (Babtista et al., 2008). A log-linear relationship between the age at first elevated SCC and the age at first positive ELISA was found, suggesting that feeding milk with high SCC to young calves could pose a risk factor for acquiring JD (Babtista et al., 2008). Remarkably, MAP in milk can be efficiently eradicated by pasteurizing milk for 15 seconds at 72°C (Pearce et al., 2001).

2.4.7.1.1 Testing and culling of infected heifers.

The use of the IFN-γ assay to measure the cell-mediated immunity triggered against MAP PPD (johnin) intradermal skin test to identify and cull infected heifers at a young age has significant limitations (Jungersen et al., 2011). The chronic nature of JD confounds definitive interpretation of the assay result. On the other hand, the testing modalities adopted to detect and cull carrier heifers are far much less sensitive. During subclinical JD, the sensitivity of fecal culture and ELISA to detect JD-infected cows is about 30 and 15%, respectively (Whitlock et al. 2000).

Eventually, the IFN-γ assay indicates primarily whether an animal has been exposed to JD infection. Indeed, the IFN-γ assay doesn’t define conclusively the progression of JD in a given animal (Jungersen et al., 2011) or otherwise the animal will continue to harbor latent JD or becomes cured and immunized against the disease.

Early culling of affected heifers has been long advocated measure to limit spread of JD within a dairy herd (Kennedy et al., 2001). A recent report proposed that heifers younger than two years that test positive for JD are not significantly different than other herdmates that test negative for JD in respect to longevity and milk production parameters (Pillars et al., 2011).
Along with the difficulty of making conclusive interpretation of the IFN-γ testing, findings of Pillars and coworkers (2011) underscore the validity of testing dairy heifers for JD early in life.

2.4.7.2 Vaccination

In addition to its economic impact on dairy industry (Ott et al., 1999; McKenna et al., 2006; NAHMS, 2008), MAP have been raising considerable public health concern because of its controversial role in the pathogenesis of CD, an inflammatory bowel disease in humans (Mendoza et al., 2010; Rosenfeld and Bressler, 2010; Tuci et al., 2011; Lee et al., 2011).

As yet, effective antibiotic therapy for JD is lacking (Rosenfeld and Bressler, 2010). Additionally, significant concern has been raised about the efficacy of the currently available oil suspension heat-killed JD bacterin that produced inconsistent results (Kalis et al., 2001; Rosseels and Huygen, 2008). Given that as many as 15 different strains of MAP could be infecting animals within a herd simultaneously (Paradhan et al., 2011), strain specificity could be the main limitation of the whole cell JD bacterin.

Additionally, the heat-killed MAP bacterin also impedes the early diagnosis and detection of tuberculosis (TB) and JD infections during the assurance programs because it gives false positive results (Muskens et al., 2002). The heat-killed bacterin also impinges on animal welfare because the bacterin is a tissue irritant that causes significant tissue reaction and granuloma formation that may result in abscessation of the injection site (Muskens et al., 2002; Huntley et al., 2005). Because of concern about these limitations, oil suspension JD bacterin use has been restricted to herds with high prevalence of clinical cases of JD (Muskens et al., 2002).
2.4.7.2.1 The use of heat-killed MAP vaccine

The efficacy of long-term vaccination with the heat-killed MAP vaccine in preventing fecal shedding of MAP was evaluated (Kalis et al., 2001). In the first study, fecal samples were collected from 25 vaccinated and 29 non-vaccinated dairy herds. Fecal culture results were positive in about 4.4% of the 25 dairy herds. No significant difference in fecal shedding was revealed among the 25 vaccinated dairy herds. In the 29 non-vaccinated herds, fecal culture results were positive in 6.7% of the herds.

In the second study, fecal samples were cultured for MAP four times at six-month intervals from two vaccinated and two non-vaccinated dairy herds from cows older than six months (Kalis et al., 2001) followed by culling of cows that cultured positive for MAP. Curiously, after culling of cows that had positive fecal culture in the two vaccinated herds, the percentage of MAP-positive fecal culture decreased from 10.9 and 5.7 to 3.5 and 0.0%, respectively. In contrast, in the two non-vaccinated herds, percentages decreased from 6.1 and 16.5 to 0 and 2.3%, respectively. Investigation of the husbandry practices adopted by the different participant herds indicated that feeding raw milk to neonate calves is strictly prohibited in the unvaccinated herds. Thus, it was concluded that a heat-killed MAP bacterin is ineffective at preventing fecal shedding of MAP in dairy cows (Kalis et al., 2001).

By using different testing modalities Muskens and colleagues (2002) evaluated induction of the cell-mediated and humoral immunities by the heat-killed MAP bacterin. For this purpose, serum IFN-γ concentrations were quantified by using ELISA. Also, serum MAP antibodies were determined by using ELISA and complement fixation (Muskens et al., 2002). Though both the cell-mediated and humoral immunity were enhanced by the vaccine, significant inconsistency in immune response was observed among animals of the same herd.
Additionally, results obtained by the ELISA and complement fixation did not correlate. Thus, conclusive conclusions about efficacy of the vaccine could not be made, and it was projected that the vaccine will induce a long-lasting interference with diagnosis of TB and JD (Muskens et al., 2002).

2.4.7.2.2 The use of adjuvanted, live, attenuated MAP vaccines

The immunologic inference with diagnosis of TB by an oil- or aqueous-adjuvanted, live, attenuated MAP strain 316F vaccines was evaluated in farmed red deer (Cervus elaphus) (Mackintosh et al., 2005). Five-months-old farmed red deer were assigned to treatment groups (n = 15) that either received no vaccine (control), received one dose of 2 mL oil-adjuvanted, live, attenuated MAP vaccine, or received two doses of 2 mL aqueous-adjuvanted vaccine six months apart (Mackintosh et al., 2005).

It was found that the oil-adjuvanted, live, attenuated, MAP vaccine induced considerable cell-mediated immunity that might confer protection against JD in red farmed deer (Makintosh et al., 2005). Subsequent evaluation of the immune responses by the deer, however, indicated that vaccination by the oil-adjuvanted vaccine caused significant cross reactivity with tuberculin skin testing. Though the cross reactivity to TB testing declined with time, its potential to cause false TB positive results cannot be ruled out because of the persistently elevated MAP antibodies in serum of deer (Kohler et al., 2001; Mackintosh et al., 2005).

On the other hand, the aqueous-adjuvanted, live, attenuated MAP vaccine triggered less cross reactivity with tuberculin skin testing but induced less significant MAP antibody concentrations.
2.4.7.2.3 The use of adjuvanted, live, non-attenuated MAP vaccine

IL-12 induces selectively the differentiation of naïve T cells into IFN-γ-producing Th1 cells (Schmitt et al., 1994). The effect of co-administration of 0.5 mL of 20 µg/mL recombinant rIL-12 on JD vaccine efficacy to confer protection against JD was evaluated in neonate Holstein calves (Uzonna et al., 2003). Human or murine rIL-12 was used as an adjuvant to non-attenuated field-isolate or strain 18 MAP vaccine. Both vaccines induced modest cell-mediated immunity as evident by the slight increase of IFN-γ (Uzonna et al., 2003). Tissue culture for MAP suggested that co-administration of rIL-12 and the non-attenuated MAP vaccine did not significantly lower the MAP burden in tissues. The study concluded that the use of rIL-12 adjuvanted non-attenuated MAP vaccine confers limited protection against bovine JD (Uzonna et al., 2003).

It became evident now that neither form (heat-killed, live attenuated and the live non-attenuated) of the MAP bacterin confers protection against future infection with MAP in cattle (Koets et al., 2006). Also, immunization of cattle with the MAP bacterin only slightly limits number of subclinically infected cattle that still shed MAP into the environment (Kohler et al., 2001; Muskens et al., 2002; Uzona et al., 2003; Mackintosh et al., 2005; Koets et al., 2006).

2.4.7.2.4 The use of recombinant MAP DNA vaccine:

Scientists from the USDA-ARS-NADC and the College of Veterinary Medicine at Iowa State University pioneered the search in the genome of MAP using DNA expression library to uncover potential DNA vaccine sequences against MAP infection (Huntley et al., 2005). Generated clone pools of sequence were injected into mice by using gene gun delivery followed by challenging mice with virulent MAP to determine sequences that would confer
immune protection against MAP (Huntley et al., 2005). Nucleotide sequencing of the clones that conferred protection to MAP revealed that 26 genes are potential vaccine subunits against MAP infections (Huntley et al., 2005).

The heat-shock protein70 (Hsp70) subunit is a chaperone protein that enhances correct protein folding and prevents protein aggregate formation (Morano, 2007). Gene and protein expression of Hsp70 is upregulated during cellular stress and in response to infection (Koets et al., 2006; Wieten et al., 2009). Also, Hsp70 is an immuno-dominant antigen in bovine JD and can trigger vigorous cell-mediated and humoral immune responses (Wieten et al., 2009).

Koets and colleagues (2006) evaluated immune responses of neonate calves to recombinant MAP Hsp70. Forty calves were assigned randomly to four treatment groups (n = 10). Group I represents animals that were neither immunized nor infected, group II contained calves that were non-infected but immunized, group III contained infected calves that were not immunized, and group IV contained calves that were both immunized and infected. Subsequently, group I and II calves were mixed together but kept separate from groups III and IV (Koets et al., 2006).

Infected calves received 20 g of feces from a known infected cow that had been mixed with 100 mL of milk daily for the first 21 days of life. For immunization, 200 µg of recombinant MAP Hsp70 suspended in 1 mL sterile PBS containing 20 mg/mL dimethyl dioctadecyl ammonium bromide (DDA) adjuvant was injected under the skin. A booster dose was given on day 308 of the study. Koets et al (2006) demonstrated, for the first time, that vaccination of cattle with Hsp70/DDA significantly decreased shedding of MAP in feces with significant implication on decreasing the spread of JD in cattle herds. Of significance, contrary to the whole cell bacterin, vaccination with Hsp70/DDA enabled the differentiation
between the vaccinated and infected animals. Whether Hsp70/DDA-vaccinated calves might exhibit false positive results on tuberculin testing, however, remained to be verified (Koets et al., 2006).

Extensive evaluation of the immunogenicity of various MAP antigens as well as their ability to induce Th1 responses when used as vaccine subunits received increased interest. Work by Park and co-workers (2008) from the College of Veterinary Medicine at Cornell University showed that the MAP 85 antigen complex (85A, 85B, and 85C), superoxide dismutase (SOD) and 35 kDa protein induces robust lymphocyte proliferation and secretion of Th1-associated IFN-γ, IL-2, IL12, and TNF-α secretion.

Motivated by these findings, MAP 85 antigens, SOD and 35 kDa proteins were cloned and recombined in eukaryotic expression plasmid pVR1020. To evaluate the vaccine efficacy, C57BL/6 mice were injected intramuscularly with the recombinant DNA vaccine cocktail three times. The control mice received the pVR1020 DNA alone (Park et al., 2008).

Following vaccination, all mice were infected with MAP. The vaccine cocktail generated robust CD4+ and CD8+ T cell responses and higher lymphocytes proliferation compared with that of control mice (Parke et al., 2008).

In vitro splenocyte cell cultures from vaccinated mice showed enhanced IFN-γ production. Also, MAP burden in livers and spleens of vaccinated mice was significantly decreased relative to the control (Park et al., 2008). Histopathological examination of tissues from the control mice unveiled severe histopathological injury evident by the increased numbers of granulomas and their content of acid-fast bacilli. The substantial Th1 responses observed coupled with reduction of MAP burden in tissue led the researcher to conclude that
MAP 85 antigens, SOD, and the 35 kDa cocktail vaccine has potential to protect mice against infection with JD (Park et al., 2008).

Similarly, the differential immune responses and the protective efficacy of these four recombinant antigens with two different adjuvants, the monophosphoryl lipid A (MPLA) or the bovine IL-12 were examined in calves (Kathaperumal et al., 2008).

Group I calves were administered the four MAP antigens together with MPLA and IL-12. Group II calves were administered with the four antigens and MPLA. Calves in group III received MPLA and IL-12. Calves in group IV received MPLA alone (Kathaperumal et al., 2008). In group I and II animals, all the MAP antigens, except for SOD, induced significant Th1 responses as indicated by the increase of IFN-γ, antigen-specific IL-2, IL-12p40, and TNF-α concentrations. Similarly, expression of CD4⁺, CD8⁺, CD25⁺, and CD3⁺ was increased dramatically, indicative of enhanced cell-mediated immunity (Kathaperumal et al., 2008).

As far as the bacterial burden is concerned, whereas 4 and 3 out of 8 calves in each of group I and II, respectively, were cultured positive, 3 out of 4 animals in each of group III and IV were cultured positive suggesting that the recombinant MAP antigens could confer protection to calves against JD infection (Kathaperumal et al., 2008).

The same research group examined the protective efficacy and differential immune response of the recombinant 85A, 85B, SOD, and the fusion protein Map 74F along with DDA as an adjuvant in week-old goat kids (Kathaperumal et al., 2009). Goat kids were chosen from herds that were JD free. Initially, goats were randomized into three treatment groups (n = 8). Animals in group I were immunized subcutaneously with DDA-adjuvanted recombinant antigen cocktail at 100 µg. Group II animals were immunized with the non-
DDA adjuvanted antigen cocktail. Goat kids in group III were administered adjuvant DDA alone to serve as a control.

All goat kids in the different groups were boostered after three weeks. Three weeks later, all goat kids were administered $5 \times 10^8$ CFU of MAP per animal in 10 mL of milk replacers for seven consecutive days.

In group I and II, T lymphocyte proliferation became significant three weeks after the booster dose and peaked after the fourth week. In group I goats, the antigen-specific IFN-γ increased significantly three weeks after the booster dose and remained significantly elevated for 10 weeks. Similarly, the CD4$^+$ subset was higher in the immunized animals (groups I and II) six to 10 weeks after the immunization compared with the control animals (Kathaperumal et al., 2009).

Remarkable reduction of MAP burden in tissues was seen at necropsy of group I (Kathaperumal et al., 2009). It was evident that immunization with the 85A and Map 74F antigens along with adjuvant DDA triggers Th1 responses and imparted protection against MAP infection in a goat model (Kathaperumal et al., 2009).

Because MAP rHsp70 subunit conferred protection against MAP infection in mice and goats (Park et al., 2008; Kathaperumal et al., 2008; 2009), it was hypothesized that Hsp70 in intact MAP must be exposed to the anti-Hsp70 antibodies (Santema et al., 2006). Two B cell linear epitopes on the cell wall of the intact MAP that are ligand for Hsp70-induced antibodies have been identified by using mAb (Koets et al., 2006). The same epitopes were identified in Hsp70-immunized goat and calves (Santema et al., 2006). Further work illustrated that the epitopes exist in the intestinal tissue lesions and in naturally infected JD animals (Santema et al., 2011). These findings further showed that vaccination by MAP
rHsp70 induces recognition by B cell of epitopes on rHspt70 and generation of protective antibodies against native Hsp70 that impart protection against JD (Santema et al., 2009).

Subsequent work to characterize the MAP Hsp70-specific T cell epitopes in cows immunized with MAP Hsp70 and cows infected with experimental JD has been conducted (Hoek et al., 2010). Several peptides of the Hsp70 induced proliferation and IFN-γ secretion by T cells from cattle infected with JD or immunized with Hsp70 (Hoek et al., 2010). Of these Hsp70 peptides, five peptides induced T cell responses in about 82% of the infected calves. Further molecular characterization unraveled that the antigenic peptides were presented to T cells by multiple BoLA cells II DRB3 alleles indicating ability of Hsp70 subunit to induce protection against JD in cattle (Hoek et al., 2010).

Overall, polarization of immune response to either Th1 or Th2 is dictated by the host-pathogen interaction (Santema et al., 2011). Infection with virulent MAP generates initial Th1 responses in the first stage of infection (Watkins et al., 2010). Despite this, infected animals are unable to overcome MAP infection and eventually succumb to the clinical stage of the JD (Souza et al., 2007). Vaccination with the Hsp70 can induce anti-MAP antibodies that can bind surface epitopes and neutralize MAP (Sentema et al., 2011). Coupled with the initial cell-mediated immunity triggered by the natural infection with MAP the Hsp70 might protect the animal against JD and lessen JD economical loss to farmers.

In conclusion, rHsp70 has been shown to express linear epitopes that are recognized by T (Hoek et al., 2010) and B cells (Santema et al., 2011). Vaccination with Hsp70 induced robust Th1 responses (Hoek et al., 2010) and decreased MAP shedding in cattle (Koetz et al., 2006). Importantly, Hsp70-induced immune responses do not interfere with the
immunodiagnosis of TB (Santema et al., 2009). Interference with TB diagnosis via ELISA has been eliminated by inclusion of a Hsp70 pre-absorption step (Santema et al., 2009).

2.4.8 Johne’s disease-related intestinal disorders

Association of MAP with chronic granulomatous disease in humans is receiving strong debate now (Sing et al., 2008; Parrish et al., 2009; Mendoza et al., 2010; Lee et al., 2011)

2.4.8.1 The Chronic inflammatory bowel disease

Inflammatory bowel disease (IBD) is unremitting intestinal inflammation that is classified into two subtypes; CD and ulcerative colitis (UC) (Podolsky, 2002; Baumgart and Carding, 2007). CD is a chronic inflammation of the gastrointestinal tract and has a poorly defined etiology (Fiocchi, 1998). Similarly, UC is a chronic inflammatory disorder of the colon mucosa whose etiology remains largely ill characterized (Bamias et al., 2011). It is likely that CD and UC develop in genetically susceptible individuals because of an overly intolerant immune response to commensal microbiota of the ileum and colon (Fiocchi, 1998; Bamias et al., 2011).

The genetically dictated miscommunication between the otherwise tolerated commensal inhabitants of the gut and GALT seemed to involve mutations of the intracellular bacterial sensors known as the NOD-2/caspase activation and recruitment domain15 (NOD-2/CARD15) (Bianchi et al., 2007) and the autophagic regulator gene (ATG16L1) (Plantinga et al., 2011). Mutations of these genes have been linked to aberrant excessive secretion of proinflammatory IL-1β, IL-6 (Plantiga et al., 2011), IL-13 from NK T cells (Mannon et al., 2011), and IL-33 (Sponheim et al., 2011) in ileal and colonic mucosa.
2.4.8.1.1 Crohn’s disease

Research findings that argue for association of MAP with CD cite significant prevalence of MAP DNA in ilea of individuals with CD (Lee et al., 2011). In mucosal biopsies taken from the terminal ilea of pediatric patients, MAP IS900 genomic DNA was detected in 1 out of 19 (5%) pediatric controls and 7 out of 20 (35%) pediatric subjects with CD. In mucosal biopsies taken from colon, MAP IS900 genomic DNA was not detected in all 19 control pediatric subjects. In contrast, MAP IS900 genomic DNA was detected in 5 of 19 (26%) pediatric subjects diagnosed with CD (Lee et al., 2011). Despite that MAP IS900 genomic DNA did not seem to associate with CD prognosis, it was significantly associated with CD in pediatric subjects with significant tropism to the ileal tissues (Lee et al, 2011).

Detection of MAP IS900 genomic DNA in a population of subjects, however, might be less revealing of the actual MAP infection. A study aimed at detecting viable MAP or MAP IS900 DNA in blood of individuals with or without CD and UC demonstrated presence of MAP IS900 genomic DNA in all blood samples from patients tested by IS900 PCR and confirmed by using the dot blot assay including the controls (Mendoza et al., 2010).

Of significance, the cell wall-deficient spheroblast MAP was detected only in blood from the CD patients by culturing blood on specific medium (Mendoza et al., 2011). Positive cultures were further identified to be negative for the Ziehl-Neelsen stain (Mendoza et al., 2011) but positive for the phenolic acridine orange fluorescent stain (Smithwick et al., 1995; Mendoza et al., 2011). Positive blood cultures of MAP neither correlated with CD-linked SNPs (Parish et al., 2009; Mendoza et al., 2010) nor with the use of immunosuppressive drugs against CD (Mendoza et al., 2010).
A striking finding was reported by Sing and coworkers (2008). In this report MAP was cultured positive from four stool samples out of five CD-diagnosed shepherds who cared for goat herds infected with JD. ELISA using indigenous MAP 'bison type' genotype of goat origin was positive on sera of all CD-diagnosed shepherds. Curiously enough, in the same study, Ziehl-Neelsen staining was positive on eight normally healthy individuals who have regular contact with MAP-infected goat herds (Sing et al., 2008).

On the other hand, research done at Johns Hopkins University examined prevalence of MAP in blood of 130 CD subjects and 130 controls (Parrish et al., 2009). All CD subjects had high rate of SNPs in their NOD-2 and CARD-15, indicating a direct association with CD. Only one individual of the CD subjects’ blood samples was cultured positive for MAP (Parrish et al., 2009).

Considerable evidence suggests that MAP is present in the gut of individuals inflicted with CD (Sing et al., 2008; Mendoza et al., 2010; Lee et al., 2011). As yet, the actual role of MAP in the pathogenesis of CD remains poorly understood. Research to unveil whether MAP is an incidental opportunistic pathogen that proliferates in immunocompromised gut of CD individuals or otherwise a malicious pathogen of the human gastrointestinal tract is warranted.

2.4.8.1.2 Ulcerative Colitis (UC)

Typically, UC thought to be a Th2-driven colon inflammation that involves upregulation of IL-4 and IL-13 cytokines (Bouma and Strober, 2003). It has been shown that IL-13 induces apoptosis of IEC and dissociation of IEC tight junction. This damaging effect of IL-13 is believed to be mediated through the TNF-like weak inducer of apoptosis (TWEAK)
and its receptor Fn14 (Kawashima et al., 2011). Also, aggravation of the UC symptoms by Th17-type responses has been implicated in the pathogenesis of UC (Kobayashi et al., 2008).

2.4.9 Interaction of Mycobacterium avium subspecies paratuberculosis with probiotics

Van Brandt and colleagues (2011) studied the survival of MAP in yoghurt and commercially fermented milk containing probiotic bacteria. For this purpose, whole and skimmed milk was pasteurized by using the UHT method, inoculated with MAP, and used to produce yoghurt and fermented milk. Subsequently, yoghurt and fermented milk were stored at 6°C for 6 weeks. During the incubation period, MAP growth was monitored frequently by using conventional culture method. It was unveiled that the fat content or type of probiotic starter culture did not significantly alter MAP growth in yoghurt. Of significance, MAP prevalence in fermented milk was lowered by 1.2 to 3.8 $\log_{10}$, depending on the probiotic starter in the fermented milk and the strain of MAP inoculum (Van Brandt et al., 2011).

*Rhodococcus maris*, previously known as *Flavobacterium maris*, is a member of the mycolic acid-containing bacteria such as the *Mycobacterium, Rhodococcus, Corynebacterium*, and *Nocardia* genera (Nesterenko et al., 1982). Based on 16S sequencing of the ribosomal DNA, Rainey et al (1995) suggested that *Rhodococcus maris* must be reclassified as the new genus *Dietzia* with the species *maris*.

*Dietzia subspecies* 79793-74, however, was isolated as *Mycobacterium gordonae* from the fecal matter of dairy cow that was diagnosed positive by using the culture and ELISA modalities (Richards, 1989). Based on 16S rRNA sequencing data, *M. gordonae* was reclassified as *Dietzia*.

*Dietzia subspecies* 79793-74 has been shown to cure JD in different breeds of 48 dairy cattle (Click and Van Campen, 2010). During the life time of the animal, close monitoring of
the seroprevalence of MAP coupled with fecal culture demonstrated that decreased MAP shedding relative to that of 22 JD-infected control cows used in the study. Also, longevity of Dietzia-treated cows was enhanced and correlated positively with the initial MAP-specific antibodies. In addition, the cured animals were only those that were given the probiotic Dietzia. The mechanism by which Dietzia subspecies 79793-74 inhibits MAP growth in vivo calls for further immunobiological elucidation.

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CHAPTER 3. TOLERANCE OF SPECIFIC PATHOGEN-FREE BALB/C MICE TO VARYING CONCENTRATIONS OF VIALBE OR HEAT-KILLED PROBIOTIC LACTOBACILLUS ACIDOPHILUS STRAIN NP51®

A paper to be submitted to the Journal of Immunobiology

RUNNING TITLE: NP51, IMMUNE RESPONSE, BALB/C MICE.

Tolerance of specific pathogen-free Balb/c mice to varying concentrations of viable or heat-killed probiotic Lactobacillus acidophilus strain NP51®

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**ABSTRACT**

We evaluated tolerance to and effects of varying doses of viable and heat-killed *Lactobacillus acidophilus* strain NP51 fed to specific pathogen-free Balb/c mice on their immune responses and intestinal histopathology. We hypothesized that feeding the probiotic NP51 would enhance innate immunity of Balb/c mice in a dose-dependent manner without inducing intestinal injury. Thus, Balb/c mice were randomized to treatment groups to consume either the viable or heat-killed NP51 (**VNP51** or **HNP51**, respectively). VNP51 or HNP51 were fed at $10^4$, $10^5$, or $10^6$ CFU·mouse$^{-1}$·day$^{-1}$ for 6 weeks along with the sterile normal mouse chow until the end of the study. On day 45, ten mice from each group were euthanized. Small intestinal tissues were excised and stained with E&H and examined for histopathological tissue injury. Spleens were dissected and used in splenocyte *in vitro* cultures that were either nonstimulated or stimulated with concanavalin A and examined for proliferation of T lymphocyte subpopulations and intracellular cytokine secretion pattern. Overall, feeding the VNP51 or HNP51 to specific-pathogen free Balb/c mice at $10^4$, $10^5$, or $10^6$ CFU·mice$^{-1}$·day$^{-1}$ did not perturb the intestinal histology. Most importantly, feeding VNP51 or HNP51 expanded CD4$^+$CD25$^+$ and CD4$^+$CD25$^+$ T reg cells and induced CD8α$^+$ immune cells-secreting IL-12, IFN-γ, and TNF-α in a dose-dependent manner. These data
suggest that feeding either the VNP51 or HNP51 up to $10^6$ CFU·mice$^{-1}$·day$^{-1}$ is well tolerable by Balb/c mice and induce CD8$^+$-oriented immunity.

Key words: PROBIOTICS, NP51, BALB/C MICE.

**INTRODUCTION**

Probiotic bacteria members of the gut microbiota prevent pathogen colonization of the gut. Probiotic Lactobacilli and Bifidobacterium fed to immunocompetent and immunodeficient human microbiota-associated mouse model in Balb/c mice challenged with either *Campylobacter jejuni* or *Salmonella enterica* completely prevented *C. jejuni* colonization and significantly decreased *S. enterica* numbers in their intestines (Wagener et al., 2009). Lactobacilli prevented *Salmonella enterica*-induced immunosuppression in immunocompetent Balb/c mice by repressing activation of caspase 3 and 7 of the B and T lymphocytes and preventing induction of immune cells apoptosis (Wagner et al., 2009).

Additionally, *Lactobacillus paracasei* fed at $10^9$ CFU for one month increased body weight gain by 50% and decreased intestinal inflammation in Rag2$^{-/-}$ murine model of inflammatory bowel disease as revealed by the significant decrease of the neutrophils infiltration and secretion of IL-1β, IL-6, and IL-12 in the colon (Oliveira, et al., 2011).

Probiotic lactobacilli decrease intestinal inflammation via inhibition of CXCL-8 production by intestinal cells and increase of glutathione production by hepatocytes (Arribas et al., 2011) suggesting that oral probiotic immune modification is not limited to the GALT.

These probiotic strain-dependent immune effects are attributed to unique interaction between the probiotics-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) on DC (Mazzoni and Segal, 2004). Specificity of DC-probiotic interaction is mediated by the affinity of the DC-specific intracellular adhesion molecule-3 (ICAM-3)-
grabbing nonintegrin (DC-SIGN) on DC to interact with certain probiotic bacteria (Smits et al., 2005).

Probiotic *lactobacillus rhamnosus* LcR35 programmed DC maturation in a dose-dependent manner. At high dose, LcR35 upregulated DC expression of CD86 (B7.2), CD83, HLA-DR, and TLR-4. Alternatively, DC-SIGN (mediates DC rolling and adhesion to endothelium) and CD14 (lipopolysaccharide-binding protein) expression was downregulated (Evrard et al., 2011), suggesting enlisting of DC to recognize certain bacterial strains. Subsequently, mature DC dictates the type of the ensuing immune response via secreting distinct cytokine profile depending on the DC phenotype (Mazzoni and Segal, 2004). Factors that destined DC to specific immune phenotype include the DC maturation-inducing stimulus, cytokines and chemokines dominate the microenvironment, and lineage of DC (Mazzoni and Segal, 2004).

In support of Mazzoni and Segal (2004) findings, treating immature DC with different strains of probiotics caused a dose-dependent alteration of their IL-10/IL-12p70 secretion. *Lactobacillus salivarius* Ls-33 and *Bifidobacterium infantis* 35624 predominantly programmed DC to secrete IL-10, an antiinflammatory cytokine with pleiotropic effects. On the other hand, probiotic *Lactobacillus acidophilus* NCFM and the probiotic cocktail VSL#3 selectively induced secretion of IL-12p70, a proinflammatory cytokine. *Escherichia coli* Nissle 1917, however, equally provoked IL-10 and IL-12p70 secretion by DC (Gad et al., 2011).

We hypothesized that feeding either the heat-killed or viable *Lactobacillus acidophilus* strain NP51 to specific pathogen-free Balb/c mice would differentially modulate their immune response in a dose-dependent manner. Our objective was to examine the intestinal
tolerance and immune response of Balb/c mice to various concentrations of viable and heat-killed NP51.

**MATERIALS AND METHODS**

**Experimental Design.**

Eighty pathogen-free, 6-week-old Balb/c mice (Jackson Laboratories) were randomly assigned to eight treatment groups that included four major groups (Table 1). Group I was fed the sterile chow meal. Group II was fed the maltodextrin (MDX) carrier at 3% of their chow meal. Group III was fed the heat-killed NP51 (HNP51) at $10^4$, $10^5$, or $10^6$ CFU·mouse$^{-1}$·day$^{-1}$. Group IV was fed the viable NP51 (VNP51) at $10^4$, $10^5$, or $10^6$ CFU·mouse$^{-1}$·day$^{-1}$ for 45 days.

**Table 1. Designation of treatment groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
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<th>IV</th>
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<tr>
<td>Probiotic</td>
<td>Cntrol</td>
<td>MDX</td>
<td>HNP51</td>
<td>VNP51</td>
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<tr>
<td>Dose</td>
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<td>3%</td>
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<td>No. of mice</td>
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Cntrl: Control mice fed the regular sterile regular chow diet. MDX: maltodextrin (carrier for HNP51 and the VNP51). HNP51 and VNP51: heat-killed and viable *Lactobacillus acidophilus* strain NP51$^\circ$.

Mice were housed in a BSL-2 rodent room in the animal facility of the Iowa State University. The environment within the room was maintained to provide a temperature of 22.2°C, a relative humidity of 35-45%, and 12 h light/dark cycle. Mice were housed in autoclaved standard mice cages fitted with raised-wire floors and High Efficiency Particulate Air (HEPA) filters.
Mice were offered *ad libitum* sterile water, but fed restricted amount of chow meal (Harlan Laboratories) throughout the study. Mice were adapted to the sterile chow meal for one week prior to the addition of the probiotic HNNP51 or VNP51 to the chow diet. Either the HNP51 or VNP51 was fed at $1 \times 10^4$, $1 \times 10^5$, or $1 \times 10^6$ CFU·mouse$^{-1}$·day$^{-1}$ contained in 3.0 g of the chow meal. To prepare the HNP51, VNP51 was incubated in a preheated oven at 85$^\circ$ C for 15 minutes. The offering of chow meal was increased by 0.5 g chow·mouse$^{-1}$ every 15 days until it reached a maximum of 4.0 g of chow·mouse$^{-1}$·day$^{-1}$ by day 30 of the study. Mice continued to be offered 4.0 g until the end of the study on day 45. The experiment was conducted in accordance with guidelines established by the Iowa State University Institutional Animal and Care Use Committee.

**Sampling and Analyses**

On day 45 of the study, 10 mice from each treatment group were euthanized. Spleens were dissected and used for splenocyte single-cell suspension preparation. Splenocytes were cultured *in vitro* in 48-well plates at $2 \times 10^6$ cells per mL in RPMI 1640 (Invitrogen, Carlsbad, CA) plus 10% heat-inactivated FCS (Fisher Scientific, Hanover Park, IL) and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, NM). Splenocytes *in vitro* cultures were either nonstimulated or stimulated with concanavalin A (**ConA**) (Sigma-Aldrich, St. Louis, MO) at 3 µg/mL.

Following stimulation with ConA, splenocyte cultures were incubated at 37$^\circ$ C and 5% CO$_2$ for 72 hours. The pelleted splenocytes were washed twice with cold sterile PBS and stained for the surface markers CD4 (anti-mouse CD PerCP-Cy5.5, eBioscience, San Diego, CA), CD8α (anti-mouse CD8α APC, eBioscience, San Diego, CA), and CD25 (anti-mouse CD25 phycoerythrin; PE, eBioscience, San Diego, CA).
Subsequently, frequencies of these T cell subpopulations were determined by using the Fluorescence-Activated Cell Sorter (BD Facsaria II; Becton Dickson Biosciences, Franklin Lakes, NJ). Data were analyzed by using the FlowJo software (Tree Star Inc., Ashland, OR) for IFN-γ, TNF-α, IL-2, IL-4, and IL-10 concentrations.

To examine tolerance of Balb/c mice to the HNP51 and VNP51, we examined the histological architecture of the small intestine. Briefly, the whole duodenum, jejunum, and ileum were fixed in 10% formaldehyde, stained with hematoxylin and eosin (H&E), and imbedded in paraffin. Subsequently, 4 µm sections were examined under the microscope by a histopathologist to determine effects of feeding the HNP51 or VNP51 on the crypt and villus areas and the villus area: crypt area ratio.

Also, upon euthanizing mice, we withdraw 0.9 mL of blood by cardiac puncture and used it to isolate blood serum. Briefly, blood in eppendorf tubes was kept at 4° C overnight to clot. Blood was then spun in eppendorf centrifuge at 5000 rpm for 15 minute. Subsequently, serum was pipetted out in a another sterile eppendorf tube and stored at -20° C until analyzed for IgG₂a and IgG₁ using commercially available kit (Kamiya Biomedical, Seattle, WA).

**Statistical Analyses**

A linear mixed-effects model was fit to the data for each response variable by using the PROC MIXED procedure of the Statistical Analysis System (SAS; SAS Institute Inc., Cary, North Carolina, USA) version 9.2. Each linear mixed-effects model included random cage effect and fixed effects for probiotic dose, stimulation of cells, and their interactions. The standard errors of the means were reported and the pairwise differences among the estimated means were considered significant when the probability (P) value was ≤ 0.05. Tendency for a trend was declared when 0.05 ≤ P ≤ 0.1.
RESULTS

Surface Markers and Intracellular Cytokines Staining

*CD4*⁺ *T helper cells*. On the whole, feeding either the VNP51 or HNP51 at different doses to pathogen-free Balb/c mice did not significantly (*P* = 0.13; Figure 1A) change CD4⁺ T helper cells frequency. On the contrary, the effects of the stimulation with ConA and its interaction with VNP51 and HNP51 feeding (treatment) were significant (*P* = 0.0001 and *P* = 0.04,
Figure 1. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their splenic CD4+ T helper cell and CD4+CD25+ T reg proliferation and CD4+ T helper cells intracellular cytokine secretion. Panel A. CD4+ T helper cell proliferation. Panel B. CD4+CD25+ T reg cell proliferation. Panel C. CD4+ T helper cell-secreting IL-2. Panel D. CD4+ T helper cell-secreting IL-4. Panel E. CD4+ T helper cell-secreting IL-10. Panel F. CD4+ T helper cell-secreting IL-12. Panel G. CD4+ T helper cell-secreting IFN-γ. Panel C. CD4+ T helper cell-secreting TNF-α. Data are mean ± SEM. (*) indicates significant difference at $P \leq 0.05$; (#) indicates tendency for a significant difference at $0.05 < P \leq 0.10$; NS, nonstimulated in vitro splenocyte cultures; ConA, concanavalin A-stimulated in vitro splenocyte cultures.
respectively; Figure 1A). In particular, feeding either the VNP51 or the HNP51 at differing dosages did not significantly ($P \geq 0.49$) alter frequency of CD4$^+$ T helper cells in the in vitro nonstimulated splenocyte cultures (Figure 1A). When the splenocytes cultures were stimulated with ConA mitogen, feeding either VNP51 or the HNP51 at $10^4$ CFU appreciably ($P = 0.04$ and $P = 0.03$, respectively) increased CD4$^+$ T helper cells proliferation (Figure 1A). Also, HNP51 fed at $10^6$ CFU tended ($P = 0.09$) to increase CD4$^+$ T helper cells proliferation (Figure 1A).

**CD4$^+$CD25$^+$ T reg cells.** Overall, feeding the VNP51 and HNP51 to Balb/c mice and stimulation with ConA had significant ($P = 0.0005$ and $P = 0.0001$) effects on CD4$^+$CD25$^+$ T reg cells proliferation (Figure 1B). Precisely, feeding the HNP51 at $10^4$ or $10^5$ CFU significantly ($P = 0.004$ and $P = 0.0007$; Figure 1B) increased CD4$^+$CD25$^+$ T reg cells repertoire. On the other hand, feeding the HNP51 at $10^6$ or feeding the VNP51 at different doses did not appreciably ($P \geq 0.14$) modify CD4$^+$CD25$^+$ T reg cells repertoire (Figure 1B).

**CD4$^+$ T helper cells intracellular cytokine secretion.** Examination of the intracellular cytokines secretion by CD4$^+$ T helper cells from splenocytes of Balb/c mice stimulated with ConA revealed no significant ($P \geq 0.32$, $P \geq 0.35$, $P \geq 0.22$, $P \geq 0.28$, and $P \geq 0.21$), effect of VNP51 or HNP51 feeding on number of IL-2-, IL-4-, IL-10-, IFN-$
$-y-, and TNF-$
$-*$
$- secreting CD4$^+$ T helper cell repertoire, respectively, (Figures 1C, D, E, G, and H). In contrast, feeding HNP51 at $10^5$ CFU tended ($P = 0.10$) to increase IL-12-secreting CD4$^+$ T helper cells (Figure 1F). Repertoire of IL-12-secreting CD4$^+$ T helper cells, however, was not altered by feeding the VNP51 at any doses ($P \geq 0.31$) or by feeding the HNP51 at $10^4$ or $10^5$ CFU ($P \geq 0.33$; Figure 1F).
Figure 2. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their splenic CD8α+ immune cells and CD8α+CD25+ T reg cell proliferation and CD8α+ immune cells intracellular cytokine secretion. Panel A. CD8α+ immune cells proliferation. Panel B. CD8α+CD25+ T reg cell proliferation. Panel C. CD8α+ immune cells-secreting IL-2. Panel D. CD8α+ immune cells-secreting IL-4. Panel E. CD8α+ immune cells-secreting IL-10. Panel F. CD8α+ immune cells-secreting IL-12. Panel G. CD8α+ immune cells-secreting IFN-γ. Panel C. CD8α+ immune cells-secreting TNF-α. Data are mean ± SEM. (*) indicates significant difference at \( P \leq 0.05 \); (#) indicates tendency for a significant difference at \( 0.05 < P \leq 0.10 \); NS, nonstimulated in vitro splenocyte cultures; ConA, concanavalin A-stimulated in vitro splenocyte cultures.
**CD8α⁺ immune cells.** Overall, there was no significant \( P = 42 \) VNP51 or HNP51 treatment effect. ConA stimulation, however, exerted significant \( P = 0.0001 \) effect. On contrast, the interaction of treatment by ConA stimulation effect was not significant \( P = 0.67 \); Figure 2A). Feeding the MDX to Balb/c mice tended \( P = 0.07 \) to decrease CD8α⁺ immune cells repertoire in their splenocyte cultures relative to that of control mice fed the chow diet only (Figure 2A). But VNP51 or HNP51 did not significantly \( P \geq 0.33 \) change CD8α⁺ immune cells repertoire in comparison with that of the MDX-fed control mice nonstimulated in vitro splenocyte culture (Figure 2A). Of note worthy, feeding the VNP51 or the HNP51 at different dosages to Balb/c mice did not significantly \( P \geq 0.22 \); Figure 2A) alter CD8α⁺ immune cells repertoire in their in vitro ConA-stimulated splenocyte cultures relative to that of the MDX-fed control mice.

**CD8α⁺CD25⁺ T reg cells.** Generally, feeding the VNP51 or the HNP51 to Balb/c mice did not significantly \( P = 0.21 \) change the CD8α⁺CD25⁺ T reg cell repertoire. Stimulation with ConA exerted significant \( P = 0.0001 \) effect. In particular, feeding the HNP51 at \( 10^4 \) CFU tended \( P = 0.08 \) to increase and at \( 10^5 \) CFU significantly \( P = 0.01 \) increased CD8α⁺CD25⁺ T reg cell repertoire (Figure 2B). Intriguingly, feeding the HNP51 at \( 10^6 \) CFU did not \( P = 0.48 \) expand CD8α⁺CD25⁺ T reg cells repertoire (Figure 2B). Also, feeding the VNP51 at any dose did not significantly \( P \geq 0.27 \) change CD8α⁺CD25⁺ T reg cell repertoire in the nonstimulated or ConA-stimulated splenocyte in vitro cultures (Figure 2B).

**CD8α⁺ immune cells intracellular cytokine secretion.** Feeding the VNP51 or the HNP51 to Balb/c mice did not significantly \( P \geq 0.16, P \geq 0.17, \) and \( P \geq 0.24 \) change IL-2-, IL-4-, and IL-10-secreting CD8α⁺ immune cells repertoire, respectively, in the nonstimulated or
ConA-stimulated in vitro cultures (Figure 2C, D, and E). Importantly, in the nonstimulated in vitro cultures, feeding VNP51 at $10^6$ CFU or HNP51 at $10^4$ or $10^5$ CFU to Balb/c mice tended ($P \leq 0.09$) to expand IL-12-secreting CD8$^\alpha^+$ immune cells repertoire (Figure 2F). Note worthy, increasing HNP51 feeding dose to $10^6$ CFU dramatically ($P = 0.04$) expanded IL-12-secreting CD8$^\alpha^+$ immune cells repertoire compared with that of the MDX-fed control mice (Figure 2F). On the contrary, stimulation of the cultures with ConA did not significantly ($P \geq 0.18$) modify IL-12-secreting CD8$^\alpha^+$ immune cells (Figure 2F).

In addition, intracellular IL-12 secretion was substantially ($P = 0.02$ and $P = 0.03$) enlarged in the CD8$^\alpha^+$ immune cells of Balb/c mice fed the VNP51 or the HNP51 at $10^6$ and $10^5$ CFU, respectively, compared with that of MDX-fed control (Figure 2F). Similarly, HNP51 fed at $10^6$ CFU tended ($P = 0.06$) to enhance IL-12 secretion in the CD8$^\alpha^+$ immune cells in in vitro cultures of Balb/c mice fed the HNP51 at $10^6$ CFU (Figure 2F).

Of note worthy, feeding the VNP51 at $10^6$ CFU coupled with ConA stimulation substantially ($P = 0.04$ and $P = 0.01$) increased intracellular production of IFN-γ and TNF-α, respectively, by the CD8$^\alpha^+$ immune cells of Balb/c mice relative to that of MDX-fed mice (Figures 2G and H). Feeding the VNP51 or the HNP51 at $10^4$ or $10^5$ CFU did not ($P \geq 0.54$ and $P \geq 0.24$) appear to cause changes in the intracellular secretion of IFN-γ or TNF-α by the CD8$^\alpha^+$ immune cells when stimulated with ConA and compared to that of the MDX-fed control mice (Figure 2G and H).
Figure 3. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their splenic T cells intracellular cytokine secretion. Panel A. T cells-secreting IL-2. Panel B. T cells-secreting IL-4. Panel C. T cells-secreting IL-10. Panel D. T cells-secreting IL-12. Panel E. T cells-secreting IFN-γ. Panel F. T cells-secreting TNF-α. Data are mean ± SEM. (*) indicates significant difference at \( P \leq 0.05 \); (#) indicates tendency for a significant difference at \( 0.05 < P \leq 0.10 \); NS, nonstimulated in vitro splenocyte cultures; ConA, concanavalin A-stimulated in vitro splenocyte cultures.

**Splenic immune cells intracellular cytokine secretion.** It was obvious that VNP51 or HNP51 fed to specific pathogen-free Balb/c mice did not significantly (\( P \geq 0.29, P \geq 0.27, P \geq 0.20, \) and \( P \geq 0.13 \)) alter their splenic T cells secretion of IL-2, IL-10, IFN-γ, or TNF-α, respectively (Figures 3 A, C, E, and F) relative to secretion by splenic T cells from MDX-fed mice. Of importance, feeding the HNP51 at \( 10^5 \) CFU to Balb/c mice tended \( (P = 0.07) \) to increase IL-4 secretion by their splenic T cell when stimulated with ConA (Figure 3B). Note
worthy, HNP51, not VNP51 \((P \geq 0.13)\), fed at \(10^4\) CFU tended \((P = 0.07)\) to increase IL-12 secretion by splenic T cells and at \(10^5\) CFU it increased it significantly \((P = 0.03)\) higher than that of the MDX-fed control mice when stimulated with ConA (Figure 3D).

![Graphs showing the effect of VNP51 and HNP51 probiotic feeding on intestinal histological structure and spleen cellularity.]

**Figure 4.** Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their intestinal histological structure and spleen cellularity. Panel A. Effect on crypt length. Panel B. Effect on villus height. Panel C. Effect on crypt to villus ratio. Data are mean ± SEM. Panel D. Effect on spleen cellularity. \((*)\) indicates significant difference at \(P \leq 0.05\); \((#)\) indicates tendency for a significant difference at \(0.05 < P \leq 0.10\).
**Histopathological effects of VNP51 and HNP51.** Compared to specific pathogen-free Balb/c mice fed the chow diet only, feeding the MDX carrier at 3% of the chow significantly ($P = 0.01$; Figure 4A) decreased the crypt length of the intestine (Figure 4A). Subsequent inclusion of VNP51 or the HNP51 at $10^4$, $10^5$, or $10^6$ CFU did not significantly ($P \geq 0.15$) decrease the length of crypt relative to that of the MDX-fed control mice (Figure 4A). In addition, feeding the VNP51 or the HNP51 at $10^4$, $10^5$, or $10^6$ CFU to pathogen-free Balb/c mice did not significantly ($P \geq 0.18$) affect the villus height in their small intestines compared to the noise of the MDX-fed control or the chow-fed control (Figure 4B). Subsequently, the ratio of the crypt to villus was not significantly ($P \geq 0.26$; Figure 4C) altered by the VNP51 or the HNP51 feeding compared with that of the MDX-fed control or the chow-fed control.

Feeding the VNP51 at different dosages did not significantly ($P \geq 0.15$) alter the cellularity of the spleen. On the contrary, HNP51 fed at $10^4$ and $10^6$ CFU tended to increase spleen cellularity by day 45 compared with that of the MDX-fed group (Figure 4D).

**DISCUSSION**

VNP51- and HNP51-induced modulation of CD4$^+$ T helper cell repertoire was dose-dependent (Figure 1A). Both VNP51 and HNP51 fed at $10^4$ CFU·mouse$^{-1}$·day$^{-1}$ significantly expanded CD4$^+$ T helper cells repertoire. Increasing the VNP51 and HNP51 concentrations to $10^5$ CFU abrogated CD4$^+$ T helper cells expansion. But HNP51 at $10^6$ CFU, not VNP51, tended to restore CD4$^+$ T helper cells expansion (Figure 1A). This finding agree with finding of Mane et al (2011) that *Lactobacillus plantarum* CECT 3715 and CECT 3716 at low dose
induce proliferation of CD4+ T helper and this immune response is abolished at higher dose in human subjects.

Feeding the VNP51 or HNP51 per se enlarged IL-12-secreting CD8α+ immune cells repertoire in the nonstimulated in vitro cultures, suggesting activation of CD8α+ DC (Mashayekhi et al., 2011; Figure 2F). Intriguingly, stimulation with ConA ablated effect of VNP51 and HNP51 on IL-12-secreting CD8α+ immune cells (Figure 2F), but expanded IFN-γ- and TNF-α-secreting CD8α+ immune cells repertoire (Figures 2G and H). Unlike plasmacytoid DC, conventional DC includes CD8α+ and CD8α- subsets. CD8α+ DC is efficient at presenting exogenous antigenic peptides on MHC class 1 to activate CD8+ cytotoxic T cells (Schnorre et al., 2006), but is less efficient at presenting antigenic peptide on MHC class II to activate CD4+ T helper cells (Kamphorst et al., 2010). We are lured to speculate that the source of IFN-γ and TNF-α stimulated by ConA is likely CD8α+ cytotoxic and NK cells.

Probiotic Lactobacillus salivarius CECT5713 and Lactobacillus fermentum CECT5716 co-cultured at 10^5, 10^6, or 10^7 CFU/mL with human PMNs activated CD8+ CD56+ NK cells and CD8+ T cells as revealed by the high expression of CD69 and CD25. Subsequently, probiotics-activated CD8+ NK and T cells secreted high concentrations of TNF-α (Perez-Cano et al., 2010). Similar to our findings, Lactobacillus salivarius CECT5713 and Lactobacillus fermentum CECT5716 did not seem to fully activate CD4+ T helper cells (Perez-Cano et al., 2010). Additionally, in splenocytes cultures, probiotic Lactobacillus casei induced IL-12 production by CD11b expressing cells (Chiba et al., 2010). CD11b has been suggested a unique marker for activated CD8+ cytotoxic T cells (Christensen et al., 2001).
Recently, Gad and colleagues (2011) showed that treating immature DC, with different strains of probiotics causes a dose-dependent alteration of their IL-10/IL-12p70 secretion. In agreement with our findings, *Lactobacillus acidophilus* strain NCFM and the probiotic cocktail VSL#3 at high dose destined DC to predominantly secrete IL-12 (Drakes et al., 2004). Signaling of IL-12 through STAT-4 promotes differentiation of naïve T cells into Th1 and CD8⁺ cytotoxic T cells lineages (Ely et al., 1999; Rao et al., 2010). Subsequently, DC-activated T cell secretes TNF-α, IFN-γ, and IL17 depending on the T cell lineage involved (Neurath, 2007). Thus, it is evident that the IL-12-secreting CD8α⁺ immune cells repertoire that was significantly expanded in the nonstimulated *in vitro* cultures by VNP51 and HNP51 feeding were mainly splenic CD8α⁺ DC (Figure 2F). In contrast, IFN-γ- and TNF-α-secreting CD8α⁺ immune cell repertoire that was enlarged by HNP51 feeding and ConA stimulation was mainly CD8α⁺ NK and cytotoxic T cells (Figure 2F). It is highly possible that IL-12 secreted by CD8α⁺ DC activated secretion of both IFN-γ and TNF-α by NK cells (Jewett et al., 1996) and CD8α⁺ cytotoxic T cells (Denton et al., 2011). Notably, feeding the VNP51 or HNP51 did not expand the IFN-γ- and TNF-α-secreting CD4⁺ T helper cells (Figures 1G and H), suggesting that Th1 cells were not induced by VNP51 and HNP51 feeding.

Thus, our findings support other laboratories findings that probiotic Lactobacilli accelerate the adaptive immunity via accelerating the innate immunity (Neurath, 2007; Gad et al., 2011; Evrard et al., 2011). We are tempted to speculate that VNP51 and HNP51 stimulate CD8α⁺ DC maturation as seen by the upregulated secretion of IL-12 in the nonstimulated *in vitro* cell cultures. Differentially, under ConA stimulation, HNP51 induces CD8α⁺ cytotoxic and NK cell expansion (Figure 2F) and upregulation of their IFN-γ and TNF-α secretion.
Typically, induction of TNF-α has been linked to induction of mucosal inflammation and tissue damage (Nenci et al., 2007). Recent evidence, however, has shown that probiotics VSL#3-induced TNF-α is pivotal for maintaining gut health and permeability. On the contrary, mucosal TNF-α deficiency is implicated in the pathogenesis of the inflammatory bowel disease (Pagnini et al., 2010).

We observed that VNP51 fed to mice increased their CD8α+CD25+ T reg cells when their splenocytes were stimulated with ConA (Figure 2B). In line with our findings, stimulation of human PMNs with probiotic Lactobacillus casei Shirota promoted expansion of CD8α+CD25+ T reg cells and 56+ NK (Dong et al., 2010).

Feeding VNP51 or HNP51 to Balb/c mice at 10^6 CFU·day^{-1} (6,000 times higher than the dose typically prescribed for human expressed per kg of body weight) had no adverse effect on intestinal mucosa (Figure 4A, B and C). This finding lend support to similar findings by Lara-Villoslada and coworkers (2007) that feeding probiotic Lactobacillus salivarius CECT5713 at 10^{10} CFU daily is not pathogenic to Balb/c mice.

**CONCLUSIONS**

We concluded that Lactobacillus acidophilus NP51 fed at 10^4, 10^5, or 10^6 CFU·mouse^{-1}·day^{-1} is not pathogenic to healthy, specific pathogen-free Balb/c mice and is well tolerated. Also, NP51 program splenic DC to secrete IL-12, however, CD8α+ cytotoxic T and NK cell- and NK T cell-secreting IFN-γ and TNF-α are upregulated only after additional stimulation with a mitogen, suggesting that NP51 induce a Th1-like immune response in context of an infection.
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CHAPTER 4. THE PROBIOTIC LACTOBACILLUS ACIDOPHILUS STRAIN NP51® CURTAILS THE ADVANCE OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) INFECTION IN BALB/C MICE

A paper to be submitted to the Journal of Immunobiology

RUNNING TITLE: NP51, MAP, AND JOHNE’S DISEASE.

The probiotic Lactobacillus acidophilus NP51® curtails the advance of Mycobacterium avium subsp. paratuberculosis (MAP) infection in Balb/c mice.2

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ABSTRACT

We examined the immunologic effects of feeding the probiotic *Lactobacillus acidophilus* strain NP51® to Balb/c mice infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) as the murine model of Johne’s disease. We hypothesized that feeding the probiotic NP51 would induce the adaptive immunity to prevent development of MAP infection in mice. Specific pathogen-free Balb/c mice were randomized to treatment groups with mice either fed the viable or heat-killed NP51 (VNP51 or HNP51, respectively) at 1 × 10^6 CFU·mice⁻¹·day⁻¹ along with normal mouse chow until the end of the study. On day 45, mice were challenged with 1 × 10^8 CFU of heat-killed or a viable MAP (VMAP or HMAP, respectively) injected intraperitonealy. Ten mice from each group were euthanized on days 45, 90, 135, and 180. At each sampling period, tissues were excised from mice and cultured for MAP. Also, the fecal pellets were collected and examined for the presence of MAP IS900 genomic DNA. Overall, feeding the HNP51 or VNP51 to mice significantly increased the cellular density of the spleens of mice infected with VMAP and decreased the acid-fast bacilli numbers in their livers. Most importantly, burden of VMAP was decreased in
the mesenteric lymph nodes, livers, ceca, and spleens of mice fed either HNP51 or VNP51 compared with the HMAP- and VMAP-infected controls on days 135 and 180. These results suggest that feeding probiotic NP51 modifies the immune responses favorably and prevents progression of MAP infection in Balb/c in mice.

Key words: MAP, NP51, Johne’s disease.

**INTRODUCTION**

Johne’s disease (JD) is a chronic granulomatous enteritis of domestic and wild ruminants. The etiologic agent of JD, *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a mycobactin J-dependent, acid-fast mycobacterial species that multiplies inside macrophages of the host (Momotani et al., 1988; Sweeney, 1996). It is well established that neonatal calves acquire JD soon after birth through the ingestion of MAP-contaminated colostrum or milk (Streeter et al., 1995). Significant transmission of MAP infection, however, occurs through consumption of MAP-contaminated colostrum rather than milk (Nielsen et al., 2008). Indeed, pasteurization of colostrum significantly decreases MAP infection in newborn calves (Stabel, 2008). In addition to peripartal infection, congenital infection with JD has been reported (Koets, 2006) and about 9% of neonatal calves could acquire MAP infection when JD prevails in 40% of the herd (Whittington and Windsor, 2009). Adult cattle are also susceptible and yearling cattle can acquire JD after intensive grazing of a MAP-contaminated pasture (Fecteau et al., 2010). Introduction of MAP-shedding cattle to the herd results in detection of viable MAP on the farm and its surroundings suggesting transmission of MAP in bio-aerosols (Eisenberg et al., 2010; 2011).

After the infection with MAP is established, MAP remains latent, causing the infected cattle to go through protracted subclinical phase that might reach 5 years (Nielsen, 2006).
During the subclinical phase, infected cattle have decreased milk production and shed MAP intermittently, increasing the intra-herd transmission of the disease. In contrast, clinically affected cattle develop chronic, intermittent diarrhea and progressive emaciation that leads to death (Chiodini et al., 1984).

In 1996, JD prevailed in about 22% of the U.S. dairy herds (NAHMS, 1996), causing an annual loss of 225 million U.S. dollars to the dairy industry (Ott et al., 1999). Currently, 68% of dairy herds in the U.S. have been declared to be infected with MAP (NAHMS, 2008). Thus, we estimate the current annual losses to the U.S. dairy farmers to approach 700 million U.S. dollars because of decreased milk production, premature culling, and death of animals infected with JD. In addition, valuable genetic traits of high milk production and disease resistance can be lost by the premature culling of cows infected with JD (Stabel, 1998).

The severity of MAP infection and shedding of MAP in feces could be attenuated by vaccination of cattle with either the killed or the live whole cell MAP bacterin (Gwozdz et al., 2000; Muskens et al., 2002; Uzonna et al, 2003). Vaccination with either the whole cell killed or live MAP bacterin, however, did not prevent the transmission of MAP within the herd (Kalis et al., 2001), and it also interfered with the diagnosis of bovine tuberculosis.

Recombinant antigen vaccines, which include MAP 85 antigen complex (85A, 85B, 85C), superoxide dismutase, and 35 kDa proteins, could enhance host immunity as demonstrated by increased lymphocyte proliferation as well as secretion of Th1-associated cytokines including interferon gamma (IFN-γ), interleukin-2 (IL-2), IL-12, and tumor necrosis factor alpha (TNF-α) in C57BL/6 mice (Park et al., 2008) and calves (Kathaperumal et al., 2008). Though MAP burden decreased in livers and spleens of mice vaccinated with antigen 85, the increased secretion of the TNF-α and IFN-γ might perpetuate further intestinal tissue
destruction and inflammation, thereby exacerbating the ileitis and further comprising nutrient absorption in carrier or subclinical animals.

In agreement, neutralization of IL-18 decreased the production of colonic IFN-γ \textit{in vivo} and TNF-α \textit{in vitro} and attenuated the severity of colitis in Balb/c and C57BL/6 mice (Siegmund et al., 2001). Similarly, antibiotic-induced amelioration of ileitis in a murine model of Crohn’s disease was accompanied by a significant decrease of IFN-γ and TNF-α concentrations (Bamias et al., 2002).

Indeed, a safer and more efficacious therapeutic for JD is a research priority. Recently, Nerstedt and colleagues (2007) showed that feeding \textit{Lactobacillus acidophilus} to mice upregulates genes involved in host defense against pathogens as well as genes that control energy homeostasis. Additionally, \textit{Lactobacilli} administration improved the immunological gut barrier via enhancement of germinal center formation and promotion of sIgA secretion. Also, feeding \textit{Lactobacilli} activated phagocytosis by macrophages and alleviated the intestinal inflammatory responses (Ibnou-Zekri et al. 2003; Wei et al., 2007).

On the basis of previous findings, we hypothesized that pre-feeding the probiotic \textit{Lactobacillus acidophilus} NP51 to Balb/c mice would induce the adaptive immunity to prevent later development of MAP infection. To test the hypothesis, we evaluated the effect of pre-feeding the viable or heat-killed probiotic NP51 on immune responses of Balb/c mice infected with virulent MAP strain. We also examined the effect of pre-feeding the NP51 on colonization resistance to MAP in tissues and MAP shedding in feces of infected Balb/c mice.
**MATERIALS AND METHODS**

**Experimental Design.**

Pathogen-free, 6-week-old 360 Balb/c mice (Jackson Laboratories) were randomized to nine treatment groups in a factorial design that included three major groups (Table 1). Group I was fed the maltodextrin (MDX) carrier at 3% of their chow diet. Group II was fed the heat-killed NP51 (HNP51) at $1 \times 10^6$ CFU·mouse$^{-1}$·day$^{-1}$ in MDX as 3% of the diet. Group III was fed the viable NP51 (VNP51) at $1 \times 10^6$ CFU·mouse$^{-1}$·day$^{-1}$ in MDX as 3% of the diet.

**Table 1. Designation of treatment groups.**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>MDX 3% of the diet</td>
<td>HNP51 $10^6$ CFU·mouse$^{-1}$·day$^{-1}$</td>
<td>VNP51 $10^6$ CFU·mouse$^{-1}$·day$^{-1}$</td>
</tr>
<tr>
<td>Code</td>
<td>Cntrl</td>
<td>HMAP</td>
<td>VMAP</td>
</tr>
<tr>
<td>HMAP</td>
<td>---</td>
<td>✓</td>
<td>---</td>
</tr>
<tr>
<td>VMAP</td>
<td>---</td>
<td>---</td>
<td>✓</td>
</tr>
<tr>
<td>No. of mice</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

*Cntrl*: Control noninfected mice fed the sterile chow diet only. **MDX**: maltodextrin (carrier for VNP51 and HNP51). **VNP51** and **HNP51**: viable and heat-killed NP51, respectively. **VMAP** and **HMAP**: viable and heat-killed MAP, respectively.

Group I contained a non-infected control (n = 40) fed chow plus MDX, heat-killed MAP-infected control (HMAP; n = 40), and viable MAP-infected control (VMAP; n = 40) subgroups. Group II included non-infected control (HNP51; n = 40), heat-killed MAP-infected (HNP51+HMAP; n = 40), and viable MAP-infected (HNP51+VMAP; n = 40) subgroups. Group III included non-infected control (VNP51; n = 40), heat-killed MAP-infected (VNP51+HMAP; n = 40), and viable MAP-infected (VNP51+VMAP; n = 40) subgroups.
subgroups (Table 1). HMAP or VMAP was injected intraperitoneally at $1 \times 10^8$ CFU·mouse$^{-1}$ once on day 45 of the study. Viability of MAP was continuously monitored before injection.

Mice were housed in a BSL-2 rodent room in the animal facility of the Iowa State University.

The environment within the room was maintained to provide a temperature of 22-25$^\circ$C, a relative humidity of 35-45%, and 12/12 h light/dark cycle. Mice were housed in autoclaved standard mice cages fitted with raised-wire floors and high efficiency particulate air (HEPA) filters.

Mice were offered ad libitum sterile water and chow meal (Harlan Laboratories) throughout the study. Mice were adapted to the sterile chow meal for a week prior to the addition of NP51. During the adaptation period, mice daily feed intake was precisely determined.

To ensure complete consumption of the probiotics, mice in the VNP51 or HNP51 treatment subgroups were fed the NP51 at $1 \times 10^6$ CFU·mouse$^{-1}$·day$^{-1}$ in 3.0 grams of the chow meal. The offering of chow meal was increased by 0.5 g chow·mouse$^{-1}$ every 15 days until it reached a maximum of 5.0 g of chow·mouse$^{-1}$·day$^{-1}$ by day 105 of the study. Mice continued to be offered 5.0 g until the end of the study. To prepare the HNP51, the viable probiotic NP51 was incubated in an oven at 85$^\circ$C for 15 minutes. Similarly, HMAP was prepared by incubating the VMAP in a water bath at 85$^\circ$C for 20 minutes (Stable, USDA-ARS-NADC, Ames, IA). The experiment was conducted in accordance with guidelines established by Institutional Animal Care and Use Committee at Iowa State University.

**Inoculation with Mycobacterium avium subspecies paratuberculosis.** MAP inoculum was prepared according to Stabel et al (2009). Briefly, 50 mL of sterile oleic acid at 0.60
mL·L⁻¹, albumin at 50 g·L⁻¹, dextrose at 20 g·L⁻¹, catalase at 0.04 g·L⁻¹, and sodium chloride at 8.50 g·L⁻¹ (OADC) enrichment were added to 450 mL of sterile Middlebrook broth 7H9 (PML Microbiology, Wilsonville, OR). Subsequently, 25 mL of a virulent MAP clinical isolate of strain 167 grown to the OD₅₄₀nm = 0.2 (USDA, National Animal Disease Center, Ames, IA) were inoculated into the broth.

After thoroughly mixing, the VMAP culture was incubated at 37°C and the OD₅₄₀nm was measured weekly. The bacteria was harvested in the log phase (OD₅₄₀nm = 0.2-0.4). The VMAP culture was decanted into sterile 500 mL Beckman bottles with O-ring caps followed by centrifugation at 1000 x g for 30 minutes in the refrigerated Beckman J2-21 centrifuge using the JA-10 rotor. The supernatant was decanted and the VMAP pellets were washed twice by suspension in 500 mL of sterile, cold PBS (pH = 7.2). Subsequently, VMAP suspension was centrifuged for 30 minutes and the supernatant discarded. The formed VMAP pellets were re-suspended in 5 mL of cold sterile phosphate buffered saline (PBS; 0.14 M NaCl, 0.003 M KCl, 0.01 M Na₂HPO₄, and 0.0018 M KH₂PO₄; pH 7.4) and placed on ice. The OD₅₄₀ was measured to verify the initial concentration of the bacteria. Then, the PBS was used to adjust the concentration to 10⁹ CFU/mL as determined by OD₅₄₀ (OD₅₄₀nm = 1.0-1.2 absorbance units). To prepare the HMAP, VMAP suspension was heated in a water bath at 85°C for 20 minutes, then cooled to room temperature, and kept on ice until used for inoculating mice.

On day 45 of the study, mice were challenged intraperitonealy with 1 × 10⁸ CFU of the virulent clinical isolate 167 as either HMAP or VMAP suspended in 100 µL of sterile PBS. Control groups received 100 µL of sterile PBS. Following inoculation with MAP, 10 mice from each treatment group were euthanized on days 45, 90, 135, and 180 of the study.
Microbiological quantification of tissue VMAP burden:

To quantify VMAP burden in tissues, tissue homogenates were cultured on Herrold’s Egg-yolk Agar with mycobactin J, amphotericin, nalidixic acid, and vancomycin (Stabel and Osman USDA-ARS-NADC). Briefly, weighed mouse tissues was added to half strength Brain Heart Infusion Broth containing 0.75% hexadecylpyridinium chloride monohydrate (Sigma-Aldrich, St. Louis, MO) in a whirl bag and stomached for two minutes. The stomached samples were then incubated in 15-mL graduated tube for 3 hours to decontaminate. Following the incubation, the samples were centrifuged at 900 × g for 10 minutes and the supernatant was discarded. The pellets were suspended in one mL of mixed antibiotic solution composed of 10 mg/mL of vancomycin, nalidixic acid, and amphotericin B (Sigma-Aldrich, St. Louis, MO) and incubated at 37° C over night.

Subsequently, for each sample, four Herrold’s egg yolk Agar slants (BD Diagnostic Systems, Franklin Lakes, NJ) were inoculated with 200 µL each of the tissue samples. The inoculated tubes were incubated tilted with loosen lids to aid excess moisture evaporation for a week at 37° C. Then, the lids were tightened and the tubes were incubated in an upright position for four months and examined every two weeks for growth of VMAP.

**Histopathological examination of the tissue injury.** To examine effect of VNP51 and HNP51 on VMAP- and HMAP-induced tissue injury, we excised the liver on days 135 and 180, fixed them in 10% formaldehyde, stained them with hematoxylin and eosin or Zeihl-Neelsen stain, and imbedded them in paraffin. Subsequently, 4 µm sections were examined under the microscope for the granulomatous reaction intensity and acid-fast bacilli numbers by a pathologist who was unaware of the designation of the treatment groups.
**ABI Prism 7900HT PCR assay for detection of MAP IS900 genomic DNA in fecal pellet.** To verify whether the decreased VMAP burden in tissues was accompanied by decreased VMAP shedding in fecal pellets, we examined the presence of MAP IS900 genomic DNA, the insertion element IS900, by PCR (Kim et al., 2002). Briefly, total nucleic acids were extracted from fecal pellets (MagMax Total Nucleic Acids Isolation Kit; Ambion, Inc., Austin, TX). To conduct the ABI Prism 7900HT PCR assay, 20 µL of assay reaction containing 12.5 µL of the TaqMan Universal Master Mix (AB Applied Biosystems, Foster City, CA), 6.75 µL of water, 0.25 µL of the 20 µM IS900 forward primer (5’-CCG CTA ATT GAG AGA TGC GAT TGG-3’), 0.25 µL of the 20 µM IS900 reverse primer (5,-AAT CAA CTC CAG CAG CGC GGC CTC G-3,) and 0.125 µL of the IS900 fluorescent probe (5,-fluorescent FAM label, 3’-TAMRA quencher; sequence TCC ACG CCC GCC CAG ACA GG-3’) were added to each well containing 5 µL of the total nucleic acid extracted from fecal pellets. A 10 ng/µL MAP genomic DNA stock solution was 10-fold serially diluted to make four standards that were used to construct the standard curve by using the ABI Prism® 7900 Sequence Detection System (AB Applied Biosystems, Foster City, CA). The thermocycler was adjusted to run in the first stage at 50°C for 2 min, in the second stage at 95°C for 10 min, and in the third stage at 94°C for 15 sec followed by 40 cycles at 66°C each for 1 min.

**Statistical Analysis:**

A linear mixed-effects model was fit to the data for each response variable by using the PROC MIXED procedure of the Statistical Analysis System (SAS; SAS Institute Inc., Cary, North Carolina, USA) version 9.2. To overcome natural immune variability among mice of the same treatment group, means were log transformed. Each linear mixed-effects model
included fixed effects for infection status, gender, and their interactions. The least squared means were computed by using the LSMEANS option of SAS. Subsequently, different among least squared means were determined by using the PDIFF CL option of SAS. The standard errors of the means were reported, and the pairwise differences among the estimated means were considered significant when the probability ($P$) value was $\leq 0.05$. Tendency for a trend was declared when $0.05 \leq P \leq 0.1$.

**RESULTS**

*Modulation of the histopathological lesions.* To evaluate the effect of feeding either the VNP51 or HNP51 on the immune responses of infected and non-infected mice, we examined the cellularity of the spleen in mice euthanized on day 45 of the study. Feeding the probiotic VNP51 or HNP51 to VMAP- or HMAP-infected mice did not significantly alter the cellularity of the spleen on day 45 ($P = 0.12$; Figure 1A). Nor it caused significant gender ($P = 0.13$) or treatment by gender interaction ($P = 0.99$) effects on the same variable.

Similarly, the histopathological examination of the granuloma formation in the hepatic tissue stained with hematoxylin and eosin (H&E) revealed no significant differences among VMAP- or HMAP-infected control mice and the VMAP- and HMAP-infected mice fed either the VNP51 or HNP51 ($P \geq 0.46$; Figure 1B) on day 45 of the study.
Figure 1. Effect of probiotic VNP51 and HNP51 feeding on the immune responses of specific pathogen-free Balb/c mice noninfected or infected with VMAP or HMAP on day 45 of the study. Panel A. Effects on the spleen cellularity (SEM = 0.54 – 3.8). Panel B. Effects on hepatic granuloma formation (SEM = 0.13 to 0.15). Panel C. Effects on acid-fast bacilli enumeration (SEM = 0.14 to 0.17). Panel D. Effects on MAP shedding in the fecal pellets. Unless otherwise specified, VMAP (controls infected with viable MAP) were compared to HNP51+VMAP or VNP51+VMAP. Also, HMAP (controls infected with the heat-killed MAP) were compared to HNP51+HMAP or VNP51+HMAP. Additionally, the cntrl (non-infected controls) were compared to HNP51 (mice fed the heat-killed HNP51) or VNP51 (mice fed the viable NP51). Similarly, HNP51+VMAP were compared to VNP51+VMAP. Data are the mean ± SEM. (*) indicate significant difference at $P \leq 0.05$; (#) indicates tendency for significant difference at $0.05 < P \leq 0.10$. 
Feeding the VNP51 or HNP51 to non-infected mice did not seem to induce significant hepatic granulomatous reaction compared with control mice fed the MDX \((P = 1.0; \text{Figure 1B})\).

Additionally, hepatic tissues stained with Ziehl-Neelsen stain showed no significant difference \((P \geq 0.2; \text{Figure 1C})\) in the acid-fast bacilli burden in livers of HMAP- or VMAP-infected mice fed either the HNP51 or VNP51 in comparison with the infected control mice on day 45. Also, MAP DNA could not be detected in all VMAP-infected groups regardless of the HNP51 and VNP51 feeding (Figure 1D).

As mice proceeded to day 90 of the study, largely, there were significant treatment, gender, and treatment by gender interaction effects \((\text{All } P = 0.0001; \text{Figure 2A})\) on the spleen cellularity. Feeding the HNP51 to VMAP- or HMAP-infected mice substantially increased their spleen cellularity \((P \leq 0.009; \text{Figure 2A})\) to be greater than that of the VMAP- or HMAP-infected control mice. Increased spleen cellularity, however, was not accompanied \((P \geq 0.17; \text{Figure 2B})\) with significantly enhanced granulomatous reactions in their livers when compared with those of the VMAP- and HMAP-infected control mice.

It was clearly demonstrable that feeding the VNP51 or HNP51 to control non-infected mice did not induce hepatic granuloma formation at any time during the study \((P \geq 0.37; \text{Figures 2B, 3B and 4B})\) compared with the noninfected nonprobitic fed control mice.

On the contrary, feeding the VNP51 increased enormously the cellular density of the spleen \((P = 0.0001; \text{Figure 1A})\) in HMAP-infected mice, but not \((P = 0.23; \text{Figure 2A})\) in VMAP-infected mice by day 90 of the study. In contrast, the increased spleen cellularity of HMAP-infected mice fed the VNP51, unlike that of VMAP-infected \((P = 0.59; \text{Figure 2B})\), was accompanied with a significant \((P = 0.009; \text{Figure 2B})\) granulomatous reaction in livers.
Also, the acid-fast bacilli number was remarkably increased ($P = 0.002$; Figure 2C) in livers of mice fed the VNP51 and inoculated with the HMAP compared with that of the
HMAP-infected control mice. Because HMAP cannot reproduce, we reasoned that the multibacillary phenotype of the granulomas observed is indicative of effective containment of HMAP in liver on day 90.

In addition, MAP IS900 genomic DNA was detected readily in the fecal pellets of 72.2%, 60%, and 60% of mice in the VMAP-infected controls, VMAP-infected fed the HNP51, and VMAP-infected fed the VNP51, respectively (Figure 2D). Feeding the HNP51 or the VNP51 to VMAP-infected mice did not significantly influence \((P \geq 0.46)\) shedding of MAP IS900 genomic DNA in their fecal pellets compared with the VMAP-infected control mice on day 90 (Figure 2D).

By day 135 of the study, the VNP51 and HNP51-induced immune modulating effect was readily detectable. Feeding the VNP51 to VMAP-infected mice amplified significantly \((P = 0.0005;\) Figure 3A) the cellularity of the spleen compared with that of the VMAP-infected controls. In contrast, VNP51 feeding decreased \((P = 0.01;\) Figure 3A) the cellularity of the spleen in the HMAP-infected mice compared with the HMAP-infected controls.

On the other hand, feeding the HNP51 to mice infected with the VMAP or HMAP did not significantly alter \((P \geq 0.14;\) Figure 3A) their spleen cellularity compared with the VMAP- and HMAP-infected controls indicating a resolution of the infection with the VMAP and HMAP (Figure 3A). On the whole, we detected probiotic-induced significant treatment, gender, and treatment by gender interaction effects (All \(P = 0.0001\)).

We also examined the intensity of the granulomatous reaction liver of infected mice on day 135. Feeding either the VNP51 or HNP51 to VMAP- or HMAP-infected mice did not significantly alter \((P \geq 0.37;\) Figure 3B) the granulomatous reaction in their livers.
Consequently, numbers of the acid-fast bacilli in the hepatic tissues stained with Ziehl-Neelsen stain did not differ ($P \geq 0.59$; Figure 3C) from that of the VMAP- or HMAP-infected control mice. Despite this, there was a robust ($P = 0.0001$) treatment effect and a tendency ($P = 0.06$; Figure 3C) for a gender effect.
Figure 3. Effect of probiotic VNP51 and HNP51 feeding on the immune responses of specific pathogen-free Balb/c mice noninfected or infected with VMAP or HMAP on day 135 of the study. Panel A. Effects on the spleen cellularity (SEM = 0.35 – 1.33). Panel B. Effects on hepatic granuloma formation (SEM = 0.10 – 0.55). Panel C. Effects on acid-fast bacilli enumeration (SEM = 0.13 – 0.63). Panel D. Effects on MAP shedding in the fecal pellets (SEM = 0.08 – 0.22). Unless otherwise specified, VMAP (controls infected with viable MAP) were compared to HNP51+VMAP or VNP51+VMAP. Also, HMAP (controls infected with the heat-killed MAP) were compared to HNP51+HMAP or VNP51+HMAP. Additionally, the cntrl (non-infected controls fed no probiotics) were compared to HNP51 (mice fed the heat-killed HNP51) or VNP51 (mice fed the viable NP51). Similarly, HNP51+VMAP were compared to VNP51+VMAP. Data are the mean ± SEM. (*) indicate significant difference at $P \leq 0.05$; (#) indicates tendency for significant difference at $0.05 < P \leq 0.10$. 
Notably, MAP IS900 genomic DNA was readily detected in the fecal pellets of 44.4, 11.1, and 31.3% of mice in the VMAP-infected controls, VMAP-infected fed the HNP51, and VMAP-infected fed the VNP51, respectively (Figure 3D). Feeding the HNP51 to VMAP-infected tended ($P = 0.08$) tended to decrease shedding of MAP IS900 genomic DNA compared with VMAP-infected controls. Overall, female mice shed less ($P = 0.01$) MAP IS900 genomic DNA in their fecal pellets compared with male mice on day 135 (data not shown).

On day 180, VNP51 fed to mice challenged with either the VMAP or HMAP decreased significantly (Both $P = 0.0001$; Figure 4A) their spleen cellularity higher than that of the HMAP- or VMAP-infected controls. Similarly, feeding the HNP51 to mice challenged with the VMAP induced a similar and substantial increase ($P = 0.0001$) in spleen cellularity. On the contrary, HNP51 feeding did not significantly alter ($P = 0.77$; Figure 4A) spleen cellularity of HMAP-infected mice on day 180.

Despite the significant overall treatment ($P = 0.0001$) and gender ($P = 0.006$; Figure 4B) effects, feeding the VNP51 or HNP51 to mice infected with either the VMAP or HMAP had no effects ($P \geq 0.44$; Figure 4B) on their liver granuloma formation on day 180. VNP51 feeding, nonetheless, moderately ($P = 0.09$) lowered the acid-fast bacilli numbers in livers of HMAP-infected mice. In contrast, the VNP51-induced acid-fast bacilli-reducing effect was significantly pronounced ($P = 0.01$) in livers of VMAP-infected mice. On the contrary, feeding the HNP51 did not alter significantly ($P \geq 0.15$) hepatic acid-fast bacilli content (Figure 4C).

Importantly, MAP IS900 genomic DNA was detected in the fecal pellets of 30% of the VMAP-infected control mice (Figure 4D). On the contrary, MAP IS900 genomic DNA was
undetectable in fecal pellets of VMAP-infected mice fed either the HNP51 or the VNP51 (Figure 4D). Thus, feeding the HNP51 or the VNP51 to VMAP-infected completely prevented (Both $P = 0.02$) shedding of MAP IS900 genomic DNA in their fecal pellets compared with VMAP-infected controls on day 180 (Figure 4D).
Figure 4. Effect of probiotic VNP51 and HNP51 feeding on the immune responses of specific pathogen-free Balb/c mice noninfected or infected with VMAP or HMAP on day 180 of the study. Panel A. Effects on the spleen cellularity (SEM = 0.58-1.50). Panel B. Effects on hepatic granuloma formation (SEM = 0.20 -0.50). Panel C. Effects on acid-fast bacilli enumeration (SEM = 0.10 -0.36). Panel D. Effects on MAP shedding in the fecal pellets (SEM = 0.12). Unless otherwise specified, VMAP (controls infected with viable MAP) were compared to HNP51+VMAP or VNP51+VMAP. Also, HMAP (controls infected with the heat-killed MAP) were compared to HNP51+HMAP or VNP51+HMAP. Additionally, the cntrl (non-infected controls) were compared to HNP51 (mice fed the heat-killed HNP51) or VNP51 (mice fed the viable NP51). Similarly, HNP51+VMAP were compared to VNP51+VMAP. Data are the mean ± SEM. (*) indicate significant difference at $P \leq 0.05$; (#) indicates tendency for significant difference at $0.05 < P \leq 0.10$. 
Amelioration of MAP burden in tissues. After observing the dramatic HNP51- and VNP51-induced modulation of the spleen cellularity and attenuation of the VMAP DNA shedding in fecal pellets (Figures 2A, 3A, 4A, 3D, and 4D) coupled with the significant decrease of the acid-fast bacilli in livers (figure 4C), we verified whether HNP51 or VNP51 decreased VMAP burden in tissues of VMAP-infected mice fed either the VNP51 or the HNP51.

Of significance, feeding either the VNP51 or HNP51 considerably lowered ($P = 0.03$ and $P = 0.003$, respectively; Figure 5A) VMAP CFUs in livers of VMAP-infected mice on day 135. Overall, feeding HNP51 or VNP51 induced a significant treatment effect ($P = 0.008$) with no ($P = 0.17$) gender effect. Similarly, VMAP burden was remarkably decreased in mesenteric lymph nodes (MLN) of VMAP-infected mice fed either the HNP51 ($P = 0.04$) or the VNP51 ($P = 0.006$; Figure 5B) compared with the VMAP-infected control regardless ($P = 0.44$) of their gender.

A similar remarkable decrease ($P = 0.0001$ and $P = 0.0001$, respectively; Figure 5C) of VMAP burden in the spleens of VNP51- or HNP51-fed mice infected with the VMAP on day 135 was readily detected. The decrease of VMAP burden, however, was greater ($P = 0.004$) in spleens of females mice than that of spleens of males. In addition, there were overall significant treatment ($P = 0.0001$) and treatment by gender interaction ($P = 0.05$; Figure 5C) effects on day 135.

To verify whether the decreased VMAP burden in tissues was accompanied by decreased shedding of VMAP in feces, we examine presence of MAP IS900 genomic DNA in the fecal pellets by using PCR assay based on the ABI Prism® 7900HT. Feeding either VNP51 or the HNP51 significantly decreased VMAP shedding in
Figure 5. Effect of probiotic VNP51 and HNP51 feeding on VMAP burden in tissues of specific pathogen-free Balb/c mice infected with VMAP on day 135. Panel A. Effect on liver VMAP burden (SEM = 5.56 -73.16). Panel B. Effect on mesenteric lymph node (MLN) VMAP burden (SEM = 1016.9-1600.2). Panel C. Effect on spleen VMAP burden (15.76-1596.44). VMAP is controls mice infected with viable MAP fed no probiotics, HNP51+VMAP is VMAP-infected mice fed the heat-killed NP51, and VNP51+VMAP is VMAP-infected fed the viable VNP51). Data are the log-transformed mean ± SEM. (*) indicate significant difference at $P \leq 0.05$; (#) indicates tendency for significant difference at $0.05 < P \leq 0.10$. 
fecal pellets ($P = 0.01$ and $P = 0.01$, respectively, Figure 4D) compared with that of VMAP-infected controls on day 135.

By day 180 of the study, feeding either the VNP51 or HNP51 maintained VMAP burden decreased significantly ($P = 0.01$ and $P = 0.01$, respectively; Figure 6A) in livers of VMAP-infected mice compared with VMAP-infected controls. In general, we observed significant treatment ($P = 0.01$) and gender ($P = 0.001$) effects, but no ($P = 0.43$; Figure 6A) treatment by gender interaction effect.

Notably, VNP51 fed to VMAP-infected mice decreased significantly ($P = 0.05$; Figure 6B) VMAP burden in their MLN compared with VMAP-infected controls. The decrease of VMAP burden in MLN of females Balb/c mice tended to be greater ($P = 0.09$; Figure 6B) than that in MLN of males.

On the contrary, feeding the HNP51 to VMAP-infected mice did not decrease ($P = 0.25$) VMAP burden in their MLN, but decreased it significantly ($P = 0.003$) in their ceca on day 180. Also, feeding the VNP51 to VMAP-infected mice decreased ($P = 0.03$; Figure 6C) VMAP burden in their ceca compared with the VMAP-infected control mice. Generally, there was significant ($P = 0.008$) treatment effect, but no significant ($P = 0.62$) gender effect (Figure 6C).
Figure 6. Effect of probiotic VNP51 and HNP51 feeding on VMAP burden in tissues of specific pathogen-free Balb/c mice infected with VMAP on day 180 of the study. Panel A. Effect on liver VMAP burden (SEM = 5.10 – 151.4). Panel B. Effect on mesenteric lymph node (MLN) VMAP burden (SEM = 644.67 – 1161.22). Panel C. Effect on cecum VMAP burden (SEM = 8.67- 13.11). VMAP (controls infected with viable MAP fed no probiotics, HNP51+VMAP is VMAP-infected fed the heat-killed NP51, and VNP51+VMAP is VMAP-infected fed the viable VNP51). Data are the log-transferred means ± SEM. (*) indicate significant difference at $P \leq 0.05$. 
**DISCUSSION**

It was no surprise that we detected acid-fast bacilli and granulomatous reactions following inoculation with HMAP and VMAP on day 45 (Figures 1B and C). It is well established that following intraperitoneal injection, bacterial inocula are drained to the celiac, mesenteric, and peripartal lymph nodes. Inocula are either phagocytosed by the intraperitoneal resident macrophages or suspended in lymph and transported towards the mediastinal lymph node and thoracic duct prior to systemic dissemination (Parungo et al., 2006; Marco et al., 1992).

Selbitz and coworkers (1986) detected considerable numbers of *Listeria monocytogenes* in the secondary lymphoid organs as early as 10 minutes after the intraperitoneal inoculation. Also, Indian ink (inert) or viable *Listeria monocytogenes* injected intraperitoneally in mice were detected in Kupffer cells in liver and in the marginal zone of the white pulp in the spleen four hours after injection (Marco et al., 1992).

The intriguingly elevated spleen cellularity owing, at least in part, to recruitment and expansion of lymphocytes and phagocytes as a result of HNP51 and VNP51 administration to HMAP- or VMAP-infected mice on day 90 indicates the adjuvant effects of HNP51 and VNP51 and its ability to augment the natural immune responses against HMAP or VMAP pathogen in Balb/c mice (Figure 2A). Decreased spleen cellularity during mycobacterial infection was associated with increased susceptibility in alcoholic rats (Li et al., 1998).

Feeding the VNP51 to HMAP-infected mice was coupled to increased number of granulomas of multibacillary phenotype as indicated by the increased acid-fast bacilli numbers (Figures 2B and C). Because HMAP lacks the ability to replicate, it is but logical to assume that the multibacillary phenotype of granulomas we observed indicates effective containment of MAP, rather than HMAP proliferation (Figures 2B and C). On the other
hand, feeding the VNP51 to VMAP-infected mice did not alter the granulomas formation or
the number of the acid-fast bacilli (Figures 2B and C) in their livers compared with that of
the VMAP-infected controls on day 90 (Figure 2B), suggesting that triggering HNP51- or
VNP51-induced anti-VMAP immune response require longer time compared with similar
immune response triggered against the HMAP.

Of noteworthy, the enhanced granulomatous reaction in livers of HMAP- or VMAP-
infected mice fed the VNP51 was associated with an increased of MAP-specific IFN-γ
secretion in their in vitro splenocyte cultures (data not shown).

By day 135, the HNP51- and the VNP51-induced anti-VMAP immune response became
overtly robust compared with similar immune response triggered against HMAP.
Administration of VNP51 dramatically increased the spleen cellularity of mice infected with
VMAP (Figure 3A), which is indicative of a continuous recruitment and proliferation of immune
cells. The increased cellularity of the spleens, however, was not coupled to an enhanced
granulomatous reaction in liver (Figure 3B) or decrease of their content of acid-fast bacilli
(Figure 3C). Secretion of MAP-specific IL-10 decreased, however, in their in vitro
splenocyte cultures (data not shown), indicating decreased infectivity and dissemination of
MAP (Denis and Ghadirian, 1993). This immune response was further validated by showing
that feeding the HNP51 tended to decrease MAP IS900 genomic DNA shedding in fecal
pellets (Figure 3D).

We confirmed the decreased infectivity of VMAP by culturing different tissues from
VMAP-infected mice fed either the HNP51 or the VNP51 on Herrold’s egg yolk media.
Feeding the HNP51 or VNP5 induced a substantial decrease in the VMAP burden in viscera and secondary immune organs of VMAP-infected mice (Figures 5A, B, and C) on day 135.

On day 180, feeding of either the HNP51 or VNP5 to VMAP-infected mice decreased remarkably the cellularity of their spleens as an indicator of a resolved immune response against VMAP infection (Figure 4A). Also, histopathological examination of the hepatic tissues revealed the prevalence of paucibacillary granulomas in livers of the VMAP-infected and HMAP-inoculated mice fed the VNP51 (Figure 4C), suggesting efficient killing of VMAP pathogen by day 180 of the study. In Bovamine JD, the paucibacillary granuloma occurs first during the subclinical stage of the disease, whereas the multibacillary phenotype occurs during the clinical stage only.

Given the complicated structure and chronicity of granulomas, it is unlikely to see differences in granulomatous reaction intensity within short-term duration (Figure 4B). On the contrary a change of the acid-fast bacilli content of the granuloma is indicative of the efficacy of the immune response (Figure 4C).

The paucibacillary phenotype of granuloma observed was associated with an active secretion of VMAP-specific IFN-γ in the in vitro splenocyte cultures from VMAP-infected mice fed either the HNP51 or VNP51 (data not shown). It seems more prudent for the proinflammatory IFN-γ to increase at this time when the ileal inflammation has waned, as showed by the decreased numbers of acid-fast bacilli (Figure 4C) and VMAP burden (Figures 5A, B, and C and 6A, B, and C).

CONCLUSIONS

These conclusive findings provide unequivocal evidence that feeding probiotic VNP51 or the HNP51 to specific pathogen-free Balb/c mice infected with VMAP decreases tissue
VMAP burden and fecal VMAP shedding through and immune mechanism yet to be unraveled. Because both VNP51 and HNP51 impeded VMAP growth *in vivo*, we speculate that the VNP51- and HNP51-mediated immunomodulation effect is dependent on the bacterial cell components and doesn’t require the viability of NP51.

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CHAPTER 5. PROBIOTIC *LACTOBACILLUS ACIDOPHILUS NP51*® RESTIRICS PROGRESSION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* (MAP) INFECTION IN BALB/C MICE THROUGH ACTIVATING THE CD8⁺ IMMUNE CELLS

A paper to be submitted to the Journal of Immunology

RUNNING TITLE: NP51, MAP, AND JOHNE'S DISEASE.

Probiotic *Lactobacillus acidophilus NP51*® restricts progression of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in Balb/c mice through activating the CD8α⁺ immune cells.

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**ABSTRACT**

The objective of this study was to examine effects of feeding the probiotic *Lactobacillus acidophilus* strain NP51 to Balb/c mice challenged with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne’s disease. We hypothesized that feeding the NP51 would accelerate the innate immunity leading to rapid activation of the adaptive immunity to impede the development of MAP infection in mice. Thus, Balb/c mice were randomized to treatment groups in a factorial design including mice that either were fed the viable or heat-killed NP51 (VNP51 or HNP51, respectively) and challenged with either a viable or heat-killed MAP (VMAP or HMAP, respectively). Mice were fed $1 \times 10^6$ CFU of either HNP51 or VNP51 · mice$^{-1}$ · day$^{-1}$ mixed with standard mouse chow until the end of the study. Subsequently, mice were challenged with $1 \times 10^8$ CFU of VMAP or HMAP injected intraperitoneally on day 45 of the study. Ten mice from each group were euthanized on days 45, 90, 135, and 180. Spleens were excised and used for *in vitro* splenocyte cell cultures that were either stimulated with sonicated MAP antigen or concanavalin A and examined for cytokine secretion pattern and frequency of T lymphocyte subpopulations. Blood was withdrawn by cardiac puncture and used for examination of immunoglobulin production. VNP51 and HNP51 differentially stimulated the adaptive immunity. With VMAP as the
inoculum, both VNP51 and HNP51 stimulated CD8α+ immune cells-mediated immunity and decreased humoral immunity. When HMAP was used as the inoculum, VNP51 stimulated both CD8α+ immune cell-mediated and the humoral immunity. In contrast, HNP51 feeding induced CD8α+ immune cells-mediated immunity only as verified by the differential cytokine and immunoglobulin secretion pattern.

Key words: MAP, NP51, Johne’s disease, innate immunity.

**INTRODUCTION**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an obligate intramacrophage inhabitant that causes Johne’s disease (JD; paratuberculosis), a chronic granulomatous ileitis of ruminant animals (Harris and Barletta, 2001). Recently, MAP has been implicated with causing Crohn’s disease, a progressive severe ileocolitis in humans (Behr and Kapur, 2008) that resembles JD in pathophysiology. Because of the intensive modern animal farming, JD has emerged as a worldwide epidemic (Hutchinson, 1996). The pathogenesis of JD, however, is dictated primarily by the genetic susceptibility of the mammalian host and the type of invading MAP strain (Weiss and Souza, 2008).

Among the macrophage surface receptors, CR and PRRs (C-type lectin mannose receptor, CD14, and TLRs) have been shown to bind Mycobacteria (Aderem and Underhill, 1999) and facilitate its phagocytosis. Complement fragment C3b-opsonized Mycobacteria mediate phagocytosis by macrophage via binding to CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) (Souza et al., 2007). In contrast, phagocytosis of non-opsonized Mycobacteria is mediated primarily through mannose receptor (Astarie-Dequeker et al, 1999). Mycobacteria also bind TLR-1, 2, and 4 on host macrophages (Heldwein and Fenton, 2002).
The cell membrane of Mycobacteria is known to express lipomannan (LM), lipoarabinomannan (LAM), mycolyl-arabinogalactan peptidoglycan complex, a 19-KDa lipoprotein (Brennan and Nikaido, 1995), and the cord factor trehalose 6,6’-dimycoly (Spargo et al., 1991).

LAM, a well characterized primary virulence factor of MAP, is a glycolipid composed of a mannopyranosol central molecule attached with arabinofuranosyl side chains, and is immobilized to the cell membrane via a mannosyl-phosphatidylinositol membrane anchor (Briken et al., 2004). The mannan core is attached to an arabinan domain that is capped by various motifs. LAMs of pathogenic Mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium avium*, are capped with mannose caps (ManLAM). In contrast, the arabinan domain of non-pathogenic *Mycobacterium smegmatis* is capped with phospho-inositol (PILAM) and the arabinan domain of *Mycobacterium chelonae* is uncapped (AraLAM).

Recognition by innate immunity PRRs is induced by the arabinan domain capping motifs. ManLAM selectively binds C-type lectin mannose receptor on macrophages or dendritic cell-specific ICAM-3 grabbing non-integrin receptor-1 (DC-SIGNR1) on dendritic cells (DC) (Schlesinger, 1993; Maeda et al., 2002). AraLAM binds the co-receptor CD14 and induces TLR signaling and production of TNF-α, IL-8, and IL-12p40. In a sharp contrast, ManLAM binding to the mannose receptor is coupled neither to macrophage activation nor to phagosome maturation, and abrogates induction of IL-12p40, a highly pivotal cytokine for Th-1 activation (Nigou et al, 2001). Thus, the mannose receptor constitutes a safe portal for entry of pathogenic Mycobacteria.

The mycobacterial 19-kDa lipoprotein, on the other hand, binds the mannose receptor and TLR-2 (Pai et al., 2004). Signaling of 19-kDa through TLR-2 and the adaptor protein
myeloid differentiation primary response gene 88 (MyD88) represses MHC class I and II expression, antigen presentation, and subsequent CD4+ T helper cell recruitment. These inhibitory machineries allow MAP to evade the immune surveillance and survive longer in the host macrophage (Noss et al. 2001; Pai et al., 2004; Weiss and Souza, 2008). Abrogation of phagosome maturation has been ascribed to cord factor trehalose 6,6’-dimycolyl (Spargo et al., 1991; Welsh et al., 2008).

The ManLAM and 19-kDa-induced immune inhibitory effects are further potentiated by signaling of the MAP cell wall mycolyl-arabinogalactan peptidoglycan via a signal transduction pathway that does not involve TLR-2 and MyD88 (Noss et al., 2001). Overall, mycobacterial invasion of macrophage inhibits phagosome maturation and phagosome-lysosome fusion and acidification (Sturgil-Koszycki et al., 1994). This mycobacterium-induced inhibition of phagosome maturation is evident by the persistence of the phagosome early maturation markers the lysosome-associated membrane proteins (Lamp-1), Rab5, and the transferrin receptors.

Suppression of the innate immunity through deactivating APC prevents subsequent generation of protective adaptive immunity. The interaction between the host innate immunity and MAP-induced inhibitory immune mechanisms aimed at subvert the innate immunity is a significant determinant of JD pathogenesis.

Recent work by Pagnini and colleagues (2010) showed that probiotics fed to a mice model of Crohn’s disease prevented the onset of intestinal inflammation by local stimulation of epithelial innate immunity, resulting in increased production of epithelial-derived TNF-α and restoration of epithelial barrier in vivo. Subsequent work indicated that this immune effect is mediated through NF-κB (Pagnini et al., 2010). Indeed, probiotic Lactobacillus casei fed to
Balb/c mice increased the expression of macrophage mannose receptor-1 (CD206) and TLR-2, markers of innate immunity (Galdeano and Perdigon, 2006) and pivotal PRRs for MAP uptake by macrophage (Pai et al., 2004; Bhatt and Salgame, 2007).

Feeding probiotics to mice can program DC to adopt certain immune phenotype that orchestrates subsequent immune response accordingly (Hart et al., 2004). *Lactobacillus rhamnosus* LcR35 fed to mice at high dose induced DC maturation as revealed by increased expression of B7.2, CD83 (sialic acid-binding Ig-like adhesion receptor), HLA-DR, and TLR-4 and decreased expression of DC-SIGN and CD14 (Evrard et al., 2011) suggesting development of a tolerogenic DC phenotype. Induction of DC phenotype by probiotics seemed to be strain-dependent. Stimulation of immature DC with different strains of probiotics caused a dose-dependent modification of their IL-10/IL-12p70 secretion. *Lactobacillus salivarius* Ls-33 and *Bifidobacterium infantis* 35624 predominantly destined DC to secret IL-10. On the other hand, probiotic *Lactobacillus acidophilus* NCFM and the probiotic cocktail VSL#3 selectively induced secretion of IL-12p70 by DC. *Escherichia coli* Nissle 1917, however, equally provoked IL-10 and IL-12p70 secretion by DC (Gad et al., 2011).

Abundant evidence outlining the ability of DC to “cross talk” with NK has rapidly evolved (Fernandez et al., 1999; Guiton et al., 2009). DC-NK cells interaction caused bidirectional activation characterized by induced DC maturation (Vitale et al., 2005) and enhanced NK cytotoxicity and secretion of effector molecules (Ferlazzo et al., 2002). CD8\(^+\) cytotoxic T cells are the dominant effector cells following DC activation and infection with intracellular pathogen, such as *Toxoplasma gondii* (Guiton et al., 2009). Previous studies conducted to identify surface receptors involved in the DC-NK cells interaction revealed that
signaling of C-type lectin-like protein NKG2D immunoreceptor is crucial to the mutual activation of DC and NK (Raulet, 2003). NKG2D is expressed by macrophage, NK cells, NK T cells, and CD8⁺ cytotoxic T cells (Raulet, 2003). Intriguingly, in CD4⁺ T helper cell-deficient mice, NK cells activated CD8⁺- cytotoxic T cell-mediated immunity against intracellular parasites (Combe et al., 2005).

Thus, mice immune system can purposely be programmed to trigger requisite immune response against specific pathogen infections. Programming of mice immune system can be accomplished by selectively feeding certain probiotic strain that induce the intended DC phenotype. We hypothesized that pre-feeding the probiotic *Lactobacillus acidophilus* NP51 to Balb/c mice infected with MAP would induce DC maturation and accelerate the innate immunity resulting in efficient induction of the adaptive immunity against MAP. Our objective is to elucidate the NP51-induced immune response that targets *in vivo* MAP growth and proliferation in a murine model of JD.

**MATERIALS AND METHODS**

**Experimental Design.**

Three hundred sixty pathogen-free, 6-week-old Balb/c mice (Jackson Laboratories) were randomized to nine treatment groups in a factorial design that included three major groups (Table 1). Group 1 was fed the maltodextrin (MDX) carrier at 3% of their chow diet. Group II was fed the heat-killed NP51 (HNP51) at $1 \times 10^6$ CFU·mouse$^{-1}$·day$^{-1}$. Group III was fed the viable NP51 (VNP51) at $1 \times 10^6$ CFU·mouse$^{-1}$·day$^{-1}$. Both VNP51 and HNP51 were mixed with a MDX carrier at $7.5 \times 10^6$ CFU of VNP51 or HNP51·g of MDX$^{-1}$. 
Table 1. Designation of treatment groups.

<table>
<thead>
<tr>
<th>Code</th>
<th>Group</th>
<th>MDX 3% of the diet</th>
<th>Heat-killed NP51 (HNP51) 10^6 CFU/mouse·day⁻¹</th>
<th>Viable NP51 (VNP51) 10^6 CFU/mouse·day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
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<td>I</td>
<td>HMAP</td>
<td>VMAP</td>
<td>HNP51</td>
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<tr>
<td>VNP51+VMAP</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of mice</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

Cntrl: Control mice fed the regular sterile regular chow diet. MDX: maltodextrin (carrier for HNP51 and the VNP51). VNP51 and HNP51: heat-killed and viable Lactobacillus acidophilus strain NP51®. VMAP and HMAP: heat-killed and viable Mycobacterium avium subspecies paratuberculosis (MAP).

Group I mice contained a non-infected control (n = 40), a heat-killed MAP-infected control (HMAP; n = 40), and a viable MAP-infected control (VMAP; n = 40) subgroups.

Group II mice included a non-infected control (HNP51; n = 40), a heat-killed MAP-infected (HNP51+HMAP; n = 40), and a viable MAP-infected (HNP51+VMAP; n = 40) subgroups.

Group III mice included a non-infected control (VNP51; n = 40), a heat-killed MAP-infected (VNP51+HMAP; n = 40), and a viable MAP-infected (VNP51+VMAP; n = 40) subgroups.

HMAP or VMAP was injected intraperitonealy at 1× 10^8 CFU·mouse⁻¹ once on day 45 of the study. Mice were housed in a BSL-2 rodent room in the animal facility of the Iowa State University. The environment within the room was maintained to provide a temperature of 22.2°C, a relative humidity of 35-45%, and 12 h light/dark cycle. Every four female or male mice were housed in autoclaved standard mice cages fitted with raised-wire floors and high efficiency particulate air (HEPA) filters.

All the experimental procedures were done in accordance with the guidelines of the Iowa State University Institutional Animal Care and Use Committee IACUC.

Mice were offered sterile water ad libitum and chow meal (Harlan Laboratories) throughout the study. Mice were adapted to the sterile chow meal for a week prior to the addition of NP51. Mice in the HNP51 or VNP51 treatment groups were fed the NP51 at 1×
$10^6$ CFU·mouse$^{-1}$·day$^{-1}$ contained in 3.0 grams of the chow meal. To prepare the HNP51, the viable probiotic NP51 was incubated in an oven preheated to 85$^\circ$C for 15 min. The offering of chow meal was increased by 0.5 g chow·mouse$^{-1}$ every 15 days until it reached a maximum of 5.0 g of chow·mouse$^{-1}$·day$^{-1}$ by day 105 of the study. Mice continued to be offered 5.0 g·mouse$^{-1}$·day$^{-1}$ until the end of the study.

**Inoculation with Mycobacterium avium subspecies paratuberculosis.** MAP was grown on Middlebrook broth 7H9 (PML Microbiology, Wilsonville, OR). MAP inoculum was prepared according to Stabel et al (2009) using strain 167 isolate (Stabel, USDA-ARS-NADC, Ames, IA). Briefly, 50 mL of oleic acid at 0.60 mL·L$^{-1}$, albumin at 50 g·L$^{-1}$, dextrose at 20 g·L$^{-1}$, catalase at 0.04 g·L$^{-1}$, and sodium chloride at 8.50 g·L$^{-1}$ (OADC) enrichment were inoculated in 450 mL of sterile Middlebrook broth 7H9. Subsequently, 25 mL of a virulent MAP clinical isolate of strain 167 (USDA, ARS, NADC, Ames, IA) were inoculated into the broth. After thoroughly mixing, the VMAP culture was incubated at 37$^\circ$C and the OD$_{540nm}$ was measured weekly. The bacteria were harvested in the log phase (OD$_{540nm}$ = 0.2-0.4). The MAP cultures were decanted into sterile 500 mL Beckman bottles with O-ring caps followed by centrifugation at 1000 × g (7500 rpm) for 30 minutes in a refrigerated Beckman J2-21 centrifuge using the JA-10 rotor. The supernatant was decanted and the MAP pellets were washed twice by suspension in 500 mL of sterile, cold phosphate buffered saline (PBS; 0.14 M NaCl, 0.003 M KCl, 0.01 M Na$_2$HPO$_4$, and 0.0018 M KH$_2$PO$_4$; pH 7.4). Subsequently, MAP suspension was centrifuged for 30 minutes and the supernatant discarded. The formed MAP pellets were re-suspended in 5 mL of cold sterile PBS and placed on ice. The OD$_{540}$ was measured to verify the initial concentration of the bacteria.
Then, the PBS was used to adjust the concentration to $10^9$ CFU/mL as determined by OD$_{540}$ (OD$_{540nm} = 1.0\text{--}1.2$ absorbance units).

To prepare the HMAP, VMAP suspension was heated in a water bath at $85^\circ$ C for 20 minutes, then cooled to room temperature, and kept on ice until used for inoculating mice (Stabel, USDA, ARS, NADC, Ames, IA).

On day 45 of the study, mice were challenged intraperitonealy with $1 \times 10^8$ CFU of either HMAP or VMAP suspended in 100 µL of sterile PBS (Stabel, USDA, ARS, NADC). Control groups received 100 µL of sterile PBS.

**Sampling and Analysis**

Following inoculation with MAP, 10 mice from each treatment group were euthanized on days 45, 90, 135, and 180 of the study. Upon euthanizing mice, about 0.9 mL of blood were withdrawn by cardiac puncture and allowed to clot at $4^\circ$ C over night before serum was separated by centrifugation at 5000 RPM for 15 min. Also, the liver, mesenteric lymph node, and small intestine were dissected aseptically and frozen at $-80^\circ$ C until used for determination of MAP burden. Spleen was dissected and homogenized in RPMI until used for *in vitro* splenocyte culture.

**Quantification of immunoglobulin production.** To quantify total immunoglobulin (Ig) G$_1$ and IgG$_{2a}$, 100 uL of 1:50,000 diluted sera were used according to the manufacturer’s instruction (Kamiya Biomedical Company, Seattle, WA).

To quantify MAP specific IgG$_1$ and IgG$_{2a}$, 96-well plates were coated with 100 µL/well of HMAP suspended in coating buffer (Sigma Chemical) at 2000 ng/mL. Plates were incubated while covered at $4^\circ$ C overnight. After the incubation, the coating buffer was decanted inside a biosafety cabinet and the plates were washed four times by filling the wells with 300 µL of
the washing buffer (Sigma Chemical). To block the nonspecific binding, 300 µL of blocking buffer (Sigma Chemical) were pipetted in each well and the plates were incubated for 30 min at room temperature. Subsequently, the blocking buffer was decanted and the plates were allowed to dry before they were stored at 4°C until the IgG₁ and IgG₂a were determined as previously mentioned.

**Quantification of cytokine secretion.** Spleens were dissected and used to prepare splenocyte single-cell suspension. Splenocytes were cultured *in vitro* in 48-well plate at 2 × 10⁶ cells per mL RPMI 1640 (Invitrogen, Carlsbad, CA) plus 10% heat-inactivated FCS (Fisher Scientific, Hanover Park, IL) and 1% antibiotic/antimycotic (Invitrogen Carlsbad; NM). *In vitro* splenocyte cultures were either nonstimulated (negative control) or stimulated with concanavalin A (Sigma-Aldrich, St. Louis, MO) at 3 µg/mL (positive control), or with sonicated MAP antigen at 10 µg/mL.

The splenocytes cultures were incubated at 37°C and 5% CO₂/95% O₂ for 72 hours. Following incubation, the cell-free supernatant was collected and stored at -70°C for later analysis of interferon (IFN)-γ and interleukin (IL)-10 concentrations by using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN).

**Determination of T lymphocyte subpopulations.** The pelleted splenocytes were washed twice with cold sterile PBS and stained for the surface markers CD4 (anti-mouse CD4 PerCP-Cy5.5, eBioscience, San Diego, CA), CD8α (anti-mouse CD8α APC, eBioscience, San Diego, CA), and CD25 (anti-mouse CD25 phycoerytherin; PE, eBioscience, San Diego, CA). Subsequently, frequencies of these T cell subpopulations were determined by using the fluorescence-activated cell sorter (BD FACSaria II; Becton Dickson Biosciences, Franklin Lakes, NJ). Data were analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR).
Commercially available ELISA kits from R&D Systems (McKinley Place NE, MN) were used to determine IFN-$\gamma$ and IL-10 concentrations.

**Statistical Analyses**

For the flow cytometric data, a linear mixed-effects model was fit to the data for each response variable by using the PROC MIXED procedure of the Statistical Analysis System (SAS; SAS Institute Inc., Cary, NC, USA) version 9.2. Each linear mixed-effects model included random cage effect and fixed effects for diets, infection status, day of euthanization, gender, and all possible interactions among these fixed effects factors.

For the cytokine and immunoglobulin data, means of response variables were log-transformed to overcome the natural variability in immune response. Then the PROC MIXED procedure of SAS was used with CLASS statement of treatment and gender and MODEL statement of log-transformed mean equal treatment, gender and their interaction. The least squared means were computed by using the LSMEANS procedure and differences among least squared means were computed by using the PDIF CL procedure. The standard errors of the means were reported, and the pairwise differences among the estimated means were considered significant when the probability ($P$) value was $\leq 0.05$. Tendency for a trend was declared when $0.05 \leq P \leq 0.1$.

**RESULTS**

**Day 45 Results**

**CD4$^+$ T helper cells.** Effect of feeding the HN51 and VNP51 on the frequency of the T cell subpopulations was examined. Overall, there were significant treatment ($P = 0.004$) and day ($P = 0.0007$) effects on frequency of CD4$^+$ T helper cells (Figure 1A). In the non-stimulated T cell cultures, feeding the VNP51 to mice infected with the HMAP or VMAP did
not significantly change \((P \geq 0.16)\) their CD4\(^+\) T helper cell frequency (Figure 1A). Feeding the HNP51, however, decreased \((P = 0.009)\) CD4\(^+\) T helper cell frequency (Figure 1A) in the VMAP-infected mice. In contrast, feeding the HNP51 to HMAP-infected mice did not alter \((P = 0.31)\) their CD4\(^+\) T helper cell frequency (Figure 1A) on day 45. We observed that feeding VNP51 to noninfected control mice significantly \((P = 0.02)\) decreased CD4\(^+\) T helper cells (Figure 1A). On the contrary, HNP51 did not significantly \((P = 0.35)\) change CD4\(^+\) T helper cells relative to that of the noninfected control mice (Figure 1A).

Stimulation of the splenocyte culture with the VMAP antigen revealed that feeding HNP51 to HMAP-infected mice decreases \((P = 0.04)\) their CD4\(^+\) T helper cells in comparison with that of the HMAP-infected control mice on day 45 (Figure 1A). Of interest, feeding the VNP51, not the HNP51 \((P = 0.17)\), tended \((P = 0.07)\) to decrease their CD4\(^+\) T helper cell repertoire in the splenocyte cultures when stimulated with VMAP antigen relative to that of the noninfected control mice (Figure 1A).

ConA stimulation of the splenocyte cultures from mice fed the HNP51 and infected with the HMAP tended to decrease \((P = 0.06;\) Figure 1A) CD4\(^+\) T helper cell frequency. Also, stimulation of the in vitro T cell cultures with sonicated MAP antigen decreased \((P = 0.04)\) CD4\(^+\) T helper cell frequency (Figure 1A). On the contrary, feeding the HNP51 to VMAP-infected mice or feeding to the VNP51 to HMAP- or VMAP-infected mice did not substantially \((P \geq 0.13)\) change their CD4\(^+\) T helper frequency (Figure 1A) when T cell culture where stimulated with ConA or MAP antigen on day 45.

**CD4\(^+\) CD25\(^+\) T helper cells.** Similarly, non-stimulation, stimulation with ConA, or simulation with MAP antigen of the splenocytes cultures from mice fed the HNP51 or the VNP51 and infected with the HMAP or VMAP did not significantly change \((P \geq 0.17;\)
Figure 1B) CD4\(^{+}\)CD25\(^{+}\) T reg cell frequency on day 45 compared with that of the VMAP- and HMAP-infected control mice. Feeding the VNP51 or HNP51 to noninfected mice significantly (\(P = 0.03\) and \(P = 0.006\), respectively) increased their CD4\(^{+}\)CD25\(^{+}\) T reg cell repertoire compared with that of the noninfected control mice (Figure 1B).

\textbf{CD8\(\alpha\)^{+} immune cells.} Overall, in the non-stimulated or ConA-stimulated splenocyte cultures, there were treatment (\(P \leq 0.07\)), day (\(P = 0.0001\)), and day by treatment interaction (\(P \leq 0.04\)) effects on CD8\(\alpha\)^{+} immune cells. Despite this, in the non-stimulated \textit{in vitro} cultures, feeding the HNP51 or the VNP51 to mice infected with HMAP or VMAP did not appear to modify (\(P \geq 0.21\)) the frequency of CD8\(\alpha\)^{+} immune cells (Figure 1C) relative to control mice infected with the HMAP or VMAP on day 45.

Feeding the HNP51 or the VNP51 to non-infected mice provoked a significant increase (\(P = 0.0001\) and \(P = 0.0001\), respectively; Figure 1C) of CD8\(\alpha\)^{+} immune cells in the MAP antigen-stimulated splenocytes cultures in comparison to the control mice. In the ConA-stimulated cultures, however, feeding the HNP51 or the VNP51 \textit{per se} did not change significantly (\(P \geq 0.37\)) the frequency of CD8\(\alpha\)^{+} immune cells relative to the control (Figure 1C).

On the other hand, stimulation of the splenocytes cultures with the sonicated MAP antigen, demonstrated that feeding HNP51 or the VNP51 to mice infected with the HMAP or the VMAP increased significantly (\(P \leq 0.05\); Figure 1C) the frequency of the CD8\(\alpha\)^{+} immune cells on day 45.

\textbf{CD8\(\alpha\)^{+}CD25\(^{+}\) T reg cells.} In the non-stimulated or MAP-stimulated \textit{in vitro} splenocyte cultures of HMAP- or VMAP-infected mice fed the HNP51 or the VNP51, frequency of CD8\(\alpha\)^{+}CD25\(^{+}\) T reg cells did not change (\(P \geq 0.21\)) in comparison with the control mice.
On the other hand, stimulation of the splenocyte cultures with the mitogen ConA increased considerably ($P \leq 0.002$) the frequency of $CD8^{\alpha +}CD25^{+}$ T reg cells in mice fed the HNP51 or the VNP51 and infected with the HMAP or the VMAP relative to the HMAP- and VMAP-infected control (Figure 1D).

Figure 1. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their in vitro splenic T cell subpopulations proliferation stimulated with ConA (open bars) or with MAP antigen (grey bars) or nonstimulated (dark bars) on day 45. Panel A. CD4$^{+}$ T helper cells proliferation. Panel B. CD4$^{+}$CD25$^{+}$ T reg cells. Panel C. CD8$^{\alpha +}$ immune cells. Panel D. CD8$^{\alpha +}$CD25$^{+}$ T reg cells. Data are mean ± SEM. (*) indicates significant difference at $P \leq 0.05$; (#) indicates tendency for a significant difference at $0.05 < P \leq 0.10$. 

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**Diagram Description**

- **Panel A**: CD4$^{+}$ T helper cells proliferation.
- **Panel B**: CD4$^{+}$CD25$^{+}$ T reg cells.
- **Panel C**: CD8$^{\alpha +}$ immune cells.
- **Panel D**: CD8$^{\alpha +}$CD25$^{+}$ T reg cells.
**IL-10 and IFN-γ.** Feeding the HNP51 to mice infected with either HMAP or VMAP did not cause significant variation in the production of IFN-γ and IL-10 ($P \geq 0.41$; Figures 2A and B, respectively) relative to that of control mice infected with either the HMAP or VMAP on day 45. In a sharp difference, feeding the VNP51 to mice challenged with the HMAP induced a significant increase in IFN-γ and IL-10 ($P = 0.03$ and $P = 0.02$; Figures 2A and B, respectively) on day 45.

On the other hand, feeding the VNP51 to mice infected with the VMAP did not significantly ($P \geq 0.33$; Figures 2A and B) alter IFN-γ or IL-10 secretion by splenocytes stimulated with MAP antigen. Feeding the VNP51 *per se*, not HNP51 ($P = 0.91$), increased ($P = 0.0003$) IL-10 secretion relative to the control mice (Figure 2B). But feeding either the HNP51 or the VNP51 to non infected mice did not significantly change ($P \geq 0.69$; Figure 2A) IFN-γ secretion pattern on day 45.

**Total and MAP-specific IgG isotypes.** We also examined effects of feeding the probiotics on immune parameters of adaptive immunity. Feeding the VNP51 to Blab/c mice infected with VMAP decreased significantly ($P = 0.0001$; Figures 2C) serum total IgG$_1$ concentration and simultaneously increased ($P = 0.0001$; Figures 1I) IgG$_{2a}$ concentration. In contrast, feeding the VNP51 to Balb/c mice infected with HMAP induced a substantial increase (Both $P = 0.0001$; Figure 2C and D) in their total IgG$_1$ and IgG$_{2a}$. Feeding the HNP51 to Balb/c mice infected with HMAP elevated significantly their total IgG$_{2a}$ ($P = 0.001$); however, total IgG$_1$ was decreased ($P = 0.04$; Figure 2C and D) in comparison to that of HMAP-infected controls (Figures 2C and D). When HNP51 was fed to mice infected with the VMAP total IgG$_1$ concentrations was not significantly altered ($P = 0.61$; Figure 2C). On the contrary, total IgG$_{2a}$ was remarkably increased ($P = 0.0001$; Figure 2D) on day 45 of the study.
Quick significant elevation ($P \leq 0.02$; Figures 2E and F) of MAP-specific IgG$_1$ and IgG$_{2a}$ was detected in sera of mice infected with either HMAP or VMAP and fed VNP51. Immune effects of HNP51 suggested dependency on viability of MAP (Figures 2E and F). Whereas feeding HNP51 to mice infected with HMAP did not significantly alter ($P \geq 0.73$; Figures 2E and F), feeding the HNP51 to VMAP-infected mice significantly raised ($P \leq 0.007$; Figures 2E and F) both MAP-specific IgG$_1$ and IgG$_{2a}$.
Figure 2. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their MAP-specific interferon (IFN)-γ and interleukin (IL-) 10 and serum total and MAP-specific immunoglobulins (Ig) concentrations on day 45. Panel A. MAP-specific IFN-γ concentrations. Panel B. MAP-specific IL-10 concentrations. Panel C. Total IgG1 concentrations. Panel D. MAP-specific IgG1 concentrations. Panel E. Total IgG2a concentrations. Panel F. MAP-specific IgG2a concentrations. Data are mean ± SEM. (*) indicates significant difference at $P \leq 0.05$; (\#) indicates tendency for a significant difference at $0.05 < P \leq 0.10$. 
Day 90 Results

*CD4⁺ T helper cells.* In the non-stimulated splenocyte cultures, feeding the HNP51 to HMAP-infected mice tended to decrease ($P = 0.09$) the frequency of CD4⁺ T helper cells. Similarly, feeding the VNP51 to HMAP-infected mice decreased ($P = 0.05$) their CD4⁺ T helper cells on day 90 (Figure 3A). On the other hand, feeding the HNP51 or VNP51 to VMAP-infected mice did not alter ($P \geq 0.58$) CD4⁺ T helper cells in their *in vitro* cultures (Figure 3A). Note worthy, infection with VMAP significantly ($P = 0.01$) decreased their CD4⁺ T helper cells in the nonstimulated *in vitro* cultures relative to that of the HMAP-infected control mice (Figure 3A).

Stimulation with ConA or MAP antigen of splenocyte cultures from mice fed the HNP51 or the VNP51 and infected with the HMAP or the VMAP did not significantly vary ($P \geq 0.23$; Figure 3A) the frequency of CD4⁺ T cells on day 90.

*CD4⁺CD25⁺ T reg cells.* Responses of CD4⁺CD25⁺ T reg cells on day 90 mimicked that of CD4⁺ T helper cells (Figure 2B). VNP51 and HNP51 fed to HMAP-infected mice, respectively, decreased ($P = 0.02$ and $P = 0.01$, respectively) the frequency of CD4⁺CD25⁺ T reg cells (Figure 3B) in the nonstimulated cell cultures. On the contrary, feeding the VNP51 or the HNP51 to VMAP-infected mice did not modify ($P \geq 0.34$) the frequency of CD4⁺CD25⁺ T reg cells repertoire. Again, infection with VMAP infection of the control mice significantly ($P = 0.03$) decreased CD4⁺CD25⁺ T reg cells numbers in their nonstimulated *in vitro* cultures relative to that of the HMAP-infected control (Figure 3B).

Also, stimulation of the splenocyte cultures with ConA mitogen or MAP antigen did not significantly alter ($P \geq 0.35$) the frequency of CD4⁺CD25⁺ T reg cells (Figure 3B).
Also, ConA stimulation of splenocyte cultures of control mice fed the HNP51 did not affect \( P = 0.11 \) the frequency of \( \text{CD}4^{+}\text{CD}25^{+} \) T reg cells frequency in their \textit{in vitro} splenocyte cultures. But, \( \text{CD}4^{+}\text{CD}25^{+} \) T reg cells frequency in the cultures from VNP51-fed mice decreased \( P = 0.02 \); Figure 2B). In contrast, \( \text{CD}4^{+}\text{CD}25^{+} \) T reg cells frequency in cultures from HNP51- and VNP51-fed mice did not change \( P \geq 0.11 \) when cultures were stimulated with MAP antigen on day 90.

\textit{CD8α}^{+} \text{cytotoxic T cells.} Non-stimulated \textit{in vitro} cultures from mice fed the HNP51 and infected with the HMAP or the VMAP revealed higher \( P \leq 0.04 \) \textit{CD8α}^{+} immune cells relative to the HMAP- and VMAP-infected controls (Figure 3C). As well, nonstimulated \textit{in vitro} cultures from HMAP-infected mice fed the VNP51 showed increased \( P = 0.01 \) \textit{CD8α}^{+} immune cells compared with those from HMAP-infected controls. Feeding the VNP51 to VMAP-infected mice did not significantly \( P = 0.25 \) alter \textit{CD8α}^{+} cells population on day 90. Feeding the HNP51 \textit{per se} to non-infected control mice did not alter \( P = 0.89 \) the \textit{CD8α}^{+} immune cells. But feeding the VNP51 to non-infected control mice, however, increased \( P = 0.03 \) the frequency of these cells.

Stimulation with ConA of \textit{in vitro} splenocyte cultures from HMAP-infected mice fed the HNP51 or the VNP51 did not change \( P \geq 0.20 \) the \textit{CD8α}^{+} immune cells frequency (Figure 2C). Similarly, feeding the VNP51 to VMAP-infected mice did not vary \( P = 0.14 \) \textit{CD8α}^{+} immune cells frequency. Conversely, feeding the HNP51 to VMAP-infected mice tended to increase \( P = 0.06 \) \textit{CD8α}^{+} immune cells frequency (Figure 3C). Feeding the HNP51 or the VNP1 to non-infected mice, however did not alter \( P \geq 0.38 \) the frequency \textit{in vitro} \textit{CD8α}^{+} immune cells frequency.
Importantly, stimulation with MAP antigen of splenocyte cultures of HMAP- or VMAP-infected mice fed the HNP51 or the VNP51 significantly increased \((P \leq 0.001)\) the \(\text{CD}8\alpha^+\) immune cells frequency on day 90. Also, stimulation of splenocyte from non-infected mice fed the HNP51 or the VNP51 substantially increased \((P \leq 0.0003)\) \(\text{CD}8\alpha^+\) immune cells frequency (Figure 3C).

**\text{CD}8\alpha^+\text{CD}25^+ \text{T reg cells.}** Feeding the HNP51 or the VNP51 to HMAP-infected mice decreased \((P \leq 0.03)\) the population of \(\text{CD}8\alpha^+\text{CD}25^+\) T reg cells in their splenocyte cultures relative to that of the control (Figure 3D). Conversely, feeding the HNP51 or the VNP51 to VMAP-infected mice did not modify \((P \geq 0.73)\) their \(\text{CD}8\alpha^+\text{CD}25^+\) T reg cells \textit{in vitro} (Figure 2D). Similarly, feeding the HNP51 or the VNP51 \textit{per se} did not alter \((P \geq 0.84)\) frequency of \(\text{CD}8\alpha^+\text{CD}25^+\) T reg cells on day 90. Notably, the frequency of \(\text{CD}8\alpha^+\text{CD}25^+\) T reg cells was higher in the HMAP-infected mice relative to that of the VMAP-infected ones (Figure 3D).

When the splenocyte cultures were stimulated with the mitogen ConA, frequency of \(\text{CD}8\alpha^+\text{CD}25^+\) T reg cells seemed significantly lower \((P \leq 0.02)\) in \textit{in vitro} cultures of HMAP- or VMAP-infected mice fed the VNP51 in comparison with HMAP- and VMAP-infected controls. Feeding the HNP51, on the other hand, decreased \((P = 0.04)\) and tended to decrease \((P = 0.06)\) the frequency of \(\text{CD}8\alpha^+\text{CD}25^+\) T reg cells in cultures of the HMAP- and VMAP-infected mice relative to the HMAP- and VMAP-infected controls (Figure 3D). Of importance, MAP antigen stimulation of splenocyte \textit{in vitro} cultures of HMAP- or VMAP-infected mice fed the HNP51 or the VNP51 lowered significantly \((P \leq 0.002)\) the population of \(\text{CD}8\alpha^+\text{CD}25^+\) T reg cells in comparison with the control mice (Figure 3D). Unlike the stimulation with ConA, stimulation with the MAP antigen did not alter \((P \geq 0.64)\) the
frequency of CD$8\alpha^+$CD$25^+$ T reg cells in non-infected mice fed the HNP51 or the VNP51 (Figure 3D).

**Figure 3.** Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their *in vitro* splenic T cell subpopulations proliferation stimulated with ConA (open bars) or with MAP antigen (grey bars) or nonstimulated (dark bars) on day 90. Panel A. CD$4^+$ T helper cell proliferation. Panel B. CD$4^+$CD$25^+$ T reg cell. Panel C. CD$8\alpha^+$ immune cells. Panel D. CD$8\alpha^+$CD$25^+$ T reg cell. Data are mean ± SEM. (*) indicates significant difference at $P \leq 0.05$; (#) indicates tendency for a significant difference at $0.05 < P \leq 0.10$

**IL-10 and IFN-$\gamma$ secretion.** With progression of MAP infection to day 90, MAP-specific IFN-$\gamma$ secretion by splenocytes from mice fed the VNP51 and inoculated with HMAP tended to increase ($P = 0.09$; Figure 4A) relative to that of the HMAP-infected control mice.
HNP51- and VNP51-induced IFN-γ secretion on day 90 was coupled to enhanced
granulomatous reaction in livers of infected mice (Osman et al. manuscript under review).

Secretion of IL-10, however, was significantly increased ($P \leq 0.003$; Figure 4B) in the *in vitro* splenocytes cultures of VMAP- and HMAP-infected mice fed the VNP51 to be higher
than that of the VMAP- and HMAP-infected control mice. Similarly, feeding HNP51 to mice
challenged with VMAP elevated significantly ($P = 0.009$; Figure 4B) IL-10 concentrations in
their *in vitro* splenocyte cultures to be greater than that of the VMAP-infected control.

**Total and MAP-specific IgG isotypes.** HNP51 or VNP51 fed to mice infected with the
VMAP remarkably decreased ($P \leq 0.01$; Figures 4C and D) their total IgG$_1$ and IgG$_{2a}$ in
comparison to control mice infected with HMAP or VMAP on day 90. On the other hand,
feeding the VNP51 to mice infected with the HMAP highly significantly increased ($P =
0.002$) increased their total IgG$_1$ but decreased ($P = 0.008$; Figure 4C and E) their total IgG$_{2a}$
relative to that of the control. Similarly, feeding the HNP51 to HMAP-infected mice
increased ($P = 0.05$) their total IgG$_1$ and simultaneously decreased significantly ($P = 0.0001$)
their total IgG$_{2a}$.

Also, the anti-MAP IgG$_1$ and IgG$_{2a}$ seemed to follow a pattern similar to that of total IgG
isotypes (Figures 4D and F). It was obvious that HNP51 fed to mice infected with HMAP did
not significantly change ($P = 0.18$) their MAP-specific IgG$_1$ (Figure 4D). In comparison,
HNP51 fed to VMAP-infected mice induced a significant decrease ($P = 0.005$) in their MAP-specific IgG$_1$ on day 90 (Figure 4D). Also, VNP51 fed to HMAP-infected mice tended to
increase ($P = 0.06$; Figure 4D) their MAP-specific IgG$_1$ compared with HMAP-infected
controls. On the contrary, feeding VNP51 to VMAP-infected mice substantially lowered ($P =
0.0001$) their MAP specific IgG$_1$ compared with VMAP-infected controls (Figure 4D).
Figure 4. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their MAP-specific interferon (IFN)-γ and interleukin (IL-) 10 and serum total and MAP-specific immunoglobulins (Ig) concentrations on day 90. Panel A. MAP-specific IFN-γ concentrations. Panel B. MAP-specific IL-10 concentrations. Panel C. Total IgG₁ concentrations. Panel D. MAP-specific IgG₁ concentrations. Panel E. Total IgG₂a concentrations. Panel F. MAP-specific IgG₂a concentrations. Data are mean ± SEM. (*) indicates a significant difference at $P \leq 0.05$ and (#) indicates a tendency for a significant difference at $0.05 < P \leq 0.10$. 
Serum concentrations of MAP-specific IgG$_{2a}$ was not significantly altered (Both $P = 0.77$) by feeding the VNP51 or the HNP51 to HMAP-infected mice relative to that of the HMAP-infected control mice (Figure 4F). Also, HNP51 fed to VMAP-infected mice did not change ($P = 0.22$) their MAP-specific IgG$_{2a}$ concentrations compared with that of the VMAP-infected control mice (Figure 4F). In contrast, MAP-specific IgG$_{2a}$ was significantly decreased ($P = 0.03$) in sera of VMAP-infected mice fed the VNP51 relative to that of VMAP-infected control (Figure 4F).

**Day 135 Results**

**CD4$^+$ T helper cells.** On day 135, in the non-stimulated, ConA-stimulated, or MAP antigen-stimulated splenocyte cultures, feeding either the HNP51 or the VNP51 to HMAP- or VMAP-infected mice did not modify ($P \geq 0.11$) the frequency of CD4$^+$ T helper cells (Figure 5A).

**CD4$^+CD25^+$ T helper cells.** Indeed, the frequency of CD4$^+$ CD25$^+$ T regulatory cells did not change ($P \geq 0.45$) by feeding either the HNP51 or the VNP51 to HMAP- or VMAP-infected mice (Figure 5B) regardless of the culture stimulant used.

**CD8$\alpha^+$ immune cells.** The frequency of CD8$\alpha^+$ immune cells increased ($P = 0.04$) in the non-stimulated *in vitro* splenocyte cultures of mice fed the VNP51 and infected with the HMAP (Figure 3C). Also, feeding the VNP51, not the HNP51 ($P = 0.36$), to control non-infected mice increased ($P = 0.01$; Figure 5C) the frequency of CD8$\alpha^+$ immune cells on day 135. On the contrary, no change ($P \geq 0.2$) in the frequency of CD8$\alpha^+$ immune cells in other treatment groups was detected on day 135. Under ConA stimulation, however, only feeding the HNP51 to VMAP-infected mice tended to increase ($P = 0.09$) CD8$\alpha^+$ immune cells frequency on day 135 (Figure 5C).
On the contrary, under MAP-antigen stimulation of the \textit{in vitro} splenocyte cultures, feeding the HNP51 or VNP51 to HMAP- or VMAP-infected mice increased ($P \leq 0.03$) the frequency of the CD8$\alpha^+$ immune cells to be higher than that of the infected control mice on day 135 (Figure 3C). Intriguingly, stimulation of splenocytes from non-infected mice fed either HNP51 or the VNP51 by MAP antigen raised ($P = 0.03$ and $P = 0.005$) the frequency of CD8$\alpha^+$ immune cells higher than that of the non-infected controls (Figure 5C) on day 135.

\textit{CD8$\alpha^+CD25^+$ T reg cells.} Frequency of CD8$\alpha^+CD25^+$ T regulatory cells on day 135 was did not differ ($P \geq 0.48$) among the various treatments in the non-stimulate splenocyte cultures (Figure 5D). Stimulation with ConA, nonetheless, tended to decrease ($P = 0.06$ and $P = 0.07$) frequency of CD8$\alpha^+CD25^+$ T regulatory cells of \textit{in vitro} cultures of mice fed the HNP51 and either infected with HMAP or VMAP, respectively (Figure 5D). Also, under ConA stimulation, feeding the VNP51 tended to decrease ($P = 0.06$) and decreased ($P = 0.007$) CD8$\alpha^+CD25^+$ T regulatory cells population in splenocyte cultures of HMAP- and VMAP-infected mice, respectively, to be significantly lower than that of the infected control mice on day 135 (Figure 5D). Of significance, under MAP antigen stimulation condition, CD8$\alpha^+CD25^+$ T regulatory cells population decreased ($P \leq 0.05$) in splenocyte cultures of mice fed the HNP51 or VNP51 and infected with the HMAP or the VMAP (Figure 5D).

Feeding the HNP51 or VNP51 \textit{per se} to non-infected mice did not alter ($P \geq 0.55$) CD8$\alpha^+CD25^+$ T regulatory cells frequency under no stimulation or MAP antigen stimulation (Figure 3D). Yet, under ConA stimulation, frequency of CD8$\alpha^+CD25^+$ T regulatory cells increased ($P \leq 0.05$) to be greater than that of non-infected control on day 135 (Figure 5D).

\textit{IL-10 and IFN-$\gamma$.} By day 135 of the study mice have been infected for 90 days. VNP51 or HNP51 fed to VMAP- or HMAP-infected mice did not change ($P \geq 0.21$; Figure 6A) IFN-
γ concentrations in vitro. Concentration of IFN-γ, on the other hand, was elevated ($P = 0.0003$) in splenocyte cultures from HMAP-infected control mice compared with that from VMAP-infected control mice (Figure 6A). Also, feeding the VNP51 ($P = 0.008$), not the HNP51 ($P = 0.77$), notably decreased secretion of IFN-γ by splenocytes in vitro compared with the control mice (Figure 6A).

![Graphs showing the effect of probiotic feeding on T cell subpopulations](image)

Figure 5. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their in vitro splenic T cell subpopulations proliferation stimulated with ConA (open bars) or with MAP antigen (grey bars) or nonstimulated (dark bars) on day 135. Panel A. CD4+ T helper cell proliferation. Panel B. CD4+CD25+ T reg cell. Panel C. CD8α+ immune cells. Panel D. CD8α+CD25+ T reg cell. Data are mean ± SEM. (*) indicates a significant difference at $P ≤ 0.05$; (#) indicates a tendency for a significant difference at $0.05 < P ≤ 0.10$. 
Of note worthy, VNP51 fed to HMAP- or VMAP-infected mice decreased significantly ($P \leq 0.02$; Figure 6B) IL-10 secretion in their *in vitro* splenocyte cultures. Conversely, VNP51 raised IL-10 concentration ($P = 0.002$; Figure 6B) *in vitro* in splenocytes of mice infected with the HMAP on day 135.

Remarkably, feeding the HNP51 *per se* did not vary ($P = 0.83$) IL-10 concentrations (Figure 6B) nor did feeding the HNP51 alter significantly ($P = 0.50$; Figure 6B) their splenocyte secretion of IL-10 in comparison to control mice not fed the probiotic. Feeding the VNP51 to mice, however, decreased ($P = 0.05$) IL-10 secretion when their splenocytes were stimulated with the sonicated VMAP antigen *in vitro* (Figure 6B) when compared with that of the control mice. Also, feeding the HNP51 to mice tended to decrease ($P = 0.08$) IL-10 secretion in their splenocyte culture when stimulated with MAP antigen compared with that of the control mice (Figure 6B).

**Total and MAP-specific IgG isotypes.** In addition, the immuneomodulating effects of HNP51 and VNP51 was mostly suppressive to CD4$^+$ T helper cell responses (Th1 and Th2 responses) on day 135. Feeding the HNP51 to mice infected with the VMAP or HMAP decreased substantially ($P = 0.0001$ and $P = 0.05$, respectively) serum total IgG$_1$ and ($P = 0.0001$ and $P = 0.0001$, respectively) total IgG$_{2a}$ concentrations (Figures 6C and E).

Also, feeding the VNP51 to VMAP-infected mice lowered significantly ($P = 0.03$ and $P = 0.0001$, respectively) their serum total IgG$_1$ and IgG$_{2a}$ concentrations (Figures 6C and E). On the contrary, feeding the VNP51 to mice infected with HMAP triggered a significant increase ($P = 0.0001$ and $P = 0.02$) in their total IgG$_1$ and IgG$_{2a}$ concentrations, respectively, compared with HMAP-infected controls (Figures 6C and E).
When HNP51 or VNP51 were fed to control non-infected mice, their total IgG_1 and IgG_2a decreased significantly ($P \leq 0.0003$) when compared with the non-infected control mice (Figure 6C and E). On the other hand, infection with HMAP or VMAP caused total IgG_1 and IgG_2a to increase ($P \leq 0.008$) on day 135 with VMAP effect being significantly greater ($P \leq 0.01$; Figure 6C and E) than that of HMAP-infected control mice.

Generally, feeding either the HNP51 or VNP51 appeared not in favor of triggering anti-MAP adaptive immune response (Figures 3H and J). It was obvious that HNP51 or VNP51 fed to VMAP-infected mice remarkably lowered ($P \leq 0.007$) their MAP-specific IgG_1 and IgG_2a (Figures 6D and F) below that of the control mice infected with either the HMAP or VMAP. In contrast, feeding the HNP51 to HMAP-infected mice did not cause ($P \geq 0.54$) their MAP-specific IgG_1 and IgG_2a to significantly change (Figures 6D and F). In contrast, feeding the VNP51 to HMAP-infected mice decreased ($P = 0.002$) their MAP-specific IgG_2a, but not MAP-specific IgG_1 ($P = 0.30$; Figures 6D and F).
Figure 6. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their MAP-specific interferon (IFN-γ) and interleukin (IL-) 10 and serum total and MAP-specific immunoglobulins (Ig) concentrations on day 135. Panel A. MAP-specific IFN-γ concentrations. Panel B. MAP-specific IL-10 concentrations. Panel C. Total IgG1 concentrations. Panel D. MAP-specific IgG1 concentrations. Panel E. Total IgG2a concentrations. Panel F. MAP-specific IgG2a concentrations. Data are mean ± SEM. (*) indicates a significant difference at $P \leq 0.05$ and (#) indicates a tendency for a significant difference at $0.05 < P \leq 0.10$. 
Day 180 Results

**CD4+ T helper cells.** On day 180, frequency of CD4+ T helper cells did not respond \((P \geq 0.27)\) to stimulation with ConA mitogen or MAP antigen (Figure 7A). Also, in the non-stimulated culture, frequency of CD4+ T helper cells was invariant \((P \geq 0.14)\) among different treatments and controls (Figure 7A).

**CD4+CD25+ T reg cells.** Similarly, non-stimulation or stimulation of splenocyte cultures with MAP antigen did not change \((P \geq 0.34; \text{Figure 7B})\) the frequency of CD4+CD25+ T regulatory cells *in vitro*. On the other hand, stimulation of the *in vitro* cultures with ConA, tended to decrease \((P = 0.06)\) CD4+CD25+ T regulatory cells in splenocytes of HMAP-infected mice fed the HNP51. In contrast, CD4+CD25+ T regulatory cells did not differ \((P = 0.39)\) in the VMAP-infected mice fed the HNP51. On the contrary, feeding the VNP51 to HMAP- or VMAP-infected mice decreased \((P \leq 0.04)\) CD4+CD25+ T regulatory cells population relative to the infected controls (Figure 7B).

**CD8α+ immune cells.** On day 180, non-stimulation of *in vitro* splenocyte cultures only tended \((P = 0.06)\) to increase CD8α+ immune cells of splenocytes cultures of HMAP-infected mice fed the VNP51 (Figure 7C). But, effects of feeding HNP51 to VMAP-infected mice or feeding the VNP51 to HMAP- or VMAP-infected mice on CD8α+ immune cells were not significant \((P \geq 0.16; \text{Figure 7C})\).

Under ConA stimulation, however, feeding the HNP51 to VMAP-infected mice tended to increase \((P = 0.1; \text{Figure 4})\) CD8α+ immune cells frequency *in vitro* (Figure 7C). In contrast, feeding the HNP51 to HMAP-infected mice or feeding the VNP51 to HMAP- or VMAP-infected mice did not modify \((P \geq 0.12)\) CD8α+ immune cells *in vitro* (Figure 7C). Of significance, when in the vitro cultures were stimulated with MAP antigen, feeding the
HNP51 or the VNP51 to HMAP- or VMAP-infected mice appreciably increased \((P \leq 0.0007;\)  Figure 7C) CD8\(\alpha^+\) immune cells population in vitro. At this point, feeding the HNP51 or the VNP51 to non-infected control mice did not cause significant change \((P \geq 0.11)\) in CD8\(\alpha^+\) immune cells repertoire in vitro splenocyte cultures stimulated with MAP antigen (Figure 7C).

**CD8\(\alpha^+CD25^+\) T helper cells.** Non-stimulation of in vitro splenocyte cultures indicated that feeding the HNP51 or VNP51 to mice infected with HMAP or VMAP did not alter \((P \geq 0.59)\) CD8\(\alpha^+CD25^+\) T regulatory cells on day 180. On the other hand, ConA or MAP antigen stimulation coupled with feeding the HNP51 or the VNP51 to HMAP- or VMAP-infected mice decreased significantly \((P \leq 0.004;\)  Figure 7D) CD8\(\alpha^+CD25^+\) T regulatory cells population to be lower than that of infected controls.

Again, feeding the HNP51 or VNP51 per se to non-infected mice did not alter \((P \geq 0.70)\) CD8\(\alpha^+CD25^+\) T regulatory cells frequency under no stimulation or MAP antigen stimulation (Figure 7D). Yet, under ConA or MAP stimulation, frequency of CD8\(\alpha^+CD25^+\) T regulatory cells increased \((P \leq 0.002)\) to be higher than that of non-infected control on day 180 (Figure 7D).
Figure 7. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their in vitro splenic T cell subpopulations proliferation stimulated with ConA (open bars) or with MAP antigen (grey bars) or nonstimulated (dark bars) on day 180. Panel A. CD4\(^+\) T helper cell proliferation. Panel B. CD4\(^+\)CD25\(^+\) T reg cell. Panel C. CD8\(^{α}\) immune cells. Panel D. CD8\(^{α}\)CD25\(^+\) T reg cell. Data are mean ± SEM. (*) indicates a significant difference at \(P \leq 0.05\); (#) indicates a tendency for a significant difference at \(0.05 < P \leq 0.10\).

**IL-10 and IFN-γ.** The modulating immune effect of NP51 was still pronounced by day 180. The MAP-specific IFN-γ secretion in the in vitro splenocyte cultures of mice infected with the VMAP tended to increase \((P = 0.07)\) and was increased significantly \((P = 0.0004)\) by HNP51 or VNP51 feeding, respectively, relative to the HMAP- or VMAP-infected controls (Figure 8A).
Conversely, feeding the HNP51 or VNP51 to mice infected with HMAP did not significantly change ($P \geq 0.16$; Figure 8A) the secretion of IFN-$\gamma$ in splenocyte culture from mice inoculated with HMAP. On the whole, there was significant treatment ($P = 0.0001$) and gender ($P = 0.02$; Figure 8A) effects on MAP-specific IFN-$\gamma$ in the in vitro splenocyte cultures.

Importantly, feeding the VNP51, not the HNP51 ($P \geq 0.57$; Figure 8A), remarkably increased the production of MAP-specific IL-10 by the splenocytes from mice infected with either the HMAP ($P = 0.0003$) or VMAP ($P = 0.0001$; Figure 8A) when stimulated with the sonicated MAP in vitro.

**Total and MAP-specific IgG isotypes.** By day180, the immune-modulating effects of HNP51 and VNP51 changed dramatically (Figures 8C, 8D, 8E and F). Feeding the HNP51 to HMAP- or VMAP-infected mice did not change significantly ($P \geq 0.14$; Figures 8C and E) their serum total IgG$_1$ or IgG$_{2a}$ relative to those of the control mice.

But feeding the VNP51 to mice infected with the HMAP considerably decreased ($P = 0.03$) their total IgG$_1$ concentration and simultaneously maintained their total IgG$_{2a}$ concentration significantly greater ($P = 0.0001$) than that of the HMAP-infected control mice. In contrast, however, feeding the VNP51 to VMAP-infected mice did affect ($P = 0.81$; Figure 8C) their total IgG$_1$ and maintained their total IgG$_{2a}$ concentration lower ($P = 0.02$) than that of the control similar to finding of day 135 (Figure 8E).

Similar to that of day 135, feeding VNP51 to non-infected mice significantly lowered their total IgG$_1$ ($P = 0.01$) and IgG$_{2a}$ ($P = 0.0001$; Figures 8C and E). Also, HNP51 feeding to non-infected mice lowered ($P = 0.002$) their total IgG$_{2a}$ but did not significantly change ($P = 0.18$) their total IgG$_1$ relative to that of uninfected control mice (Figures 8C and E). Notably,
Figure 8. Effect of VNP51 and HNP51 probiotic feeding to Balb/c mice on their MAP-specific interferon (IFN)-γ and interleukin (IL-10) and serum total and MAP-specific immunoglobulins (Ig) concentrations on day 180. Panel A. MAP-specific IFN-γ concentrations. Panel B. MAP-specific IL-10 concentrations. Panel C. Total IgG1 concentrations. Panel D. MAP-specific IgG1 concentrations. Panel E. Total IgG2a concentrations. Panel F. MAP-specific IgG2a concentrations. Data are mean ± SEM. (*) indicates a significant difference at $P \leq 0.05$ and (\#) indicates a tendency for a significant difference at $0.05 < P \leq 0.10$. 
whereas the effect of HNP51 and VNP51 on total IgG1 and IgG2a was identical ($P = 0.21$), feeding the VNP51 decreased ($P = 0.0001$) total IgG2a lower than that of the HNP51-fed mice (Figures 8C and E).

Examination of the MAP-specific immunoglobulins revealed a pattern that coincides with the total IgG1 and IgG2a (Figures 8C, D, E and F). Feeding the HNP51 to HMAP- or VMAP-infected mice decreased dramatically ($P \leq 0.02$) their MAP-specific IgG1 and IgG2a (Figures 8D and F). Curiously enough, feeding the VNP51 to HMAP-infected animal raised significantly their MAP-specific IgG1 and IgG2a higher ($P \leq 0.002$) than that of the HMAP- and VMAP-infected controls (Figures 8D and F).

**DISCUSSION**

This paper describes novel findings outlining the efficacy of the probiotics NP51 to curtail progression of JD in a murine model. The most important finding of this work is the discovery that NP51 prevents progression of MAP infection through upregulation of the CD8α+ immune cell-mediated immunity. These findings provide substantial evidence that future MAP vaccines should be designed to mediate CD8α+ immune cell-mediated immunity, rather than Th1-mediated adaptive immunity.

Overall, feeding the VNP51 to HMAP-infected mice elicited a sustained secretion of MAP-specific IFN-γ and its counter-regulatory MAP-specific IL-10 (Figures 2A and B, 4A and B, 6A and B, and 8A and B). It also increased total and MAP specific IgG1, but mostly, decreased total and MAP specific IgG2a suggesting attenuation of the inflammatory response (Figures 2C, 2D, 2E, and 2F, 4C, 4D, 4E, and 4F, 6C, 6D, 6E, and 6F, 8C, 8D, 8E, and 8F).
Nerstedt and colleagues (2007) has shown that feeding *Lactobacillus acidophilus* to mice upregulates genes involved in host defense against pathogens infection as well as genes that control energy homeostasis.

Feeding probiotics to mice orchestrates DC maturation and activation of NK cells (Fernandez et al., 1999; Guiton et al., 2009; Gad et al., 2011; Evrard et al., 2011). Activation of DC during intracellular pathogens, such as *Toxoplasma gondii* and *Mycobacterium tuberculosis* infections augmented CD8⁺ T cell-mediated immunity (Guiton et al., 2009; ) in mice. Report by Rauelt and colleagues (2003) clearly showed that NKG2D immunoreceptor mediate DC, NK cells, and CD8⁺ cytotoxic T cells mutual activation.

Also, infection with MAP decreases abrogate CD4⁺ Th1 responses during the incubation period that extend for 2-5 years (Noss et al. 2001; Pai et al., 2004; Weiss and Souza, 2008). Intriguingly, in CD4⁺ T helper cell deficient mice, NK cells activated CD8⁺- cytotoxic T cell-mediated immunity against intracellular parasites (Combe et al., 2005).

In this study, no change, however, was detected in CD4⁺ T helper cells population (Figure 1A, 3A, 5A, and 7A) suggesting that the source of the IFN-γ is not CD4⁺-mediated adaptive immunity. Following infection with intracellular pathogen, CD8⁺ T cells were the primary source of IFN-γ secretion (Bou-Bahnem et al., 2009). IFN-γ augments the ability of macrophages to express MHC class I and crosspresent exogenous antigen to CD8⁺ cytotoxic T cells (Trost et al., 2009). In contrast, signaling of the 19-kDa lipoprotein on the cell wall of MAP via TLR-2 on macrophage inhibits IFN-γ-induced upregulation of macrophage genes involved in the immune response against intracellular pathogens (Pai et al., 2004).

Of significant interest, is the finding that the CD8α⁺ immune cells (cytotoxic T cells, DC, NK cells, and NK T cells) have increased substantially (Figures 1C, 3C, 5C, and 7C, and
8C), but not the CD4\(^+\) T helper cells. Works by Perez-Cano and colleagues (2010) showed that *Lactobacillus salivarius* CECT5713 and *Lactobacillus fermentum* CECT5716 stimulation of PMNs did not seem to activate CD4\(^+\) T helper cells (Perez-Cano et al., 2010). In contrast, *L. salivarius* CECT5713 and *L. fermentum* CECT5716 induced the activation of the CD8\(^+\) immune cells.

Our data indicate that VNP51- and HNP51-orchestrated CD8\(\alpha\)\(^+\) immune response is characterized by a Th1-like immunity. The data also point significant evidence that probiotic VNP51 and HNP51 do not mediate proliferation of CD4\(^+\) Th2 helper cell repertoire instead VNP51 and HNP51 modulate the activity of immunoglobulin-secreting plasma cells (Figures 2C, 2D, 2E, and 2F, and 4C, 4D, 4E, and 4F, and 6C, 6D, 6E, and 6F, and 8C, 8D, 8E, and 8F).

CD8\(\alpha\)\(^+\)CD25\(^+\) T regulatory cell population decreased by HNP51 or VNP51 feeding (Figures 1D, 3D, 5D, and 7D) to VMAP- or HMAP-infected mice or VMAP-infected controls. Similar to CD4\(^+\)CD25\(^+\) T reg cells, CD8\(\alpha\)\(^+\)CD25\(^+\) T reg cells inhibit proliferation of CD25\(^+\) T cells via cell-to-cell contact that requires expression of CTLA-4 and TGF-\(\beta\) (Cosmi et al., 2003). Induction of CD8\(^+\)CD25\(^+\) T reg cells during infection with pathogenic simian immunodeficiency virus (SIV) in cynomolgus macaques correlated negatively with SIV antigen-specific T cell proliferation but correlated positively with increased virus burden in cynomolgus macaques (Karlson et al., 2007) and in human (Nigam et al., 2010).

Curiously enough, when HNP51 or VNP51 was fed to VMAP-infected mice on day 90, not only secretion of MAP-specific IFN-\(\gamma\) was not stimulated, but also total and MAP specific IgG\(_1\) and IgG\(_{2a}\) production was inhibited, suggesting that probiotic NP51, whether
heat-killed or viable, mediate its anti-VMAP immune responses through a pathway that does not involve Th1 and Th2 responses in murine model of JD.

It was established that up to 29 days postinfection with intracellular pathogens, the primary sources of IFN-\(\gamma\) in mice is chiefly the CD8\(^+\) immune cells, cytotoxic and NK T cells (Thale and Kiderlen, 2005; Bou Gahnem et al., 2009), not MHC-II-expressing CD4\(^+\) T helper cells. Thus, it is rational to suggest that the simultaneous increase of the anti-inflammatory IL-10 is the MAP-infected macrophages, not CD4\(^+\) Th-2 T cells whose frequency did not change under mitogen or MAP antigen stimulation (Figures 1A, 3A, 5A, and 7A). Also, infection with MAP is known to increase the production of IL-10 primarily from the macrophage lineage (Fiorentino et al., 1991).

The immunoglobulin profile indicates that HNP51 and VNP51 differentially modulate the immune system depending on viability of MAP. We observed that HNP51 did not increase MAP-specific IgG\(_1\) and decreased MAP-specific IgG\(_2a\) in HMAP-infected mice. On the contrary, HNP51 sustained a robust IgG\(_1\) and IgG\(_2a\) decrease in VMAP-infected mice (Figures 1C and E, 2C and E, 3C and E, 4C and E).

By day 135, the anti-VMAP immune response induced by either the HNP51 or VNP51 became distinct than that triggered against HMAP. Remarkably, HNP51 or VNP51 administration to VMAP-infected mice did not elevate IFN-\(\gamma\) concentration but decreased VMAP-specific IL-10 production in their \textit{in vitro} splenocyte cultures when stimulated with the sonicated VMAP antigen on day 135 (Figures 6A and B). The decrease of IL-10 production is associated with decreased infectivity and dissemination of MAP (Denis and Ghadirian, 1993).
On the contrary, stimulation of the in vitro splenocyte culture of HMAP-infected mice fed the VNP51 increased IL-10 production. It can be speculated that the immunologic goal of the increased IL-10 in the splenocytes culture of HMAP-infected mice is to alleviate the unnecessary IFN-γ produced against an already dead pathogen and to counter-regulate the tissue destructive effects of IFN-γ (Figure 6A and B).

To verify whether this immune response was associated with decreased infectivity of MAP, we cultured different tissues from VMAP-infected mice fed either the HNP51 or the VNP51 on Herrold’s egg yolk agar medium. Of significance, administration of the HNP51 or VNP51 remarkably decreased the VMAP burden in livers, MLNs, and spleens of mice infected with the VMAP on days 135 (Osman, et al. unpublished data).

The decreased VMAP burden in tissues on day 135, when the IFN-γ concentration was not elevated and IL-10 concentration was decreased, provided evidence that killing of VMAP has been accomplished by the HNP51- and VNP51-induced CD8α+ immune machineries (cytotoxic T cells and NK cells) that bypasses the need for high IFN-γ induction.

Abrogation of CD8+ T cells cytotoxicity and production of IFN-γ, not CD4+ T helper cell function, increased mortality of Mycobacterium tuberculosis-infected mice deficient in IL-15 (Rausch et al., 2005). Impaired CD8+ T cell cytotoxicity and increased mortality because of M. tuberculosis infection were associated with down-regulation of NKG2D on CD8+ cytotoxic T cells (Rausch et al., 2005) suggesting a role for NK and DC in induction of CD8+ T cell-mediated immunity triggered against M. tuberculosis.

Depletion of endogenous IL-10 in Balb/c mice infected with the virulent Mycobacterium avium subspecies avium (MAA) diminished the bacterial growth in their spleens. Also, addition of anti-IL-10 antibodies to the supernatants of MAA-infected macrophages
remarkably enhanced their bactericidal activity (Denis and Ghadirian, 1993). Thus, it is possible that down regulation of IL-10 secretion to decrease pathogenicity of VMAP seems to be one immune mechanism that features NP51 immunologic action.

By day 180, histopathological examination of VMAP-infected mice demonstrated presence of paucibacillary granulomas suggesting significant killing of MAP. Also, the decreased spleen cellularity indicated a subsided immune response against VMAP infection (Osman et al., manuscript under review). The paucibacillary phenotype of granuloma observed was associated with an active production of VMAP-specific IFN-γ in the in vitro splenocyte cultures from VMAP-infected mice fed either the HNP51 or VNP51 (Figure 8A).

It seems more prudent for the proinflammatory IFN-γ to increase at this time in the VMAP-infected mice fed the VNP51. We are lured to speculate that an increase of IFN-γ at this stage is unlikely to exacerbate the already subsiding ileitis, especially when VMAP-specific IL-10 is elevated (Figure 8A and B). We validated the histopathological evidence of MAP remission on day 180 by culturing tissues of VMAP-infected mice fed or not the HNP51 or VNP51 on Herrold’s egg yolk agar medium supplemented with Mycobactin J. Of interest, MAP burden was remarkably attenuated in livers, MLNs, and ceca of VMAP-infected mice fed the HNP51 or the VNP51 (Osman et al., manuscript under review).

The most important finding of this work is the discovery that the VNP51- and HNP51-induced immune response targeting MAP is accomplished through the CD8α+ immune cell-mediated immunity.

Our data suggest that VNP51 and HNP51 caused DC maturation and activation of NK cells leading to induction of CD8α+ immune cell-mediated immunity. These findings lend support to Guiton et al (2009) that CD8+ cytotoxic T cells are the dominant effector cells
following DC activation and infection with intracellular pathogen. Also our findings are in agreement with observation of Rault and colleagues that NKG2D immunoreceptor orchestrate the interaction of DC, NK, and CD8\(^+\) T cell to activate CD8\(^+\) immune cell-mediated immunity against intracellular pathogens.

**CONCLUSIONS**

The data suggest that probiotic VNP51 and HNP51 induce CD8\(\alpha^+\) immune cells upregulation that hinders MAP growth *in vivo*. The important finding that feeding the VNP51 to HMAP-infected mice increases significantly their CD8\(\alpha^+\) immune cell repertoire and serum MAP-specific IgG\(_1\) and IgG\(_2a\) higher than that of the HMAP- and VMAP-infected control mice exquisitely reveal and persuasively advocate the efficacy of VNP51 as a potent adjuvant to second generation MAP vaccines. The data also provide considerable evidence that the second generation MAP vaccines should be designed to induce CD8\(^+\) T cell mediated, not Th1, immunity.

**ACKNOWLEDGMENT**

The authors cordially thank the Nutrition Physiology Company, LLC, (Guymon, OK) for donation of the NP51 and for generously financing the study. Our thank is extended to Dr. Jane Leedle (JL Microbiology, Inc, Hartland, WI) for the logistic assistance with conducting the study

**REFERENCES**


CHAPTER 6. TRANSFORMATION OF LACTATING DAIRY COW DIGESTIVE SYSTEM MICROBIOME BY FEEDING BOVAMINE®: EFFECTS ON PRODUCTIVITY AND IMMUNE RESPONSES

A paper to be submitted to the Journal of Dairy Science

INTERPRETIVE SUMMARY: Modification of immune responses and digestive system microbiota of lactating dairy cows by feeding Bovamine®. By Osman et al., page. Ruminal and digestive system microbiota is known to influence animal productivity. A novel direct-fed microbial that positively modifies the digestive system microbiota to enhance the production efficiency of dairy animals was evaluated. Herein, we show that Bovamine® modifies the ruminal and digestive system microbiota to increase concentrations of ruminal volatile fatty acids that may lead to enhanced production efficiency.

RUNNING TITLE: BOVAMINE®, MICROBIOME, AND PRODUCTIVITY

Transformation of lactating dairy cow digestive system microbiome by feeding Bovamine®: Effects on productivity and immune responses.

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Mandatory disclaimer: Brand names are necessary to report accurately on available data. The USDA-NADC, however, neither guarantees nor warrants the quality of the biological product used in this study. The use of the name by the USDA implies no approval of the product, or barring of other alternatives.”

ABSTRACT

We evaluated the effect of Bovamine®, Lactobacillus acidophilus strain NP51 and Probionibacterium freudenreichii strain NP24, feeding on the bacterial population composition of the digestive system microbiota of Jersey and Holstein dairy cattle during late lactation (average DIM = 202.44 days on time 0). To unveil the underlying mechanisms, we examined the Bovamine® immune modulative effects as well as effects on productivity. Dairy cows were randomized to treatment groups that were either fed the Bovamine® (1 ×10⁹ CFU of Lactobacillus acidophilus strain NP51 plus 2 × 10⁹ CFU of Probionibacterium freudenreichii· cow⁻¹·day⁻¹) or the lactose carrier mixed with the total mixed ration (TMR) for six weeks. Feeding Bovamine® favorably modified the digestive system microbial ecology as seen by the bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP)
compared with that of the control cows fed the lactose carrier only. Fecal *Firmicutes : Bacteroides* was decreased suggesting decreased shedding of potentially pathogenic bacteria. On the contrary, ruminal *Firmicutes : Bacteroides* was increased, which is indicative of increased energy harvest and improved ruminal digestibility. As a result, concentrations of the total volatile fatty acids were increased significantly in the rumen. These results suggest that feeding Bovamine® to lactating dairy cows during late lactation favorably alters their digestive system microbiota and improves the nutrient digestibility and energy availability with significant implication to transition and early lactation dairy cows.

**INTRODUCTION**

The digestive system of dairy cattle is inhabited by a multitude of microorganisms that profoundly influence the whole dairy cow physiology, metabolism, and productivity (Ozutsumi et al., 2005; Dowd et al., 2008; McGarvey et al., 2010). It is well established that dairy cattle serve as primary reservoir for *E. coli* O157:H7 (Chapman et al., 2001) and *Salmonella* (Steinmuller et al., 2006). Thus, transformation of dairy cattle digestive system microbiota to decrease prevalence of pathogenic bacterial species in their fecal matter is central to biosecurity, food safety, and production efficiency. Microbiota of the bovine digestive system can be transformed in a noteworthy manner, not only through the alterations of the diet chemical composition (Callaway, 2010), but also through feeding probiotics (Vamanu et al., 2008).

Bovamine® is a patented formulation of *Lactobacillus acidophilus* strain NP51, lactic acid-producing bacteria and *Probionibacterium freudenreichii* NP24, lactic acid-utilizing bacteria. These microbes are mixed with lactose as a carrier to make the commercially available Bovamine®.
It has been shown that feeding Bovamine® to beef cattle appreciably decreases *E. coli* O157:H7 shedding in feces (Younts-Dahl et al., 2006; Stephens et al., 2007). Also, *E. coli* O157:H7 population was decreased significantly on the hide of Bovamine®-fed cattle. In addition, feeding Bovamine® decreased shedding of *Salmonella* in feces of beef steers (Stephens et al., 2007).

Feeding *Lactobacillus acidophilus* strain NP51 alone decreased shedding of *E. coli* O157:H7. Also, feeding *Lactobacillus acidophilus* strain NP51 to beef steers decreased the average thickness of the lamina properia (Elam et al., 2003) with excellent implication to inflammation reduction and digestive system diseases. Additionally, feeding the viable or heat-killed NP51 to murine model of Johne’s disease decreased shedding of *Mycobacterium avium* subspecies *paratuberculosis*, the causitive agent of Johne’s disease, in their fecal pellets (Osman et al., unpublished data). Also, supplemental Bovamine® feed to beef steers on steam-flacked corn diet increased their gain:feed by about 2% (Vasoconcelos et al., 2008).

The biological mechanisms that underly the effects of Bovamine® on the productivity and immunity of dairy cow are understood poorly and are yet to be unravelled. We hypothesize that feeding Bovamine® to dairy cattle differentially alter the relative abundance of the bacterial composition of their digestive system microbiota to inhibit growth of pathogenic bacteria, improve nutrient digestibility and energy homeostasis, and favorably modify the immune system responses. To test this hypothesis, we fed Bovamine® to late lactation dairy cattle and evaluated ruminal microbial population fermentation and immune responses.
MATERIALS AND METHODS

Experimental Design

Dairy cows (24 Holsteins and 24 Jerseys) in late lactation (average DIM = 202.4 days) were assigned randomly to one of two treatment groups: Bovamine®-fed (n = 24) or control (n = 24). Each treatment group contained 12 Holstein and 12 Jersey dairy cows housed separately in a pen at the Iowa State University Research Dairy Farm in Ames, IA. Cows were adapted to the pen and total mixed ration (TMR; Table 1) diet for two weeks prior to the administration of the treatment.

The Bovamine® group was fed $1.04 \times 10^9$ CFU of *Lactobacillus acidophilus* strain NP51 plus $2.04 \times 10^9$ CFU of *Probionibacterium freudenreichii* mixed with lactose (Bovamine® commercial dairy formula; Nutrition Physiology Company, LLC, Guymon, OK) at a rate of 1.04 g·cow$^{-1}$·day$^{-1}$ for six wk. Bovamine® was mixed with 471.7g per cow daily of ground corn and top-dressed onto the TMR that fed *ad libitum*. The control group was fed the lactose carrier (1.04 g per cow) mixed with 471.1 g of ground corn per cow daily for six wk. Cows were fed once a day at 9:00 h and milked twice a day at 18:00 h. All the experimental procedures were done in accordance with the guidelines of the Iowa State University Institutional Animal Care and Use Committee (IACUC).

Sampling and Analysis.

Ruminal and Fecal Samples. At the end of the two-wk adaptation period (time 0) and at the end of wk 3, and wk 6 of the experiment, ruminal liquor (100 mL) was collected by using a stomach tube. Simultaneously, about 15 g of feces were collected directly from the rectum of each cow. Both ruminal and fecal samples were used for examining the relative abundance of major bacterial phyla and taxa within the ruminal and fecal microbiota. We used the 16S
rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP; Roche 454 Pyresequencer; Dowd et al., 2008) to perform the diversity analyses.

Also, separate aliquots of the ruminal liquor and fecal samples were used to determine the pH by using the pH meter Accumet AR15 (Fisher Scientific, Pittsburg, PA) and the concentration of different VFAs by using the Varian 3900 GC Gas Chromatograph (Varian Analytical Instruments, Walnut Creek, CA).

**Blood Samples.** Sixty milliliters of blood were collected from 16 cows (eight Holsteins and eight Jerseys) in each treatment group by jugular venipuncture on time 0, wk 3, and wk 6. Blood samples were used to isolate the peripheral blood mononuclear cells (PMNs; Karcher et al., 2008). Briefly, 60 mL of blood were mixed with 6 mL of 2 × acid-citrate-dextrose anticoagulant (ACD, 1:10) and diluted with sterile phosphate-buffered saline (PBS; 0.14 M NaCl, 0.003 M KCl, 0.01 M Na₂HPO₄, and 0.0018 M KH₂PO₄; pH 7.4). After centrifugation at 4000 × g for 30 min, the PMN layer was collected and overlaid onto 5 mL of Ficoll-Hypaque (Sigma-Aldrich, Saint Louis, MO).

After centrifugation at 4000 × g for 30 min, PMNs were harvested into a 50 mL sterile graduated tube (Corning Inc, Corning, NY) and RBCs were lysed hypotonically. The PMNs were collected by centrifugation at 3000 × g for 10 min. PMNs then were washed twice using sterile PBS and counted by using the Z1 Coulter Counter Analyzer (Beckman Coulter, Brea, CA).

Subsequently, cells were diluted to 2 × 10⁶ cells/mL and cultured in vitro in RPMI-1640- GlutMax (Invitrogen, Carlsbad, CA) plus 10% heat-inactivated fetal calf serum (Fisher Scientific, Hanover Park, IL) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA).
In vitro cell cultures were either nonstimulated or stimulated with concanavalin A (Sigma-Aldrich) at 10 µg/mL. The cell cultures were incubated at 37°C and 5% CO₂: 95% O₂ for 72 h. Following incubation, the pelleted cells were washed twice with cold sterile PBS and stained for surface markers with primary antibodies CD4 IL-2II, IgG₂a (ABD Serotec), CD8 BÀQIII, IgM (ABD Serotec), γδ TCR, 6B21A (ABD Serotec), CD25 LCTBA, IgG₂b (ABD Serotec), and CD335 ACSI Ig₂b (ABD Serotec, Raleigh, NC).

The cells in wells were incubated for 20 min at 4°C in the dark followed by brief centrifugation and decanting of the supernatant. Pelleted cells were suspended in a secondary antibodies cocktail (Alexa 350 IgM, FITC IgG₂a, PE IgG₁, Cy5 IgG₂b, and APC Cy7 IgG₃, ABD Serotec, Raleigh, NC) at 100 µL per well.

Subsequently, frequencies of cells were determined by using the fluorescence-activated cell sorter (BD FACSaria II; Becton Dickinson Biosciences, Franklin Lakes, NJ). Cell frequency data were analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR).

**Statistical Analysis**

For volatile fatty acids production and flow cytometric statistical analyses, we used the PROC MIXED of SAS version 9.2 with covariance structure variance component and containment method for determining the degrees of freedom. The Class Statement specified breed, treatment, cow ID, and time. Also, the Model Statement specified the fixed effect of interest equals breed, treatment, time, and all their possible interactions. Cow nested in treatment by breed interaction was considered a random effect. The least squared means of the response variable of interest at different time points for both the Jersey and Holstein breeds were computed using the LSMEANS statement in PROC MIXED. Subsequently, pairwise differences among least squared means were computed by using the PDIFF option.
and adjusted by using the Tukey method. Probability (P-) values ≤ 0.05 indicate significant difference, and P values between 0.05 and 0.10 indicate tendency for the effect to be different or significant.

For the microbiome and metagenomics data analyses, treatment effects on the relative abundances of all phyla were evaluated by using a mixed-effects model. Relative abundances were transformed prior to analysis, and analyses were conducted separately for each of the sampling sites (rumen vs. fecal) and breeds (Jersey vs. Holstein). In addition, the ratio of Firmicutes to Bacteroidetes (log transformed) was determined. Treatment and week were considered fixed effects. Repeated measurements from the same individual was considered random effects. Given the study design (measurements at baseline time 0 and at wk 6), the designation of a treatment effect is based on a significant (i.e., P < 0.10) treatment by week interaction. Overall error rates were controlled using a false discovery rate of 5%. Uncontrolled P values were used to screen taxa, and α = 0.10 was used as a reference.

RESULTS

Effect on ruminal total volatile fatty acid concentrations. At time 0 of the study, total volatile fatty acids (VAFs) concentrations did not differ (P = 0.50) between treatment groups of Holstein and Jersey dairy cows (Table 1). On wk 3, feeding Bovamine® to dairy cows substantially increased (P = 0.0001; Table 1) total VFAs by 34% compared with those of the control dairy cows. Feeding Bovamine® to dairy cows increased their total VFAs by 68.4 and 14.5% on wk 3 and 6, respectively, compared with those of time 0 baseline (Table 1).

At the end of wk 6, most dairy cows approached the end of the 305-d lactation period. Considering each breed separately, feeding the Bovamine® at the end of the lactation period to Holstein dairy cows did not appreciably change (P = 0.93; Table 1) total VFAs
concentrations. On the contrary, Bovamine® fed to Jersey dairy cows increased substantially \((P = 0.01)\) total VFAs in comparison with those of the Jersey control cows fed the lactose carrier only (Table 1). Regardless of the breed, feeding Bovamine® to the dairy cows tended to elevate \((P = 0.06)\) total VFAs by 30% greater than that of the control dairy cows on wk 6 of the study (Tabel 1).

**Table 1. Effects of Bovamine® feeding to dairy cattle on ruminal total VFAs production at time 0, wk 3, and wk 6 relative to Bovamine® feeding. Data are mean ± SEM. (SEM = 6.03-8.44).**

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Holsteins</th>
<th>Breeds</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC</td>
<td>JC</td>
<td>HB</td>
<td>JB</td>
</tr>
<tr>
<td>Time 0</td>
<td>69.6±7.3</td>
<td>69.8±6.6</td>
<td>68.5±8.4</td>
<td>61.05±6.6</td>
</tr>
<tr>
<td>Wk 3</td>
<td>81.0±6.03</td>
<td>82.5±6.6</td>
<td>114.1±6.3</td>
<td>104.2±6</td>
</tr>
<tr>
<td>Wk 6</td>
<td>60.9±6.3</td>
<td>51.1±6.6</td>
<td>74.3±6.3</td>
<td>74.1±6</td>
</tr>
</tbody>
</table>

VFAs, volatile fatty acids; HC, Holstein control cows; JC, Jersey control cows; HB, Holstein dairy cows fed Bovamine®; JB, Jersey dairy cows fed Bovamine®.

**Acetate : propionate in the rumen.** At time 0 of the study, acetate : propionate was significantly \((P = 0.0003)\) in the Bovamine®-fed Holstein cows as compared with that of the control Holstein cows. As a result, the acetate:propionate in the Bovamine®-fed dairy cattle became significantly \((P = 0.04)\) higher than that of the control (Table 2). On the other hand, the acetate : propionate ratio did not significantly differ \((P = 0.80)\) between the control and Bovamine®-fed Jersey dairy cows at time 0 baseline (Table 2). Bovamine® feeding, however, did not significantly \((P \geq 0.068)\) alter the acetate : propionate in rumens of Holstein and Jersey dairy cows on wk 3 or wk 6 (Tabel 2).
Table 2. Effects of Bovamine® feeding to dairy cattle on ruminal acetate : propionate on time 0, wk 3, and wk 6 relative to Bovamine® feeding. Data are mean ± SEM. (SEM = 0.67-0.73).

<table>
<thead>
<tr>
<th>Time</th>
<th>Breed</th>
<th>Valu</th>
<th>Bovamine®-fed</th>
<th>Breed</th>
<th>Overall</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Statistic difference (P value)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetate:propionate, mM</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>Control</td>
<td>JC</td>
<td>HB</td>
<td>JB</td>
</tr>
<tr>
<td>Time 0</td>
<td>HC</td>
<td>9.4±0.73</td>
<td>2.84±0.73</td>
<td>13.9±0.94</td>
<td>3.10±0.73</td>
</tr>
<tr>
<td>Wk 3</td>
<td>JC</td>
<td>2.14±0.67</td>
<td>3.08±0.73</td>
<td>2.94±0.8</td>
<td>3.1±0.67</td>
</tr>
<tr>
<td>Wk 6</td>
<td>HB</td>
<td>2.45±0.70</td>
<td>2.83±0.73</td>
<td>2.51±0.70</td>
<td>3.08±0.67</td>
</tr>
</tbody>
</table>

HC, Holstein control cows; JC, Jersey control cows; HB, Holstein dairy cows fed Bovamine®; JB, Jersey dairy cows fed Bovamine®.

Effect on T lymphocyte subpopulations.

CD25+ T cells. At time 0, CD25+ T cells frequency in peripheral blood of dairy cows did not differ significantly (P = 0.61; Table 3) between the Bovamine®-fed and control groups.

Stimulation of the in vitro T cell cultures with ConA expanded significantly (P = 0.001) the CD25+ T cells population relative to the nonstimulated cell cultures in both the control and Bovamine®-fed cows.

Table 3. Effects of Bovamine® feeding to dairy cattle on proliferation of CD25+ T cell repertoire in PMNs at time 0, wk 3, and wk 6 relative to Bovamine® feeding. Data are mean ± SEM. (SEM = 0.41-0.52).

<table>
<thead>
<tr>
<th>Time</th>
<th>Stim</th>
<th>Control</th>
<th>Bovamine®-fed</th>
<th>Breed</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td>JC</td>
<td>HB</td>
<td>JB</td>
</tr>
<tr>
<td>Time 0</td>
<td>NS</td>
<td>8.8±0.44</td>
<td>10.1±0.39</td>
<td>9.2±0.52</td>
<td>9.4±0.52</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>15.3±0.44</td>
<td>15.8±0.39</td>
<td>15.3±0.52</td>
<td>16±0.52</td>
</tr>
<tr>
<td>Wk 3</td>
<td>NS</td>
<td>9.5±0.41</td>
<td>9.12±0.39</td>
<td>9.6±0.44</td>
<td>9.0±0.41</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>15.1±0.41</td>
<td>13.6±0.39</td>
<td>17.5±0.44</td>
<td>15.9±0.41</td>
</tr>
<tr>
<td>Wk 6</td>
<td>NS</td>
<td>8.9±0.41</td>
<td>10.23±0.41</td>
<td>9.7±0.41</td>
<td>9.0±0.41</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>13.2±0.41</td>
<td>14.53±0.41</td>
<td>19.6±0.4</td>
<td>17.3±0.41</td>
</tr>
</tbody>
</table>

Stim, stimulation with concanvalin (ConA) or nonstimulation (NS); HC, Holstein control cows; JC, Jersey control cows; HB, Holstein dairy cows fed Bovamine®; JB, Jersey dairy cows fed Bovamine®.
By wk 3 and 6, feeding the Bovamine® significantly increased \( (P = 0.001) \) the frequency of CD25\(^+\) T cells when stimulated with ConA relative to that of the control group (Table 3).

**CD4\(^+\) T helper cells.** CD4\(^+\) T helper cell frequency did not differ \( (P = 0.20) \) on time 0 between the Bovamine® feeding and control group (Table 4). Also, Bovamine® feeding did not significantly \( (P \geq 0.26) \) change the frequency of CD4\(^+\) T helper cells population in the peripheral blood of dairy cows on wk 3 and wk 6 (Table 4). Note worthy, Bovamine® fed to Jersey, not Holstein \( (P = 0.68) \), dairy cattle increased \( (P = 0.05) \) CD4\(^+\) T helper cell repertoire under ConA stimulation (Table 4).

<table>
<thead>
<tr>
<th>Time</th>
<th>Stim</th>
<th>CD4(^+) T helper cells repertoire frequency</th>
<th>Statistics ( (P ) value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Bovamine(^{\circledast})-fed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td>JC</td>
</tr>
<tr>
<td>Time 0</td>
<td>NS</td>
<td>50.1±1.8</td>
<td>51±1.6</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>69.1±1.8</td>
<td>70.1±1.6</td>
</tr>
<tr>
<td>wk 3</td>
<td>NS</td>
<td>46.9±1.7</td>
<td>50.7±1.6</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>72±1.7</td>
<td>70.5±1.6</td>
</tr>
<tr>
<td>wk 6</td>
<td>NS</td>
<td>49.1±1.7</td>
<td>48.2±1.7</td>
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<tr>
<td></td>
<td>ConA</td>
<td>78.8±1.7</td>
<td>69.3±1.7</td>
</tr>
</tbody>
</table>

*Stim*, stimulation with concanavalin (ConA) or no stimulation (NS); **HC**, Holstein control cows; **JC**, Jersey control cows; **HB**, Holstein dairy cows fed Bovamine\(^{\circledast}\); **JB**, Jersey dairy cows fed Bovamine\(^{\circledast}\).

**CD4\(^+\)CD25\(^+\) T reg cells.** T reg cell frequency was not significantly different \( (P \geq 0.98) \) between the Bovamine\(^{\circledast}\) fed and the control groups when non-stimulated or stimulated with ConA on time 0 (Table 5). Bovamine\(^{\circledast}\) feeding, however, increased \( (P \leq 0.02) \) the frequency of CD4\(^+\)CD25\(^+\)T reg cells by wk 3 and 6 (Table 5) under ConA stimulation relative to that of the control cows.
Table 5. Effects of Bovamine® feeding to dairy cattle on CD4+CD25+ T reg cell repertoire in PMNs at time 0, wk 3, and wk 6 relative to Bovamine® feeding. Data are mean ± SEM. (SEM =0.94-1.20).

<table>
<thead>
<tr>
<th>Time</th>
<th>Stim</th>
<th>CD4+CD25+ T reg cells repertoire frequency</th>
<th>Statistics (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (HC)</td>
<td>JC</td>
</tr>
<tr>
<td>Time 0</td>
<td>NS</td>
<td>10.4±1.0</td>
<td>12.0±0.89</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>17.6±1.0</td>
<td>17.8±0.89</td>
</tr>
<tr>
<td>wk 3</td>
<td>NS</td>
<td>10.0±0.94</td>
<td>10.2±0.89</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>13.5±0.94</td>
<td>13.7±0.89</td>
</tr>
<tr>
<td>wk 6</td>
<td>NS</td>
<td>11.5±0.94</td>
<td>13.8±0.94</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>15.5±0.94</td>
<td>14.50±0.91</td>
</tr>
</tbody>
</table>

Stim, stimulation with concanvalin (ConA) or no stimulation (NS); HC, Holstein control cows; JC, Jersey control cows; HB, Holstein dairy cows fed Bovamine®; JB, Jersey dairy cows fed Bovamine®.

**CD8+ cytotoxic T cells.** At time 0, when nonstimulated *in vitro*, frequency of CD8+ cytotoxic T cells repertoire did not significantly (P = 0.97) change between the control and Bovamine®-fed group (Table 6). When stimulated with ConA, CD8+ cytotoxic T cells expanded equally (P = 0.60) in the Bovamine®-fed and control groups, but significantly higher (P = 0.0001) than the expansion of the nonstimulated cultures from the two control groups (Table 6).

By wk 3 and 6, on the other hand, Bovamine® feeding *per se* substantially increased (P ≤ 0.09) the CD8+ cytotoxic T cells population in the nonstimulated *in vitro* cell cultures to be greater than that of the control cows (Table 6). Expansion of CD8+ cytotoxic T cells of Bovamine®-fed cows under ConA stimulation was significantly higher (P ≤ 0.002) than that of cell culture from control cows (Table 6).
Table 6. Effects of Bovamine® feeding to dairy cattle on CD8+ cytotoxic T cell repertoire in PMNs on time 0, wk 3, and wk 6 relative to Bovamine® feeding. Data are mean ± SEM. (SEM = 0.75–1.01).

<table>
<thead>
<tr>
<th>Time</th>
<th>Stim</th>
<th>Control</th>
<th>Bovamine®-fed</th>
<th>Breed</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td>JC</td>
<td>HB</td>
<td>JB</td>
</tr>
<tr>
<td>Time 0</td>
<td>NS</td>
<td>22.9±0.9</td>
<td>21.5±0.9</td>
<td>22.0±1.0</td>
<td>22.4±1.0</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>29.4±0.8</td>
<td>28.3±0.8</td>
<td>29.5±1.0</td>
<td>29.7±1.0</td>
</tr>
<tr>
<td>wk 3</td>
<td>NS</td>
<td>24.4±0.8</td>
<td>23.6±0.8</td>
<td>28.7±0.9</td>
<td>29.2±0.8</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>29.1±0.8</td>
<td>29.1±0.8</td>
<td>35.3±0.9</td>
<td>35.7±0.8</td>
</tr>
<tr>
<td>wk 6</td>
<td>NS</td>
<td>24.6±0.8</td>
<td>24.9±0.8</td>
<td>28.8±0.8</td>
<td>26.8±0.8</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>32.4±0.8</td>
<td>34.7±0.8</td>
<td>39.5±0.8</td>
<td>38.2±0.8</td>
</tr>
</tbody>
</table>

*Stim*, stimulation with concanvalin (ConA) or nostimulation (NS); *HC*, Holstein control cows; *JC*, Jersey control cows; *HB*, Holstein dairy cows fed Bovamine®; *JB*, Jersey dairy cows fed Bovamine®.

*CD8+CD25+ T reg cells*. Following a pattern disimilar to that of the CD8+ cytotoxic T cells, CD8+CD25+ T reg cells did not differ (*P* ≥ 0.47) at time 0 and wk 3 and 6 between control and the Bovamine®-fed groups in the nonstimulated cultures (Table 7). Also, by time 0 and wk 3, Bovamine® feeding and stimulation with ConA did not significantly vary CD8+CD25+ T reg cells differently from that of the control (Table 7).

Table 7. Effects of Bovamine® feeding to dairy cattle on CD8+CD25+ T reg cell repertoire in PMNs on time 0, wk 3, and wk 6 relative to Bovamine® feeding. Data are mean ± SEM. (SEM = 1.10-1.39).

<table>
<thead>
<tr>
<th>Time</th>
<th>Stim</th>
<th>Control</th>
<th>Bovamine®-fed</th>
<th>Breed</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td>JC</td>
<td>HB</td>
<td>JB</td>
</tr>
<tr>
<td>Time 0</td>
<td>NS</td>
<td>13.8±1.2</td>
<td>14.3±1.0</td>
<td>14.7±1.4</td>
<td>14.3±1.4</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>23.9±1.2</td>
<td>22.9±1.0</td>
<td>23.2±1.4</td>
<td>23±1.4</td>
</tr>
<tr>
<td>wk 3</td>
<td>NS</td>
<td>11.8±1.1</td>
<td>12.6±1.0</td>
<td>11.5±1.2</td>
<td>11.3±1.1</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>19.2±1.1</td>
<td>17.3±1.0</td>
<td>21.3±1.2</td>
<td>17.1±1.1</td>
</tr>
<tr>
<td>wk 6</td>
<td>NS</td>
<td>15.2±1.1</td>
<td>14.7±1.1</td>
<td>15.6±1.1</td>
<td>14.4±1.1</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>23.1±1.1</td>
<td>21.2±1.1</td>
<td>30.7±1.1</td>
<td>25.1±1.1</td>
</tr>
</tbody>
</table>

*Stim*, stimulation with concanvalin (ConA) or nostimulation (NS); *HC*, Holstein control cows; *JC*, Jersey control cows; *HB*, Holstein dairy cows fed Bovamine®; *JB*, Jersey dairy cows fed Bovamine®.
On wk 6, on the other hand, feeding Bovamine® and stimulation with ConA increased CD8\(^+\)CD25\(^+\) T reg cells proliferation to be significantly \((P = 0.001)\) higher than that of the ConA-stimulated control (Table 7).

**CD335\(^+\) NK cells.** At time 0, nonstimulated NK cells expressing CD335\(^+\) of Bovamine®-fed cows did not significantly \((P \geq 0.17)\) differ from that of the control cows on any wk tested (Table 8). Stimulation of CD335\(^+\) NK cells with ConA, however, nondiscriminately \((P = 0.33\) for the difference) and significantly \((P = 0.0001)\) elevated CD335\(^+\) NK cells repertoire in the Bovamine®-fed and control cows to be higher than that of the nonstimulated cultures of both groups on time 0 (Table 8).

By wk 3 and 6, Bovamine® feeding and ConA stimulation appreciably \((P = 0.0001)\) increased expansion of CD335\(^+\) NK cells population to be greater than that of CD335\(^+\) NK from control cow cell cultures (Table 8).

### Table 8. Effects of Bovamine® feeding to dairy cattle on CD335\(^+\) NK cell repertoire in PMNs on time 0, wk 3, and wk 6 relative to Bovamine® feeding. Data are mean ± SEM. (SEM = 0.11-0.13).

<table>
<thead>
<tr>
<th>Time</th>
<th>Stim</th>
<th>Control</th>
<th>Bovamine®-fed Breed</th>
<th>Statistics (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td>JC</td>
<td>HB</td>
</tr>
<tr>
<td>Time 0</td>
<td>NS</td>
<td>1.6±0.11</td>
<td>1.8±0.10</td>
<td>1.8±0.11</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>2.5±0.11</td>
<td>2.4±0.10</td>
<td>2.6±0.13</td>
</tr>
<tr>
<td>wk 3</td>
<td>NS</td>
<td>1.7±0.11</td>
<td>1.6±1.0</td>
<td>1.7±0.11</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>2.5±0.11</td>
<td>2.5±0.10</td>
<td>3.6±1.1</td>
</tr>
<tr>
<td>wk 6</td>
<td>NS</td>
<td>1.7±0.11</td>
<td>2.1±0.11</td>
<td>1.8±0.11</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>2.7±0.11</td>
<td>2.6±0.11</td>
<td>4.1±0.11</td>
</tr>
</tbody>
</table>

*Stim,* stimulation with concanvalin (ConA) or nonstimulation (NS); *HC,* Holstein control dairy cows; *JC,* Jersey control dairy cows; *HB,* Holstein dairy cows fed Bovamine®; *JB,* Jersey dairy cows fed Bovamine.

**γδ T cells.** The repertoire of γδ T cells did not significantly \((P \geq 0.19)\) differ between the nonstimulated *in vitro* cell cultures from Bovamine®-fed and control cows during time 0 and wk 3, and 6 (Table 9). Intriguingly, feeding Bovmaine and stimulation with ConA of γδ T
cells did not significantly \((P \geq 0.52)\) expand their repertoire relative to the nonstimulated cultures (Table 9). Bovamine\textsuperscript{®} feeding, however, decreased \((P = 0.03)\) \(\gamma\delta\) T cells in the nonstimulated splenocyte cultures of Jersey dairy cows, but not \((P = 0.56)\) of Holstein dairy cows (Table 9).

**Table 9. Effects of Bovamine\textsuperscript{®} feeding to dairy cattle on \(\gamma\delta\) T cell repertoire in PMNs on time 0, wk 3, and wk 6 relative to Bovamine\textsuperscript{®} feeding. Data are mean ± SEM.**

\[(SEM = 0.21-0.26)\]

<table>
<thead>
<tr>
<th>Time</th>
<th>Stim</th>
<th>Control</th>
<th>Bovamine\textsuperscript{®}-fed</th>
<th>Breed</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td>JC</td>
<td>HB</td>
<td>JB</td>
</tr>
<tr>
<td>Time 0</td>
<td>NS</td>
<td>2.8±0.22</td>
<td>3.0±0.21</td>
<td>2.6±0.26</td>
<td>2.6±0.24</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>3.1±0.22</td>
<td>2.9±0.21</td>
<td>2.9±0.26</td>
<td>3.0±0.24</td>
</tr>
<tr>
<td>wk 3</td>
<td>NS</td>
<td>3.6±0.21</td>
<td>3.7±0.20</td>
<td>3.7±0.22</td>
<td>3.3±0.21</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>3.5±0.21</td>
<td>3.5±0.2</td>
<td>3.7±0.22</td>
<td>3.6±0.21</td>
</tr>
<tr>
<td>wk 6</td>
<td>NS</td>
<td>3.5±0.21</td>
<td>3.9±0.21</td>
<td>3.4±0.21</td>
<td>3.2±0.21</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>3.8±0.21</td>
<td>3.8±0.21</td>
<td>3.7±0.21</td>
<td>3.6±0.21</td>
</tr>
</tbody>
</table>

**Stim**, stimulation with concanvalin (ConA) or nostimulation (NS); **HC**, Holstein control cows; **JC**, Jersey control cows; **HB**, Holstein dairy cows fed Bovamine\textsuperscript{®}; **JB**, Jersey dairy cows fed Bovamine\textsuperscript{®}.

**Effect on digestive system microbiome.** Feeding Bovamine\textsuperscript{®} to late lactation Jersey and Holstein dairy cattle tended to maintain the relative abundance of *Firmicutes* (Bacilli and Clostridia) high \((P = 0.06\) and \(P = 0.07\), respectively; figure 1) in comparison with the control cows on wk 6 (Figure 1).
Figure 1. Effects of Bovamine® feeding to dairy cattel on the ratio of the relative abundance of *Firmicutes* to *Bacteroidetes* in rumen. Points represent individual observations, and subjects are linked with a line. Average changes are denoted by the black lines.

In the rumens of the control group, relative abundance of *Anaerofilum, Rhodomicrobium, Caldilinea, Desulfobulbus, Leptolyngbya,* and *Erysipelothrix* genera correlated positively
(r ≥ 0.50; P ≤ 0.05) with production of all VFA at wk 6 (Figure 2). In rumens of Bovamine®-fed cows, production of valerate and butyrate correlated positively with Succinovebrio, Bulleidia, Cattonella, Bacteroidaceae, and Verrucomicrobiales genera (r ≥ 0.5; P ≤ 0.05; Figure 2). Acetate production correlated positively with the relative abundance of Verrucomicrobiales, Mitsuokella, Burkholderia, and Roseomonas genera (r ≥ 0.5; P ≤ 0.05; Figure 2). Also, propionate production correlated positively with the relative abundance of the Caldilinea, Escherichia, Peptostreptococcus, Clostridium, Mollicutes, Solirubrobacter, Proteus, Pseudomonas, Dorea, Staphylococcus, Massilia, Selenomonas, Paracoccus, and Roseomonas genera (r ≥ 0.5; P ≤ 0.05; Figure 2). Isobutyrate correlated positively with relative abundance of Anaerovibrio, Anaerofilum, Escherichia, Proteus, Pseudomonas, Staphylococcus, and Roseomonas genera (r ≥ 0.5; P ≤ 0.05; Figure 2). Valerate correlated positively with the relative abundance of Succinivibrio and Verrucomicrobiales genera in rumens of Bovamine®-fed cows (r ≥ 0.5; P ≤ 0.05; Figure 2).
Figure 2: Effects of Bovamine® feeding to dairy cattle on the Spearman Correlation among genera and individual VFAs in rumens of control and Bovamine®-fed Jersey and Holstein dairy cows on wk 6.
In feces of Jersey dairy cows, feeding Bovamine® significantly decreased \( (P \leq 0.04) \) shedding of *Turicibacter* and tended \( (P = 0.09) \) to decrease shedding of *Streptococcus* genera (Figure 3). Additionally, feeding Bovamine® increased shedding of *Pyramidobacter, Thauera, Synergistetes* (not shown) genera (Figure 3). Effect of the treatment by week interaction was significant \( (P \geq 0.04) \) regarding the relative abundance of *Veillonella* and *Microbacterium* genera as they decreased in feces of Bovamine®-fed Jersey relative to that of the control cows (Figure 3).

![Figure 3](image)

**Figure 3.** Effects of Bovamine® feeding to dairy cattle on the relative abundance of bacterial genera in the Jersey fecal samples. Points represent individual observations, and subjects are linked with a line. Changes in average relative abundance per treatment are denoted by the thick lines with a shaded 95% confidence region.

In feces of Holstein dairy cows, Bovamine® feeding significantly \( (P \leq 0.04; \text{Figure 4}) \) decreased the relative abundance of *Lactobacillus* genus relative to that of the control cows on wk 6. The effect of treatment by week interaction was significant on the relative
abundance of the Ruminococcus, Pseudobutyrivibrio, Dorea, Moryella, and Gordonibacter as they decreased \((P \leq 0.05)\) in the feces of Bovamine\(^\circledR\)-fed Holsteins in comparison with that of the control cows (Figure 4). Also, Bovamine\(^\circledR\) feeding increased relative abundance of Paludibacter and maintained relative abundance of Paenibacillus, Comamonas, and Placentomycets (not shown) genera stable whereas they were decreased in the feces of the control Holstein cows on wk 6 of the study (Figure 4).

Figure 4. Effects of Bovamine\(^\circledR\) feeding on the relative abundance of bacterial genera in the fecal matter of Holstein dairy cows. Points represent individual observations, and subjects are linked with a line. Changes in average relative abundance per treatment are denoted by the thick lines with a shaded 95% confidence region.

Feeding Bovamine\(^\circledR\) to Jersey dairy cows tended \((P = 0.08;\) Figure 5) to decrease the relative abundance of Anaerofilum genus in their ruminal liquor and tended \((P = 0.05\) and \(P = 0.10)\) to maintain the relative abundance of Citrobacter and TM7 (not shown) genera relatively stable in comparison with that of the control cows by wk 6. The effect of treatment
by week interaction was significant ($P \leq 0.03$) on the relative abundance of the *Treponema* and *Robinsonella* genera, which were increased in the ruminal liquor of Bovamine®-fed Jersey cows relative to that of the control cows (Figure 5). In contrast, the relative abundance of *Brevibacillus* genus was decreased ($P = 0.03$), whereas that of the *Acidobacter* genus was maintained relatively unchanged ($P = 0.03$) in comparison with those of the control (Figure 5).

**Figure 5.** Effects of Bovamine® feeding on the relative abundance of bacterial genera in the rumen liquor of Jersey dairy cows. Points represent individual observations, and subjects are linked with a line. Changes in average relative abundance per treatment are denoted by the thick lines with a shaded 95% confidence region.

In the rumen of Holstein dairy cows, feeding Bovamine® significantly ($P = 0.001$) increased the relative abundance of *Sharpea* and *Synergistetes* (not shown) genera and tended ($P = 0.09$ and $P = 0.07$, respectively) to increase the relative abundance of the *Pedobacter*
and *Propionibacteria* genera in their ruminal liquor (Figure 6). Also, Bovamine® feeding maintained the relative abundance of *Lactobacillus* genus relatively unchanged whereas abundance of *Lactobacillus* genus was significantly ($P = 0.01$) decreased in the ruminal liquor of the control Holstein cows (Figure 6). Also, Bovamine® feeding tended ($P = 0.06$) to decrease the relative abundance of *TM7* genus in the ruminal liquor of Holstein dairy cows (data not shown).

**Figure 6.** Effects of Bovamine® feeding on the relative abundance of bacterial genera in the rumen liquor of Holstein dairy cows. Points represent individual observations, and subjects are linked with a line. Changes in average relative abundance per treatment are denoted by the thick lines with a shaded 95% confidence region.
DISCUSSION

We verified whether Bovamine® feeding at the prescribed dose is efficacious at advantageously transforming the digestive system microbiome of lactating dairy cattle. We conducted a microbiome-wide metagenomic analyses to trace a Bovamine®-induced massive transformation in the ruminal and fecal microbiome to the genera level (Figures 1-6). The data exquisitely provided convincing evidence that feeding Bovamine® favorably alters the digestive system microbiome of dairy cattle (Figures 1-6). We also verified whether the Bovamine®-provoked transformation of the ruminal microbiome is coupled to increase energy harvest in the rumen (Tables 1 and 2). Chromatographic analyses of the ruminal liquor content of VFA indicated that Bovamine® feeding significantly increased the concentrations of VFA by wk 3 and 6 (Tables 1 and 2). Overall, feeding Bovamine® tended ($P = 0.06$ and $P = 0.07$) to maintain the ratio of Firmicutes to Bacteroides in the rumens of Holstein and Jersey dairy cows to be higher than that of the control cows, suggesting that Bovamine® mediates greater energy capture and feed efficiency (Figure 1).

In obese human subjects, the Firmicutes to Bacteroides ratio is significantly greater than that of lean subjects (Armougom and Raoult, 2008). Of significance, the energy capture by human subjects was increased by 150 kcal when the relative abundance of the genus Firmicutes were increased by 20% and that of Bacteroides genus was decreased by the same percentage in the intestine (Jumpertz et al., 2011). In this study, feeding Bovamine® to dairy cows significantly increased ruminal Firmicutes relative abundance and simultaneously increased the production of VFAs concentration by 68.4 and 14.5% on wk 3 and wk 6, respectively (Figure 1; Table 1). In support of our findings, feeding microbial constituents of
Bovamine® to feedlot cattle fed steam-flaked corn-based diets increased their gain : feed by about 2% (Vasoconcelos et al., 2008).

The acetate : propionate inversely correlates with methanogenesis in rumens of cows (Russell et al., 1998). Because Bovamine® did not alter the acetate : propionate it is likely that Bovamine® doesn’t increase methane emission (Table 2). This finding indicates that feeding Bovamine® to dairy cattle does not adversely impact global warming.

Dominance of particular bacteria species and their metabolism in the rumen contributes to differences in cattle feed efficiency (Hernandez-Sanabria et al., 2010). We verified whether the increase of VFAs concentration is associated with Bovamine®-induced alteration of the microbiome. In rumens of dairy cattle those fed Bovamine®, Spearman rank correlation coefficient for the association of individual genera with individual VFA indicated that individual VFAs correlated with different genera in the ruminal microbiome of the Bovamine®-fed cows (Figure 2). Probiotics feeding causes significant modification of the host gut microbiome leading to significant changes in metabolism of amino acids, VFAs, bile acids, and also accelerates glycolysis (Martin et al., 2008). Metagenomic analyses conducted to correlate the ruminal microbiome composition with feed efficiency phenotype in cattle illustrated that microbiota of feed-efficient steers cluster together differently than that of feed inefficient steers (Guan et al., 2008). Also, feed efficiency correlated positively with increased concentrations of butyrate and valerate in the rumen of feed-efficient cattle (Guan et al., 2008).

In feces of Jersey and Holstein cows, feeding Bovamine® significantly ($P = 0.04$) decreased *Turicibacter* genus shedding. *Turicibacter* has been linked to digestive system inflammation (Bosshard et al., 2002). Also, Bovamine® feeding tended to decrease ($P = 0.10,$
relative abundance of *Citrobacter*, an opportunistic pathogen in humans (Whalen et al., 2007), *Streptococcus*, and *Comamonas* and increased ($P = 0.05$) *Thauera* (Figures 3 and 4). It has been shown that *T. selenatis* decreases selenate to form selenium nanospheres that is secreted into the microenvironment (Charles et al., 2011). In addition, feeding Bovamine® to dairy cows increased ($P = 0.01$) the relative abundance of *Lactobacillus* and tended to increase the relative abundance of *Propionibacterium* in their rumen liquor, which is indicative of the ability of Bovamine® microbial constituents to transiently colonize the rumen (Figures 5 and 6).

The Bovamine®-induced microbiome shift seemed to be accompanied with modification of the immune responses. Feeding Bovamine® to dairy cattle significantly increased expression of CD25+ T cells by wk 3 and wk 6 (Table 2), CD4+ T cells, and CD4+CD25+ T reg cells in PMNs of Jersey dairy cow by wk 6 under ConA stimulation (Table 3). In agreement, stimulation of PMNs with probiotic mixture *in vitro* increased expression of CD4, CD25, CD69, and CD11b (Hua et al., 2010) and increased CD4+CD25+ T reg cell repertoire (Baroja et al., 2007).

The increase of CD8+ T cells by Bovamine® feeding agrees with findings of Hua et al (2010) that probiotic stimulation of PMNs increases expression of CD11b. It is well established that expression of CD11b is upregulated on effector CD8+ T cells (Christensen et al., 2001). It is possible that probiotics mediate differential activation of T cell subpopulations via modifying DC phenotype and function (Hart et al., 2004).

Bovamine®-triggered expansion of CD335+ repertoire of NK cells in peripheral blood of dairy cattle lends support to findings of Gill and coworkers (2001) who fed *Lactobacillus rhamnosus* HN001 or *Bifidobacterium lactis* HN019 to elderly and observed expansion of
CD56\(^+\) NK cells in their peripheral blood and increased tumoricidal activity. Cattle fed Bovamine\(^\text{®}\) demonstrated increased serum IgA and increased granulocytes and monocytes in peripheral blood (Guillen et al., 2009).

We did not detect a Bovamine\(^\text{®}\)-caused expansion of resting nonstimulated \(\gamma\delta\) T cells cells in \textit{in vitro} cultures, nor did ConA stimulation expand their repertoire (Table 8). It is likely that the extreme sensitivity of \(\gamma\delta\) T cells to mitogen-induced apoptosis (Guo et al., 2001) has confounded their stimulation with Bovamine\(^\text{®}\). Unlike activated \(\alpha\beta\) T cells that continue to divide after activation (Nobholz and Macdonald, 1983), activated \(\gamma\delta\) T cells, however, proliferate instantly and then die by apoptosis (Spaner et al., 1993). The short proliferation and rapid death of \(\gamma\delta\) T cells probably did not allow us to detect their proliferation under effect of Bovamine\(^\text{®}\) and ConA stimulation (Table 8).

**CONCLUSIONS**

In conclusion, Bovamine\(^\text{®}\) feeding to dairy cows significantly transformed their digestive system microbiota, and increased the energy capture via increasing the \textit{Firmicutes} to \textit{Bacteroides} ratio. The increase energy capture resulted in increased production of VFAs in the rumen, suggesting the efficacy of Bovamine\(^\text{®}\) to increase feed efficiency in dairy cows. These findings have far-reaching implications to fresh and transition dairy cattle as well as finishing beef cattle.

**ACKNOWLEDGMENT**

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REFERENCES


CHAPTER 7. GENERAL CONCLUSIONS

General Discussion

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis, also known as Johne’s disease (JD) in ruminant animals. JD is a chronic wasting ileitis that is characterized by a prolonged incubation period that lasts two to five years followed by a profuse, watery, intermittent diarrhea that concludes with the remise of the affected animal.

Once described in 1885 by John and Forthingham, JD was a sporadic disease that posed unimportant economical loss to dairy and beef industries. Adoption of the modern animal farming technology has enabled increasing the animal density in farms, resulting in increased JD incidence and increased economic losses to dairy farmers in particular. In addition, the ability of MAP to form biofilm makes MAP highly ubiquitous in the environment.

Currently, 68% of US dairy herds are infected with JD, and economic losses because of JD have been estimated by the USDA National Animal Health Monitoring System to approach US$ 250 million annually.

Up to date, vaccines are designed against JD to trigger Th1 responses that are characterized by induction of the proinflammatory cytokines IFN-γ and TNF-α. In our laboratory, we are concerned that induction of proinflammatory response in an ileum of a JD-infected cow could exacerbate symptoms of JD by causing further mucosal tissue damage that impairs nutrient absorption and hasten death of subclinically infected animals.

Our experiments have grown out of the curiosity that NP51 has completely inhibited MAP growth in *in vitro* co-culture. To examine the efficacy of NP51 to inhibit MAP growth *in vivo*, we recruited 360 specific pathogen-free Balb/c mice and randomly assigned them to
consume sterile standard mouse chow supplemented with or without the probiotic HNP51 or VNP51 and either noninfected or infected with HMAP or VMAP 45 days after the onset of NP51 feeding. The study exquisitely illustrated that VNP51 and HNP51 probiotic feeding decreased MAP burden in various tissues of infected Balb/c mice including livers, spleens, mesenteric lymph node, and ceca. This result was further confirmed by sufficiently revealing that VNP51 and HNP51 also decreased shedding of MAP IS900 genomic DNA in fecal pellets of VMAP-infected mice.

We also elucidated the immune mechanism whereby NP51 targets MAP growth in vivo. By using flow cytometric analyses, we provided persuasive evidence that NP51 potently links the innate immunity to the CD8α+ cytotoxic T cell adaptive immunity via activating the CD8α+ immune cell repertoire that includes dendritic cells (DC), natural killer cells (NK), natural killer T cells (NK T cells), and cytotoxic T cells. Intriguingly, VNP51- and HNP51-caused immune response bypassed the Th1 immune responses and the potential local mucosal damage it might induce.

Whereas the NK cells kills MAP-infected cells that do not express MHC class 1 molecules on the surface (MAP suppresses expression of MHC molecules on macrophages), cytotoxic T cells kills cells that express peptide antigen on MHC class I molecules. Additionally, because MAP infects and suppresses activation of macrophages, antigen-presenting cells, it is pivotal that DC has to be activated to switch the innate immunity to a CD8+ T cell-mediated immunity through cross presentation of MAP-derived antigenic peptide on MHC class I. Additionally, activation of NK T cells is required to secrete IL-12 and IFN-γ to induce maturation of DC.
Thus, the VNP51- and HNP51-orchestrated anti-MAP immune response elegantly unveils the “economy” and “wisdom” of the immune system by inducing the NK and cytotoxic T cells that kill MAP-infected cells only and spare adjacent healthy cells in a very precise “immunological microsurgery”. This VNP51- and HNP51-induced “immunological microsurgery” preserves the healthy ileal intestinal epithelial cells to ensure adequate nutrient absorption and survival of the JD-infected mouse while concurrently ridding the ileum of its MAP-infected cells.

The exquisite observation that VNP51 fed to HMAP-inoculated mice activate both the CD8α+ cell-mediated immunity and MAP-specific humoral immunity provided influential evidence that VNP51 has considerable adjuvant efficacy to potentiate potency of next generation MAP vaccines.

**Recommendations for Future Research**

In these experiments, we have presented persuasive evidence that VNP51 and HNP51 adequately inhibited MAP growth *in vivo* and prevented progression of JD in Balb/c mice. Because the VNP51- and HNP51-induced anti-MAP immune responses are independent of the viability of MAP, it is cost-effective to use HNP51 to treat mice infected with MAP.

I propose that a treatment study be conducted to further elucidate whether co-administration of probiotic NP51 and VMAP to specific pathogenic-free Balb/c mice would inhibit VMAP infection. The study would also provide an opportunity to elucidate the molecular interaction that underlies the VNP51- and HNP51-orchestrated inhibition of MAP growth *in vivo*. To achieve these goals, the following research can be conducted:
Experimental Design

**Mice.** Two hundred and twenty-four 6-week-old Balb/c mice (112 MHC class 1a<sup>−/−</sup> and 112 wild type mice) will be randomized to two main treatment groups: viable MAP-infected controls (**VMAP**; n = 112, 56 MHC class 1a<sup>−/−</sup> and 56 wild type) and VMAP-infected mice fed the heat-killed probiotic NP51 (**VMAP+HNP51**, n = 112, 56 MHC class 1a<sup>−/−</sup> and 56 wild type). Mice will be housed in a BSL-2 rodent room. The environment within the room will be maintained to provide a temperature of 22.2°C, a relative humidity of 35-45%, and 12 h light/dark cycle. Four female or male mice will be housed in each autoclaved standard mice cage fitted with raised-wire floors and High Efficiency Particulate Air (**HEPA**) filters.

**Infection with VMAP.** Mice in the VMAP control group and the VMAP+HNP51 will be inoculated with 10<sup>8</sup> CFU of VMAP suspended in 100 µL of sterile PBS (Stabel, USDA, ARS, NADC) intraperitonealy on day 1 of the study.

**Diet.** Mice in the VMAP control will be fed the sterile mouse chow meal mixed with maltodextrin (**MDX**) carrier at 3% of their chow diet starting on day 1 of the study. On the other hand, mice in the VMAP+HNP51 will be fed the HNP51 at 1× 10<sup>6</sup> CFU·mouse<sup>−1</sup>·day<sup>−1</sup> contained in 3.0 g of the sterile mouse chow meal for the first 15 days. The offering of chow meal will be increased by 0.5 g·mouse<sup>−1</sup> every 15 days until it reaches a maximum of 5.0 g·mouse<sup>−1</sup>·day<sup>−1</sup> by day 91 of the study but continues to provide 1× 10<sup>6</sup> CFU of HNP51·mouse<sup>−1</sup>·day<sup>−1</sup>.

Following inoculation with MAP, 14 mice (7 MHC class 1a<sup>−/−</sup> and 7 wild type) from each treatment group will be euthanized on days 1, 15, 30, 45, 90, 135, 180, and 225 relative to inoculation with MAP and HNP51 feeding.
Sample collection and intended analyses.

**Blood.** About 0.9 mL of blood will be withdrawn by cardiac puncture and allowed to clot at 4° C over night before serum is separated by centrifugation at 10,000 × g for 15 min. To quantify total immunoglobulin (Ig) G and IgG2a, 100 uL of appropriately diluted sera will be used according to the manufacturer’s instruction (Kamiya Biomedical Company, Seattle, WA).

To quantify MAP specific IgG1 and IgG2a, 96-well plates will be coated with 100 µL/well of HMAP suspended in coating buffer (Bethyl Laboratories, Inc., TX, USA) at 2000 ng/mL. Plates will be incubated while covered at 4° C overnight. After the incubation, the coating buffer will be decanted inside a biosafety cabinet and the plates will be washed four times by filling the wells with 300 µL of the wash buffer (Bethyl Laboratories, Inc., TX, USA). To block the nonspecific binding, 200 µL of block buffer (Bethyl Laboratories, Inc) will be pipetted in each well and the plates shall be incubated for 30 min at RT. Subsequently, the blocking buffer will be decanted and the plates will be dried in the hood and stored at 4° C until the IgG1 and IgG2a were determined as previously mentioned by using reagents from Kamiya Biomedical Company.

**Tissues.** Mesenteric lymph nodes (MLN), livers, and ilea will be dissected and used to quantify tissue MAP burden on Herrold’s yolk agar media. Part of the livers will be used to quantify acid-fast bacilli.

Spleens will be dissected and used to prepare splenocyte single-cell suspension. Splenocytes will be cultured *in vitro* in 48-well plate at 2 × 10⁶ cells per mL RPMI 1640 (Invitrogen, Carlsbad, CA) plus 10% heat-inactivated FCS (Fisher Scientific, Hanover Park, IL) and 1% Antibiotic/antimycotic (Invitrogen Carlsbad; NM).
Splenocyte in vitro cultures will be either nonstimulated (negative control) or stimulated with concanavalin A (Sigma-Aldrich, St. Louis, MO) at 3 µg/mL (positive control), with sonicated MAP antigen at 10 µg/mL, or with HNP51 at 10 µg/mL and then incubated at 37° C and 5% CO₂/95/O₂ for 72 hours. Following incubation, the cell-free supernatant will be collected and stored at -70° C for later analysis for interferon (IFN)-γ and interleukin (IL)-10, IL-12, and tumor necrosis factor (TNF)-α concentrations.

Splenocytes will be washed twice with cold sterile PBS and stained for the surface CD8α (anti-mouse CD8α APC, eBioscience, San Diego, CA), CD25 (anti-mouse CD 25 phycoerytherin; PE, eBioscience, San Diego, CA), CD11b, CD11c, DC-SIGN, CD 56, CD3. Subsequently, cells will be fixed, permeabilized, and stained for the intracellular transcription factors Tbet1, RORγ-2, and, GATA-3, and Foxp1.

Frequencies of T cell subpopulations and expression of the intracellular transcription factors will be determined by using the fluorescence-activated cell sorter (BD FACSAria II; Becton Dickson Biosciences, Franklin Lakes, NJ). Data will be analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR). For IL-10, IL-12, IL-17, IFN-γ, and TNF-concentrations, commercially available ELISA kits from R&D Systems (McKinley Place, MN) will be used to determine their concentrations.

Statistical Analyses

A linear mixed-effects model will be invoked to analyze the response variable by using the PROC MIXED procedure of the Statistical Analysis System (SAS; SAS Institute Inc., Cary, North Carolina, USA) version 9.2. The model will account for the fixed effects of diets, infection status, day of euthanization, and their interactions. Differences among
treatment means will be deemed significant when the probability ($P$) value $\leq 0.05$. Tendency for a difference will be declared at $0.05 \leq P \leq 0.1$.

**Expected results:**

1. **VMAP-infected MHC class 1a$^{-/-}$:** we expect from this group to:
   - Succumb to severe MAP infection characterized by progressive emaciation and increased fatalities.
   - Exhibit increased IL-10 and decreased IFN-$\gamma$ and TNF-$\alpha$ secretion in their *in vitro* splenocyte cultures.
   - Have extremely decreased frequency of CD3$^+$CD8$\alpha^+$ cytotoxic T cells, CD25$^+$ T cells, and CD11b, but increased frequency of CD3$^+$Foxp3$^+$ T cells.
   - Have normal CD56 and CD11c, but decreased CD3, and CD25.
   - Have increased MAP burden in tissues and acid-fast bacilli in liver higher than that of other groups.
   - Have the highest increase of MAP IS900 genomic DNA in fecal pellets relative to other groups

2. **VMAP-infected wild type:** We expect from this group to:
   - Succumb to a protracted chronic MAP infection. Fatalities are expected by day 260 but less severe than that of the HMC class 1a$^{-/-}$ group.
   - Exhibit increased IL-10 and moderately decreased IFN-$\gamma$ and TNF-$\alpha$ secretion in their *in vitro* splenocyte cultures relative to that of the VMAP-infected MHC-class 1$^{-/-}$ mice.
- Have normal frequency of CD8α⁺CD3⁺ T cells, but elevated frequency of CD3⁺Foxp3⁺ T cell will be as high as that of the VMAP-infected MHC-class 1⁻/⁻ mice.
- Have normal CD3⁺CD56⁺, CD3⁺CD56⁺ and CD11c⁺, but moderately decreased CD3⁺CD25⁺ cells compared with that of the VMAP-infected MHC class 1⁻/⁻.
- Have increased MAP IS900 genomic DNA in fecal pellets but less than that of the VMAP-infected MHC class 1⁻/⁻ group.

3. HNP51+VMAP-infected MHC-class 1a⁻/⁻: We expect from this group to:
   - Succumb to severe MAP infection characterized by progressive emaciation and increased fatalities.
   - Exhibit increased IL-10 and decreased IFN-γ and TNF-α secretion in their in vitro splenocyte cultures.
   - Have extremely decreased frequency of CD3⁺CD8α⁺ cytotoxic T cells and CD11b, but increased frequency of CD3⁺Foxp3⁺ T cells
   - Have normal CD3⁺CD56⁺, CD3⁺CD56⁺ and CD11c⁺, but decreased CD3⁺CD25⁺ cells.
   - Have increased MAP burden in tissues and increased acid-fast bacilli in liver similar to that of the VMAP-infected MHC-class 1⁻/⁻.
   - Have increased MAP IS900 genomic DNA in fecal pellets comparable to that of the VMAP-infected MHC class 1⁻/⁻ group.

4. HNP51+VMAP-infected wild type: We expect from this group to:
   - Have milder MAP infection that does not induce emaciation or cause fatalities.
- Exhibit increased IL-10, IFN-γ, and TNF-α secretion in their *in vitro* splenocyte cultures.

- Have the highest frequency of activated CD3⁺CD8⁺ cytotoxic T cells and CDb11⁺ cells, but decreased frequency of CD3⁺Foxp3⁺ T cells.

- Have normal CD3⁻CD56⁺, CD3⁺CD56⁺ and CD11c⁺, but the highest frequency of CD3⁺CD25⁺ cells.

- Have significantly decreased MAP burden in tissues and decreased acid-fast bacilli in liver in comparison to that of all other groups.

- Decreased MAP IS900 genomic DNA in fecal pellets.
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