PROBIOTIC-SUPPLEMENTED SOY BAR EFFECTS ON RESISTANCE TO INFECTION BY *LISTERIA MONOCYTOGENES*

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by
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

PROBIOTIC-SUPPLEMENTED SOY BAR EFFECTS ON RESISTANCE TO INFECTION BY *LISTERIA MONOCYTOGENES*

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ABSTRACT

Probiotics are living microorganisms that help regulate the gastrointestinal tract. The aim of this project was to investigate the benefits of probiotics and their inhibitory effect on *Listeria monocytogenes* EGD. The well diffusion assay was conducted to screen the probiotics for inhibition of *L. monocytogenes* EGD. A diet was created out of a soy protein bar supplemented with the probiotics to be used in our in vivo test using a mouse model.

Three groups of mice were fed for two weeks with three different diets, one diet each, Control diet with no probiotic, ADH diet with *Lactobacillus acidophilus* ADH, and B6 diet with *Bifidobacterium animalis* B6. Each group was subdivided into two different groups, one of them would be challenged with *L. monocytogenes* EGD and the other group would not (control group). After 14 days of feeding, the mice were challenged intragastrically with ~10^8 CFU/ml *L. monocytogenes* EGD. At day 3 post-infection, the mice were euthanized. Of the samples collected, the colon and cecum were tested for probiotic concentration. The spleen and liver were tested for the presence of *L. monocytogenes*. In the first replication, a half a log reduction of *L. monocytogenes* EGD in the liver was

observed in the *Bifidobacterium* group as compared with the control group, but no significant reduction in the pathogen was seen in the *Lactobacillus* group for any of the samples collected. For the second trial, a one log reduction of *L. monocytogenes* EGD in the liver was achieved in the *Bifidobacterium* group, but at the same time, a one log reduction of the pathogen in the spleen was also observed in the *Lactobacillus* group. We also collected evidence showing that the probiotics colonized the colon and the cecum with concentrations of ~10^7 CFU.

According to our results we believe that the probiotic supplemented soy protein bar holds promise to prevent listeriosis.

CHAPTER 1

INTRODUCTION

The foodborne pathogen, *Listeria monocytogenes*, causes an invasive human infection called listeriosis. This infection is predominant among immunosuppressed individuals, such as pregnant women, HIV-positive and cancer patients, and the unborn fetus. Its symptoms are severe and include meningitis, and miscarriage, among others. This pathogen is an intracellular microorganism that is capable of crossing the intestinal barrier, fetoplacental barrier, and blood-brain barrier. *L. monocytogenes* has an overall mortality rate of ~30%.

Microorganisms have become resistant to antibiotic medications in such a short time that researchers are having trouble finding new antibiotics. Another disadvantage of antibiotics is that they can affect the natural microflora in the body. This is why scientists are trying to find alternative ways to fight pathogens. Using probiotics is one way to do that. Probiotics are microorganisms that affect the intestinal tract in a positive way when ingested in an appropriate amount. Some studies have shown that benefits of ingesting probiotics can include: stimulation of the host immune system, lowering cholesterol, expressing anti-cancer activities, and suppressing the growth of undesirable microorganisms, including pathogens. By suppressing undesirable microorganisms, probiotics assist in maintaining a balanced microflora in the human gastrointestinal tract. The documentation of these benefits has increased the interest and demand for probiotic-supplemented food products. Some probiotics in the current market

include: *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* GG and *Bifidobacterium longum*. These and other probiotics are marketed in a variety of forms, including powders, milk, yogurts, and capsules.

This experiment was conducted to investigate the benefits of probiotic bacteria invoking immune responses and eradicating the foodborne pathogen *L. monocytogenes* EGD in a mouse model by supplementing a soy protein diet containing probiotics. This diet contains soy protein which, according to some researchers, also has some benefits for reducing the risk of heart disease.

CHAPTER 2

LITERATURE REVIEW

2.1 Soybeans

2.1.1 Health benefits of soy

The physical and chemical characteristics of soy allow us to define it as a functional food. The Food and Drug Administration (FDA) has announced that 25 g of soy protein per day, as part of a diet low in saturated fatty acids and cholesterol, can result in a lower serum cholesterol level and may reduce the risk of heart disease (FDA 2000). Later, the American Heart Association increased this amount to 50 grams (FDA 2000). Soy foods contain proteins that reduce bad LDL cholesterol levels while raising good HDL cholesterol levels (Anderson and others 1995; Potter 1996). Other studies have shown that soy can also be used to prevent cancer and osteoporosis (Ikeda and others 2006; Lechner and others 2005).

Soybeans are rich in proteins, and contain significant amounts of all the amino acids, vitamins, and minerals essential to human nutrition. These nutrients must be supplied in the diet since they are not synthesized by the human body (FDA 2000). Soybeans contain a significant amount of omega-3 fatty acid, which has been shown to inhibit blood clotting and reduce the risk of heart attacks (Rodriguez-Cruz and others 2005). This essential polyunsaturated fatty acid is not synthesized by the body and therefore needs to be obtained from food (Wardlaw and Kessel 2002). Soybeans are rich in isoflavones, which include phytoestrogens. There is evidence that due to the similarity in chemical

structure between phytoestrogens and human estrogen, that phytoestrogen can interfere with the action of our own natural estrogen (Kaufman and others 1997). Some researchers have shown that isoflavones can protect against hormone-related disorders, such as bone density loss (Potter 1998) and breast and prostate cancers (Setchell and Cassidy 1999). Despite its putative health benefits, the soy produced in the U.S. is still mostly used for animal feed and other non-food uses. In the U.S., soy is consumed in many different foods and beverages, but only in small quantities. Soy is mostly used during formulation to associate a product with health benefits or utilize the functional properties of soy without actually using significant amounts of it. However, there are a few foods and drinks marketed in the U.S. where soy is the main ingredient.

2.1.2 Examples of functional soy foods

The demand for functional foods has been increasing. Many food products that have been proven to have health benefits have been approved by the FDA. For example, the approval of labeling soy protein containing foods as protective against coronary disease in 1999 (FDA 2000). This contributed to the increase in soy foods demand in the market from $300 million to $3.9 billion from 1992 to 2006 (U.S. Soyfoods Market Report 2007). Some of these foods and food ingredients include oat products, soy protein, sugarless candies, cranberry juice, garlic, green tea, tomatoes, probiotics and prebiotics (Meister 2002). In the U.S., food manufacturers introduced over 1600 new foods, from 2001 to 2004, containing soy as an ingredient (Soyatech LLC and others 2004).

2.2 Probiotics

Probiotics are living microorganisms which, “upon ingestion, given in adequate amounts, exert health benefits beyond inherent nutrition” (Fuller 1989). Probiotics must survive the host’s natural digestive processes. Probiotics assist in maintaining a balanced microflora in the human gastrointestinal tract, reduce cancer risks, reduce blood cholesterol, and prevent chronic constipation and diabetes (Lee and others 1999). Evidence shows that probiotics can suppress the growth of pathogenic and opportunistic microorganisms in the colon and small intestine. This helps to stabilize or regulate the digestive system. Probiotics can have a direct or indirect effect in the host through modulation of the endogenous flora or stimulation of the immune system (Fuller 1989). These organisms can prevent bacterial infections, lower serum cholesterol, express anti-cancer activities, improve lactose utilization, stimulate the immune system, and allow for efficient mineral absorption and metabolism (Rolfe 2000; Roos and Katan 2000). In-vitro studies have documented that some probiotics may have anti-pathogenic effects (Mack and others 1999), such as eradication of *Salmonella* typhimurium (Silva and others 2004) and *Helicobacter pylori* (Felley and others 2001). This anti-pathogenic effect might be achieved by decreasing the pH in the lumen by the production of short chain fatty acids (acetic acid, lactic acid or propionic acid), producing hydrogen peroxide, or producing inhibitory substances, like bacteriocins (Kailasapathy and Chin 2000). The recommended level of probiotics in order to exert any health benefit is at least 10^7 cfu of living

microorganism per milliliter or gram at the time of consumption (Guarner and Schaafsma 1998).

The most commonly used probiotics are strains of lactic acid bacteria, including *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. These three strains can survive the host's barriers, gastric acid, bile salts and pancreatic enzymes, adhere to the intestinal mucosa, and colonize the intestinal tract. Certain disorders that are treated with probiotics include antibiotic-induced diarrheal disease, *Clostridium difficile*-associated intestinal disease, infectious diarrhea, traveler's diarrhea, *H. pylori* gastroenteritis, hepatic encephalopathy, HIV/AIDS diarrhea, irritable bowel syndrome, carcinogenesis, lactose intolerance, and many others (Rolfe 2000). Because of these applications, products containing probiotics are increasing in number.

According to the FDA, it is necessary to know the genus and species of the probiotic strain because probiotic effects are strain-specific. Strain identity is important to link a strain to a specific health effect as well as to enable accurate surveillance and facilitate epidemiological studies. Many new products, such as yogurt, smoothies, spreads, cream cheese, and cereals are available in the market today. Probiotics are sold in the U.S. in two different categories, food and dietary supplements (Lee and others 1999). Probiotic strains that are the most popular or commonly used are lactobacilli and bifidobacteria (Lee and others 1999). Some examples of probiotic products currently in the market include lyophilized probiotic cultures in the form of capsules and powder, manufactured by Nutraceutix, Inc. (Redmond, WA) (Table 2.1) (FDA 2006), fermented dairy

products manufactured by Yoplait (Minneapolis, MN) containing probiotics, such as traditional yogurts containing *L. acidophilus* and Yo-Plus™ yogurt containing *Bifidobacterium* sp. Dannon Co. (Allentown, PA) also markets several products, including Activia®, a low fat yogurt containing *Bifidus regularis*™, which is a trade name for *Bifidobacterium animalis* DN – 173 010 and *DanActive*™ containing *Lactobacillus casei*. Further, Culturelle® probiotic dietary supplement manufactured by Amerifit Brands, Inc. (Cromwell, CT), is a commercial capsule containing *Lactobacillus rhamnosus* GG.

Table 2.1. Bulk probiotic organisms currently available from Nutraceutix, Inc.

Lactobacillus acidophilus	*Bifidobacterium bifidum*
Lactobacillus brevis	*Bifidobacterium breve*
Lactobacillus casei	*Bifidobacterium infantis*
Lactobacillus fermentum	*Bifidobacterium longum*
Lactobacillus gasseri	*Enterococcus faecium*
Lactobacillus helveticus	*Lactococcus lactis lactis*
Lactobacillus paracasei	*Pediococcus acidilactici*
Lactobacillus plantarum	*Pediococcus pentosaceus*
Lactobacillus reuteri	*Streptococcus thermophilus*
Lactobacillus rhamnosus	
Lactobacillus salivarius	

2.2.1 *Lactobacillus species*

The lactobacilli are a major member of the lactic acid bacteria group, whose primary fermentation end product is lactic acid (Tannock 2004). *Lactobacillus* spp. is a regular gram positive, non-spore forming rod with a size of 0.5-1.2 × 1.0-10.0 μm (Holt and others 1994). Lactobacilli are usually facultative anaerobes but can sometimes be facultative aerobic (Jay and others 2005). *Lactobacillus* serves as an important indicator of gastrointestinal and vaginal tract health and is heavily used by consumers, either intentionally as probiotic supplements, or unintentionally in microbially fortified foods.

2.2.2 *Bifidobacterium species*

The “lactic acid bacteria” were a group set aside by Orla-Jensen in the 1920's whose major fermentation product was lactic acid. Further research showed that the bifidobacteria are considered part of the lactic acid bacteria group on a “physiological” basis because they produce lactic acid as a major fermentation product. However, more recently, as 16S rRNA profiles became available, it became clearer by taxonomic, morphological, and genetic criteria, that the bifidobacteria are distinct from the lactobacilli, streptococci, leuconostocs, lactococci and other lactic acid bacteria (Stiles and Holzapfel 1997). *Bifidobacterium* sp. is an irregular, gram-positive, non-motile and non-spore-forming rod-shaped bacterium. This species is indigenous to the intestinal tract of infants, adults, and various animals. Bergey’s Manual of Systemic Bacteriology (Sneath and others 1986) describes bifidobacteria as anaerobic

microorganisms. Different species and strains of bifidobacteria exhibit different sensitivity to oxygen in the presence of CO_2 (Sneath and others 1986). Most strains only grow at 25 to 45˚C and pH 5 to 8 (Jay 2005). Some studies indicate that *Bifidobacterium infantis* and *Bifidobacterium longum* have antimicrobial effects against potentially pathogenic microorganisms, such as *Salmonella, L. monocytogenes, Campylobacter, Shigella* and *Vibrio cholerae* (Gibson and Wang 1994).

2.2.3 Other probiotics

There are other lactic acid and non-lactic acid bacteria that exhibit probiotic effects. Few studies have been carried out on spore-forming lactic acid producing bacteria (SFLAB) as probiotics. Many factors make SFLAB good candidates for probiotic use: (i) they are easily cultured in 'bulk'; (ii) they produce organic acids; and (iii) they possess the capacity to sporulate. In addition, in the spore form, SFLAB are more resistant to heat, which facilitates the pelleting process used in the mass production of probiotic animal feeds (Hyronimus and others 2000**)**. *Leuconostoc mesenteroides* and *Sporolactobacillus inulins* are lactic acid bacteria that have been used as probiotics (Holzapfel and Botha 1988). *S. inulins* is acid and bile salt tolerant, has adhesiveness, and is antagonistic against pathogenic *Salmonella* enteritidis BCRC 10744 (Huang and others 2007). Some of the non-lactic acid bacteria that exhibit probiotic qualities are *Bacillus cereus* Toyoi (mainly used in animal feed), *Escherichia coli* Nissle 1917, *Propionibacterium freudenreichii*, *Saccharomyces cerevisiae*, and *Saccharomyces boulardii*, all four of which are mainly used as pharmaceutical

preparations (Holzapfel and others 2001). For example, *S. boulardii* was used in a premature infant formula which contributed to a fecal flora that was comparable to that of a breast fed formula (Costalos and others 2003).

2.3 Probiotic preservation

A variety of methods have been used to preserve and store microorganisms for use in food products and drugs. These techniques make cells stable and transportable at ambient temperatures. Probiotics should be able to retain their characteristic properties and a sufficient number that make them beneficial to consume. Some of these methods include: spray drying (Ananta and others 2005), encapsulation (Kailasapathy 2006), and freeze drying (Carvalho and others 2004). When the method of freeze drying or lyophilization is mentioned in relation to the preservation of microorganisms, it is nearly always with regards to long term storage of cell suspensions that contain greater than 10^8 cells ml^{-1} (Miyamoto-Shinohara and others 2000; Costa and others 2000). This technique involves three steps or processes: freezing, primary drying (sublimation), and secondary drying (desorption) (FDA, 2008). More specifically, it is a process in which water is removed from a product after it is frozen (solid phase) and placed under vacuum, allowing the ice to change directly from solid to vapor (gas state) without passing through a liquid phase (Heldman and Singh 1981). The moisture is removed without exposing the product to excessively high temperatures and preventing damage in the structure of the product. When the moisture content of a product is reduced, microorganisms and enzymatic activities can be inhibited. Freeze drying is the most complex and expensive

form of drying, and because of this, it is usually used only for delicate, heat-sensitive materials of high value, such as microorganisms. When this method is used to preserve microbial cells, a freezing bacterial suspension with a cryoprotectant is necessary.

A cryoprotectant is a substance that, when added to a cell suspension, protects the cells from freeze damage by preventing the formation of large ice crystals. The principal site of damage in the bacterial cell during freezing is the cytoplasmic membrane. According to the Handbook of Food Preservation (Rahman 2007), fast cooling of bacterial cells can lead to more lipid crystallization before any rearrangement of intramembrane particles can occur. On the other hand, when the samples' freezing process is slow, phase separation of the external and cytoplasmic membranes can occur, causing the external membrane to be split off by extracellular ice crystal formation. It is possible to decrease this damage by adding a cryoprotectant. If the membrane is injured, it can cause leakage or release of cellular materials, such as amino acids, potassium ions, low molecular solutes, RNA, and single and double strand DNA. This damage is related to non viability of cells. A different type of cell injury is osmotic dehydration. This is caused by the ice formation on the outside of the cell and it can produce a movement of the intracellular macromolecules, causing a deformation and phase separation (Rahman 2007).

Some cryoprotectants, such as glycerol and dimethyl sulphoxide (DMSO), are differently permeable to the cells which, in turn, affect the mechanisms of their protective effects by penetrating both the cell wall and the cytoplasmic

membrane. Other mechanisms would be penetration of the cell wall but not the membrane (e.g. oligosaccharides and amino acids), and no penetration of the cell wall or no direct interaction with the cell wall or the membrane (e.g. polysaccharides) (Carvalho and others 2004).

Examples of cryoprotectants are trehalose and sucrose. Trehalose is a disaccharide that consists of two glucose units linked by a 1, 1-α–glycosidic bond (Figure 2.1a) (Hetzer 2008). Trehalose is a naturally occurring reducer of cell stress, by protecting cells from extremes in heat shock and osmotic stress (Crowe 2002). Trehalose is thought to work by altering or replacing the water shell that surrounds lipids and protein macromolecules (Colaco and others 1995). Sucrose is a disaccharide composed of one glucose and one fructose molecule (Figure 2.1b). The cryoprotective capacity of sucrose has been tested with several bacteria and viruses at concentrations from 1-68% (median 10%) (Hubalek 2003). The Maillard Browning reaction between reducing sugars and proteins in the dry state has often been invoked as a major source of damage (Li 1996), and the fact that both sucrose and trehalose are non-reducing sugars may explain at least partly why they are the natural products accumulated by anhydrobiotic organisms (Crowe 2002).

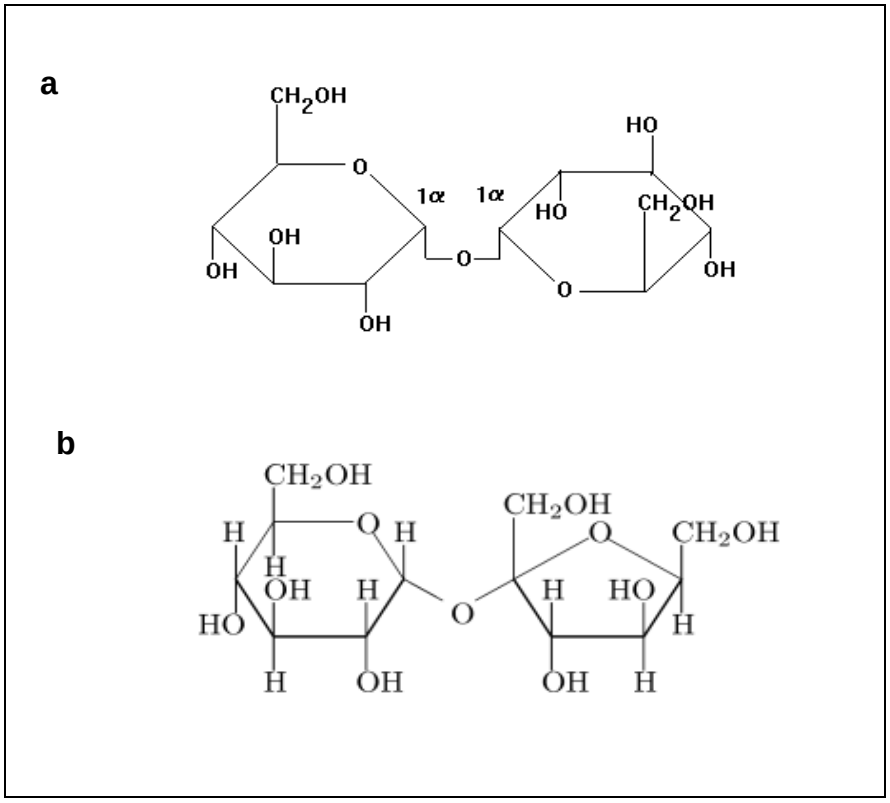

Figure 2.1. Trehalose (a) and sucrose (b) structures.

2.4 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, facultative anaerobic, intracellular, nonspore-forming rod with a tumbling motility and an optimum growth temperature of 30-37˚C. It resists heat, salt, nitrite and acidity much better than many organisms. This bacterium survives on cold surfaces and it can survive and multiply slowly at 0˚C (FDA 2003). *L. monocytogenes* is responsible for a disease called listeriosis. This is a human infection with an overall mortality rate of 30% (Gahan and Hill 2004; Lecuit 2005). This infection may have different degrees of severity. Some symptoms include fever, muscle aches, and sometimes gastrointestinal symptoms, such as nausea or diarrhea. However, if

infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance, or convulsions can occur. This infection may lead to meningeal infection, putting *L. monocytogenes* as the fourth most common cause for this disease. Infected healthy pregnant women may experience only a mild, flu-like illness. However, infections during pregnancy can lead to miscarriages or stillbirths, premature delivery, or infection of the newborn (CDC 2008; Gahan and Hill 2004).

According to the FDA, the infective dose of *L. monocytogenes* is unknown but is believed to vary with the strain and susceptibility of the victim (FDA 2007). This pathogen predominately affects pregnant women, newborns and immune-compromised individuals, such as HIV and immunosuppressive therapy patients, individuals with diabetes, cancer and kidney disease (Park and others 2004). Listeriosis can only be positively diagnosed by culturing the organism from blood, cerebrospinal fluid, or stool, although the latter is difficult and of limited value (FDA 2007). According to the FDA, there could be a substantial delay between the time of ingestion of contaminated food and the onset of serious symptoms. The average time from exposure to illness is approximately 30 days, but symptoms can appear as late as 90 days after exposure. It is important for consumers to know that the infection can occur as much as 90 days later, so that they can seek appropriate treatment if they have symptoms of the infection (FDA 2005; USDA 2005).

2.4.1 Distribution

L. monocytogenes is widely distributed in the environment, which makes it easier to be transmitted to all types of food sources or food contact surfaces. An important property of this pathogen is that it can grow at refrigeration temperatures and low oxygen conditions. These characteristics make *L. monocytogenes* a problem for manufacturers of ready-to-eat (RTE) food products, especially RTE meats. It can also be found in milk products, fruits, vegetables, seafood, water, soil, animal gastrointestinal (GI) tract, and feces (Jay and others 2005: CDC 2008).

2.4.2 Incidence and treatment

L. monocytogenes causes an estimated 2,500 cases of listeriosis, resulting in 500 deaths each year in the U.S (Hurd 1998; Notermans and others 1998; CDC 2008). The mortality rate depends on how far along the infection is. When listeric meningitis occurs, the overall mortality rate may be as high as 70%, while in septicemia rates may reach as high as 50%, and for perinatal/neonatal infections rates may exceed 80% (FDA, 2007). If the infection is diagnosed early enough, antibiotic treatment can prevent serious consequences of the disease. Unfortunately, early diagnosis of listeriosis does not happen often. To achieve an effective treatment, penicillin or ampicillin must be combined with gentamicin. However, patients allergic to penicillin must be treated with trimethoprim-sulfamethoxazole (FDA 2007). Vaccines are not available for this infection.

According to the USDA, one of the largest outbreaks of *L. monocytogenes* occurred in 1998 with a large hot dog manufacturer. The result was 15 adult

deaths, six stillbirths, and over one million pounds of products recalled (FDA, USDA 2005). According to the Minnesota Department of Health (2007), in 2006, seven cases were reported, ending with hospitalizations and one death. In the previous year, the same state had 15 cases (all hospitalized) and two deaths. The organism was isolated from different sources. In eleven out of fifteen cases, *L. monocytogenes* was isolated from blood, two from cerebral spinal fluid, one from joint fluid, and one from peritoneal fluid. However, these cases were not associated with a recognized outbreak. According to the CDC (2000), a multistate outbreak of *L. monocytogenes* infections with 29 cases ended in four deaths and three stillbirths or miscarriages in ten states. This outbreak was linked to sliceable turkey deli meat. Fifteen cases have been reported from New York, three from Georgia, two from each Ohio, Connecticut, and California, and one from each of the following states, Pennsylvania, Tennessee, Utah, and Wisconsin (Table 2.2).

Table 2.2. Outbreaks of listeriosis in the United States (1970-2002) with known food vehicle(s) (FDA 2005).

Year	Food Vehicle	State	Case	Perinatal case (% of total)	Death (% of total)	Serotype
1979	Raw vegetables or cheese	MA	20	0 (0)	3 (15.0)	4b
1983	Pasteurized fluid milk	MA	32	7 (21.9)	14 (43.8)	4b
1985	Mexican-style cheese (raw milk)	CA	142	93 (65.5)	48 (33.8)	4b
1986-1987	Ice cream, salami, brie cheese	PA	36	4 (11.1)	16 (44.4)	4b,1/2b, 1/2a
1986-1987	Raw eggs	CA	2	Unknown	Unknown	4b
1987	Butter	CA	11	Unknown	Unknown	Unknown
Not specified	Frozen vegetables	TX	7	3 (42.9)	Unknown	4b
1998-1999	Hot dogs, deli meats	22 states	101	Unknown	21 (20.8)	4b
1999	Pâté	CT, MD, NY	11	2 (18.2)	Unknown	1/2a
2000	Deli turkey meat	10 states	29	8 (27.6)	7 (24.1)	unknown
2000-2001	Homemade Mexican-style cheese (raw milk)	NC	12	10 (83.3)	5 (41.7)	unknown
2002	Deli turkey meat, sliceable	8 North Eastern states	63	3 (4.8)	7 (11.1)	unknown

2.4.3 Pathogenicity

Mice and cell biology studies using tissue culture cells have given the most information on the pathogenesis of *L. monocytogenes* (Kuhn and others, 1999). As a food borne pathogen, this bacterium penetrates the GI tissues when ingested. From there, it is exposed to phagocytic cells. *L. monocytogenes* can evade the immune system mechanisms, survive, and multiply within host phagocytes. This is possible because of its virulence factors that protect the organism within the host cells, or allowing it to escape from it. *L. monocytogenes* moves throughout the host via the blood or lymphatic circulation to various tissues, breaking the three significant barriers in the human body (intestinal, blood-brain and fetoplacental barriers) (Lecuit 2005). Once inside a tissue, it can invade cells, multiply within them, and use cytoskeletal acting filaments to spread to neighboring cells. The number of ingested cells, virulence of the strain, and host susceptibility are factors to be considered in the outlook of tissue invasion (FDA 2005). Most cases of listeriosis occur in fetuses or neonates and individuals with a predisposing condition that impairs the immune system (FDA 2007).

2.4.4 Virulence factors

The GI tract is the primary route of *L. monocytogenes* infection of the host. From the GI tract, it can invade tissues (placenta) and enter the blood stream, from where it is able to reach adjacent cells. The mechanism to enter the cell varies with the type of cell. In the case of phagocytic cells, *L. monocytogenes* enters directly into phagosomes. However, for non-phagocytic cells, this

pathogen needs surface-bond proteins called internalin A (InlA) and internalin B (InlB) (Lingnau and others 1995). Internalin A helps the bacterium enter intestinal epithelial cells by interaction with the calcium-dependent glycoprotein E-cadherin, which is its receptor in mammalian cells (Wollert and others 2007; Lecuit and others 2001). This glycoprotein is species-specific because of a single amino acid in position 16 of the molecule: proline in permissive species such as humans, guinea pigs, ovines, and bovines, and glutamic acid in non-permisive species such as mice and rats (Lecuit 2005). Internalin B (InlB) helps in the uptake into hepatocytes and some endothelial or fibroblast cells (Drevets and others 1995; Wollert and others 2007).

Once *L. monocytogenes* is inside the host cell, specifically, a phagocytic vacuole, it needs to break that vacuole to escape from it. Listeriolysin O (LLO), a pore-forming protein, acts together with two phospholipases (PlcA and PlcB) to allow *L. monocytogenes* to escape from the host vacuole into the cytosol. This protein is encoded by a hemolysin gene (*hly*). These pores do not kill the cell. This protein has been compared with perfringolysin O (PFO) and streptolysin O, which are normally secreted by extracellular pathogens and apparently act on cells from outside (Jones and Portnoy, 1994).

After the vacuole is broken, *L. monocytogenes* needs to move out of the cell to spread to other cells. Once in the cytosol, the actin filaments (*ActA*) mediate an actin-based movement that drives the bacterium across the intracellular pathways to reach the surface of the cell to spread from cell to cell.

2.4.5 Cytokine induction

The immune system has different types of cells that react to infections and tainted cells or substances. These reactions include physical and chemical barriers, such as epithelial cells, phagocytic cells, such as macrophages, and cytokines (Abbas and Litchman, 2003). This last defense activity responds to specific microbes and antigens. Cytokines are glycoproteins produced by leucocytes and other types of cells acting as communicators between cells that are required to activate the immune system (Callard and Gearing 1994). *L. monocytogenes* infection, as any other infection induces the production of several types of cytokines, some of which are essential for host protection and resistance against this pathogen, especially those synthetized by T-helper 1 (Th1) cells. Some of them include tumor necrosis factor alpha (TNF-α) (Nakane and others 1988), interleukin-6 (IL-6) (Nakane and others 1999; Liu and others 1995), interferon gamma (IFNγ) (Yamamoto and others 2005) and monocyte chemoattractant protein-1 (MCP-1).

Tumor necrosis factor-alpha (TNF-α) is also known by several names, such as cachectin, macrophage cytotoxin, necrosin, and others. This cytokine is one of the first cytokines to be produced by *L. monocytogenes*-infected macrophages (Liu 2008). Some of its activities include growth regulation and differentiation of several types of cells, and causes tumor cell death necrosis *in vivo*. Many of TNF-α reactions occur in combination with other cytokines (Callard and Gearing, 1994). Another cytokine is interleukin-6 (IL-6) also referred to as B-cell stimulatory factor-2 (BSF-2) and interferon beta-2 (Callard and Gearing

1994). According to the Handbook of *L. monocytogenes* (Liu 2008), immediately following a *L. monocytogenes* infection, IL-6 is produced by Kupffer cells inducing a “peripheral blood neutrophilia and synthesis and release of acute phase proteins by hepatocytes”. This cytokine may protect the liver from apoptosis in *L. monocytogenes* infection (Miura and others 2000).

IFNγ is also known as immune interferon, and T cell interferon. It is involved in the regulation of immune and inflammatory responses, such as activating T cells, B cells, macrophages, and control their growth and differentiation. This cytokine is important for fighting *L. monocytogenes* infections because it enhances the macrophage antimicrobial activity at killing phagocytosed mircroorganisms (Abbas and Litchman 2003). The other cytokine that is secreted in response to listerial infection is MCP-1 (Liu 2008). It is also known as human JE, monocyte chemoattractant and activating factor (Callard and Gearing 1994).

2.5 Probiotic effects on *Listeria monocytogenes*

It is well known that probiotics can inhibit the in vitro growth of several pathogens (Rolfe 2000), as well as prevent gastrointestinal infections in animal models (Corthier 1997). Some mechanisms that may be the cause for these antimicrobial activities include: competition for nutrients, stimulation of the immune system, and production of acids and antimicrobial substances (Marteau and others 2001). Some researchers suggested that lactic acid bacteria or probiotics can exhibit antimicrobial activity against pathogens, such as *Salmonella* (Alm 1983), *Campylobacter* (Tojo and others 1986), and *L.*

monocytogenes (Harris and others 1989). One of the probiotics that have been tested for its antimicrobial effects is *Lactobacillus*. Chateau and others (1992) reported that some strains of *Lactobacillus* spp. were able to inhibit some of these pathogens including *L. monocytogenes* in vitro.

Food products should be stable before going into the market, but sometimes even with preservatives and different treatments, spoilage or pathogenic bacteria can still survive and grow. According to Työppönen and others (2003), practicing the hurdle-concept in dry sausages is not enough to prevent the survival of two major foodborne pathogens, *L. monocytogenes* and *Escherichia coli* O157:H7. Some researchers are looking for probiotic strains that are able to reduce the risk and count of these pathogens in the food. Winkowski and others (1993) was able to find a strain that, depending on the inoculum concentration, was able to reduce the *L. monocytogenes* count in most of the beef systems that were studied. Another example of *L. monocytogenes* inhibition in food was shown by De Martinis and Franco (1998) when they demonstrated that *Lactobacillus sake* 2a was able to inhibit this pathogen in a pork product by producing a bacteriocin-like substance.

According to Mahoney and Henriksson (2003) probiotics can inhibit *L. monocytogenes* in vivo. They have proven that *Lactobacillus acidophilus* LAFT® L10 had a 2.5 log reduction of the foodborne pathogen. *L. monocytogenes* LM3, when compared to the control group. In-vivo testing is expensive and time consuming, it also requires approval by ethical committees;

therefore, there is not enough information about in-vivo probiotic inhibition activity against pathogens such as *L. monocytogenes*.

CHAPTER 3

MATERIAL AND METHODS

3.1 Probiotic selection

Eight different probiotic strains were tested via a well diffusion assay for the inhibition of *L. monocytogenes* EGD. These probiotics, all from the culture collection of the University of Missouri, Food Microbiology Laboratory, included *Bifidobacterium infantis* 25962, *Bifidobacterium animalis* B6, *Lactobacillus acidophilus* NCFM, *L. acidophilus* LA-1, *L. acidophilus* LA-2, *L. acidophilus* ADH, *Lactobacillus rhamnosus* GG, and *Lactobacillus paracasei*. The probiotics were propagated in deMann, Rogosa, Sharpe (MRS) broth (Difco Labs., BD Diagnostics, Sparks, MI) at 37˚C under anaerobic conditions, and *L. monocytogenes* were grown in Brain Heart Infusion broth (Difco Labs.) at 37˚C under aerobic conditions. There are differences in the nutritional requirements and incubation conditions between the probiotics and the pathogen, but the well diffusion assay can be conducted on only one type of agar, so both the probiotics and the pathogen must be able to growth on it. Thus, before performing the well diffusion assay, the probiotics were plated in Brain Heart Infusion Agar (BHIA) (favoring the pathogen), and *L. monocytogenes* was plated in MRS agar (favoring the probiotics) to see if they were able to overcome the unfavorable conditions.

Because the aim of this experiment was to test the inhibition activity of the probiotic cells and not the fermentation end products of the probiotics in the growth medium, fresh overnight grown probiotic cultures were centrifuged at 700

x *g* for 15 min at 4˚C, the supernatant discarded, and the cells resuspended in 9 mL of peptone water. Then, 100 µL of *L. monocytogenes* were spread on the surface of both BHI and MRS agar, and five wells were made in the agar using a 5 mm sample corer. Each probiotic, in the amount of 100 µL or 50 µL was placed in the four outer wells and the control (peptone water) was placed in the center well. The plates were incubated at 37˚C for 24 h. Each set was incubated in different conditions (aerobic or anaerobic), having a total of eight sets plus duplicates (Figure 3.1). The diameter of the inhibition zones around each well was measured in centimeters.

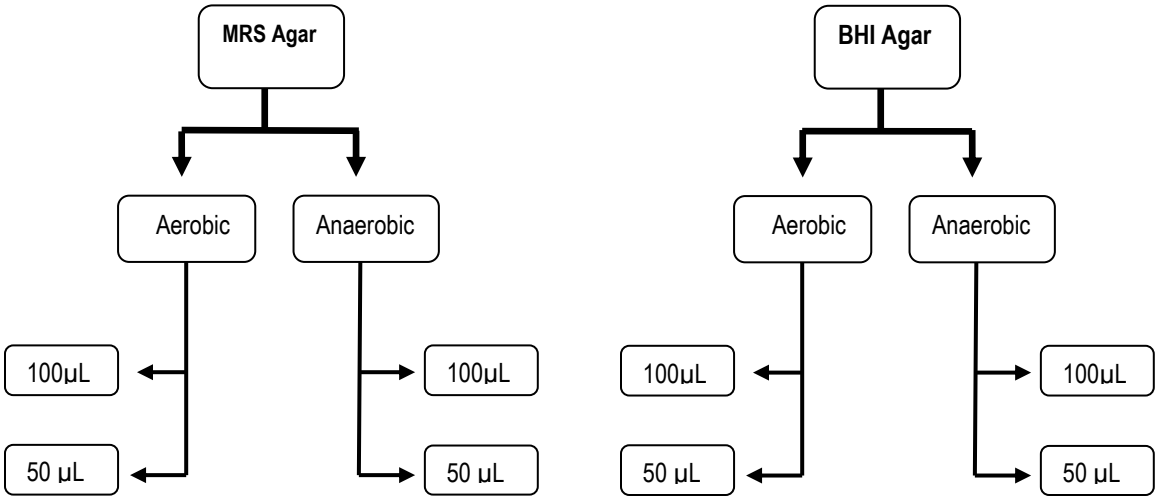

Figure 3.1. Probiotic selection/well diffusion assay scheme.

3.2 Design of selective media for probiotics

Because the mice tissue samples to be processed in the in vivo studies of the project would contain natural flora, suitable selective media for the probiotics used in this study first had to be designed. Although a good medium for growing lactic acid bacteria, MRS agar is not selective enough to allow for good estimation of the numbers of the *Lactobacillus* and *Bifidobacterium* probiotics that were delivered to the mice. Beerens' Agar (Table 3.1) (Silvi and others, 1996) is a known selective medium for bifidobacteria, but not selective enough for isolating bifidobacteria from the colon, cecum, or feces of mice. Therefore, several published selective media were modified to improve their selectivity towards the probiotics used in this study. Some of these included five different modifications of Beerens' Agar (Table 3.2). Other selective media tested included Columbia Agar, Brain Heart Broth, Trypticase-Peptone-Yeast extract medium, Blood-Glucose-Liver Agar, Neomycin-Paramomycin-Nalidixic-Lithium chloride (NPNL) Agar, and MRS Agar with different concentrations of nalidixic acid (Table 3.2). Various different strains of bacteria, including *B. animalis* B6, *Escherichia coli* K12, *L. acidophilus* ADH, *L. acidophilus* LA-2, *L. acidophilus* NCFM, *B. infantis*, *L. paracasei*, *L. rhamnosus* GG, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *L. monocytogenes* EGD, and a fresh sample of mice feces were used to test the selectivity of each medium. Each test strain or fecal sample, in the dilution range of 10^{0} to 10^{-3}, was pour-plated in the different agar media and anaerobically incubated at 37˚C for 24 h.

Table 3.1 Beerens' agar[1] original recipe (Silvi and others, 1996).

Ingredient	Amount per Liter
Columbia agar	42.5 g
Glucose	5.0 g
Cysteine hydrochloride	0.5 g
Agar	1.5 g
Propionic acid	5.0 mL
Distilled water	1,000 mL

Table 3.2 Beerens' agar[1] modified recipes.

Ingredient	Amount per Liter (g or mL)		
Beerens' Agar[1]	**Modification #3**	**Modification #4**	**Modification #5**
Columbia Agar	42.5	42.5	42.5
FOS	5.0	-	-
Glucose	-	5.0	-
Lactulose	-	-	5.0
Cysteine hydrochloride	0.5	0.5	0.5
Agar	1.5	1.5	1.5
Propionic acid	5.0	5.0	5.0
Lithium chloride	1.0	1.0	1.0
Sodium chloride	2.0	2.0	2.0
Paramomycin sulphate	0.3375	-	-
Distilled water	1,000	1,000	1,000

[1] Beerens' agar is not autoclaved but boiled. The pH is adjusted to 5.0 after boiling.

3.3 Experimental diet

3.3.1 Probiotic freeze-drying

The two probiotic strains selected for this experiment (*L. acidophilus* ADH and *B. animalis* B6) were freeze-dried according to the protocol of Zayed and Roos (2004). The probiotics were transferred from frozen stocks to 10 mL of MRS broth (Difco Labs) supplemented with 0.05% L-cysteine hydrochloride (Fisher Biotech., Fair Lawn, NJ) and incubated anaerobically at 37˚C for 24 h. Then, 1 mL of the cultures was used to inoculate 50 mL of MRS broth, supplemented with 0.05% of cysteine hydrochloride, and incubated anaerobically at 37˚C for 24 h. The tubes were centrifuged at 1400 x g for 20 min in a Beckman GPR centrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was discarded and the cells resuspended in 15 mL of Ringer buffer solution (0.6% NaCl, 0.0075% KCl, and 0.01%$CaCl_2$) to be washed. These steps were performed two more times. The samples were centrifuged and the supernatant discarded, but this time, the cells were resuspended in a cryoprotectant solution (5% trehalose and 5% sucrose) (Simoes 2006). Then, the samples were stored at -18˚C for 24 h, and freeze-dried for 48 h using a pilot plant scale freeze drying system (Model Lyph Lock12, Labconco, Kansas City, MO). Freeze-dried cells were stored in a dessicator in the dark at room temperature until used for diet treatments.

3.3.2 Soy protein energy bar raw materials

The raw materials used to prepare the soy bar were those used by Simoes (2006), and included a soy protein isolate, PRO-FAM® 873 (Archer Daniels Midland Co., Decatur, IL), non-fat dried milk (NFDM, Carnation, Nestle Inc., Solon, OH), high fructose corn syrup (HFCS, International Food Products, St. Louis, MO), brown sugar (C&H Sugar, Crokett, CA), gum guar Bland (TIC Gums, Belcamp, MD), canola oil (Crisco, J.M Smucker Co. Inc., Hunt Valley, MD), 40% citric acid (Sensient Flavors, Inc., Amboy, IL), vanilla extract (McCormick & Co., Inc., Hunt Valley, MD), and soy flour provided by the Missouri Soybean Merchandising Council. All ingredients were stored at room temperature in closed receptacles until used. Table 3.3 shows the soy protein energy bar formulation. Note that for these experiments the probiotics were added during preparation of the mouse diets.

Table 3.3. Soy protein energy bar formulation (Simoes 2006).

Ingredient	Amount (g/kg)
Soy flour	45.0
Soy protein isolate (Pro-Fam® 825)	180.0
Non-fat dry milk (NFDM)	35.0
Gum guar blend	57.0
Canola Oil	31.5
Brown sugar	180.0
High fructose corn syrup	333.0
Vanilla	14.0
Citric acid 40%	14.0
Probiotic (or sucrose, 40 g / trehalose, 40 g)	111.0

3.3.3 Soy protein energy bar preparation

Using the amounts from Table 3.3**,** the soy flour, soy protein isolate and non-fat dry milk were evenly mixed and the mixture baked in an oven for 3 h (mixing every 30 min) at 88˚C (190˚F) to rid the product of any bacterial contamination. After 3 h, the mixture was mixed for 3 min with the guar gum, canola oil, 40% citric acid, and vanilla extract (mix #1), using a commercial mixer (Hobart Corporation Model Automix D330, Troy, OH). High fructose corn syrup (HFCS), brown sugar, and water were mixed by hand, heated up to 85˚C for 15 s, and removed from the hot plate. Upon cooling to 65˚C, this mixture was added to mix #1. All ingredients were then mixed in the Hobart mixer for 5 min on high speed and vacuum packed in pouches (Doug Care Equipment, Inc. Springville, CA) using a food sealer (Rival Seal-a-Meal VS220, 2006, Milford, MA). The packaged soy protein energy bar was stored in the dark at 4˚C for approximately 1 week prior to being incorporated into the mouse diets.

3.3.4 Mice diet ingredients

A soy bar/sucrose pre-mix was prepared by combining one part soy bar with two parts of sucrose. The basal mouse diet contained (per kg) soy bar/sucrose pre mix (500 g), casein (200 g), mineral mix (AIN93M) (70 g), vitamin mix (AIN93) (20 g), L-cysteine (3.6 g), choline bitartrate (5 g), cornstarch (21.4 g), fiber (100 g), and soybean oil (80 g).

3.3.5 Diet preparation

The soy bar/sucrose premix was prepared using a two-step grinding process, to make the premix as homogeneous as possible. The premix was first ground with a mortar and pestle, and then pulsed in a food processor until a fine granular powder was obtained.

A basal diet dry premix was prepared as follows. Approximately one-half of the casein was transferred to a stainless steel mixing bowl. The mineral mix (AIN93M), vitamin mix (AIN93VX), L-cysteine, and choline bitartrate were individually weighed and ground together using a mortar and pestle. This mixture was ground with a portion of the remaining casein and added to the mixing bowl, followed by the remainder of the casein. The cornstarch and fiber were individually weighed and added to the bowl. The dry premix ingredients were then mixed using a Hobart mixer on a low setting for 5 min.

To prepare the basal diet, the soy/sucrose premix and the basal diet dry premix were combined and the soybean oil was poured into the dry ingredients while mixing at low speed. The completed diet was then mixed at high speed for an additional 5 min, and stored at 4˚C until needed (approximately 1-2 weeks).

3.3.6 Storage conditions

A total of 5 kg of the basal diet (without the freeze-dried probiotics) was made and stored in the dark at 4˚C. The probiotics were added to the basal diet in batches to keep their numbers as high as possible and to maintain their concentration throughout the feeding phase of the experiment. Experimental diets were prepared every 4 days. For each probiotic treatment, 270 g of diet

was mixed with 30 g of freeze-dried probiotic. For the control treatment, 300 g of basal diet was used. Each of these batches was then divided into four aliquots of 75 g, sufficient for one day's feeding. These aliquots were vacuum-packaged and stored at 4˚C.

3.3.7 Microbial analysis

A new batch was prepared every four days. Each new batch of diet was analyzed for microbial count. Five grams of each diet (B6, ADH, and Control) were diluted in 225 mL of peptone water and dilutions from 10^{-4} to 10^{-8} (duplicates) were plated on MRS and Beerens' modification #5 and incubated at 37˚C under anaerobic conditions for 24 h for MRS and 48-72 h for Beerens' modification #5.

3.4 *L. monocytogenes* strain selection

Two different *L. monocytogenes* strains (EGD and Scott A) were tested to determine which exhibited a more consistent infection in the mice. One set of five 5-7 week-old female BALB/c mice per strain was orally inoculated with a concentration of 10^8 cfu/mL of each pathogen. Tissue samples (small intestine, gallbladder, and cecum) were collected at 5 days post-infection. For two of the samples, the entire organ was taken, but for the small intestine, only 20 cm were taken. The samples were homogenized using a Tissumizer Model SDT-1810 (Tekmar Co., Cincinnati, Ohio) (~1 min turning on and off to avoid over-heating of the samples), and respective dilutions were made in peptone water (small intestine and gallbladder, 10^{-2}-10^{-3}; cecum, 10^{-3}-10^{-5}). Samples were plated in

Modified Oxford (MOX) agar (Difco Labs.) and incubated at 37˚C in aerobic conditions for 48 h.

3.5 Animal treatments

3.5.1 Preliminary diet study

A preliminary study was performed to determine whether the mice would eat the diets containing the soy protein bar. Specific pathogen-free, 5-7 week-old BALB/c mice were used for this study. There were two dietary treatments, the basal (control) diet containing no probiotics, and the control diet containing *B. animalis* B6. Both male and female mice were used in each dietary group. The mice were fed for a 14-day period and the weight of each mouse was measured on days 0, 7, and 14.

3.5.2 Dietary treatment groups

For all three trials, the mice were divided into three dietary groups. They were fed three different soy protein bar diets: Control diet (no probiotics), ADH diet (containing *L. acidophilus* ADH), and B6 (containing *B. animalis* B6). The mice were fed once a day for 14 days, before receiving an oral inoculation of 10^9 cfu/ mL *L. monocytogenes* EGD. Each cage had 4 to 5 mice, and the food was replaced every day. The dishes in which the food was served were cleaned with soap and hot water everyday even though the sets of dishes were separated by treatments.

3.5.3 *L. monocytogenes* challenge

First trial

For the first replication, the experiment had a total of 38 mice. Specific pathogen-free female A/J mice were placed in polycarbonate box cages, four mice per cage, with aspen-shaving bedding. The environment in the room was controlled: 12:12 h light:dark cycle at 23-25˚C and 40-50% relative humidity. The mice were acclimated for 1 week in the facilities before being used in the experiment. The mice were randomly divided into three groups: control containing no probiotic, B6 containing *B. animalis* B6, and ADH containing *L. acidophilus* ADH. Each group was subdivided into two groups: control which was not inoculated with *L. monocytogenes* EGD, and experimental which was inoculated with the pathogen. The animals were given free access to distilled water and respective treatment food. The mice were fed the respective energy bar diets once a day for 11 days, before an oral inoculation with 10^9 cfu/ mL *L. monocytogenes* EGD. At 3 days post-infection while still on the respective diets (2 weeks total), the animals were euthanized and samples (small intestine, colon, cecum, gallbladder, liver and spleen) were collected, plated, and incubated at their respective conditions as described above.

Second trial

For the second replication, the experiment had 41 mice. The mice were divided in the same way as the first replication, but this time, the control group had 13 mice, and the experimental groups had 14 mice each, instead of 13. The mice were fed the respective diets for two weeks before the *Listeria* inoculation.

This time, the mice were inoculated with a lower concentration (10^8 cfu/mL) of *L. monocytogenes* EGD while still on the diet. The same tissues (colon, small intestine, cecum, liver, and spleen) were plated on day 3 post-infection and incubated at their respective conditions. Gallbladders were not collected because the tissue was too delicate, releasing the fluids that were suspected to contain the pathogen when it was being collected. This problem led to inconsistent results in the first replication.

Third trial

For the third replication, the experiment had 42 mice. The control group had 12 mice and the experimental groups had 15 mice each. The mice were fed the respective diets for two weeks before the *Listeria* inoculation. The mice were inoculated with 10^8 cfu/mL of *L. monocytogenes* EGD while still on the diet. The same tissues (colon, small intestine, cecum, liver, and spleen) were plated on day 3 post-infection.

3.5.4 Mice weights

For all three replications the mice were individually weighed on days 0, 7, 14, and 17 (3 days post-infection).

3.5.5 Sample analyses

3.5.5.1 Colon and cecum analyses

The colon and cecum samples were each collected as whole organs. Each organ was weighed and the measured amount in grams multiplied by nine gave the total amount in mL of peptone water that was added to the organ. This assumed a 1.0 g/mL density of the peptone water. The ratio of peptone water

added created a dilution of 10^{-1}. These organs were homogenized, then serial 10 fold dilutions were plated in MRS agar and Beerens' modification #5 to determine if the probiotics present in the diet were able to colonize. The samples were incubated at 37˚C under anaerobic conditions, for 24 h for MRS agar and 48-72 h for Beerens' agar.

3.5.5.2 Liver and spleen analyses

The liver and spleen were collected and weighed, and only one-half of each organ was used for the experiment. These organs were homogenized and serial dilutions were then plated on Blood agar to determine the presence of *L. monocytogenes.* Samples were incubated at 35˚C aerobically for 24-48 h.

3.5.5.3 Gallbladder analyses

The gallbladder was collected to verify the presence of the pathogen. The whole organ was collected, including the bile. This organ is so fragile that it was difficult not to break it in the collection process. The gallbladder was plated in MOX agar and incubated at 35˚C for 24 h.

3.5.5.4 Cytokine analyses

For this experiment, the serum concentration of six cytokines, TNF-α, IL-6, IFNγ, MCP-1, IL-10, IL-12p70 were determined. The cytokine analysis was performed following the method of Fritsche and others (2005). The concentration of IL-12p70, TNF-α, IFN*γ*, MCP-1, IL-10, and IL-6 in sera samples were determined simultaneously using a commercial multiplexing assay, cytometric bead array (CBA kit 552364, BD Biosciences, San Jose, CA). Blood was collected by cardiac puncture and transferred into sterile vacutainer tubes with

clot activator (BD Biosciences). After 1 h at room temperature, tubes were spun down (1000 × g for 10 min) and serum was decanted into microcentrifuge tubes. Sera samples were stored at -70˚C until assayed. Prior to analysis by CBA, sera samples were thawed and diluted 1:4 with assay diluents. Samples and standards were prepared and analyzed as per the manufacturer's instructions. The flow cytometer used to analyze these samples was a BD FACScan equipped with BD CellQuest and CBA soft-ware. According to the manufacturer, the intra- and inter-assay precision (%CV) for this kit was: 3.7, 4.0, 3.7, 6.6, 2.3, 6.3, and 7.3, 6.7, 8.0, 8.0, 5.7, 9.0, respectively, for IL-12p70, TNF-α, IFN*γ*, MCP-1, IL-10, and IL-6.

3.5.5.5 Identification of colon and cecum colonies

To confirm that the colonies collected from the colon and the cecum on Beerens' modification #5 Agar were probiotics, the API® 50 CH System (bioMérieux® Inc, Durham, NC) was used. This system studies lactic acid bacterial carbohydrate metabolism profile and can be used in combination with the API 50 CHL medium specifically for the identification of *Lactobacillus* and related genera. Samples were prepared according to the manufacturer's instructions.

3.6 Statistical analysis

Fisher's Protected Least Significant Difference of microbial data and mice body weight was performed using the PROC GLM SAS 9.1 (Littell et al., 2006). The analysis of variance was conducted using the Statistical Analysis System

(SAS 9.1, NC, USA). Significant differences between treatments were established at a level of $P = 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Probiotic selection

The probiotic strains tested for the inhibition of in-vitro growth of *Listeria monocytogenes* EGD showed variable results depending on the growth media and incubation conditions (aerobic or anaerobic incubation). The inhibition activity was measured using the well diffusion assay as shown in Figure 4.1. This figure shows how the experiment was arranged. The center well contained peptone water (control) which resulted in no inhibition zone. On the other hand, outer wells contained a different probiotic each, with evidence of inhibition of the pathogen. Two probiotic strains, *Bifidobacterium animalis* B6 and *Lactobacillus acidophilus* ADH, showed more consistent results (Figure 4.2). Other probiotics, such as *Bifidobacterium infantis* 25962 *and Lactobacillus paracasei*, showed less inhibition but consistent results for both incubation conditions. *B. animalis* B6 and *L. acidophilus* ADH were chosen because they not only had the highest and consistent inhibition activity, but according to our previous research (Simoes 2006), these two probiotic strains also showed long-term viability in freeze-dried conditions.

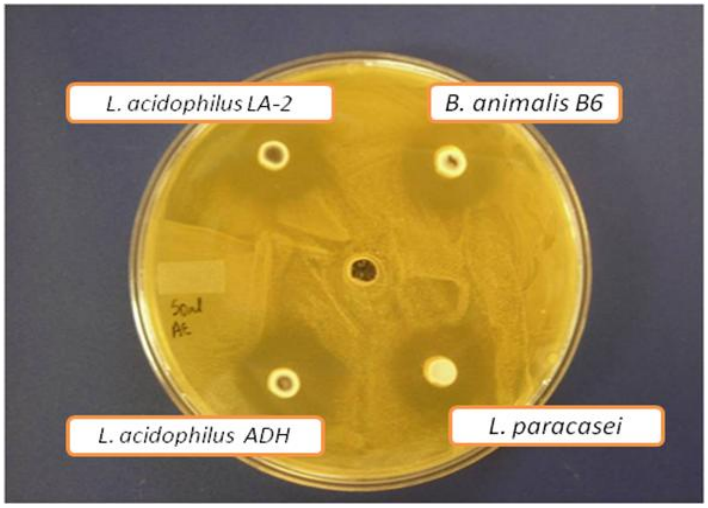

Figure 4.1. Well diffusion assay showing *L. monocytogenes* EGD inhibition zones by some of the probiotics tested. The center well contained peptone water (control).

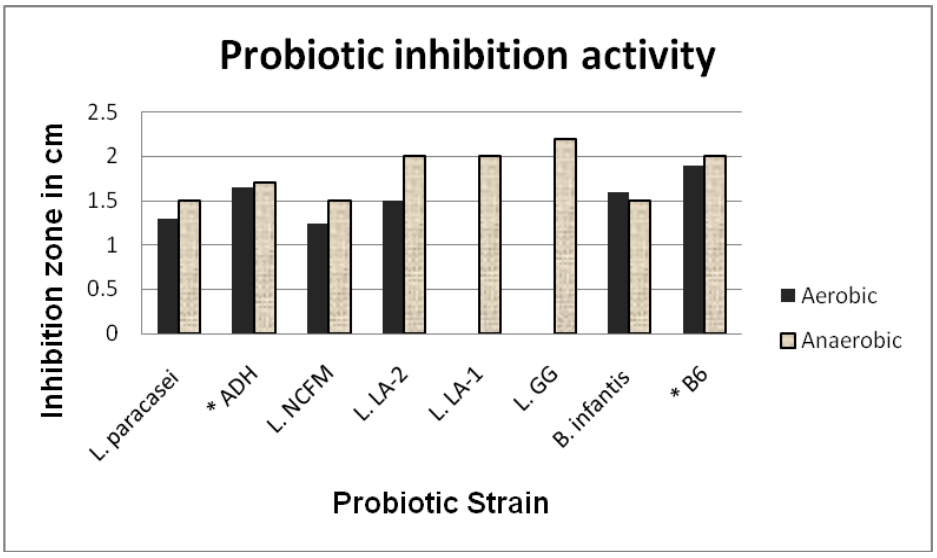

Figure 4.2. Probiotic inhibition of *L. monocytogenes* EGD via the agar diffusion test. * = probiotics chosen as the strains to be used in this experiment.

4.2 Selective media

Due to the origin of the samples (cecum, colon and small intestines), a more selective medium was necessary to allow the enumeration of the probiotics provided. After testing numerous media formulations, it was found that Beerens' agar, which is a known selective medium for bifidobacteria, was not selective enough for growing the *B. animalis* B6 from the colon, cecum or fecal samples. Therefore, the ingredients in Beerens' agar were modified and adjusted, in order to make it selective enough for the probiotics in this study. Some of the ingredients used to modify this medium included propionic acid, lactulose and lithium chloride (Nebra and Blanch 1999).

According to Beerens (1991), propionic acid at the right concentration, inhibits bacteria from the family Enterobacteriaceae and other gram positive bacteria, including *Staphylococcus*. Propionic acid has also been used as a selective agent in the detection of bifidobacteria (Faculty of Pharmacy 1991). Propionic acid was used by Ogden and others (1997) as a preservative in pork meat stored at room temperature. Another modified ingredient was lactulose, a semi synthetic disaccharide. According to Tabatabaie and Mortazavi (2008), lactulose is known for its stimulating effect on intestinal lactic acid bacteria, especially *Bifidobacterium* species. It has been shown that lithium chloride can inhibit the growth of other lactic acid bacteria (Lapierre and others 1992).

As shown in Table 4.1, Beerens' modifications #4 and #5 were the best for *B. animalis* B6, but when comparing the growth of the two probiotics in these two modifications, it was clear that modification #5 also resulted in better growth of *L.*

acidophilus ADH. Even though bacteria in the mice fecal samples grew in both media #4 and #5, growth was only seen at a low dilution of 10^{-1} which was not plated during the actual study. Further, neither gram negative bacterium, *Escherichia coli* nor *Enterobacter cloacae* grew in these two media, indicating the selectivity of the media for gram positives, particularly the lactobacilli and bifidobacteria. Thus, Beerens' modification #5 was subsequently selected as the medium of choice for enumerating the probiotics in the study. The diet and feces from mice fed each probiotic diet were plated on this medium and counts of each probiotic bacterium were found to be high as expected (Table 4.21). The mice used for this experiment were not germ-free, therefore the feces always contain lactic acid bacteria, but when comparing the control feces (no probiotic in the diet) with B6 and ADH diets, a 3-log reduction was observed in the feces of mice fed the control diet, versus a 2-log reduction in that of mice fed the B6 and ADH diets. Although the Beerens' modification #5 did not completely inhibit the fecal microflora, it showed a greater selectivity towards the probiotics out of all eleven media tested.

Table 4.1 Selective media for *Bifidobacterium* and *Lactobacillus*[1].

Organism	Beerens' modification #4	Beerens' modification #5	BMF[2]	MRS[3]
B. animalis B6				
10^{0}	++	++	++	TNTC
10^{-3}	+	+	+	TNTC
L. acidophilus ADH				
10^{0}	+	++	+	++
10^{-3}	+	+	-	++
Mice feces				
10^{-1}	+	++	++	TNTC
10^{-3}	-	-	+	TNTC
E. coli				
10^{0}	-	-	-	+
10^{-3}	-	-	-	+
E. cloacae				
10^{0}	-	-	-	+
10^{-3}	-	-	-	+
L. acidophilus LA-2				
10^{0}	+	+	+	+
10^{-3}	+	+	+	+

[1]TNTC= Too numerous to count; (++) = high growth; (+) = growth; (-) = no growth.
[2]BMF = *Bifidobacterium* medium.
[3]MRS = de Man, Rogosa and Sharpe

Table 4.2 Selective medium confirmation[1].

Sample	Beerens' modification #5	MRS[2]
B. animalis B6 - diet	1.3×10^6	4.0×10^7
L. acidophilus ADH – diet	8.7×10^7	3.3×10^8
B. animalis B6 - feces	7.5×10^6	2.0×10^8
L. acidophilus ADH – feces	3.5×10^6	2.1×10^8
Control feces (No probiotics)	7.2×10^5	1.1×10^8

[1]Counts in CFU/ml.
[2]MRS = de Man, Rogosa and Sharpe agar.

4.3 Microbial analysis of the diet

Each of the three diet treatments, consisting of the soy protein bar, was made in five different batches, vacuum packaged and stored at 4˚C in the dark. The samples were analyzed for microbial counts every four days (Table 3.4), by plating in two different agar media, MRS Agar and Beerens' Modification #5 Agar. For the second and third trials, the initial numbers of probiotics were almost one log higher than that in the first trial. For the third trial, pour plating on Beerens agar was not conducted due to the lack of one ingredient (lactulose). Instead, we continued this part of the experiment using MRS agar. Since the diet was made under controlled conditions, the microbial population in the diet only represents the probiotics that were added to the diet. Thus, the colony counts from MRS agar should not be significantly different from that on the selective

media. In each trial, a one log reduction in probiotic counts was observed after the freeze-dried probiotic was added into the diet. Over a period of two weeks, there was a stable probiotic count for all five batches from each of the three treatments. Thus, the only variable that was not controlled was the exact amount of diet that each mouse was eating, but the weight gained indicated that the animals were, in fact, eating the respective diet.

Table 4.3 Diet probiotic counts (CFU/g) for first trial.

Sample	Freeze Dried	Diet Batch #1		Diet Batch #2		Diet Batch #3		Diet Batch #4		Diet Batch #5	
	MRS	MRS	Beerens	MRS	Beerens	MRS	Beerens	MRS	Beerens	MRS	Beerens
Control	-	0	0	0	0	0	0	0	0	0	0
ADH	6.5×10^9	6.9×10^8	3.2×10^8	4.5×10^8	2.4×10^8	5.8×10^8	2.6×10^8	3.7×10^8	3.0×10^8	4.3×10^8	3.2×10^8
B6	6.9×10^9	3.4×10^8	2.3×10^8	4.7×10^8	3.7×10^8	5.0×10^8	4.3×10^8	4.5×10^8	1.7×10^8	4.1×10^8	1.2×10^8

Table 4.3.1 Diet probiotic counts (CFU/g) for second trial.

Sample	Freeze Dried	Diet Batch #1		Diet Batch #2		Diet Batch #3		Diet Batch #4		Diet Batch #5	
	MRS	MRS	Beerens	MRS	Beerens	MRS	Beerens	MRS	Beerens	MRS	Beerens
Control	-	0	0	0	0	0	0	0	0	0	0
ADH	3.9×10^{10}	3.1×10^9	2.6×10^9	4.3×10^9	2.8×10^9	2.6×10^9	1.1×10^9	3.0×10^9	1.3×10^9	2.1×10^9	1.0×10^9
B6	1.1×10^{10}	1.3×10^9	9.3×10^8	1.2×10^9	7.2×10^8	6.1×10^8	4.2×10^8	2.9×10^9	8.6×10^8	2.2×10^9	8.9×10^8

Table 4.3.2 Diet probiotic counts (CFU/g) for third trial.

Sample	Freeze Dried	Diet Batch #1	Diet Batch #2	Diet Batch #3	Diet Batch #4	Diet Batch #5
	MRS	MRS	MRS	MRS	MRS	MRS
Control	-	0	0	0	0	0
ADH	9.8×10^{10}	4.1×10^9	2.9×10^9	2.0×10^9	1.8×10^9	1.7×10^9
B6	3.7×10^{10}	3.2×10^9	1.7×10^9	1.2×10^9	1.0×10^9	8.9×10^8

4.4 *L. monocytogenes* strain selection

There were five mice per treatment (treatment #1: *L. monocytogenes* EGD and treatment #2: *L. monocytogenes* Scott A) in this study. The oral inoculum concentration for both strains was 10^8 cfu/ml per mouse. The samples were plated in Modified Oxford (MOX) agar and incubated for 48 h at 37˚C under aerobic conditions. The results revealed that some inconsistencies were found for both strains although they were similar in their infection of the mice (Table 4.4). Since the EGD strain is known to be a suitable strain for infecting mice, and it has been used successfully in the past for numerous mouse studies, this strain was selected for use in this study. According to Irons and others (2003), the route and dose of the *L. monocytogenes* EGD strain must be chosen carefully because the route of inoculation and dose can affect the results of the experiment. For all their previous experiments, the route of infection was intraperitoneal (i.p.), but for the experiment cited here, the bacterial challenges were given intravenously (i.v.), at a concentration of 10^5 or 10^6 cfu of bacteria per mouse.

For the current study, an oral challenge was conducted, due to the nature of the experiment described herein. The mice were fed a probiotic-supplemented diet for two weeks. It was expected that the probiotic would colonize the colon during this time and reduce a listerial infection. The pathogen had to go through a number of barriers, such as stomach acids, natural colonic microflora and/or in this case, probiotics, and survive to be able to infect the host. This is why the

challenge concentration given to the mice was higher than that used by Irons and others (2003).

Table 4.4 Isolation of *L. monocytogenes*[1] from mice.

EGD Strain	**Scott A Strain**
Small Intestine **E1** = 2.7 x 10^4 **E2** = <25 **E3** = 1.8 x 10^4 **E4** = <25 **E5** = <25	**Small Intestine** **S1** = 5.4 x 10^4 **S2** = <25 **S3** = 2.2 x 10^3 **S4** = <25 **S5** = 1.5 x 10^4
Gallblader **E1** = 2.5 x 10^3 **E2** = <25 **E3** = 2.5 x 10^3 **E4** = 1 x 10^2 **E5** = <25	**Gallblader** **S1** = 2.5 x 10^3 **S2** = <25 **S3** = 2.5 x 10^3 **S4** = <25 **S5** = 2.5 x 10^3
Cecum Tissue **E2** = 1.1 x 10^4 **E3** = 1.0 x 10^5 **Cecum Content** **E1** = 4.0 x 10^5 **E2** = 5.2 x 10^3 **Cecum (whole)** **E4** = 2.6 x 10^4 **E5** = 6.3 x 10^4	**Cecum Tissue** **S1** = 3.9 x10^4 **S2** = 1.2 x 10^3 **Cecum Content** **S2** = 1.7 x 10^5 **S3** = 2.5 x 10^3 **Cecum (whole)** **S4** = 5.0 x 10^4 **S5** = 3.0 x 10^5

[1]E= *L. monocytogenes* EGD; S= *L. monocytogenes* Scott A.

4.5 Mice weight

4.5.1 Weights from preliminary study

A group of 17 mice were divided into two diet treatments, one treatment without probiotics and another treatment with probiotics, to see if there would be a change in the body weight of the animals due to a difference in the diet. Each treatment group was then divided into males and females and weighed on days 0, 7 and 14. Mice fed the probiotic treatment gained weight as did those fed the control treatment, indicating the suitability of the formulated diets (Table 4.5). It was also confirmed that the mice, in fact, ate the soy protein diet.

4.5.2 Body weight from trials

Each trial was divided into three treatment groups, control diet group containing no probiotics, B6 diet group containing *B. animalis* B6, and ADH diet group containing *L. acidophilus* ADH. The mice from all three trials were weighed on days 0, 7 and 14, to see if there would be a change in their weight due to the presence or absence of probiotics in the diets. The mice were 5-7 week old females at the start of the experiment. According to Poiley (1972), the range of average body weight of a 5-7 week female mouse is 14.2 to 21.5 g. Each treatment had a group of 4-5 mice that were not challenged with the pathogen, but fed with the respective diet treatments. As shown in Tables 4.6 to 4.8.2 (APPENDIX), the mice from all of the treatments in this category gained weight, and were between the average weight range of approximately 18 -21 g. On the other hand, the mice from all three trials that were challenged with the pathogen lost weight (1-3 g) but they were still in the average range.

Table 4.5 Weight gain of mice fed optimized probiotic diets.

Animal weight (g)			
Treatment – Gender	**Day 0**	**Day 7**	**Day 14**
Control – Females	19.1	17.5	18.6
	17.9	16.2	19.9
	16.2	18.3	18.5
	18.0	17.7	18.9
	17.9	17.9	17.1
Average weight	***17.8***	***17.5***	***18.6***
Control – Males	14.7	20.6	23.5
	14.7	20.2	23.6
	16.6	20.7	23.5
Average weight	***15.3***	***20.5***	***23.5***
+Probiotic –Females	21.8	21.3	21.6
	21.5	22.7	20.4
	22.4	21.0	21.5
	20.5	21.3	23.7
	23.6	20.1	21.6
Average weight	***21.9***	***21.3***	***21.8***
+Probiotic – Males	24.3	23.9	26.6
	23.9	24.3	26.3
	21.8	25.2	24.5
	23.8	24.9	27.4
Average weight	***23.5***	***24.6***	***26.2***

4.5.2.1 First trial

The statistical analysis showed no significant differences in mice weight between treatments before the *L. monocytogenes* EGD challenge. No significant differences in weight ($P > 0.05$) between the treatments were observed even at 3 days post infection (P.I.) when compared with the control group. Specifically, when the two probiotic treatments were compared to the control group, the P values were $P = 0.1032$ and $P = 0.7646$, respectively. Table 4.5.1 shows the average daily gain mean for all three treatments (Control, B6 and ADH). This table shows that the mice did not loose any considerable weight as a symptom of disease.

Table 4.5.1 ADG Average daily gain (ADG) mean (first trial)

	ADG LS Mean per treatment			**ADG Mean all treatments**
Treatment	*B. animalis* B6	Control	*L. acidophilus* ADH	
Challenged	0.03823529	0.01470588	-0.02573529	**0.00906863**
Unchallenged	0.13393665	0.08235294	0.06470588	**0.22901961**
Average Overall value	***0.13393665***	***0.08235294***	***0.06470588***	

4.5.2.2 Second trial

When compared with the control group, no significant differences in weight between the treatments were observed ($P > 0.05$). Table 4.5.2 shows that both

probiotic treatments maintained the weight for both challenged and unchallenged groups, while control group lost some weight.

Table 4.5.2 Average daily gain (ADG) mean (second trial)

	ADG LS Mean per treatment			**ADG Mean all treatments**
Treatment	*B. animalis* B6	Control	*L. acidophilus* ADH	
Challenged	0.04640523	-0.02125000	0.06764706	0.03294118
Unchallenged	0.16352941	0.21058824	0.15411765	0.17607843
Average Overall value	***0.08823529***	***0.06791855***	***0.10270982***	

4.5.2.3 Third trial

The statistical analysis shows that, when compared with the control group, no significant differences in weight between the treatments ($P > 0.05$) were observed. This may possibly mean that the diet did not aggravate weight lose or induce gain weight in the mice. As shown in table 4.5.3.

Table 4.5.3 Average daily gain (ADG) mean (third trial)

	ADG LS Mean per treatment			**ADG Mean all treatments**
Treatment	B. animalis B6	Control	L. acidophilus ADH	
Challenged	0.05441176	0.12941176	0.11229947	0.10313725
Unchallenged	0.19411765	0.22058824	0.220588424	0.21176471
Average Overall value	***0.10098039***	***0.15372549***	***0.14117647***	

4.6 Samples analyses

4.6.1 Colon and cecum analyses

It has been shown that *L. monocytogenes* is able to survive the acid conditions within the stomach and the bile salts in the small intestine (Gahan and Hill 2004). The low pH of the stomach should be significantly effective against *L. monocytogenes* infections, but according to Donnelly (2001), individuals that are treated with antacids may have a reduced resistance to this pathogen. Once the pathogen crosses these barriers, it is able to colonize the gastrointestinal tract. *L. monocytogenes* has been isolated from the feces of 1-5% of healthy human individuals (Hof 2001).

4.6.1.1 First trial

For the first trial, the samples were plated on both MRS Agar and Beerens' modification #5, but the dilutions made for Beerens' Agar were not low enough to be able to get a credible count. Thus, for this trial, the data presented are only the counts on MRS Agar from both the colon and cecum (Figures 4.3 and 4.4). In other words, only the lactic acid bacterial counts were obtained instead of both lactic acid bacteria and probiotics. There were no significant differences between the lactic acid bacterial count for the three diet treatments.

The *L. monocytogenes* challenge for this trial had a higher concentration (10^9 cfu) than that from the preliminary study (10^8 cfu). This may be the factor that changed the expected probiotic inhibition activity. As shown in Figure 4.5,

the listerial count in the cecum was not significantly different between the treatments, but the *B. animalis* B6 diet was able to decrease the pathogen by almost half a log when compared with the control group. However, this does not represent a significant statistical difference. When the two treatments were compared with the control group, the P values were 0.4011 for the B6 diet and 0.8026 for the ADH diet. The *L. monocytogenes* count in the cecum was not significantly different between the treatments, but the *B. animalis* B6 diet was able to decrease the pathogen almost half a log when compared with the control group.

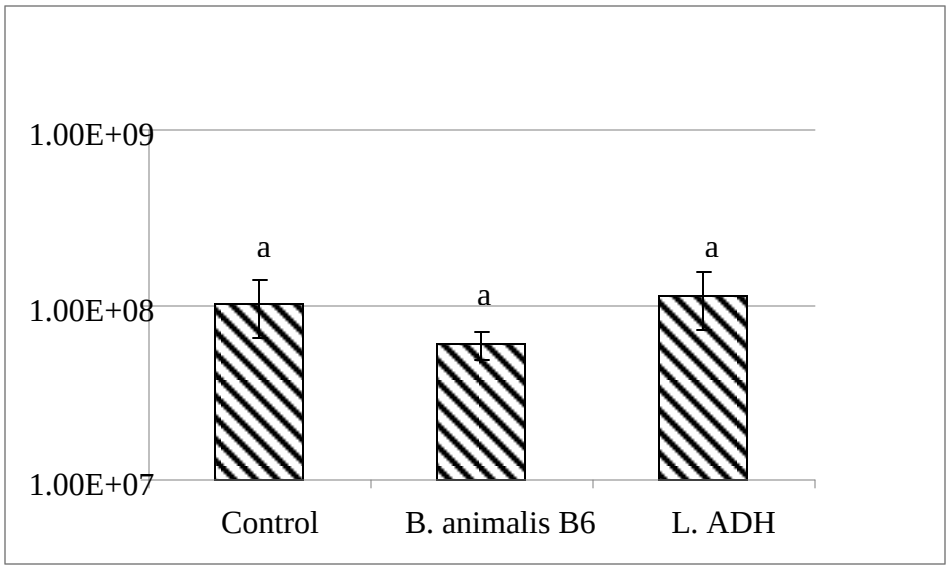

Figure 4.3. Counts of lactic acid bacteria in the colon of mice on MRS Agar. Means with same letters indicate no significant differences (*P* > 0.05).

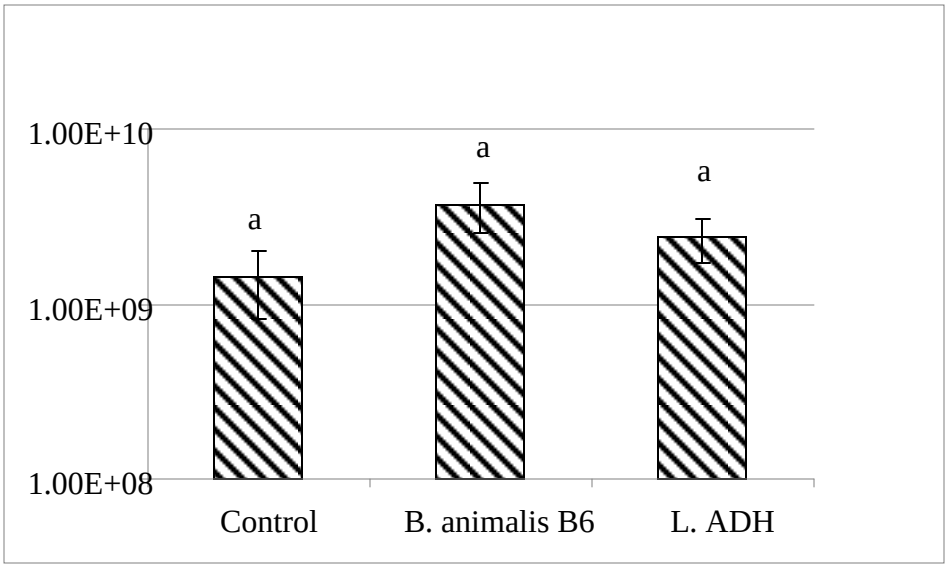

Figure 4.4. Counts of lactic acid bacteria in the cecum of mice. Means with same letters indicate no significant differences (*P* > 0.05).

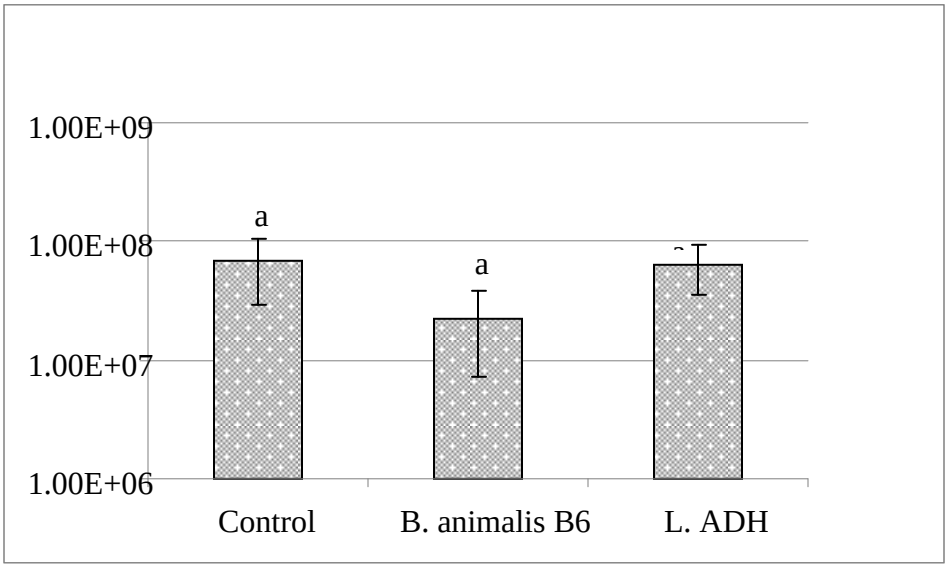

Figure 4.5. Counts of *L. monocytogenes* EGD in the cecum of mice. Means with same letters indicate no significant differences (*P* > 0.05).

4.6.1.2 Second trial

For the second trial, then number of dilutions made for Beerens' Agar was decreased, and the pathogen inoculum was lowered to (10^8 cfu). Thus, probiotic counts were obtained on this selective media and compared with the lactic acid bacterial counts on MRS. As shown in Figure 4.6, no counts were found for the control group on Beerens' modification #5. This meant that the probiotics were not detected in the colon of the control group, in contrast to the experimental groups where probiotic colonization was evident. This shows a significant difference between the treatment groups and the control group ($P \leq 0.05$). A significant difference in counts between the control group and the two treatment groups in the cecum were also found (Figure 4.7).

The *L. monocytogenes* count in the cecum was significantly different from that of the control group. Figure 4.8 shows that the ADH group had a complete log reduction of the pathogen when compared to the control treatment ($P \leq 0.05$). In the first trial, the B6 treatment showed a small reduction. However, in this trial, the ADH treatment reduced the pathogen count the most.

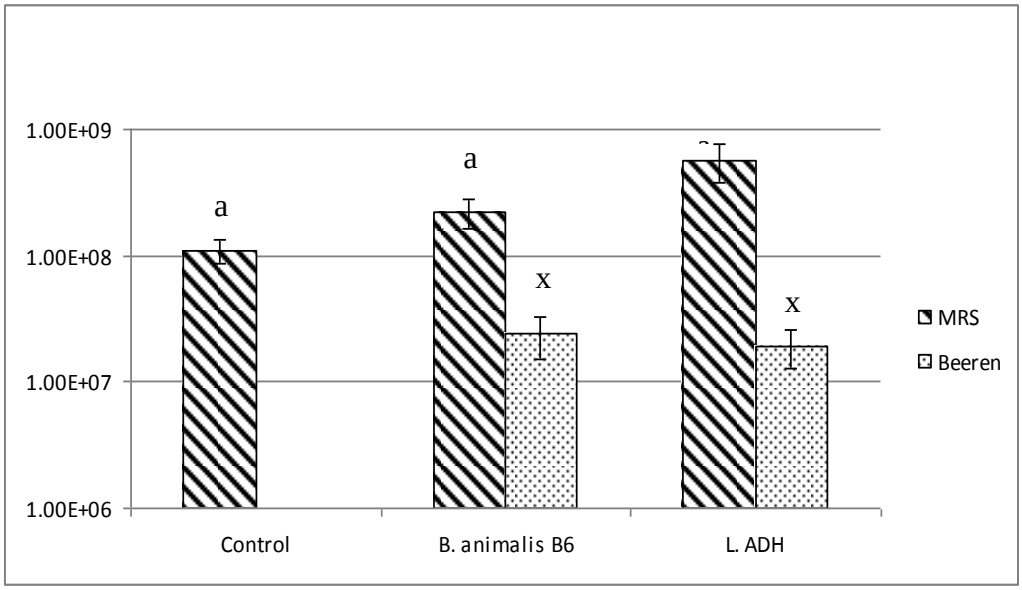

Figure 4.6. Counts of probiotics in the colon of mice. Means with same letters indicate no significant differences (*P* > 0.05).

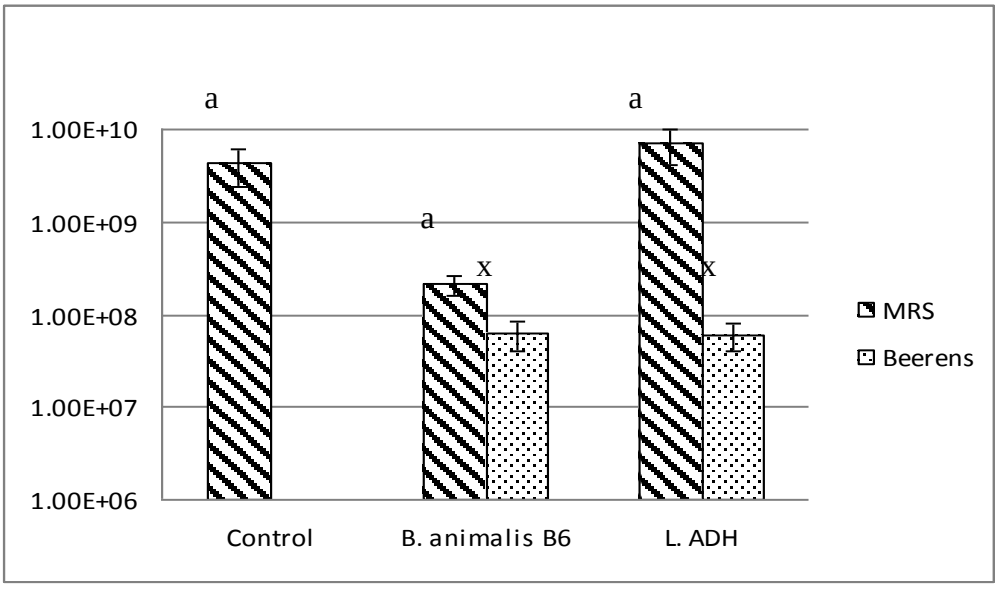

Figure 4.7. Counts of probiotics in the cecum of mice. Means with same letters indicate no significant differences (*P* > 0.05).

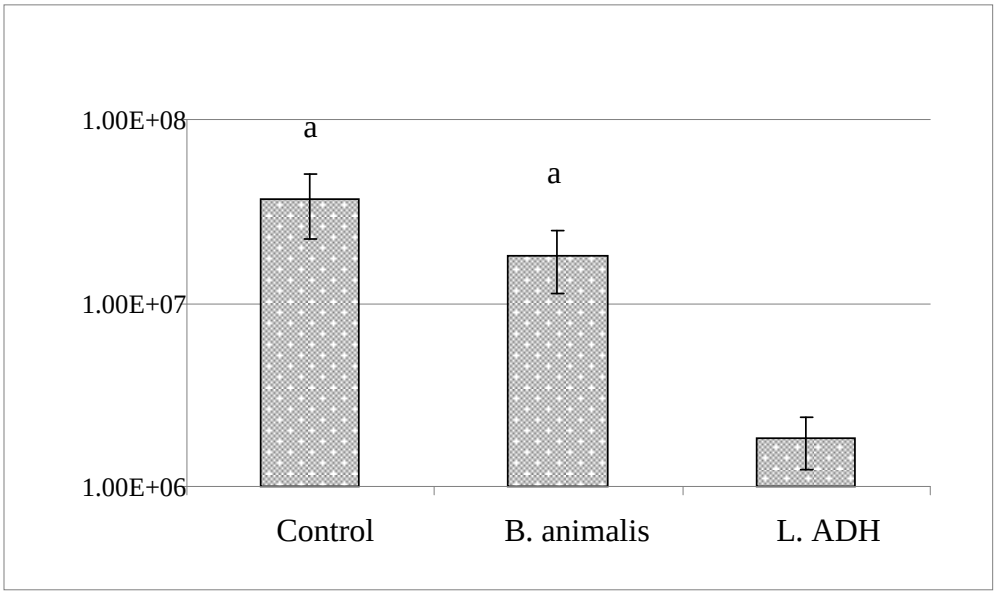

Figure 4.8. Counts of *L. monocytogenes* EGD in the cecum of mice. Means with same letters indicate no significant differences (*P* > 0.05).

4.6.1.3 Third trial

For the third trial, the dilutions made for Beerens' modification #5 Agar and MRS Agar included one dilution lower and one dilution higher of those from the second trial. Therefore, countable plates were generated for both media, allowing for the enumeration of both lactic acid bacterial and probiotic counts. No counts were generated on Beerens' modification #5 for the control group (Figure 4.9). On the other hand, both treatments showed considerably high counts as evidence of probiotic colonization. The same occurrence was observed in the cecum (Figure 4.10), and a significant difference ($P \leq 0.05$) between the control group and both probiotic treatment groups were observed. The ADH group resulted in a complete log reduction in listerial counts. The difference between

the control group and the B6 group was not as significant even though it was a reduction in numbers. This was consistent with the results from the second trial.

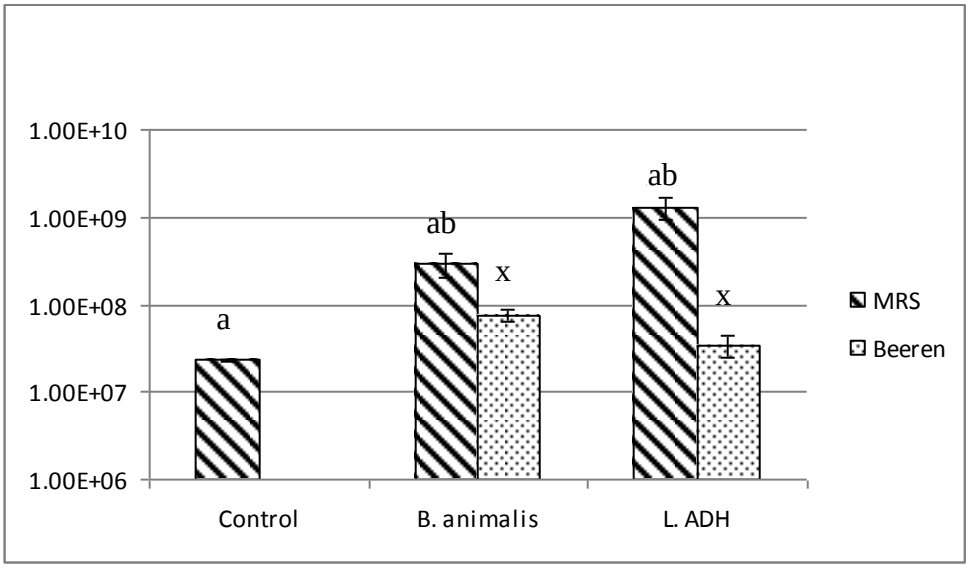

Figure 4.9. Counts of probiotics in the colon of mice. Means with same letters indicate no significant differences (*P* > 0.05).

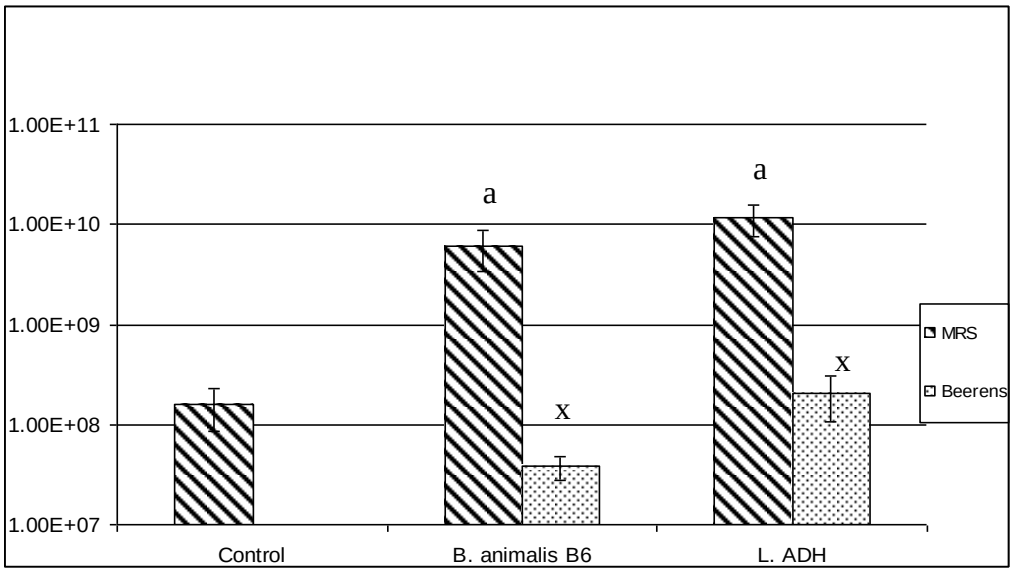

Figure 4.10. Counts of probiotics in the cecum of mice. Means with same letters indicate no significant differences (*P* > 0.05).

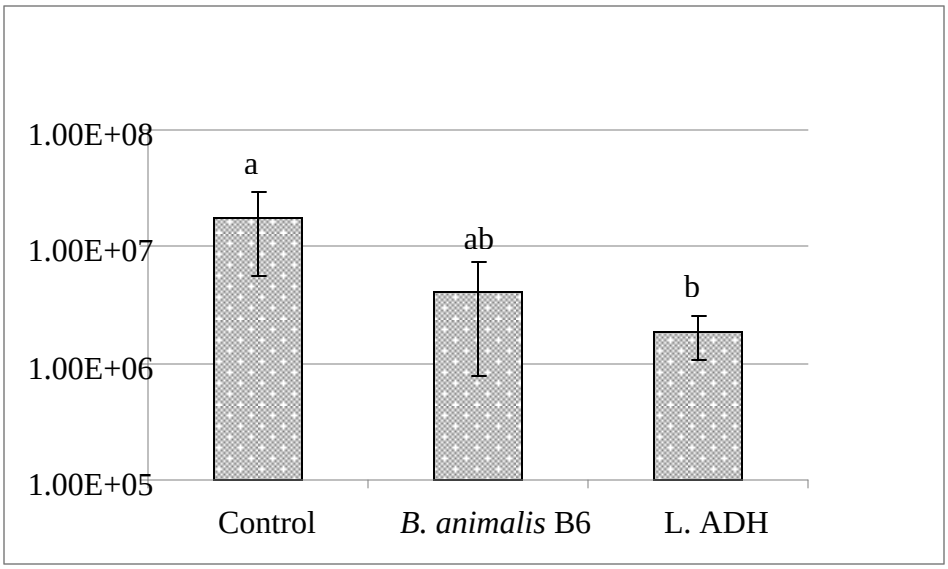

Figure 4.11. Counts of *L. monocytogenes* EGD in the cecum of mice. Means with same letters indicate no significant differences (*P* > 0.05).

4.6.2 Liver and spleen analyses

According to Lecuit and others (2001), once *Listeria* survives the intestinal barriers, it is able to multiply in the small intestine followed by dissemination to lymph nodes, liver and spleen. In the liver, this pathogen can invade and multiply extensively in hepatocytes (Conlan and North 1992).

4.6.2.1 First trial

For the first trial, a significant reduction of *L. monocytogenes* counts was not observed for the spleen in any of the three dietary treatments. However, the *B. animalis* diet decreased the *Listeria* load by almost half a log even though the difference was not significant ($P > 0.05$) (Figure 4.12). The same happened in the liver, where no significant differences were observed.

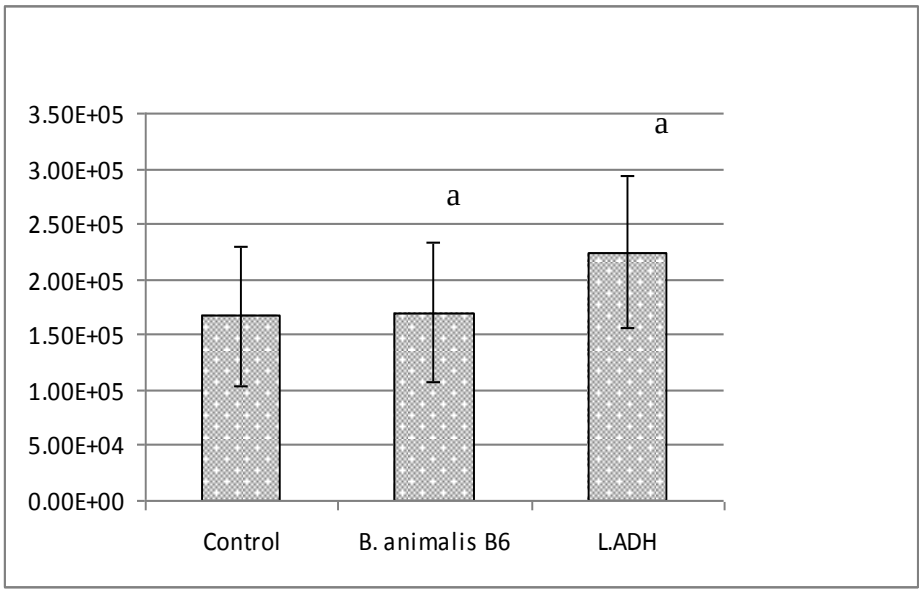

Figure 4.12. *L. monocytogenes* count in the spleen of mice. There were no significant differences between treatments (*P* > 0.05)

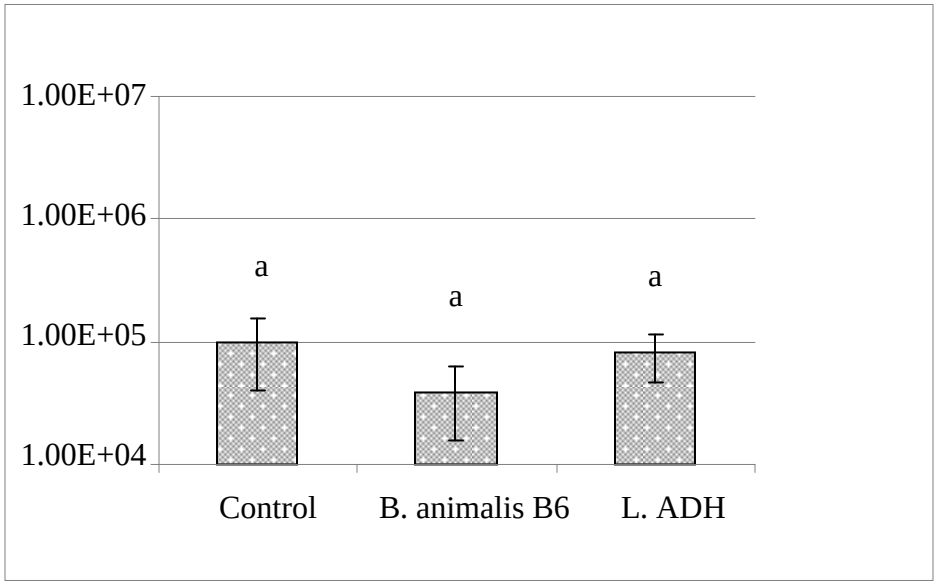

Figure 4.13. *L. monocytogenes* count in the liver of mice. No significant difference among treatments were seen (*P* > 0.05).

4.6.2.2 Second trial

For the second trial, a reduction in *L. monocytogenes* counts for both of the treatments in both organs (liver and spleen) were seen when compared to the control group (Figures 4.14, 4.15). The two probiotics, *B. animalis* B6 and *L. acidophilus* ADH, had different outcomes: *B. animalis* B6 had a significant effect in reducing listerial counts in the liver. On the other hand, *L. acidophilus* ADH had more impact in the spleen, with a complete log reduction of the pathogen ($P \leq 0.05$).

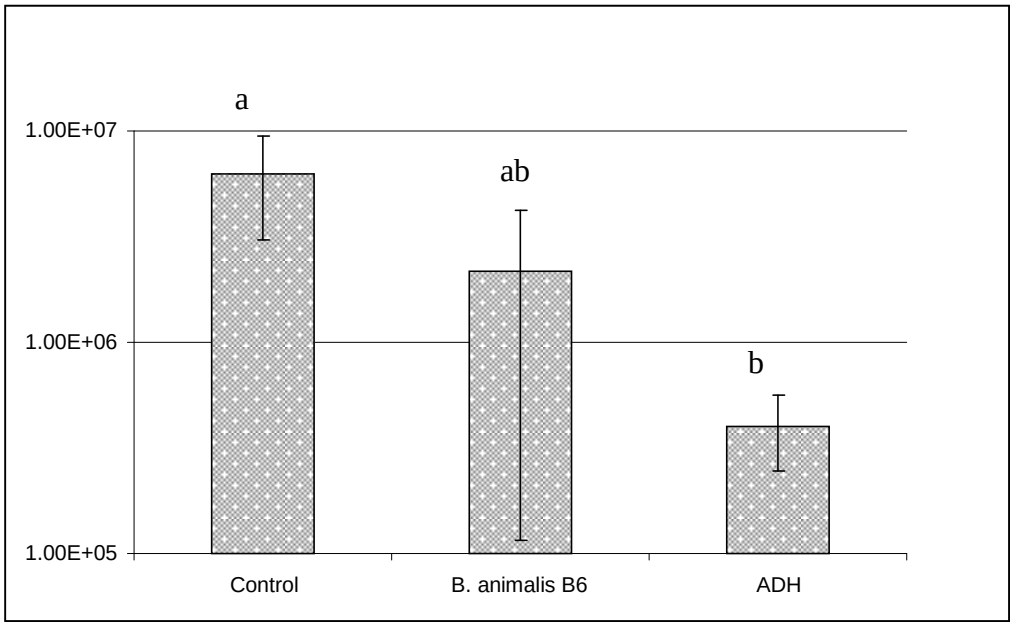

Figure 4.14. *L. monocytogenes* count in the liver. Means with same letters indicate no significant differences ($P > 0.05$; n= 8-9).

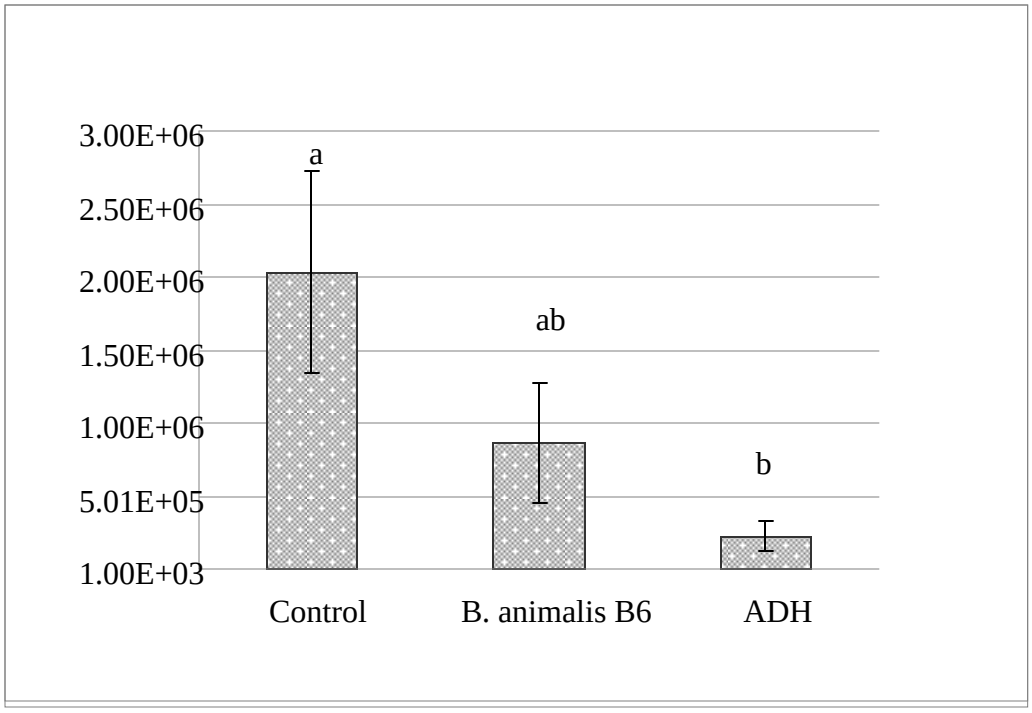

Figure 4.15. *L. monocytogenes* count in the spleen. Means with same letters indicate no significant differences (*P* > 0.05; n=9).

4.6.2.3 Third trial

For the third trial, the inoculum concentration was the same as that from the second trial. However, no significant differences in the reduction of the pathogen in the spleen or the liver were observed, as shown in Figures 4.16 and 4.17.

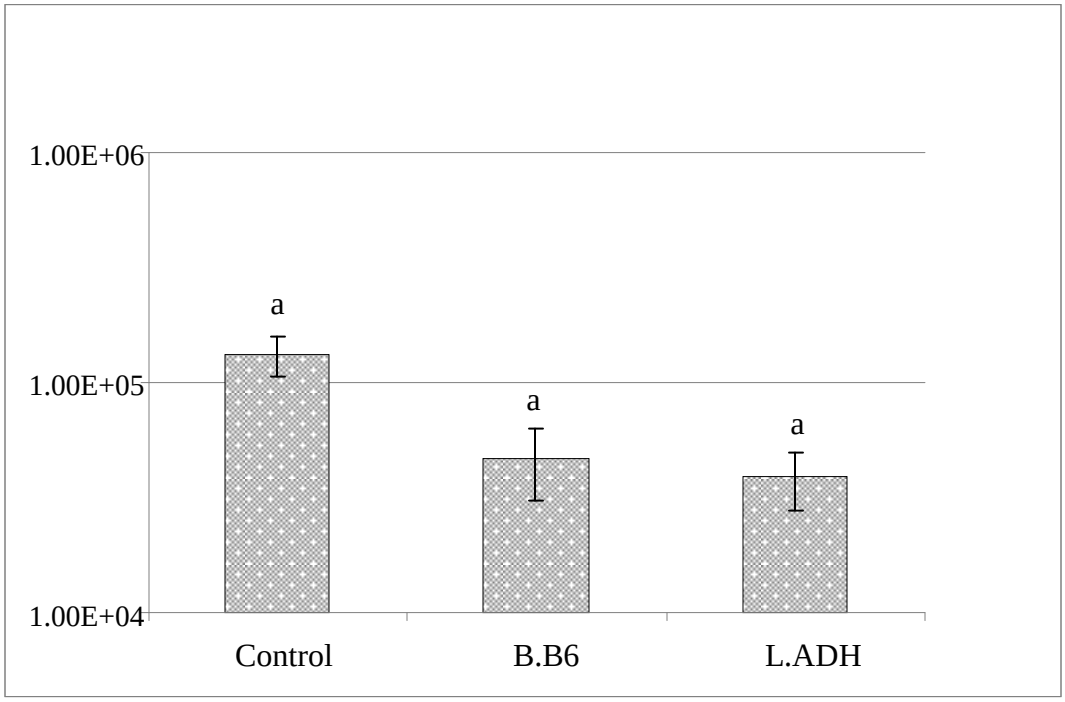

Figure 4.16. *L. monocytogenes* count in the liver. Means with same letters indicate no significant differences (*P* > 0.05; n= 8-11).

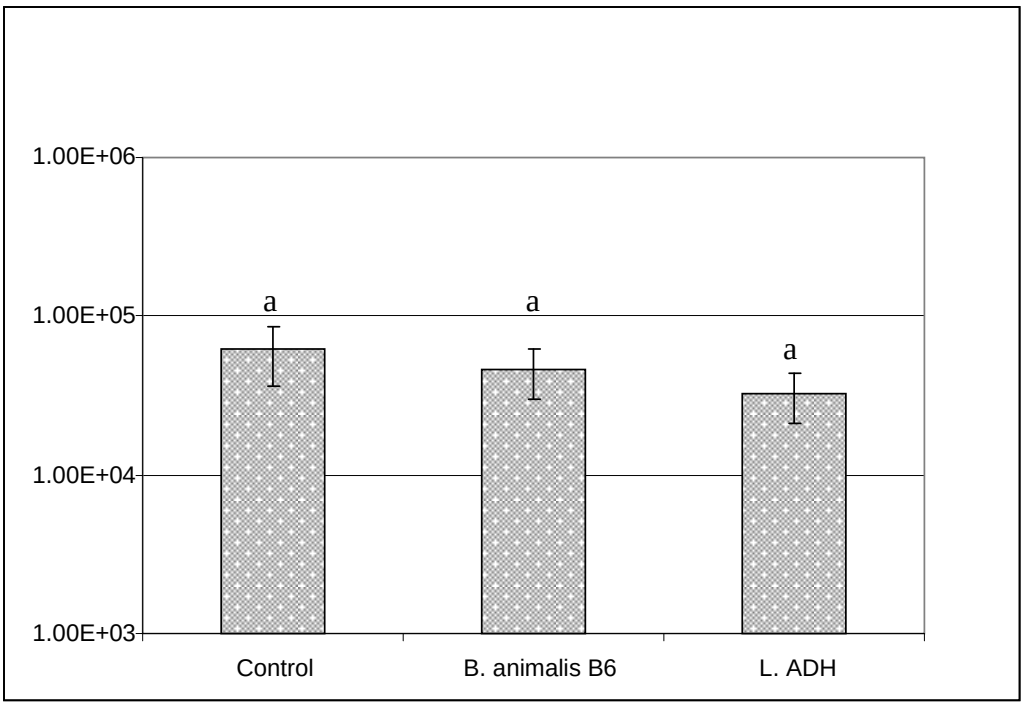

Figure 4.17. *L. monocytogenes* count in the spleen. Means with same letters indicate no significant differences (*P* > 0.05; n= 8-11).

4.6.3 Gallbladder analyses

According to Hardy and others (2004), *L. monocytogenes* colonizes the gallbladder of mice. This organ may be a source of chronic shedding of the pathogen in a similar way to *Salmonella* Typhi. According to their study, in either intravenous or oral routes of infection the pathogen can replicate extracellularly within the lumen of this organ. This shows that *L. monocytogenes* can survive the high concentrations of bile. This organ is so fragile that it was difficult not to rupture it during the collection process. Thus, the results from the first trial were very inconsistent. Taking into consideration how many organ samples were collected from each mouse, it was decided not to collect the gallbladder for the other two trials, thus making the lapse of time between collection and plating

shorter. Studies indicate the culturing of the gallbladder but not as a whole and integral organ (Peter and others 1996).

4.6.4 Cytokine analyses

Cytokines are proteins that act as chemical communicators between cells when an infection is taking place. Listerial infection activates some cytokines that are essential to activate the immune system and subsequently the survival of the host. The presence of these cytokines confirms the infection of the host. In other words, when these proteins are in lower amounts, the infection is not as invasive. Some of the cytokines that are produced to protect the host during a listerial infection, include tumor necrosis factor alpha (TNF-α) (Nakane and others 1988), interleukin-6 (IL-6) (Nakane and others 1999: Liu, and others 1995), interferon gamma (IFNγ) (Barber and others 2005) and monocyte chemoattractant protein-1 (MCP-1).

4.6.4.1 Second trial

For this trial, the mice fed the *L. acidophilus* ADH diet produced significantly less pro-inflammatory cytokines at the peak of infection. Thus, lower production of interferon-gamma was seen. This cytokine is an important biomarker of the host response to *Listeria* infection. There was no detectable TNF-α, IL-6 or IFNγ in any of the non-challenged mice. Plasma cytokines were measured by cytometric bead array (BD Biosciences). Values represent means $\pm$ SEM (n = 12); * $P < 0.05$ vs. control (Figure 4.18).

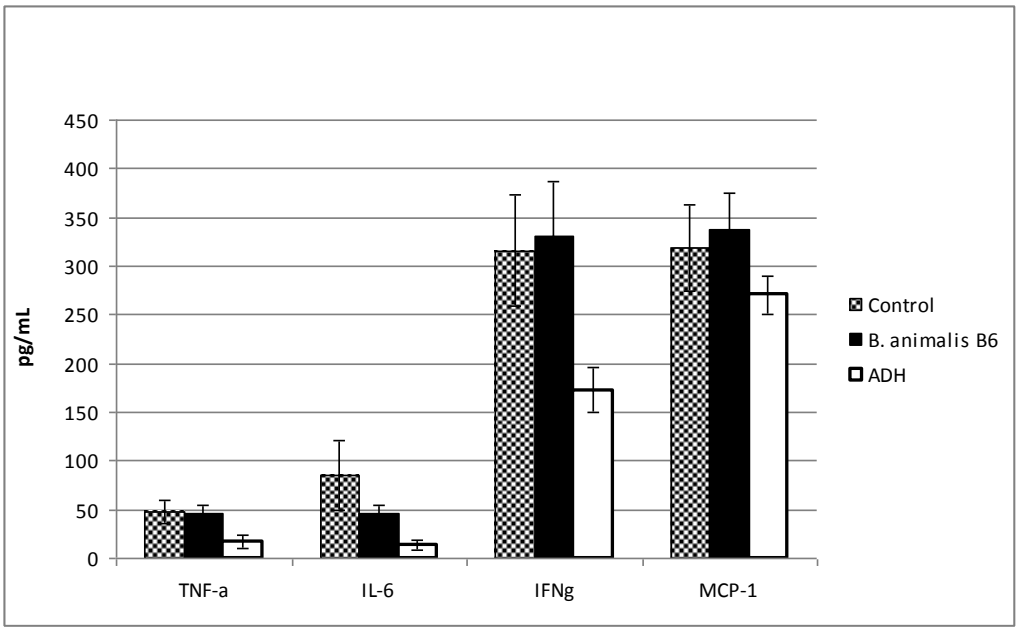

Figure 4.18. Cytokines in the blood. Mice fed the diets with *Lactobacillus* tended to have lower circulating pro-inflammatory cytokines at the peak of infection. Values represent means ± SEM (n = 12); * *P* ≤ 0.05 vs. control.

4.6.5 Identification of colonies in the colon and cecum

To confirm that the colonies collected from the selective media were, in fact, the probiotics fed in the diet, some colonies were tested for identification. For all three trials, 5 different colonies from the two agar media (MRS Agar and Beerens' #5 Agar) used for lactic acid bacterial and probiotic count, respectively, were isolated and tested for Gram stain, and the fermentation of a series of 49 carbohydrates. The API® 50 CH System (bioMérieux® Inc, Durham, NC) was used to perform the carbohydrate test. Specifically, API 50 CHL medium was used. Pure cultures of *L. acidophilus* ADH and *B. animalis* B6 were used as controls. The results of some of these colonies are shown in Table 4.9. All the

colonies were gram positive rod. The API 50 CHL results showed that colonies collected from Beerens' #5 Agar fermented the same carbohydrates and those colonies collected from MRS Agar had several differences when compared to the two controls. One colony (Beerens' #5, colon, 415) had one difference in the carbohydrate N Acetyl glucosamine when compared with the two controls.

Table 4.9 Colonies tested for identification by API.

Medium Agar	Organ	Number of the mouse
Beerens' #5	Cecum	424
Beerens' #5	Colon	415
Beerens' #5	Colon	1014
MRS	Colon	1062
MRS	Colon	406

Table 4.9.1 Carbohydrate fermentation of different colonies.

Carbohydrates Test	L. ADH	B. B6	424	415	406	1062
Control	-	-	-	-	**?**	**-**
Glycerol	-	-	-	-	**+**	**+**
Erythritol	-	-	-	-	-	-
D-Arabinose	+	+	+	+	**?**	**+**
L-Arabinose	-	-	-	-	-	-
Ribose	-	-	+	-	**+**	**+**
D-Xylose	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-
β Methyl-xyloside	-	-	-	-	-	-
Galactose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-
Dulcitol	+	+	+	+	+	+
Inositol	?	?	?	?	**-**	?
Mannitol	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+
α Methyl-D-mannoside	-	-	-	-	-	-
αMethyl-Dglucoside	-	-	-	-	-	-
N Acetyl glucosamine	-	-	-	**+**	**+**	**+**
Amygdaline	+	+	+	+	+	+
Arbutine	+	+	+	+	+	+
Esculine	+	+	+	+	+	+

Salicine	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Maltose	+	-	-	-	+	+
Lactose	-	-	-	-	-	?
Meliobiose	-	-	-	-	-	-
Saccharose	-	-	-	-	+	+
Trehalose	+	+	+	+	+	+
Inuline	-	-	-	-	-	-
Melezitose	+	+	+	+	+	+
D-Raffinose	-	-	-	-	-	-
Amidon	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-
β Gentiobiose	-	-	-	-	?	-
D-Turanose	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-
D-Tagatose	+	+	+	+	+	+
D-Fucose	-	-	-	-	-	-
L-Fucose	+	+	+	+	+	+
D-Arabitol	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-

CHAPTER 5

CONCLUSIONS

In this study, a suitable mice diet that included a probiotic-fortified soy energy bar was successfully manufactured. A suitable recovery medium to enumerate the probiotics in mice feces and intestinal tracts was designed utilizing Beerens' agar with several modifications.

Animal studies have shown that *Listeria monocytogenes* can be inhibited by probiotics upon consumption (Mahoney and Henriksson 2003). The two probiotics used in this study were able to colonize the GI tract as shown by the difference in counts between the MRS agar and the modified Beerens' agar, which is selective for *Bifidobacterium animalis* B6 and *Lactobacillus acidophilus* ADH. Samples from mice fed the control diet showed no growth in Beerens' agar because their diets did not contain any probiotics. In contrast, colon and cecum samples from mice fed the B6 and ADH diets showed considerable colony counts on this agar medium. The first trial showed that *B. animalis* B6 was the more effective probiotic to inhibit the pathogen, but the opposite result was observed in the second trial. This may have been due to various factors. For one, the *L. monocytogenes* EGD inoculum in the first trial was higher (10^9 cfu/ml) than the one for the second trial (10^8 cfu/ml). The inoculum in the first trial may have been too high to allow for sufficient survival of the probiotics. Further, numbers of *L. acidophilus* ADH in the ADH diet for the second trial was generally higher than the numbers of *B. animalis* B6 in the B6 diet, which may have also affected the results in the second trial. The third trial had the same *L.*

monocytogenes inoculum as the second. These two trials had some consistencies between each other. Mice fed the ADH diet decreased by a log in listerial counts in the cecum and the liver.

In conclusion, this study demonstrates that both probiotics, *Bifidobacterium animalis* B6 and *Lactobacillus acidophilus* ADH, were capable of diminishing the in vitro growth of the common food-borne pathogen, *L. monocytogenes*. It was also demonstrated that both probiotics successfully colonized the large intestine (i.e. colon and cecum) when provided to mice in a diet containing the soy protein bar. *L. acidophilus* ADH tended to improve in vivo host resistance to an oral challenge with *L. monocytogenes* EGD, as shown by a lower bacterial load in the liver and/or spleen. The tendency for circulating pro-inflammatory cytokines to be lower in mice fed probiotics suggests that these "good" bacteria diminish the ability of a pathogenic bacterium to infect mice via the oral route. All these results suggest that a soy protein bar enriched with probiotics may hold promise for reducing the risk of human listeriosis.

APPENDIX A
ANOVA and GLM Program for SAS

SAS program to determine effects of probiotic treatments on pathogens

For all three trials

```
options ls=100 ps=70;
data one;
input trt$ count;
lcnt=log10(count);
cards;
DATA DELETED
;
proc print;

proc glm; class trt;
model count lcnt=trt;
means trt;
lsmeans trt/s p;

run;;
```

SAS program to determine change in weight due to treatments

For all three trials

```
Data wt1;  infile input1.csv' dsd firstobs=2 missover ;
input cu$ trt$ id$ bw0 bw7 bw14 bw17;
tgain=bw17-bw0;
adg=tgain/17;
proc print;
proc glm; class cu trt;
model bw0 bw7 bw14 bw17 tgain adg=cu|trt;
means cu|trt;
lsmeans cu|trt/s p;


Data wt2;  infile input2.csv' dsd firstobs=2 missover ;
input cu$ trt$ id$ bw0 bw7 bw14 bw17;
trial=2;
tgain=bw17-bw0;
adg=tgain/17;
proc print;
proc glm; class cu trt;
```

```
model bw0 bw7 bw14 bw17 tgain adg=cu|trt;
means cu|trt;
lsmeans cu|trt/s p;

Data wt3;  infile input3.csv' dsd firstobs=2 missover ;
input cu$ trt$ id$ bw0 bw7 bw14 bw17;
trial=3;
tgain=bw17-bw0;
adg=tgain/17;
proc print;
proc glm; class cu trt;
model bw0 bw7 bw14 bw17 tgain adg=cu|trt;
means cu|trt;
lsmeans cu|trt/s p;
run;

data next; set wt2 wt3;

proc glm; class trial cu trt;
model bw0 bw7 bw14 bw17 tgain adg=trial|cu|trt;
means trial|cu|trt;
lsmeans trial|cu|trt/s p;
run;
```

APPENDIX B

Table 4.1. Diameter (cm) of zone of *L. monocytogenes* EDG growth inhibition on MRS agar by probiotic bacteria after a 24-h incubation.

Probiotic strain	Aerobic incubation	Anaerobic incubation
L. paracasei	1.3	1.5
L. acidophilus ADH	1.65	1.7
L. acidophilus NCFM	1.25	1.5
L. acidophilus LA-2	1.5	2.0
L. acidophilus LA-1	0	2.0
L. rhamnosus GG	0	2.2
B. infantis 25962	1.6	1.5
B. animalis B6	1.9	2.0

APPENDIX C

Selective medium tested for the isolation of probiotic from feces

I. Columbia Agar

Ingredients	Amount
Columbia Agar	42.5g
Glucose	5.0g
Cysteine hydrochloride	0.5g
Agar	5.0g
Propionic Acid	5 or 10mL

*All Beerens' medium tested in this project were not autoclaved and the pH was adjusted to 5 with NaOH. Propionic acid was added after the media was cooled down.

II. Beerens' modification #1

Ingredients	Amount
Columbia	42.5g
Glucose	5.0g
Cysteine hydrochloride	0.5g
Agar	1.5g
Propionic acid	5.0 mL
Lithium chloride	2.25g
Parmomycin sulphate	0.3375g

III. Beerens' modification #2

Ingredients	Amount
Columbia	42.5g
Cellobiose	5.0g
Cysteine hydrochloride	0.5g
Agar	1.5g
Propionic acid	5.0 mL
Lithium chloride	2.25g
Paramomycin sulphate	0.3375g

IV. Beerens' modification #3

Ingredients	Amount
Columbia	42.5g
FOS	5.0g
Cysteine hydrochloride	0.5g
Agar	1.5g
Propionic acid	5.0 mL
Lithium chloride	2.25g
Paramomycin sulphate	0.3375g

V. Trypticase-peptone-yeast-extract medium

Ingredients	Amount
Trypticase soy broth	10g
Peptone	5.0g
Glucose	5.0g
Yeast Extract	2.5g
Tween 80	1.0mL
Cysteine hydrochloride	0.5g
K_2HPO_4	2.0g
$MgCl_2$-$6H_20$	0.5g
$ZnSO_4$-$7H_2O$	0.25g
$CaCl_2$	0.15g
$FeCl_3$	0.041g
Agar	15.0g

VI. Lithium chloride-sodium propionate agar

Ingredients	Amount
Liver Infusion	35.0g
Lactose	20.0g
Bacto-peptone	10.0g
Sodium chloride	2.0g
Lithium chloride	1.0g
Sodium propionate	3.0g
Agar	20.0g

*Adjust pH to 6.7

VII. Lithium chloride-sodium propionate agar modification #1

Ingredients	Amount
Liver Infusion	35.0g
Lactose	20.0g
Bacto-peptone	10.0g
Sodium chloride	2.0g
Lithium chloride	1.0g
Sodium propionate	3.0g
Agar	20.0g

*Same ingredients s the original recipe but pH was adjusted to 5.

VIII. BFM

Ingredients	Amount
Meat Extract	2.0g
Yeast Extract	7.0g
Starch	2.0g
L-cysteine hydrochloride	0.5g
Sodium chloride	5.0g
Peptone	5.0g
Tryptone	2.0g
Lactulose	5.0g
Thiamine chloride hydrochloride	1.0mg
Methylene blue	16mg
Lithium chloride	2.0g
Propionic acid	5.0mL
Agar	15.0g

*Autoclave for 20 min, add propionic acid after cooling down, adjust pH to 5.5

APPENDIX D

Mouse body weight for all three trials

First Trial

Table 1.1.1 Weight of mice fed with control diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged - *Listeria* - Probiotic	1071	17.0g	19.7g	21.3g	20.9g
	1072	19.1g	19.7g	20.7g	22.5g
	1073	19.0g	19.6g	20.6g	21.8g
	1074	18.5g	19.7g	20.9g	21.3g
	1076	16.0g	18.0g	19.2g	19.3g
	Average Weight	*17.9g*	*19.3g*	*20.5g*	*21.2g*
Challenged + *Listeria* - Probiotic	1077	17.5g	19.0g	20.3g	20.1g
	1078	19.2g	20.7g	21.5g	19.5g
	1079	16.9g	17.5g	18.9g	19.4g
	1080	18.1g	19.7g	21.5g	19.5g
	1081	17.6g	18.9g	20.2g	16.1g
	1082	18.3g	20.6g	21.5g	18.1g
	1083	19.3g	20.6g	21.2g	17.5g
	1084	17.7g	19.8g	20.3g	16.4g
	Average Weight	*18.1g*	*19.6g*	*20.7g*	*18.3g*

Table 1.1.2 Weight of mice fed with *B. animalis* B6 diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	1014	17.6g	19.7g	20.7g	21.2g
	1015	16.9g	21.5g	22.5g	25.5g
- *Listeria*	1016	18.5g	21.0g	22.5g	22.6g
+ Probiotic	1017	18.0g	20.6g	21.8g	22.0g
	1018	17.6g	19.3g	21.3g	21.7g
	Average Weight	*17.7g*	*20.4g*	*21.8g*	*22.6g*
Challenged	1019	18.8g	21.0g	22.6g	Died
	1020	18.3g	19.3g	20.9g	17.9g
	1051	16.7g	18.3g	19.6g	17.4g
	1052	19.2g	21.6g	23.5g	21.9g
+ *Listeria*	1053	18.7g	19.7g	21.0g	17.0g
+Probiotic	1054	17.5g	18.7g	19.7g	20.1g
	1055	20.2g	21.3g	22.8g	19.5g
	1056	18.2g	18.8g	20.5g	18.2g
	1057	17.8g	19.3g	20.4g	19.8g
	Average Weight	*18.5g*	*19.8g*	*21.3g*	*18.9g*

Table 1.1.3 Weight of mice fed with *L. acidophilus* ADH diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	1058	19.0g	21.2g	22.8g	23.5g
	1059	18.4g	19.2g	20.4g	21.5g
- *Listeria*	1060	17.9g	20.1g	20.7g	21.6g
+ Probiotic	1061	18.3g	19.8g	21.1g	21.2g
	1062	18.5g	20.2g	22.2g	22.1g
	Average Weight	*18.4g*	*20.1g*	*21.4g*	*22.0g*
Challenged	1063	18.6g	20.3g	21.2g	16.7g
	1064	19.7g	22.6g	23.3g	18.4g
	1065	18.8g	20.9g	22.0g	18.6g
+ *Listeria*	1066	17.5g	19.1g	20.1g	16.5g
+ Probiotic	1067	19.3g	20.3g	21.3g	17.5g
	1068	16.8g	18.7g	19.6g	18.7g
	1069	18.5g	19.9g	21.1g	17.7g
	1070	17.8g	19.8g	21.5g	19.4g
	Average Weight	*18.4g*	*20.2g*	*21.3g*	*17.9g*

Second Trial

Table 1.2.1 Weight of mice fed control diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	443	19.7g	20.3g	21.7g	22.0g
	402	19.1g	20.7g	22.4g	23.1g
- *Listeria*	403	18.9g	22.0g	23.6g	23.7g
- Probiotic	404	17.7g	20.1g	21.4g	22.0g
	452	17.6g	18.7g	19.7g	20.1g
	Average Weight	*18.6g*	*20.4g*	*21.8g*	*22.2g*
Challenged	406	18.2g	18.5g	20.0g	20.9g
	407	19.0g	20.0g	21.1g	21.2g
	453	18.6g	19.4g	21.2g	16.94g
+ *Listeria*	409	18.0g	20.5g	21.4g	19.4g
+ Probiotic	454	20.0g	20.9g	21.8g	17.97g
	411	16.8g	18.3g	19.2g	16.7g
	412	18.9g	19.5g	20.8g	16.7g
	413	17.2g	18.1g	18.6g	14.0g
	Average Weight	*18.3g*	*19.4g*	*20.5g*	*18.0g*

Table 1.2.2 Weight of mice fed *B. animalis* B6 diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	414	20.3g	21.3g	23.0g	22.8g
	415	18.4g	20.7g	22.1g	21.5g
- *Listeria*	455	19.2g	20.4g	21.7g	22.0g
+ Probiotic	417	18.4g	20.1g	21.4g	21.3g
	418	19.1g	20.2g	21.8g	21.7g
	Average Weight	*19.1g*	*20.5g*	*22.0g*	*21.9g*
Challenged	444	20.1g	21.0g	21.8g	19.6g
	420	19.7g	21.4g	22.4g	21.2g
	421	19.1g	20.2g	21.6g	22.0g
	422	17.8g	18.2g	19.5g	19.3g
+ *Listeria*	423	17.9g	19.9g	20.9g	17.3g
+ Probiotic	424	17.7g	19.8g	21.4g	20.0g
	425	18.8g	19.5g	21.2g	20.3g
	426	17.9g	19.3g	21.5g	18.2g
	427	18.6g	18.8g	20.2g	16.8g
	Average Weight	*18.6g*	*19.9g*	*21.3g*	*19.7g*

Table 1.2.3 Weight of mice fed *L. acidophilus* ADH diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	428	17.3g	18.6g	19.6g	19.4g
	429	18.5g	19.8g	21.1g	21.3g
- *Listeria*	430	19.4g	20.9g	22.2g	22.3g
+ Probiotic	431	17.5g	18.9g	19.0g	19.4g
	432	17.6g	18.9g	21.0g	21.0g
	Average Weight	*18.1g*	*19.4g*	*20.6g*	*20.7g*
Challenged	433	18.0g	19.3g	20.5g	18.4g
	445	17.4g	21.3g	23.4g	22.4g
	435	20.4g	20.3g	20.7g	19.5g
+ *Listeria*	446	20.0g	18.9g	20.9g	19.8g
+ Probiotic	437	18.0g	19.1g	20.2g	19.95g
	438	19.2g	20.2g	21.5g	20.6g
	439	18.1g	19.8g	21.2g	21.0g
	440	19.0g	19.3g	20.5g	20.1g
	441	18.5g	20.2g	19.4g	17.2g
	Average Weight	*18.5g*	*19.7g*	*20.8g*	*20.2g*

Third Trial

Table 1.3.1 Weight of mice fed with control diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	372	16.3g	17.4g	18.9g	20.0g
	373/353	18.1g	19.9g	22.5g	22.1g
- *Listeria*	374	16.5g	18.7g	21.2g	21.0g
- Probiotic	375	18.6g	19.0g	21.5g	21.4g
	Average Weight	*17.4g*	*18.8g*	*21.0g*	*21.1g*
Challenged	376/350	19.0g	19.9g	23.1g	22.5
	377 /351	16.6g	17.5g	20.0g	15.8
	378	17.3g	19.5g	22.4g	21.6
	379	17.7g	18.9g	21.2g	20.4
	380	16.6g	18.3g	20.5g	19.2
+ *Listeria*	381/ 349	17.9g	19.7g	21.9g	19.5
+ Probiotic	382	16.6g	18.3g	20.0g	19.3
	383	16.2g	17.9g	19.7g	16.0
	358	17.1g	18.1g	20.5g	20.3
	359/ 348	17.8g	18.4g	21.3g	20.4
	399	16.9g	17.9g	19.6g	18.9
	Average Weight	*17.3g*	*18.6g*	*21.0g*	*19.3g*

Table 1.3.2 Weight of mice fed *B. animalis* B6 diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	360	18.8g	19.5g	21.4g	20.5g
	361	17.6g	19.6g	21.3g	21.5g
- *Listeria*	362	17.8g	19.4g	21.7g	21.0g
+ Probiotic	363	17.5g	19.6g	21.8g	21.9g
	Average Weight	*17.9g*	*19.5g*	*21.6g*	*21.2g*
Challenged	364 / 357	16.9g	18.3g	19.5g	19.7
	365	17.7g	19.4g	20.6g	16.2
	366 / 356	17.2g	18.7g	21.3g	17.7
+ *Listeria*	367	18.7g*	17.8g*	19.8g	15.8
+ Probiotic	368/ 355	19.0g	21.0g	22.2g	22.1
	369 /347	17.1g	18.3g	20.3g	20.4
	370 / 354	16.9g	18.3g	20.0g	19.6
	371	16.5g	16.5g	18.8g	15.9
	Average Weight	*17.6g*	*18.9g*	*20.7g*	*18.4g*

Table 1.3.3 Weight of mice fed *L. acidophilus* ADH diet.

Treatment	Mouse ID#	Body Weight Day 0	Body Weight Day 7	Body Weight Day 14	Body Weight Day 3 P.I.
Unchallenged	384	15.3g	17.3g	19.3g	19.2g
	385	17.6g	19.1g	22.0g	21.5g
- *Listeria*	386	15.9g	17.7g	19.7g	19.8g
+ Probiotic	387	18.4g	19.4g	22.3g	21.7g
	Average Weight	*16.8g*	*18.4g*	*20.8g*	*20.6g*
Challenged	388	18.2g	18.1g	20.9g	20.1
	389	16.3g	16.2g	18.8g	15.7
	390	17.9g	18.7g	21.9g	17.2
	391	17.0g	18.2g	21.6g	19.9
+ *Listeria*	392	15.8g	16.2g	18.7g	17.5
+Probiotic	393	16.1g	17.8g	21.0g	20.3
	394	17.1g	19.1g	20.7g	20.9
	395	17.7g	19.0g	22.2g	20.3
	396	18.7g	19.9g	22.2g	20.5
	397	18.4g	20.3g	22.8g	21.0
	398	16.9g	18.7g	21.3g	17.7
	Average Weight	*17.2g*	*18.4g*	*21.0g*	*19.0g*

REFERENCES

Abbas AK, Lichtman AH. 2003. Cellular and Molecular Immunology. 5th ed. Elsevier Science. Philadelphia, PA: Elsevier,10 p.

Alm L. 1983. The effect of *Lactobacillus acidophilus* administration upon the survival of *Salmonella* in randomly selected human carriers. Prog Food Nutr Sci 7:13-7.

Ananta E, Volkert M, Knorr D. 2005. Cellular injuries and storage stability of spray-dried *Lactobacillus rhamnosus* GG. Int Dairy J 15(4):399-409.

Anderson JW, Johnstone BM, Cook ME. 1995. Meta-analysis of the effects of soy protein intake on serum lipids. New Engl J Med 333:276-82.

Barber EM, Fazzari M, Pollard JW. 2005. Th1 cytokines are essential for placental immunity to *Listeria monocytogenes.* Infection and Immunity 73(10): 6322-31.

Beerens H. 1991. Detection of bifidobacteria by using propionic acid as a selective agent. Appl Envir Microbiol 57(8):2418-9.

Callard R, Gearing A. 1994. The Cytokine Facts Book. San Diego CA : Academic Press. p 2-3.

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P. 2004. Relevant factors for the preparation of freeze-dried lactic acid bacteria. Int Dairy J 14(10):835-47.

CDC. 2000. Multistate Outbreak of Listeriosis, United States. Morbidity and Mortality Weekly Report (MMWR).

CDC. 2008. Division of Foodborne, Bacterial and Mycotic Diseases (DFBMD) Website. Atlanta GA. Accessed October 2008.

Chateau N, Castellanos I, Deschamps AM. 1992. Distribution of pathogen inhibition in the Lactobacillus isolates of a commercial probiotic consortium. J Applied Bacteriol 74: 36-40.

Colaco C, Kampinga J, Roser B.1995. Amorphous stability and trehalose. Sci 268(5212):788.

Conlan JW, North RJ. 1992. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. Infect Immun 60(12):5164–71.

Corthier G. 1997. Antibiotic-associated diarrhea: treatments by living organisms given by the oral route (probiotics). In: Fuller R, editor. Probiotics 2: Applications and Practical Aspects. New York: Chapman & Hall. p 40-64.

Costa E, Usall J, Teixido N, Garcia N, Vinas L. 2000. Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. J Appl Microbiol 89**:**793-800.

Costalos C, Skouteri V, Gournaris A, Sevastiadou S, Triandafilidou A, Ekonomidou C, Kontaxaki F, Petrochilou V. 2003. Enteral feeding of premature infants with Saccharomyces boulardii. Early Human Devel 74(2):89-96.

Crowe LM. 2002. Lessons from nature: the role of sugars in anhydrobiosis. Comp Biochem Physiol A Mol Integr Physiol 131(3):505-13.

De Martinis ECP, Franco BDGM. 1998. Inhibition of *Listeria monocytogenes* in a pork product by a *Lactobacillus sake* strain. Int'l J Food Microbiol 42(1):119-26(8).

Donnelly CW. 2001. *Listeria monocytogenes*: a continuing challenge. Nutr Reviews 59:183-94.

Drevets DA, Sawyer RT, Potter TA, Campbell PA. 1995. *Listeria monocytogenes* infects human endothelial cells by two distinct mechanisms. Infect Immun 63:4268-76.

Faculty of Pharmacy, 3,rue du Professeur Laguesse. 1991. Detection of bifidobacteria by using propionic acid as a selective agent. Appl Environ Microbiol 57(8):2418-9.

FDA. 2000. Soy: Health Claims for Soy Protein, Questions About Other Components. FDA Consumer magazine.

FDA. 2003. *Listeria monocytogenes* Risk Assessment Questions and Answers website. Accessed September 2008.

FDA website. 2006. Inventory of Notifications Received under 21 U.S.C. 343(w)(7**)** for Exemptions from Food Allergen Labeling. Accessed August 2008.

FDA website. 2007. Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. Accessed August 2008.

FDA website. Guide to inspections of lyophilization of parenterals. Accessed August 5, 2008.

FDA, USDA, CDC. 2005. Quantitative Assessment of Relative Risk to Public Health from Foodborne *Listeria monocytogenes* Among Selected Categories of Ready-to-Eat Foods. Website accessed August 2008.

Felley CP, Corthesy-Theulaz I, Rivero JLB, Sipponen P, Kaufmann M, Bauerfeind P, Wiesel PH, Brassart D, Pfeifer A, Blum AL, Michetti P. 2001. Favourable effect of an acidified milk (LC-1) on *Helicobacter pylori* gastritis in man. Eur J Gastroenterol Hepatol 13(1):25-9.

Fritsche KL, Irons R, Pompos L, Janes J, Zheng Z, Brown C. 2005. Omega-3 polyunsaturated fatty acid impairment of early host resistance against *Listeria monocytogenes* infection is independent of neutrophil infiltration and function. Cel Immun 235(1):65-71.

Fuller R. 1989. Probiotics in man and animals. J Appl Bacteriol 66:365-78.

Gahan C G M, Hill C. 2004. Gastrointestinal phase of *Listeria monocytogenes* infection. J Appl Microbiol 98:345-53.

Gibson GR, Wang X. 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J Appl Bacteriol 77:412-20.

Guarner F, Sachaafsma GJ.1998. Probiotics. Int J Food Microbiol 39:237-8.

Hardy J, Francis KP, De Boer M, Chu P, Gibbs K, Contag CH. 2004. Extracellular replication of *Listeria monocytogenes* in the murine gall bladder. Sci 303:851-3.

Harris LJ, Daeschel MA, Stiles ME, Klaenhammer TR. 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. J Food Protection 52: 384-7.

Heldman DR, Singh RP. 1981. Food Process Engineering, 2nd ed. Westport, CT:AVI Publishing Co. 68 p.

Hetzer F. TGS Chemie Online. Kohlenhydrate http://www.tgs-chemie.de/kohlenhydrate.htm Accessed September 2008.

Hof H. 2001. *Listeria monocytogenes*: a causative agent of gastroenteritis? European J Clin Microbiol Infectious Diseases 20:2094-100.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. 1994. Bergey's Manual of Determinative Bacteriology, 9th ed. Baltimore MD: Williams & Wilkins. 3 p.

Holzapfel WH, Haberer P, Geisen R, Björkroth J, Schillinger U. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr 73:365S-73S.

Holzapfel WH, Botha SJ. 1988. Physiology of *Sporolactobacillus* strains isolated from different habitats and the indication of in vitro antagonism against *Bacillus* sp. Int J Food Microbiol 7:161–8.

Huang HY, Huang SY, Chen PY, King V, Lin YP, Tsen JH. 2007. Basic characteristics of *Sporolactobacillus inulinus* BCRC 14647 for potential probiotic properties. Current Microbiol 54(5):396-404(9).

Hubalek Z. 2003. Protectants used in the cryopreservation of microorganisms. Cryobiol 46:204-29.

Hurd S. 1998. Multistate outbreak of listeriosis – United States. Morb Mortal Wkly Rep 47:1085-6.

Hyronimus B, Le Marrec C, Hadj Sassi A, Deschamps A. 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. Int J Food Microbiol 61(2-3):193-7.

Ikeda Y, Iki M, Morita A, Kajita E, Kagamimori S, Kagawa Y, Yoneshima H. 2006. Intake of fermented soybeans, natto, is associated with reduced bone loss in postmenopausal women: Japanese Population-based osteoporosis (JPOS) Study. J Nutr 136(5):1323-8.

Irons R, Anderson MJ, Zhang M, Fritsche KL. 2003. Dietary fish oil impairs primary host resistance against *Listeria monocytogenes* more than the immunological memory response. J Nutr 133:1163-9.

Jay JM, Loessner MJ, Golden DA. 2005. Modern Food Microbiology. 7th ed. p 591-612.

Jones S, Portnoy DA. 1994. Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. Infect Immun 62(12):5608-16.

Kailasapathy K. 2006. Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yogurt. Food Sci Technol 39(10):1221-7.

Kailasapathy K, Chin J. 2000. Survival and therapeutic potencial of Probiotic organisms with referente to *Lactobacillus acidophilus* and *Bifidobacterium* spp. Immunol. Cell Biol 78:80-8.

Kaufman PB, Duke JA, Brielmann H, Boik J, Hoyt JE. 1997. A comparative survey of leguminous plants as sources of the isoflavones genistein and daidzein: Implications for human nutrition and health. J Alt Complement Med 3:7-12.

Kuhn M, Pfeuffer T, Greiffenberg L., Goebel W. 1999. Host cell signal transduction during *Listeria monocytogenes* infection. Archives of Biochemistry and Biophysics 372(1):166-72(7).

Lapierre L, Undeland P, Cox LJ. 1992. Lithium chloride-sodium propionate agar for the enumeration of bifidobacteria in fermented dairy products. J Dairy Sci 74:1192-6.

Lechner D, Kallay E, Cross HS. 2005. Phytoestrogens and colorectal cancer prevention. Vitamins & Hormones 70:169-98.

Lecuit M. 2005. Understanding how *Listeria monocytogenes* targets and crosses host barrier. Clin Microbiol Infect 11:430-6.

Lecuit M, Vandormael-Pournin S, Lefort J, Huerre M, Gounon P, Dupuy C, Babinet C, Cossart P. 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Sci 292(5522):1722 -5.

Lee YK, Nomoto K, Salminen, Gorbach SL. 1999. Handbook of Probiotics. New York: John Wiley & Sons, Inc. 8 p.

Li JC. 1996. Inelastic neutron scattering studies of hydrogen bonding in ices. J Chem Phys 105:6733-55.

Lingnau A, Domann E, Hudel M, Bock M, NichterleinT, Wehland J, Chakraborty T. 1995. Expression of the *Listeria monocytogenes* EGD *inl* A and *inl* B genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and independent mechanisms. Infect Immun 63:3896-903.

Little R, Milliken W, Stroup W, Wolfinger R, Schabenberger O. 2006. SAS® for mixed models. 2nd ed. North Carolina. SAS Publication.

Liu D. 2008. Handbook of *Listeria monocytogenes*. CRC Press. 412-7.

Liu Z, Simpson RJ, Cheers C. 1995. Interaction of interleukin-6, tumor necrosisfactor and interleukin-1 during *Listeria* infection. Immunology 85(4): 562-7.

Mack DR, Michail S, Wei S, McDougall L, Hollongsworth MA. 1999. Probiotics inhibit enteropatogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. Am J Physiol Gastr L 276(4):G941-50.

Mahoney M, Henriksson A. 2003. The effect of processed meat and meat starter cultures on gastrointestinal colonization and virulence of *Listeria monocytogenes* in mice. Int'l J Food Microbiol 84(1):255-61.

Marteau PR, Vrese Md, Cellier CJ, Schrezenmeir J. 2001. Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nutr 73:430S-6S.

Meister K. 2002. Website: Facts about "Functional Foods". Am Coun Sci Health New York. NY. Accessed August 2008.

Minnesota Department of Health. 2007. http://www.health.state.mn.us/divs/idepc/diseases/listeriosis/index.html. Accessed July 2008.

Miura T, Nishikawa S, Sasaki S, Yamada K, Hasegawa S, Mizuki D, Mizuki M, Hatayama I, Sekikawa K, Tagawa Y, IwakuraY, Nakane A. 2000. Roles of endogenous cytokines in liver apoptosis of mice in lethal *Listeria monocytogenes* infection. FEMS Immunology and Medical Microbiol 28(4):335-41.

Miyamoto-Shinohara Y, Imaizumi T, Sukenobe J, Murakami Y, Kawamura S, Komatsu Y. 2000. Survival rate of microbes after freeze-drying and long-term storage. Cryobiology 41:251-5.

Nakane A, Minagawa T, Kato K. 1988. Endogenous necrosis factor (Cachectin) is essential to host resistance against *Listeria monocytogenes* infection. Infect Immun 56(10):2563–9.

Nakane A, Yamada K, Hasegawa S, Mizuki D, Mizuki M, Sasaki S, Miura T. 1999. Endogenous cytokines during a lethal infection with *Listeria monocytogenes* in mice. FEMS microbiology letters 175:133-42.

Nebra Y, Blanch AR. 1999. A new Selective Medium for *Bifidobacterium* spp. Appl and Environmental Microbiol 65(11):5173-6.

Notermans S, Dufrenne J, Teunis P, Chackraborty T. 1998. Studies on the risk assessment of *Listeria monocytogenes*. J Food Prot 61:244-8.

Ogden SK, Taylor AJ, Dodd CER, Guerrero I, Buendia H, Gallardo F. 1997. Preservative effect of combined propionic and ascorbic acids on pork meat stored at 25°C. J Food Prot 60(8):935-42(8).

Park JH, Kim DJ, Park YH, Seok SH, Cho SA, Baek MW, Lee HY, Park JH. 2004. Characteristics of the gastritis induced by *Listeria monocytogenes* in mice: microbiology, histopathology, and mRNA expression of inflammation mediators with time course of infection. J Microbiol Pathogenesis 37:87-94.

Peter RHPC, French PJ, Van Doorninck JH, Lamblin G, Ratcliff R, Evans MJ, Colledge WH, Bijman J, Scholte BJ. 1996. CFTR expression and mucin secretion in culture mouse gallbladder epithelial cells. Am J Physiol 271(34):Gl074-83.

Poiley SM. 1972. Growth tables for 66 strains and stocks of laboratory animals. Lab Anim Sci 22:759.

Potter SM. 1996. Soy protein and serum lipids. Curr Opinion Lipidol 7:260-4.

Potter SM. 1998. Soy protein and isoflavones: Their effects on blood lipids and bone density in postmenopausal women. Am J Clin Nutr 68(6 Suppl):1375S-79S.

Rahman MS. 2007. Handbook of food preservation. 2nd ed. Food Preservation by Freezing. Lincoln: CRC Press. p 638-9.

Rodriguez-Cruz M, Tovar AR, del Prado M, Torres N. 2005. Molecular mechanisms of action and health benefits of polyunsaturated fatty acids. Revista de Investigacion Clinica 57(3):457-72.

Rolfe RD. 2000. The role of probiotic cultures in the control of gastrointestinal health. J Nutr 130:396S-402S.

Roos NM, Katan MB. 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of paper published between 1988 and 1998. AM J Clin Nutr 71:405-11.

Setchell KD, Cassidy A. 1999. Dietary isoflavones: Biological effects and relevance to human health. J Nutr 129(3):758-67.

Silva AM, Barbosa FHF, Duarte R, Vieira LQ, Arantes RME, Nicoli JR. 2004.

Effects of *Bifidobacterium longum* ingestion on experimental salmonellosis in mice. J Appl Phycol 97(1):29-37.

Silvi S, Rumney CJ, Rowland IR. 1996. An assessment of three selective media for bifidobacteria in faeces. J Appl Bacteriol 81:561-4.

Simoes I. 2006. Development Of A Novel Probiotic Fortified Protein Bar. MS Thesis, University of Missouri.

Sneath PH, Mair NS, Sharpe ME, Holt JG. 1986. Bergey's Manual of Determinative Bacteriology. Baltimore, MD: Williams & Wilkins. 3 p.

Soyatech LLC, SPINS Inc, Golbitz P. 2004. Soyfoods: The U.S. Market 2004 U.S. Soyfoods Market Report. Soyfoods Association of North America. http://www.soyfoods.org/ products/sales-and-trends/. Accessed July 2008.

Stiles ME, Holzapfel WH. 1997. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol 36(1):1-29.

Tabatabaie F, Mortazavi A. 2008. Influence of lactulose on the survival of probiotic strains in yoghurt. World Appl Sci J 3(1):88-90.

Tannock GW. 2004. A Special fondness for lactobacilli. Appl Environ Microbiol 70(6):3189-94.

Tojo M, Oikawa T, Morikawa Y, Yamashita N, Iwata S, Satoh Y, Hanada J,Tanaka R. 1986.The Effects of *Bifidobacterium breve* administration on *Campylobacter enteritis*. Official J Japan Pediatric Society 29(1):160-7.

Työppönen S, Petäjä E, Mattila-Sandholm T. 2003. Bioprotectives and probiotics for dry sausages. Int J Food Microbiol 8(3):233-44.

USDA website. 2005. Pathogens and Contaminants. Food Safety Research Information Office. Accessed June 2008.

Wardlaw GM, Kessel M. 2002. Perspectives in Nutrition. 5th ed. Meyers LM, editor, New York: McGraw-Hill. 207 p.

Winkowski K, Crandall AD, Montville TJ. 1993. Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus* MN in beef systems at refrigeration temperatures. Appl Environ Microbiol 59(8):2552-7.

Wollert T, Pasche B, Rochon M, Deppenmeier S, Heuvel J, Gruber DA, Heinz DW, Lengeling A, Wolf-Dieter Schubert. 2007. Extending the host range of

Listeria monocytogenes by rational protein design. Cell 129:891-902.

Yamamoto S, Russ F, Teixeira HC, Conradt P, Kaufmann SH. 1993. *Listeria monocytogenes*-induced gamma interferon secretion by intestinal intraepithelial gamma/delta T lymphocytes. Infect Immun 61(5): 2154-6.

Zayed G, Roos YH. 2004. Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. Process Biochemistry 39:1081-6.