DIFFERENTIATION OF STRAINS OF BIFIDOBDATERIUM ANIMALIS SUBSP. LACTIS BY ABILITY TO MODULATE AN IMMUNE RESPONSE

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ABSTRACT

Irritable Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS) are two gastrointestinal disorders that are increasing in prevalence in the United States. Symptoms such of these disorders such as vomiting, bloating, and diarrhea inhibit patients from leading normal, social lives. Currently, there are a number of drugs on the market to treat these diseases but they are not effective for all patients. The use of probiotics is one possible alternative to drug therapy. Probiotics, commonly delivered through fermented dairy products and supplemental tablets, are becoming more widespread in usage as a treatment of symptoms caused by IBD and IBS. One bacterial species, *Bifidobacterium animalis*, is often used as a probiotic in commercial products. However, not all strains of *B. animalis* are thought to have the same immunomodulatory effect on the human gut. The World Health Organization has determined that the health benefits of probiotics are strain-specific and that not all members of a species have the same beneficial health effects. Using a model created during this experiment, an inflammatory response will be simulated using RAW 264.7 murine macrophages and treated with eight strains of *Bifidobacterium animalis* ssp. *lactis* and ssp. *animalis*. Production of Interleukin-10, an anti-inflammatory cytokine, and Tumor Necrosis Factor α, a pro-inflammatory cytokine, will be quantified using ELISA to determine if different strains of *B. animalis* ssp. *lactis* have differing immunomodulatory effects on RAW 264.7 macrophages.
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Chapter 1
Introduction

Lower Gastrointestinal Disease

Lower gastrointestinal diseases have become increasingly common and the public is becoming more aware of their existence. Two common lower gastrointestinal (GI) disorders are Irritable Bowel Syndrome (IBS) and Irritable Bowel Disease (IBD), which include the conditions Ulcerative Colitis (UC) and Crohn's Disease (CD). An individual with IBS will experience some mild symptoms. Typically, these symptoms are abdominal pain/discomfort that lessens after passing stool and also a change in the type and frequency of stool (Longstreth et al., 2006). IBD is a more severe disorder, with an individual experiencing inflammation of the intestine, weight loss, diarrhea and abdominal pain. The difference between UC and CD is the type of inflammation. CD has inflammation deeper into the walls of the colon while UC causes epithelial cells and other cells near the surface to be inflamed (Neuman, 2007).

While a direct cause for IBS and IBD has not been identified, some factors have been reported to influence the development of these lower GI disorders. The first is whether or not an individual has a genetic predisposition for the disease. A mutation found in the nucleotide oligomerization domain 2/caspase recruitment domain 15 (NOD2/CARD15) on chromosome 16 was commonly found in individuals with CD (Hugot et al., 1996). Other mutations, such as on the IL-23 receptor and in the ATG16L1/IRGM1 gene, have also been found to be associated to the development of IBD (Mayer, 2010). It is important to note that having one of these mutations does not guarantee that the individual will develop UC or CD.
Environmental factors within the colon have also been associated with IBD and IBS. Infections by bacteria and viruses along with the use of different types of drugs, especially antibiotics can cause changes in the microflora of the gut. Changes in diet and starting or stopping smoking have also been associated with the onset of IBD (Mayer, 2010).

The final factor in the causation of a lower GI disorder is dysfunction of the immune system. During the onset of a lower GI disorder, the adaptive immune system will cause an immune response against bacteria in the colon. In UC and CD, the immune response is not properly regulated and overproduction of inflammatory cytokines such as interferon gamma, interleukin 12, and interleukin 13 produced by T cells can cause the characteristic inflammation and onset of the disease (Mayer, 2010).

As a society, IBD and IBS have become more important as they have increased in prevalence. During the five-year span from 1999 to 2004, there was a 68% increase in hospitalization due to UC. Reports of CD increased 74% between 1992-1993 and 2003-2005. The incidence of IBS has been relatively stable over the past 20 years. (Everhart & Ruhl, 2009a).

With increasing rates of these diseases, there are increased social effects. Individuals are less able to interact socially as they require easy access to bathroom facilities because of their symptoms. These same symptoms also cause decreased productivity in the work place as well as increased absences due to symptoms in comparison to individuals without these diseases (Agarwal & Spiegel, 2011).

These three lower GI disorders cause a large financial burden on the healthcare system. In 2004, CD resulted in over $1 billion worth of costs for treatment costs, while treatment costs for both IBS and UC exceeded $1.72 billion, costing $949.8 million and $767.9 million respectively. Combined, there was a total of $3.5 billion in direct costs with an additional $385.5 million in indirect costs for the three disorders. Direct costs are the funds that go towards treating illnesses, such as doctors’ visits and medications. Indirect costs are the other costs from an illness,
such as taking time off from work to go to an appointment. In total, the costs of these diseases are close to $4 billion (Everhart & Ruhl, 2009b).

There are currently some medications that can be used to treat CD, IBS, and UC. UC is typically treated with an anti-inflammatory drug such as mesalamine or balsalazide. CD can be treated with mesalamine as well, but patients may also be prescribed drugs like mercaptopurine or azathioprine, which are immuno-suppressors. Hyoscyamine and dicyclomine are the two most commonly prescribed drugs for patients with IBS. Both of these drugs block the acetylcholine receptors in the GI tract, limiting activation of smooth muscles (Everhart & Ruhl, 2009a). There has been some doubt about the effectiveness on many of these drugs. Kornbluth et. al. (1995) found that most anti-inflammatory drugs and steroids decreased symptoms, but only a little over half of the patients went into remission. With the high costs of some of the current drugs, for example over $468.5 million was spent on 2.45 million prescriptions mesalamine alone in 2004, another option is desired. The use of probiotics to treat IBS and IBD may provide an alternative solution.

**Probiotics**

According to the World Health Organization (WHO), probiotics are defined as live microorganisms that when consumed in adequate amounts provide a health benefit. These probiotics are typically added to dairy products to be consumed by both populations at risk for gastrointestinal diseases and the general public (FAO/WHO, 2001). Currently, American consumers can buy products containing probiotics, such as Activia® yogurt by Dannon or purchase supplemental pill forms. Advertising for these products suggests these products will increase stool regularity and improve general gut health (http://www.activia.us.com/probiotics-benefits).
Probiotic bacteria, typically lactic acid bacteria, have been associated with multiple effects on the human body. First, probiotics are able to modulate the immune response of epithelial and immune cells to decrease the amount of inflammation (Grimoud et al., 2010). Some probiotics can interact with immune cells to decrease inflammatory cytokines and increase anti-inflammatory cytokines. Probiotics can influence the maturation of CD4⁺CD25⁺ regulatory T cells (T_{reg}), a regulatory immune cell, increasing the amount of cells present in circulation. T_{reg} cells regulate the production of immune cells in response to antigens. In the absence of T_{reg} cells, other immune cells mature, such as cytotoxic T cells and dendritic cells that produce inflammatory cytokines, increasing the immune response (Madsen, 2006). Probiotics also interact with T_{reg} cells to increase production of interleukin (IL) 10 among other cells (De Moreno de Leblanc et al., 2011). A second major effect of probiotics on the GI tract is the modification of the microflora within the colon. Some probiotic bacteria are able to colonize the colon and occupy the niche pathogens would otherwise take. Certain probiotic bacteria may inhibit pathogens by the production of bactericidal proteins or by causing a decrease in pH (Sartor, 2004).

**Bifidobacterium animalis**

*Bifidobacterium* is one genus that contains many common probiotic bacteria. Members of this genus are anaerobic, Gram-positive organisms that have a unique Y or bifid shape. One species that is typically employed as a probiotic is *B. animalis*. Currently, two subspecies have been identified: *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis*. These two subspecies were isolated from yogurt and animal feces, respectively. Strains within the *B. lactis* subspecies are used as a probiotic, such as *B. animalis* subsp. *lactis* Bb-12 (Lee & O’Sullivan, 2010). Genetically, strains of *B. animalis* subsp. *lactis* are highly similar with little variation (Briczinski
et al., 2009). The majority of the strains of *B. animalis* have a genome of just under two million base pairs, about 60% G+C content, with about 90% of the genomes consists of coding DNA (Barrangou et al., 2010).

Both members of the genus *Bifidobacterium*, as well as individual strains of *B. animalis* ssp. *lactis*, have been shown to have probiotic effects. In a case study, an individual with UC was undergoing treatment using steroids to treat the symptoms along with eventual antibiotics used to treat a *Staphylococcus epidermis* infection. As the patient's condition worsened, the doctors put the patient on a regiment of 6 mg/day of *Bifidobacterium* (method of treatment was not described). Colonoscopy results showed improvement of the colon wall and reduced bleeding after one week. Doctors were then able to reduce levels of steroid use without relapse of the disease (Nagasaki et al., 2010).

One method of the reduction of symptoms observed in the previous case study implemented by *B. animalis* ssp *lactis* and other *Bifidobacterium* species is their ability to manipulate the expression of cytokines by immune cells in the colon. Kim et al. (2010) demonstrated that *B. lactis* KCTC 5727 (now considered *B. animalis* ssp. *lactis* (Masco, Ventura, Zink, Huys, & Swings, 2004)) was able to inhibit the production of the transcription factor NF-κB produced by human HT-29 cells. The bacteria were able to block NF-κB activation by limiting the production of TNF-α and IL-1b. Restricting NF-κB activation inhibited production of gene products from genes such as “MMP, VEGF, and COXs”, all known tumorigenic genes. In a different study comparing species of *Bifidobacterium*, including *B. animalis*, it was found the organism was able to modulate the expression of several cytokines and signaling molecules, such as nitric oxide, IL-1B, IL-6, IL-12, and TNF-α produced by RAW 264.7 macrophages (D. W. Kim et al., 2007a).

Another activity that allows *B. animalis* ssp. *lactis* to have probiotic capabilities is the ability to alter the gut microflora. By giving mice a fermented milk product containing *B.*
animalis ssp. lactis DN-173, Veiga et al. were able to observe changes in levels of bacteria observed in fecal samples. Pathogenic bacteria such as Klebsiella pneumoniae and Proteus mirabilis were found in decreased levels after treatment, while certain butyrate-producing bacteria that are beneficial to the gut such as Eubacterium hallii and Anaerostipes caccae increased in quantity (Veiga et al., 2010). Changes observed in the microflora are most likely caused by the ability of some B. animalis strains to produce bacteriocins, a class of molecules that inhibit and kill bacterial cells (Martins et al., 2009). Short chain fatty acids produced by these strains were found to increase in the colon due to the presence of B. animalis ssp. lactis DN-173 010 and are suspected to be the bactericidal agent (Veiga et al., 2010).

It is important to be specific when discussing B. animalis ssp. lactis. Previous work has demonstrated that strains of B. animalis ssp. lactis are very similar genetically and are difficult to differentiate (Briczinski et al., 2009). However, it appears that not all strains can be used as probiotics. FAO and WHO indicate probiotic effects are to be considered “strain specific.” In a study by Lopez et. al. (2010) the B. animalis ssp. lactis strains Bb-12, IPLA 4549, and 4549dOx were put in co-culture with RAW 264.7 murine macrophages separately and different levels of cytokines were expressed by the macrophages, especially IL-10 and IL-4. This phenomenon has also been observed in other species of Bifidobacterium (Medina, Izquierdo, Ennahar, & Sanz, 2007).

Cytokines

Probiotics are considered to exert two effects on the production of cytokines: 1) to inhibit inflammatory cytokines and 2) to increase anti-inflammatory cytokines. Two important cytokines in these fields are TNF-α (inflammatory) and IL-10 (anti-inflammatory).
TNF-α is an important component of the immune response when the body is infected. Through the receptors Fas and TNFR1, this cytokine can activate apoptosis corresponding with an anti-inflammatory response, or it can activate an anti-apoptosis pathway that has an inflammatory response by binding to TNFR2 (Baud & Karin, 2001). During an infection, TNF-α is essential in mounting a proper immune response. In mice that are TNF-α deficient, a bacterial infection will quickly kill them through over activity of T cells and overproduction of Interferon (IFN)-Gamma (Zganiacz et al., 2004). While experimenting with different Bifidobacterium species to treat IBD or IBS, it has been observed that they can cause an increase in TNF-α production by macrophages (D. W. Kim et al., 2007b; Park et al., 1999).

Oppositely, IL-10 is an anti-inflammatory cytokine produced by macrophages, T cells, and other immune cells. It is used by the immune system to stop an immune response, specifically by preventing the presentation of antigens by dendrites and macrophages and down-regulating the production of cytokines such as TNF-α, IL-12, and inducible nitric oxide synthetase (Donnelly, Dickensheets, & Finbloom, 1999). Researchers have demonstrated that in the presence of certain probiotic strains, immune cells up-regulate the production of IL-10 (Gad et al., 2011). In an overactive immune system as seen in IBD and IBS, increased levels of IL-10 should limit the response and cause decreased symptoms for diagnosed individuals.

Probiotics are another treatment option for patients with IBD and IBS who wish to limit the symptoms they experience. While some species of Bifidobacterium have been identified as having probiotic capabilities, not all strains have the same potential. By comparing eight sequenced strains of B. animalis ssp. lactis and ssp. animalis, differences in their abilities can be used to modulate immune responses. By exposing RAW 264.7 murine macrophages to strains of B. animalis ssp. lactis, different amounts of TNF-α and IL-10 will be produced by the macrophages depending on the strain of the bacterium used as a treatment.
Statement of the Problem

The beneficial health effects of probiotic bacteria are considered to be strain specific by WHO. It is unclear how these strains of *Bifidobacterium animalis* ssp. *lactis* will influence immune cells, specifically RAW 264.7 murine macrophages. By measuring the production of IL-10 and TNF-α of RAW 264.7 macrophages when exposed to various strains of *B. animalis*, one will be able to determine whether or not the immunomodulatory effects of *B. animalis* strains are strain specific. It is expected that there will be a difference in IL-10 and TNF-α productions by RAW 264.7 when exposed to different strains of *B. animalis* ssp. *lactis* and *B. animalis* ssp. *animalis*. 
Chapter 2
Materials and Methods

Bacterial Cultures

Strains of *B. animalis* subsp. *lactis* and ssp. *animalis* were obtained from the culture collection of Dr. Robert Roberts. The strains used for cytokine modulation comparisons are listed in Table 4-1. *B. animalis* subsp. *animalis* ATCC 27672 was used to develop the co-culture system used in cytokine production experiments. Bacterial cells were revived from -80°C storage by transferring 100 µl of stored cells into 10 ml of de Man, Rogosa and Sharpe (MRS) broth containing 0.5% Cysteine (Cys). These cells were grown in an anoxic environment at 37°C. Sequence data was obtained from the National Center for Biotechnology Information website (ncbi.nlm.nih.gov).

SNP Typing

DNA was first isolated from bacterial cultures using the Wizard Genomic DNA Purification Kit (Promega, Fitchburg, Wisconsin, USA). The DNA concentration was measured using Quant-iT DNA Assay (Invitrogen, Carlsbad, California, USA). The DNA was then prepared for PCR. Each reaction mixture contained 5 µl GoTaq Buffer, 0.5 µl forward primer, 0.5 µl reverse primer, 0.75 µl DNTPs, 2 µl sample DNA at 25 ng/µl, 0.15 µl Taq polymerase, and 16.1 µl distilled water. Primers were designed by Briczinski et al. (2009) surrounding each SNP. Fifteen different SNP sites were used to identify the 8 strains of *B. animalis* subsp. *lactis*. Primer sequences are listed in Appendix 1. The PCR program began by raising the temperature to 95°C
for 5 min. A cycle began while temperature was kept at 95°C for an additional 30 sec before cooling to 1 min at 58°C, then raised to 72°C for 2 min. The cycle was repeated a total of 30 times. After the final cycle, the samples were held at 72°C for 7 min before holding at 4°C indefinitely. The amplified samples were subjected to gel electrophoresis in 1% agarose gels for 70 min at 120V to separate amplicons. Gels were then stained in ethidium bromide for 20 min and viewed under UV light. Visible bands were extracted using a QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands) and sequenced at the Penn State Genomics Core Facility (University Park, PA, USA). Nucleotide data was compared to the known genomes using SeqMan (DNASTAR, Madison, WI, USA).

Table 2-1: Strains and reference of _B. animalis_ strains being compared with their reference and Gene Bank Accession number.

All of the strains listed are of the _lactis_ subspecies except for ATCC 25527, which is in the _animalis_ subspecies.

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<tr>
<td>B420</td>
<td>Stahl &amp; Barrangou, 2012</td>
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</tr>
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**Bacterial Growth Curve**

Each of the 8 strains of bacteria were streaked for isolation on an MRS +0.5% Cys agar plate and incubated anoxically for 48 h at 37°C. A single colony was transferred to a tube of MRS +0.5% Cys broth, incubated for 24 h at 37°C and then 100 µl were transferred to a new tube of MRS +0.5% Cys broth to grow for another 24 h before beginning the growth curve. Tubes containing 10 ml of MRS +0.5% Cys broth were then inoculated with 100 µl of cells. Turbidity was measured using a MicroScan Turbidity Meter (Dade Behring, Deerfield, Illinois, USA) over a period of 18 h at 37°C.

**Macrophage Cultures**

RAW 264.7 murine macrophage cells were obtained from Dr. Joshua Lambert. To maintain the cells, the macrophages were grown in 25 ml flasks for 48 h at 37°C in 5 ml of DMEM with 50 ml fetal bovine serum, 0.1 mg/ml streptomycin and 100 U/ml penicillin added. After 48 h, trypsin (Life Technologies, Carlsbad, California, USA) was used to release the macrophages from the bottom of the flasks through a 5 min incubation at 37°C. Cells were then placed in a centrifuge tube and pelleted at 650 x g for 10 min. The supernatant was removed and 3 ml Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies, Carlsbad, California, USA) was added to the pellet. A new flask was filled with 5ml DMEM and 100 µl of the pellet was transferred. The macrophages were not kept past the 30th passage.

**Cell Co-Culture**

RAW 264.7 macrophages were removed from the flask and centrifuged. Next, the number of cells were counted and diluted to 10^5 cells per well of a Falcon 96 well plate (Becton
Dickson Labware, Franklin Lakes, NJ, USA). After 24 h of growth at 37°C in 5% CO₂, bacteria and LPS treatments were added to the RAW 264.7 macrophages. Bacteria for these experiments were grown for 14 h in MRS + 0.5% Cys broth in anoxic conditions at 37°C and prepared for the co-culture by adding 1ml of culture to a microfuge tube. The cells were then centrifuged twice for 1 min at 650 x g. After each cycle, the supernatant was removed and 1ml of DMEM without antibiotics was added to the tube and the bacteria were resuspended. The bacteria were diluted 1:10 before being added at a ratio of 1 µl of diluted cells to 1ml media in reservoir. Each well in the 96-well plate contained 100 µl DMEM and were incubated for an additional 24 h at 37°C in 5% CO₂.

**Methylthiazoltetrazolium Assay**

The methylthiazoltetrazolium (MTT) assay was used to quantify the viability of eukaryotic cells. The supernatant was removed from the 96-well plate and 100 µl DMSO was added to each well. After incubation for 20 min at 37°C, DMSO was removed and 100 µl of MTT solution was added to each well. The absorbance of each well was then read using a MultiskanGo plate reader (Thermo Scientific, Waltham, Massachusetts, USA) at 540 nm.

**Nitric Oxide Assay**

The nitric oxide (NO) assay was used to measure the amount of inflammation by the macrophages. From a cultured 96-well plate, 50 µl of medium was transferred from each well and was put into a new 96 well plate. In the new plate, 100 µl of Griess Reagent was added (Guevara et al., 1998). After incubating for 7 min in the dark at room temperature, the absorbance was
measured on a MultiskanGo plate reader (Thermo Scientific, Waltham, Massachusetts, USA) at 540 nm.

**ELISA for TNFα and IL-10**

ELISAs were performed to measure quantities of TNF-α and IL-10. The procedure was followed for Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA). Samples were prepared by centrifuging the 96-well plates containing a co-culture for 10 min at 650 x g. Medium was removed and added to the ELISA wells. Absorbance was measured at 450 nm using a MultiskanGo plate reader (Thermo Scientific, Waltham, Massachusetts, USA) with wavelength correction.

**Statistical Analysis**

Data was analyzed using Minitab Software (Minitab, State College, PA, USA). Bacterial treatments on macrophages were compared using ANOVA with differences represented by Tukey Groupings that had a confidence level of 5%.
Chapter 3

Results

SNP Typing

In an attempt to demonstrate differences in the strains of *B. animalis* ssp. *lactis*, SNP typing was employed. Primers used in a previous experiment in the Roberts lab were used and are listed in Table APP-1 of the Appendix (Briczinski et al., 2009). When performing the PCR, several of the primer sets did not work for specific strains although they have been shown to work previously. The age of the primers or the quality of the isolated DNA may have been a factor in the lack of PCR products.
Table 3-1: SNPs identified from sequences of PCR products.

SNPs were identified in Briczinski, et. al. 2009. (E) is the expected result, (A) is the observed result. (+) represent the production of a PCR product. (-) represent a deletion in the sequence; (*) represents no PCR product. (1) represents having the same InDel as DSM 10140. (2) represents having the same InDel as Bi-04. (3) represents having the same InDel as ATCC 27673. (4) represents having the same InDel as ATCC 25527.

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PCR products that were produced were sequenced and compared to the full genome of *B. animalis* ssp. lactis DSM 10140 using SeqMan. SNPs were called only if the nucleotide change was found for both the forward and reverse primers. A SNP report was generated and some SNPs are identified on Table 3-2. With the primers used in this experiment, ATCC 27673, HN019, Bb-
12, DSM 10140, and Bl-04 cannot be distinguished by SNP typing. By analyzing the Trace Data, it was found that Bl-04 was contaminated with foreign DNA. The data also suggests that many of the strains were contaminated with \textit{B. animalis} ssp. \textit{animalis}, specifically Bl-04, Bi-07 and B420, due to the presence of Ban2/23si PCR product, which is not expected to be present in any of the \textit{B. animalis} ssp. \textit{lactis} strains. The primers used in the experiment may also be a source of contamination, as they may contain DNA from some of the bacteria instead of only the primers. Another factor for error causing incorrect SNP typing and lack of PCR products is the concentration of the DNA used for PCR. The DNA was used at a high concentration and could have caused incorrect sequences to be formed because of secondary structures or any contamination present.

\textit{Bifidobacterium animalis} Growth Curve

An 18 h study was performed to compare the growth of the eight strains of \textit{Bifidobacterium animalis} (Figure 3-1). All strains reached stationary phase by 16 h of growth. In this experiment, strains ATCC 27673, DSM 10140, and BL 04 grew to lower densities than the other five strains, ending at turbidity values of 0.82, 0.82, and 0.69, respectively. The remaining five strains grew to a turbidity of approximately 0.92.

\textbf{Co-Culture Model}

Although there are literature reports that co-culturing macrophages with probiotic bacteria does not inhibit macrophages, strains chosen for this study have not been evaluated. To evaluate the influence of \textit{B. animalis} on RAW 264.7 cell viability initially,
MTT assays were conducted on RAW 264.7 cells co-cultured with *B. animalis* ssp. *animalis* ATCC 27672 (Figure 3-2). The experiment was replicated twice and no statistical difference was observed between the treatment and the control. However, the cell morphology of the RAW 264.7 macrophages was altered. The cells were elongated instead of spherical (Figure 3-3).

**Figure 3-1:** Growth curve of *B. animalis* strains over 18h.

Cells were grown in MRS +0.5% Cys anaerobically at 37°C. Growth was measured by turbidity of the broth ever hour. There was only one replication of this experiment.

**Figure 3-2:** MTT assay comparing of RAW 264.7 macrophages to RAW 264.7 macrophages with *B. animalis* ssp. *animalis* 27672.

Co-culture was grown in a 96 well plate for 48h in 37°C with 5% CO₂. Cells of *B. animalis* ssp. *animalis* 27672 were added after the RAW 264.7 cells were grown in the plates for 24h.
Co-culture was grown in a 96 well plate for 48h in 37°C with 5% CO₂. Cells of *B. animalis* ssp. *animalis* 27672 were added after the RAW 264.7 cells were grown in the plates for 24h.

Results shown in Figure 3-2 and Figure 3-3 represent the influence of *B. animalis* ssp. *animalis* ATCC 27672 and spent MRS +0.5% Cys broth. To determine if the bacterial cells were producing a metabolite during growth that affected the macrophages
or if the bacterial cells themselves had an effect, three treatments were evaluated: *B. animalis* ssp. *animalis* 27672 washed in DMEM, in MRS +0.5% Cys broth it grew in, and spent MRS +0.5% Cys broth alone. In addition to evaluating viability of the macrophages using MTT assays, nitric oxide assays were performed to determine if any of the treatments induced inflammation in the macrophages. When measured, the three treatments resulted in similar RAW 264.7 cell viability as measured by the MTT assay and were found to be statistically different from the viability observed using an LPS treatment (Figure 3-4a). The washed probiotic treatment induced the greatest average amount of NO produced by the macrophages (Figure 3-4b). The Tukey Groupings of each treatment show the Washed Probiotic is statistically greater than the treatment in spent MRS broth or spent MRS broth alone. Although it caused the most NO production, immune cells were only exposed to the bacterial cells and not MRS broth when being treated for GI diseases, so washed cells will be used for co-culture. Another possible source of NO in these experiments was production by the bacterial culture (Sobko et al., 2005). The bacterial treatment could also affect the measurement for the MTT assay. Measurement in the NO assay for *B. animalis* ssp. 27672 is similar to both RAW only and no treatment (Tukey Groups A, A, A) and in the MTT assay, *B. animalis* ssp. 27672 treatment was statistically similar to the negative control wells (Tukey Groups B, B) (Figure 3-5).
Figure 3-4: NO production and MTT absorbance of RAW 264.7 macrophages after exposure to different forms of *B. animalis* ssp. *lactis* ATCC 27672.

A) MTT Assay for cell viability comparing the RAW 264.7 cell viability after exposure to different *B. animalis* ssp. *lactis* ATCC 27672 treatments. B) NO Assay for inflammation comparing the RAW 264.7 cell viability after exposure to different *B. animalis* ssp. *lactis* ATCC 27672 treatments. Cells were grown in DMEM in a 96 well plate for 48h at 37°C and 5% CO₂. Treatments were added after 24h.
Figure 3-5: Effect of B. animalis ssp. 27672 on both the NO assay and the MTT assay.

A) MTT Assay comparing absorbance during assay between RAW 264.7 cells and BAA 27672 cells. These cells were not in co-culture. B) NO assay comparing the individual observations of RAW 264.7 cells and BAA 27672. Cells were grown in DMEM in a 96 well plate for 48h at 37°C and 5% CO₂. Treatments were added after 24h.

An important factor affecting the results of the experiment is the length of co-culture. Lipopolysaccharide (LPS) is known to induce inflammation in macrophages,
similar to the inflammation that would be experienced during IBD (Raschke, Baird, Ralph, & Nakoinz, 1978). Adding LPS in addition to a bacterial treatment will simulate the immune status of the macrophages during IBD and IBS. To finish creating the model, the optimal time for adding the treatment needed to be found. By measuring NO production and macrophage cell viability after adding LPS and *B. animalis* ssp. 27672 at different times, the optimal immunomodulatory effects of the bacteria should be apparent (Table 3-3). The optimal time for *B. animalis* ssp. 27672 co-culture should be used when performing ELISAs to test for cytokine production by the macrophages. Figure 3-6 is a timeline of two experiments in which either the time of LPS or of *B. animalis* ssp. 27672 addition was varied. Shorter LPS exposure time resulted in lower NO production (Figure 3-6). One explanation for this observation is that *B. animalis* 27672 had an inhibitory effect on the production of NO. The longer the pretreatment time to *B. animalis* ssp. 27672 by RAW 264.7 macrophages, the less NO was detected. The decrease in NO observed may be because RAW 264.7 cells are being exposed to LPS for a shorter amount of time. Another explanation is that *B. animalis* 27672 consumed some of the NO produced by RAW 264.7 cells. However, this effect is negligible if existent and is not normally controlled (Park et al., 1999). The absorptions measured for the addition of LPS are not as high as expected. Normally, there should be a greater increase in NO production by RAW 264.7 macrophages when exposed to LPS than the two fold increased observed in Figure 3-6 (Lambert et al., 2006). This decreased response may be due to using too low of a concentration of LPS. A similar experiment was performed by varying the addition time of the probiotic and adding the LPS after 42hr (Figure 3-7). The absorbances measured are much lower than expected when adding LPS to RAW 264.7
cells and are equivalent to no response to the treatment from the RAW 264.7 cells. The time of exposure for the LPS was most likely too short to elicit a response. The differences calculated between the treatments were due to natural variation. In order to have enough of an immune response from the macrophages and for the bacteria to have a great enough effect, it was concluded that it was optimal to add the treatments after 24 h of RAW 264.7 cell growth.

Table 3-2: Times of addition of *B. animalis* ssp. *lactis* ATCC 27672 and LPS to RAW 264.7 macrophages for time variable experiment.

Results of the experiments are in Figures 5-6 and 5-7. At 48 hr, MTT and NO assays were performed. Co-culture was grown in a 96 well plate for 48 h in 37°C with 5% CO₂.

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Figure 3-6: NO production by RAW 264.7 macrophages influenced by the addition of *B. animalis* ssp. 27672 at 24hr and varying the time of addition of LPS. Co-culture was grown in a 96 well plate for 48h in 37°C with 5% CO₂.

Figure 3-7: NO production of RAW 264.7 macrophages influenced by the addition of *B. animalis* ssp. 27672 at varying times and addition of LPS at 42 h. Co-culture was grown in a 96 well plate for 48h in 37°C with 5% CO₂.
TNF-α and IL-10 Production by Macrophages

Using the model established with RAW 264.7 macrophages using *B. animalis* ssp. *animalis* ATCC 27672, the production of IL-10 and TNF-α by RAW 264.7 cells when exposed to different strains of *B. animalis* was evaluated. None of the strains were observed to significantly alter production of IL-10 from the basal level with no treatment (Figure 3-8). When RAW 264.7 cells are exposed to LPS in addition to a bacterial treatment, differences are observed between the strains of *B. animalis*. The presence of HN019 caused the greatest production of IL-10 with an average of 177.62 µg/ml while Bb-12 and Bl-04 were the only two strains of bacteria in the same Tukey grouping as LPS alone, averaging 86.61 µg/ml and 93.67 µg/ml of IL-10, respectively.

![Figure 3-8: IL-10 production as influenced by the addition of strains of *B. animalis* ssp. *lactis*. Co-culture was grown in a 96 well plate for 48h in 37°C with 5% CO₂. Bacterial cells were added after 24h.](image-url)
Exposing RAW 264.7 macrophages to strains of *B. animalis* greatly increases the production of TNF-α when in co-culture (Figure 3-9). RAW 264.7 cells alone produced 126.2 pg/ml and is the only sample in the D Tukey Grouping. In contrast, the average TNF-α produced by macrophages was 7151 pg/ml. RAW 264.7 cells produced 7157 pg/ml of TNF-α when in the presence of only LPS. Treatments of BI-04 and ATCC 25527 modulated RAW 264.7 cells to produce statistically less TNF-α than the addition these strains with LPS. Bacterial treatments that were statistically different from LPS alone caused decreased production.

**Figure 3-9:** TNF-α production by RAW 264.7 influenced by exposure to strains of *B. animalis* ssp. *lactis*.

Co-culture was grown in a 96 well plate for 48h in 37°C with 5% CO₂. Bacterial cells were added after 24h.
The impact of the *B. animalis* strains can best be compared when comparing a ratio of the concentration of TNF-α to the concentration of IL-10 (Figure 3-10). Lower ratios represent decreased inflammation in comparison to greater ratios. Bb-12 and Bl-04 both had ratios greater than no bacterial treatment. HN019, DSM 10140 and B420 had the three lowest ratios at 40.2, 45.7, and 48.9 respectively. The data suggests that because the strains that had lower ratios of TNF-α/IL-10 than that of RAW 264.7 + LPS, the treatment would cause a decrease in inflammatory activity. Additional replications should be performed to confirm this conclusion.

*Figure 3-10: Ratio of (concentration of TNF-α)/(concentration of IL-10) produced by RAW 264.7 cells when exposed to a B. animalis and LPS.*
Chapter 4

Discussion

By measuring the amount of IL-10 and TNF-α, strains of *B. animalis* ssp. *lactis* were differentiated by their immunomodulatory effects on RAW 264.7 murine macrophages. For both cytokines, there were groups of bacterial strains that were statistically different. These findings agree with the hypothesis and WHO findings that probiotic effects are strain-specific. The bacterial strains seemed to modulate the production of IL-10 more effectively than production of TNF-α. LPS alone caused RAW 264.7 macrophages to produce less IL-10 than when LPS was added in addition to a bacterial treatment. Since IL-10 reduces the immune response, these bacteria would have an alleviating effect in the colon during IBD or IBS. The addition of bacteria with LPS did not alter TNF-α production by the macrophages compared to LPS. Macrophages would still produce an inflammatory immune response, even in the presence of these probiotics. The ratio of TNF-α/IL-10 production demonstrates a possible mechanism for the effect *B. animalis* on the immune system. Although the treatments do not decrease the amount of TNF-α produced during an inflammatory response (when RAW 264.7 macrophages are exposed to LPS), these bacteria do increase the amount of IL-10 produced when LPS is present. The data suggests the probiotics cause the production of IL-10 at the same time as TNF-α is being produced by RAW 264.7 macrophages. *In vivo*, this effect would be expected to cause a shorter immune response compared to when only TNF-α is produced.

To improve the experiments, some modifications could be made. For SNP typing, new primers should have been used to prevent the age of the primers from inhibiting the sequencing. Another improvement would be to increase the number of replications for the NO, MTT, and ELISA experiments. Although multiple wells were used for each experiment, improved statistical power would be generated by using more than two plate replications. During the bacterial growth
curve, turbidity was used because the focus was on the stage of growth the cells were in and not the actual quantity of cells. Bacterial cells added to the co-culture with RAW 264.7 macrophages were all grown for the same length of time. In hindsight it was have been better to normalize the number of bacterial cells for each organism.

The use of probiotics in vivo is a new area of research for the Roberts laboratory. Most work is currently done in bacterial genomics and dairy food processing. Through this experiment, different techniques had to be learned. One of the essential techniques for the experiment was how to grow eukaryotic cells, specifically RAW 264.7 macrophages. These cells do not grow in the same conditions as that the bacteria that the laboratory uses. A model needed to be established to be able to test the influence of different strains of B. animalis on cytokine production by RAW 264.7 cells. Because these bacterial cells are thought to have a beneficial effect, the model was designed so that the addition of B. animalis cells did not cause great stress on the macrophages, causing cell morphology alterations. Techniques for three different assays were learned in order first to design the model using the MTT assay and the NO assay, and second to differentiate the strains of B. animalis ssp. lactis and B. animalis ssp. animalis by quantifying IL-10 and TNF-α production by RAW 264.7 cells using sandwich ELISA. The experiment also utilized techniques already performed in the Roberts laboratory for B. animalis characterization such as bacterial growth and SNP typing.

The beneficial health effects of probiotic bacteria need to be further explored to understand how these bacterial treatments can be best used for clinical applications (Vanderpool, Yan, & Polk, 2008). To expand from this experiment, additional strains of B. animalis ssp. lactis could be tested to have a wider understanding of strain-specific probiotic effects. Using these eight closely bacterial strains, it might be possible to identify specific genes that modulate the immune system and compare them to other sequenced bacteria that have beneficial or harmful effects on colon health. Other cytokines could also be quantified in addition to IL-10 and TNF-α
such as IL-6 and IL-12. These bacteria could also be tested *in vivo* against simulated IBD or IBS in mice. Immunological and histological effects could be measured to quantify the differences between probiotic effects of different strains.

IBD and IBS are difficult diseases to cope with due to the symptoms that limit normal everyday functions. Although there are medicinal treatments available to patients with these diseases, they are not effective for all patients. The use of probiotics to treat the symptoms provides an alternative to expensive medications. *B. animalis* is a possible probiotic species and this experiment has demonstrated that the health benefits of these bacteria are strain-specific.
## Appendix I

Table APP-1: Primers used for SNP typing *B. animalis* with their melting temperatures. Primers were provided by Joseph Loquasto.

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BIBLIOGRAPHY


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