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Investigation of the Beneficial Effect of Enterobacter Cloacae Strain JD6301 on Mice Challenged with Escherichia Coli O157:H7

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Investigation of the beneficial effect of *Enterobacter cloacae* strain JD6301 on mice challenged with *Escherichia coli* O157:H7

By

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Investigation of the beneficial effect of *Enterobacter cloacae* strain JD6301 on mice challenged with *Escherichia coli* O157:H7

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The environment of the gastrointestinal (GI) tract is an extremely complex system made up of not only host cells, but also many beneficial microbes. Disruption of this environment can often lead to disorders and health issues. To help balance this system, probiotics have often been administered to both humans and animals, such as livestock. This study aimed to determine what beneficial effects a novel strain of *Enterobacter cloacae*, strain JD6301, could offer to a host in the presence of an enteric infection with *E. coli* O157:H7. Upon administration of JD6301, supplemented animals had overall less *E. coli* present in the colon and caecum. Moreover, these animals shed more *E. coli* than control groups. Supplemented animals also had increased concentrations of serum triglycerides one day prior to challenge. Together, these data suggest that *Enterobacter cloacae* JD6301 could perform as a novel probiotic providing energy and protection to the host.

DEDICATION

I would like to dedicate this work to my friends and family. This completion of this work would not have been possible without the constant encouragement and comradery of my family and friends. I would particularly like to thank my husband Cody Wilson, my parents Renae Grissett, Alan and Laura Grissett, Kurt and Cindy Aldy, and Richard Wilson. I am also extremely grateful to all my previous and current lab mates in both the Donaldson and Thornton labs.

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CHAPTER I

LITERATURE REVIEW

1.1 Environment of the gastrointestinal tract

The gastrointestinal (GI) tract of mammals is a complex environment composed of a variety of cells, including host cells and many commensal microflora (Gaggia et al. 2010). In mammals, the amount of commensal bacteria in the gut can exceed the number of mammalian cells by nearly ten-fold (Sears 2005). The colonization of the mammalian GI tract with these commensal bacteria begins after birth and is dependent upon a variety of factors including environment and diet (Guarner and Malagelada 2003, Holzapfel et al. 1998). The bacteria that inhabit the gut play an extremely important role in the overall health of the host, as they offer both nutritional and protective benefits, and work with the host to promote immunity (Gaggia et al. 2010, Lebeer et al. 2010, Ohashi and Ushida 2009).

The benefits conferred to the host by the gut microbiota are varied and many times essential. In regards to nutrition, commensal bacteria are important for the host since they are responsible for the synthesis of vitamins, the breakdown of certain substances, and the production of enzymes that aid in digestion (Chaucheyras-Durand and Durand 2010, Holzapfel et al. 1998). In particular, one study found that animals whose normal flora have been removed require more calories to maintain their weight than animals whose normal flora was still intact (Wostmann et al. 1983). This could be

due to the fact that, as other studies have found, the gut microbiota also seems to play a role in the absorption of carbohydrates and lipids, as well as potentially regulating fat storage (Backhed et al. 2005, Xu et al. 2003).

Microbes in the GI tract are able to offer physically protective benefits to the host through a variety of mechanisms. For example, bacteria in the gut are capable of the fermentation of undigested food, which can produce beneficial byproducts, such as short chain fatty acids (Ohashi and Ushida 2009). These short chain fatty acids can lead to the production of butyrate that can increase growth of epithelial cells. This epithelial regulation is lost if the balance of the gut flora is disrupted (Ohashi and Ushida 2009). The bacteria that reside in the gut are also thought to aid in the upkeep of the mucosal barrier of the GI tract by promoting mechanisms that increase mucosal defense (Gaggia et al. 2010, Resta-Lenert and Barrett 2003). Commensal bacteria also protect the host by blocking binding sites in the GI tract that could be used by pathogens to colonize as well as by producing antibacterial bacteriocins, which inhibit potential invading pathogens (Guarner and Malagelada 2003, Ohashi and Ushida 2009). This is emphasized in studies where mice whose normal flora had been removed were overall more susceptible to infections with enteric pathogens (Neish 2009).

The gut microbiota also has a profound effect on host immunity, particularly mucosal immunity (Guarner and Malagelada 2003, Sears 2005). The health of the intestinal mucosa is extremely important, as it is one of the largest and first physical barriers between the host and the outside world (Guarner and Malagelada 2003). Some studies suggest that a mutually beneficial co-evolution occurred, benefiting the host via the addition of new epitopes to the antigen repertoire, in turn protecting commensals from

destruction by host immune cells (Ghosh 2013). Moreover, it has been shown that in mouse models where the commensal bacteria have been removed from the gut, animals suffer from a less developed immune system, again with an emphasis on mucosal immunity (Guarner and Malagelada 2003, Sears 2005).

In innate immunity the normal flora plays a role in the production of angiongen-4, a bactericidal compound that targets Gram-positive organisms specifically; this not only plays a role in immunity, but also the development of the gut microbiota (Ghosh 2013, L. Hooper 2005, Sears 2005). The normal flora of the GI tract influences adaptive immunity through stimulation of Peyer's patches and development of lymphocytes in the intestine (Ghosh 2013, L. Hooper 2005, Sears 2005). Development of Peyer's patches is particularly important, as these are germinal centers for B and T cells in the GI tract (Ghosh 2013, L. Hooper 2005). Stimulation of immunoglobulin A (IgA) production is another key factor in which the gut microbiota influence the adaptive immunity of the host, and this effect is lost if the gut microbiota is absent (L. Hooper 2005, Sears 2005).

Because the relationship between the host and gut microbiota is so complex, and gut microbes play such an important role in host health, dysfunction in the GI tract environment can lead to disorders and disease (Guarner and Malagelada 2003, Ng et al. 2009). Stress in the forms of physiological and psychological can cause this disruption in the gut, which in turn leads to potential health issues for the host (Gaggia et al. 2010, Gareau et al. 2011). Some complications of this disruption include inflammatory bowel disease, ulcerative colitis, Crohn's disease, enteric infections and some believe, colon cancer and autoimmune diseases (Gaggia et al. 2010, Guarner and Malagelada 2003, Ng et al. 2009). In the past, treating and preventing these disorders often included the use of

antibiotics (Gaggia et al. 2010, Hardy 2002, Reid and Friendship 2002, Rolfe 2000). However, there is growing concern in the wake of an increasing number of antibiotic resistant strains of dangerous pathogens, which has led to an increase in the use of probiotics (Gaggia et al. 2010, Hardy 2002, Reid and Friendship 2002, Rolfe 2000).

1.2 Probiotics

Probiotics are usually live yeast or bacterial strains or cell wall components that provide a benefit to the host when administered, usually by colonizing and balancing the gut environment via interaction with resident flora (Chaucheyras-Durand and Durand 2010, Fuller 1989, Hakansson and Molin 2011, Ohashi and Ushida 2009). The FAO/WHO describe probiotics as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance". Initially, probiotics were used mainly as feed supplements for livestock to promote growth (Galdeano et al. 2007). This use came in response to an increasing number of antibiotic resistant pathogens, which led to a call for cessation of the use of antibiotics as growth promoters in the livestock industry (Gaggia et al. 2010, Hardy 2002). Along with functioning as a replacement for antibiotic growth promoters in livestock, probiotics are also now widely used in humans, as they also provide benefits to human hosts (Gaggia et al. 2010, Galdeano et al. 2007, Hardy 2002).

The concept of using non-pathogenic bacteria as a way to influence health of the host was first proposed by Metchnikoff in 1907 (Dunne et al. 2001). Metchnikoff suggested that the ingestion of these helpful bacteria would have a stabilizing effect on the normal flora of the GI tract, as he believed that the endogenous normal flora might actually function in a way that is detrimental to the host (Dunne et al. 2001, Holzapfel et

al. 1998). Some of the bacterial genera most commonly used as or in probiotics preparations include *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*, although many more genera are now being studied for probiotic potential (Galdeano et al. 2007, Holzapfel et al. 1998). In order for a microorganism to be a successful probiotic, it must be able to survive in the host environment (Rolfe 2000). Organisms considered for potential probiotics must be able to survive decreased pH, enzymes in the GI tract, and bile acids, among other things (Rolfe 2000). Moreover, potential probiotics must also be able to be produced on a large scale, remain viable for a certain amount of time and must be safe to the host (Ohashi and Ushida 2009).

Many commercially available probiotic bacterial strains were originally isolated from humans and exposure to these bacteria can naturally occur very soon after birth during initial colonization of the GI tract via breast milk (O'Hara and Shanahan 2006). One of the genera of bacteria most predominately found in the GI tract of breastfed infants, and found in lesser numbers in the GI tract of formula fed infants, is *Bifidobacterium* (Mountzouris et al. 2002, Parracho et al. 2007). Other genera of common commercially available probiotics that can be isolated directly from breast milk include *Lactobacillus*, *Lactococcus*, and *Enterococcus* (Lara-Villoslada et al. 2007). Many studies have found that breastfed infants are better protected from common enteric pathogens and have overall better health and immunity than infants fed formula (Mountzouris et al. 2002, Parracho et al. 2007). This natural example of the role of probiotics and the effect they can have at an integral stage of gut development and immunity suggests that regular administration of beneficial microbes is a practical application (Mountzouris et al. 2002, Parracho et al. 2007).

1.3 Human use of probiotics

Humans have been consuming preparations containing probiotics for nearly a century through the consumption of natural remedies (Reid and Friendship 2002). Modern probiotic preparations have been available to humans for years, with most of their original success in a small niche in Japan (Dunne et al. 2001). After gaining popularity, probiotics for human consumption can now be found in many commercially available foods and dietary supplements including yogurts and cheeses (Bayoumi and Griffiths 2012, Hakansson and Molin 2011). In Europe alone, there are greater than 1 million people who consume probiotic preparations (Saxelin 2008). Various countries, including the United States, Canada, and European countries have established legislation to ensure safety of probiotics used for human consumption (Gaggia et al. 2010). The U.S. Food and Drug Administration has established that probiotics should meet the Generally Regarded as Safe ("GRAS") standards while Europe has enacted similar legislation known as Qualified Presumption of Safety for probiotics (Gaggia et al. 2010).

Once a bacterial or yeast strain is deemed safe and beneficial for human consumption, a way to manufacture and deliver the probiotic product must be developed (Ross et al. 2005). Probiotics must be prepared in a way that they will have an adequate shelf life, remain viable during preparation, as well as survive inside the host after ingestion (Ross et al. 2005). To prepare dietary supplements, often taken in the form of a pill, probiotic strains are often freeze dried (Ross et al. 2005). Along with freeze dried products in pills, probiotics can also come in the forms of powders or gel capsules (Saxelin 2008). The most common food preparations of probiotics are usually fermented foods and dairy products, like yogurts, milk, cheeses and some deserts (Saxelin 2008).

The consumption of these probiotic preparations can offer many beneficial effects to the human host, and perhaps the most prominent is the ability of probiotics to positively affect the environment and resident flora of the GI tract as well as preventing colonization of enteric pathogens (Holzapfel et al. 1998). Asahara et. al found that when certain strains of *Bifidobacterium* were administered to mice, the presence of these bacteria in the GI environment decreased the pH of the intestine and increased the acetic acid concentrations of the gut (Asahara et al. 2004). This change in the gut environment attenuated the shiga toxin producing strain *E. coli* O157:H7 by inhibiting shiga toxin production (Asahara et al. 2004). Another study examining the effect of probiotics on *E. coli* O157:H7 found that the presence of *Bifidobacterium* strains was able to significantly reduce the amount of *E. coli* bound to Vero cells, kidney cells isolated from an African green monkey (Tahamtan et al. 2011). Studies have also shown the probiotic species may serve to help treat colonization of *Helicobacter pylori*, a gram-negative bacterium responsible for gastric distress, ulcers, and potentially cancer (Parsonnet et al. 1991, Wang et al. 2004). *In vitro* studies have found that various strains of *Lactobacillus* were able to inhibit growth of *Helicobacter pylori* (Midolo et al. 1995).

Along with improving gastrointestinal health by inhibiting pathogens, probiotic species also have the ability to beneficially affect other conditions including diabetes, allergies, and eczema in children (Allen et al. 2014, Gomes et al. 2014). Probiotics have been shown to be potential adjuvants in treatments for insulin resistance (Andreasen et al. 2010, Ejtahed et al. 2012, Moroti et al. 2012). People with type 2 diabetes have also been reported to have lower concentrations of two probiotic strains *Bifidobacterium* and *Faecalibacterium* in their GI tract, suggesting their presence may play a role in

maintaining the balance that can lead to development of diabetes (Furet et al. 2010). Studies have also shown that the incidence of eczema in infants can be reduced when probiotics are administered and that overall, probiotic usage decreases the likelihood that individuals will develop eczema (Mansfield et al. 2014, West et al. 2009). An *in vivo* study found that supplementation with a *Lactobacillus* strain was also able to ease the symptoms of children with Crohn's disease (Guandalini 2002). These results have been confirmed by countless other studies examining the efficacy of probiotics in treating various forms of inflammatory bowel diseases, like Crohn's disease and ulcerative colitis (Bai and Ouyang 2006, Ng et al. 2009).

1.4 Probiotic use in livestock

Like humans, the GI tract of domestic livestock is a complex environment that can be regulated and maintained with the use of probiotics (Chaucheyras-Durand and Durand 2010). Probiotic bacterial strains have been used in the livestock industry as a way to promote growth and overall health of animals for decades (Fuller 1989). Initially, the use of probiotics came amid growing fear of an increasing number of antibiotic resistant strains of dangerous pathogens and downstream human exposure to these resistant bacteria (Fuller 1989, Gaggia et al. 2010). As early as the 1970s, organizations around the world began banning the use of antibiotics as growth promoters in the industry (Thormar 2012). By 2000, the World Health Organization had recommended the full cessation of all antibiotic therapies that were used as growth promoters alone (Thormar 2012). Although antibiotic therapies are still used, particularly in the United States, pressure and new alternatives have encouraged those in the industry to discontinue use slowly (Thormar 2012). To ensure safe products for consumers and to keep animals

healthy, administration of probiotics has become a common replacement for antibiotic therapy in order to promote animal immunity, growth, and help prevent infection and colonization of animals with pathogens (Gaggia et al. 2010). The last point is of extreme importance as foodborne illnesses account for nearly 76 million illnesses a year (Mead et al. 1999).

Many of the same genera of bacteria used as probiotics for human consumption are administered to livestock as well, including *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* (Dunne et al. 2001). Along with bacterial probiotics, various forms of yeast probiotic preparations are also often used for domestic livestock (Chaucheyras-Durand and Durand 2010, Gaggia et al. 2010). Some of the yeast strains most commonly used as probiotics include *Saccharomyces cerevisiae* and *Aspergillus sp.* (Gaggia et al. 2010). Probiotics are administered to a variety of domestic livestock, most often pigs, ruminants (such as cattle), and chickens (Dunne et al. 2001). Probiotics are thought to be particularly effective in these animals, as they are thought to aid in the prevention of successful colonization of enteric pathogens like *Salmonella*, *Campylobacter* and *E. coli* (Gaggia et al. 2010, Reid and Friendship 2002). Along with preventing pathogen colonization, probiotics can also contribute to the overall health of the animal, which can lead to increased production of milk or production of better meat (Gaggia et al. 2010, Reid and Friendship 2002). Preventing the initial colonization of livestock with these pathogens by using probiotics is a major step in the prevention of downstream infection of humans and subsequent foodborne disease (Gaggia et al. 2010).

There have been many successful studies on the efficacy of probiotics in various livestock (Chaucheyras-Durand and Durand 2010, Gaggia et al. 2010). In dairy cattle,

yeast like *Saccharomyces cerevisiae* have been shown to increase milk production, while in beef cattle, probiotic yeast supplementation led to increased feed to gain ration, final weight and increases in other growth parameters (Chaucheyras-Durand and Durand 2010). Bacterial probiotics have also been used in cattle and some *Lactobacillus acidophilus* strains have been shown to decrease the amount of *E. coli* O157 in cattle feces (Chaucheyras-Durand and Durand 2010). Zhao et. al found that after administration of a probiotic mixture to calves, *E. coli* was only detected in the rumen fluid for 14 days compared to 26 days in control calves (Zhao et al. 1998).

Concerning swine, probiotics are found to be particularly useful in pregnant sows as administration during this time can positively affect the litter (Chaucheyras-Durand and Durand 2010). After birth, probiotics have also been found to be useful during and after the weaning process (Chaucheyras-Durand and Durand 2010, Gaggia et al. 2010). Immediately after weaning, piglets are typically moved to new locations and provided antibiotics to prevent disease (Reid and Friendship 2002). The antibiotics are now thought to be a determining factor in many diseases that develop in weaned pigs due to an insufficient gut microbial community (Reid and Friendship 2002). Probiotics, rather than antibiotics, may be able to offer protection during the weaning process by balancing and not disrupting the gut community (Reid and Friendship 2002). Moreover, administration of probiotics to healthy weaned piglets has led to many beneficial effects, such as weight gain and more efficient feed utilization (Fuller 1989, Reid and Friendship 2002).

In poultry, *Lactobacillus* and *Bacillus* sp. seem to be effective probiotics. In a study examining the effectiveness of a mixture of three *Bacillus* strains, birds provided the probiotic mix had increased digestibility and nutrient retention (Waititu et al. 2014).

Other studies found that administration of *L. salivarius* by oral gavage or by inclusion in feed led to clearance of *Salmonella* from chickens tested (Reid and Friendship 2002). Other studies have found that hens fed probiotics produce eggs with lower yolk cholesterol levels (Kurtoglu et al. 2004). Probiotic use in chickens has also been shown to be able to decrease mortality rates due to necrotic enteritis when administration was begun immediately after birth (Hofacre CL 2003).

1.5 Mechanism of action

Probiotics are able to affect the health of the host in many ways, and as such, there is not one definitive mechanism of action through which probiotics are able to provide these benefits (Barzegari et al. 2014, Bayoumi and Griffiths 2012). There are three general classes in which probiotics activity can be classified: direct antagonism, immunomodulation, and exclusion (Preidis et al. 2011). These classifications can themselves contain more than one mechanism by which probiotics are able to benefit the host, and as such it is essential to understand these mechanisms in order to continue developing effective products (Ng et al. 2009, Preidis et al. 2011).

By directly antagonizing pathogens, probiotics are able to protect the host from potentially dangerous enteric infections (Preidis et al. 2011). Probiotic antagonists of pathogens are usually secreted molecules, such as antimicrobials, or byproducts, like lactic acid (Asahara et al. 2004, Forestier et al. 2001). Forestier et. al found that the supernatant of *L. casei rhamnosus* contained a molecule that was able to inhibit the growth of a combination of aerobic, anaerobic, Gram-negative and Gram-positive pathogens by 5 hours *in vitro* (Forestier et al. 2001). This inhibitory molecule was determined to be a bacteriocin-like compound secreted by the *Lactobacillus* strain

(Forestier et al. 2001). Others have found that acid by-products of lactic acid bacteria reduce the pH of the intestine, decreasing the concentration of pathogens like *E. coli* in the gut (Asahara et al. 2004, Hutt et al. 2006). Moreover, one study found that acid produced by probiotics may also act to permeabilize pathogen membranes, and thus make them more susceptible to other harmful compounds (Alakomi et al. 2000). Bayoumi et. al. found that molecules secreted by probiotics were actually able to attenuate pathogenic strains without affecting the growth of these strains, although the mechanism and the composition of these molecules involved has not been determined (Bayoumi and Griffiths 2012).

Immunomodulation is another important way in which probiotics are able to provide benefit to the host (Preidis et al. 2011). Studies have found that children provided lactic acid bacteria orally had a more robust IgA response to gastroenteritis caused by rotavirus (Majamaa et al. 1995). Adults receiving an attenuated version of *Salmonella typhi* as a vaccination were also provided probiotics had an increased IgA response to the vaccination (Link-Amster et al. 1994). A study that provided two groups of healthy adults fermented milk products containing either a *Lactobacillus* or *Bifidobacterium* strain indicated that the presence of these organisms in the intestine increased the phagocytic activity of cells in peripheral blood against *E. coli* (Schiffrin et al. 1995). There does seem to be variation in the effect a strain has on the immune system (Preidis et al. 2011). One study found that *Lactobacillus rhamnosus* GG led to an increase in tumor necrosis factor (TNF), while other studies have found that different strains of *Lactobacillus* lead to a decrease in TNF (Iyer et al. 2008, Miettinen et al. 2000, Petrof et al. 2004). A non-pathogenic strain of *E. coli* used as a probiotic produces indole, which

can modulate the immune system by balancing the production of pro- and anti-inflammatory cytokines as well as by increasing secretion of IL-10 (Bansal et al. 2010).

Through competitive exclusion, or simply making the environment of the GI tract unfavorable to pathogens, probiotics are also known to offer protection to the host (Preidis et al. 2011). One very common example of exclusion of pathogens occurs when probiotic strains bind to many sites within the epithelium, leaving less space for pathogens to attach and colonize (Tuomola et al. 1999). Many strains of commonly used probiotic organisms are members of the normal flora from either a human or animal host, and as such most have an affinity to bind to the epithelium of the GI tract making them effective barriers against pathogens (Gill et al. 2001, Servin and Coconnier 2003). Strains of two of the most common genera, *Lactobacillus* and *Bifidobacterium*, were both found to adhere well to Caco-2 cells *in vitro* as well as adhering well *in vivo* (Servin and Coconnier 2003). Indole produced by probiotic strain of *E. coli* increases the expression of genes responsible for forming tight junctions and the actin cytoskeleton, improving the intestinal barrier (Bansal et al. 2010).

1.6 Future applications

Modern probiotics can provide many benefits to the host, however, there are limitations in the knowledge we currently have of these probiotics and mechanisms responsible for their effectiveness; moreover, little is known regarding how probiotics may serve as a source of energy to the host (Hakansson and Molin 2011, Ng et al. 2009). Many probiotics are able to defend the host against pathogenic infection, however, in the event that infection does occur, the host will go through the metabolically demanding process of mounting an immune response (Demas et al. 1997). During infection, energy

reallocation occurs as the organism must move energy from other biological functions to promote immunity (Sheldon and Verhulst 1996). This energy demand is also evident when an animal is undergoing a stressful period which can also require a reallocation of energy, leaving the animal with less energy to mount an immune response, and thus more susceptible to illness (Chaucheyras-Durand and Durand 2010, Demas et al. 1997).

Some common stressors of animals can include a change in food, such as when animals are weaned, a change in environment, or a change in community, among others (Chaucheyras-Durand and Durand 2010, Demas et al. 1997). Studies have found that stressors like those mentioned previously can actually cause an animal to initiate an acute immune response, again sequestering energy away from other essential biological functions (Colditz 2002, van Heugten et al. 1996) .*In vivo* studies in chickens whose immune system had been activated by sheep red blood cells showed that those animals had higher energy usage coupled with weight loss, despite the fact that those animals consumed more food than control groups (Demas et al. 1997). Overall, data on energy usage during stress or immune activation suggest that reallocation of energy may sometimes be detrimental to the animal and that providing an alternative energy source to the host may be able to stabilize this allocation process (Demas et al. 1997).

Our lab has recently identified a strain of *Enterobacter cloacae*, JD6301, that is capable of producing lipids, stored in inclusion bodies in the cell, at nearly 47% of its cell weight, classifying it as an oleaginous bacterium (Wilson et al. 2014). Bacteria that are considered oleaginous accumulate lipids at least 20% of their cell weight in some form of lipid, such as triglycerides (TAGs) (Alvarez and Steinbuchel 2002). Triglycerides have long been a known form of stored energy source for eukaryotic organisms, and recent

research has shown that some bacteria, like *Rhodococcus opacus*, also store TAGs as a form of emergency energy (Alvarez et al. 2000). *Enterobacter cloacae* strain JD6301 has many features that suggest it could be utilized as a probiotic, including its ability to grow at 37°C, tolerate the environment of the host GI tract, and provide energy via TAGs stored in inclusion bodies near the membrane of the cell (Wilson et al. 2014).

Knowledge of lipids and the role they play in immunity and health is ever growing (de Pablo and Alvarez de Cienfuegos 2000, Im et al. 2011). The influence of various forms of lipids on the immune system can be varied ranging from regulating lymphocyte proliferation to effecting cytokine production (de Pablo and Alvarez de Cienfuegos 2000). Studies have shown that unsaturated fatty acids may modulate the proliferation of T lymphocytes, leading to an overall reduction in their circulation (de Pablo and Alvarez de Cienfuegos 2000). Because of their ability to potentially reduce lymphocyte production, it has been suggested that these unsaturated fatty acids could serve to help relieve symptoms of some autoimmune diseases (de Pablo and Alvarez de Cienfuegos 2000). Although this application could be useful, it would require diligent supervision as administration of lipids capable of causing immunosuppression in too large a quantity could be dangerous (de Pablo and Alvarez de Cienfuegos 2000).

Lipids have also been shown to play a more direct role in immunity via cytokine regulation and protection against pathogens (Barcia and Harris 2005, Im et al. 2011). *In vivo* studies have shown that supplementation of fish oil to mice increased serum concentrations of interferon gamma when challenged with *Listeria monocytogenes* (de Pablo and Alvarez de Cienfuegos 2000). During infection, concentrations of lipids circulating in the bloodstream can also increase, a condition which can be dangerous if

not controlled (Barcia and Harris 2005). The presence of circulating lipids in the bloodstream, as long as controlled, is also extremely beneficial to the host (Barcia and Harris 2005). Studies have shown that lipoproteins rich in triglycerides are capable of binding and neutralizing lipopolysaccharide (LPS) produced by pathogens, and this complex can act to modulate an immune response (Barcia and Harris 2005). Moreover, *in vivo* studies have shown that mice that have inactivated SREBP-1a, a lipogenic transcription factor, have a deficient innate immune response (Im et al. 2011).

Available data on the role of lipids in immunity and health suggests that extra lipids made available to the host may be able to offset the metabolic shift during stress and illness (Barcia and Harris 2005, Demas et al. 1997). The identification of JD6301 presents an opportunity to develop a novel probiotic that would not only function to protect the host in the traditional aforementioned ways, but also supply an extra source of potentially useable energy in the form of stored TAGs. Development of this bacterium as a novel probiotic could bridge the current gap in modern applications of probiotics and have impacts not only on human and animal health but also industry, with many potential uses in the food and livestock industries.

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CHAPTER II

GENOME SEQUENCE OF *ENTEROBACTER CLOACAE* STRAIN JD6301

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2.1 Introduction

Enterobacter is a genus of the family *Enterobacteriaceae.* This family is composed of Gram-negative rods, which can be found in many different environments, ranging from soil to humans. Many strains of *Enterobacter* are known to be resistant to an array of antibiotics and some are considered nosocomial pathogens (Ren et al. 2010). Here we report the genome sequence of novel *Enterobacter cloacae* strain JD6301. This novel strain was isolated from a co-culture of wastewater collected from a municipal treatment facility and oleaginous bacteria. The bacterial strain was isolated by subsequent dilutions into fresh media and finally by culturing onto nutrient agar under aerobic conditions at 30°C. The bacteria were found to grow in a wide variety of conditions, including temperatures ranging 28-39°C and were also found to tolerate slightly acidic conditions (pH 4.5). On blood agar JD6301 exhibits alpha hemolysis. MacConkey and Eosin Methylene Blue agar confirm that it is capable of utilizing lactose. It is oxidase

negative, and exhibits gas production in triple sugar iron agar. The most unique feature of JD6301 is its ability to form inclusion bodies, which has not been previously characterized for this species. The lipid weight of this strain was found to constitute approximately 50% of the total cellular weight, suggesting that these inclusion bodies may contain lipids.

A draft of the genome of *Enterobacter cloacae* JD6301 was generated at the DOE Joint Genome Institute (JGI) using Illumina HiSeq 2000 technology. To remove any artifacts following Illumina sequencing, raw data was passed through DUK, a program developed by JGI. Illumina reads were assembled using Velvet v. 1.104 (Zerbino and Birney 2008), 1-3 kb simulated paired ends were created from Velvet contigs using wgsim (https://github.com/lh3/wgsim), and Illumina reads were then assembled with simulated pairs using Allpaths-LG v. r42328 (Gnerre et al. 2011). The assembly yielded 53 contigs in 49 scaffolds. For genome annotation, Prodigal was used to identify genes (Hyatt et al. 2010). This was followed by manual curation using GenePRIMP (Pati et al. 2010). tRNAScanSE (Lowe and Eddy 1997) was used to find tRNA genes, while rRNA genes were identified using SILVA (Pruesse et al. 2007). INFERNAL (http://infernal.janelia.org) was used to identify non-coding RNAs. Other gene predictions and manual function annotation were performed using the Integrated Microbial Genomes (IMG) platform (http://img.jgi.doe.gov) developed by JGI (Markowitz et al. 2009).

The completed genome of strain JD6301 is 4,772,910 bp in length. There are an estimated 4,288,696 coding bases and the G+C content is near 53%. There are a total of 4,509 protein-coding genes, with 84.91% predicted to have a function. Of the genes

identified, 4,246 match to *Enterobacter cloacae.* Multiple multidrug efflux pumps were identified, which is common among *Enterobacter cloacae* (Ren et al. 2010). Genes associated with a Type IV secretion system were also identified, another common feature of *Enterobacter cloacae* (Liu et al. 2013). Proteins associated with pilus production were also identified, which may have contributed to the inclusion bodies observed in this novel strain via exchange of genetic material. The novel aspect of an *Enterobacter sp.* strain producing large quantities of lipids warrants further investigation.

2.2 Nucleotide sequence accession number

This draft genome sequence has been deposited in the IMG system as accession number 20133 and GenBank under accession number JDWH00000000.

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CHAPTER III

ENTEROBACTER CLOACAE JD6301 ALTERS THE METABOLIC RESPONSE OF MICE WHEN CHALLENGED WITH ENTERIC PATHOGEN *E. COLI* O157:H7

3.1 Introduction

The gastrointestinal (GI) tract of mammals is a complex microbial ecosystem that is involved in providing many benefits to the host, including aiding in digestion, contributing to the gut immune system, and preventing the successful colonization of potential pathogens (Chaucheyras-Durand and Durand 2010). The gut also plays an important role in overall health of mammals (Gaggia et al. 2010). Stress that affects the gut microbiota can lead to an imbalance in the gut and subsequently increased susceptibility to pathogens or gastrointestinal disorders, such as irritable bowel syndrome (Gaggia et al. 2010). This delicate balance between the commensal microbes and the host is often regulated by probiotics in both humans and livestock (Chaucheyras-Durand and Durand 2010, Ohashi and Ushida 2009).

Probiotics are classified as viable cells or cell components that provide a health benefit to the host (Asahara et al. 2004). These supplements are typically used to influence the gut mucosal immunity and may provide protection against invading pathogens. However, limitations exist in the usefulness of probiotics during infections. This is primarily due to the energy required by the host to mount an efficient immune response, which is provided through diet and fat (lipid) reserves (Segerstrom 2007).

Infections increase the energy demand upon the host, which leads to shifts in energy metabolism and utilization, and often results in increases in glucose utilization and the synthesis of very low-density lipoproteins (van Heugten et al. 1996). Loss of appetite or insufficient energy reserves can affect overall energy availability, thereby negatively impacting the outcome of disease (van Heugten et al. 1996). This is commonly observed in piglets during the weaning process; these animals often experience periods of decreased feed and water intake, limiting the energy available to them (Lalles et al. 2007). This can also lead to disruption of the gut environment, making these animals more susceptible to infection (van der Peet-Schwering et al. 2007).

Along with their vital role in metabolism, it has also become clear that lipids have an integral role in innate immunity. In response to infections, particularly the presence of lipopolysaccharides (LPS), the liver increases synthesis of lipoproteins rich in triglycerides (Barcia and Harris 2005). Lipids are capable of binding and neutralizing endotoxin (LPS), reducing infectious capacity. Additionally, lipids have also been shown to have an activating effect on the innate immune response (Barcia and Harris 2005). *In vivo* studies in mice have shown that animals lacking a key lipogenic transcription factor have deficient innate immune responses. While these mice were able to resist a challenge with LPS, they were unable to mount an efficient immune response when challenged with a bacterial pathogen orally (Im et al. 2011). These responses correlate with deficient innate immunity and exhibit the important role lipids play in immunity (Im et al. 2011).

Here, we utilized a novel approach of providing additional lipids through bacteria that could improve the availability of energy and also potentially act as a traditional probiotic. The *Enterobacter cloacae* strain JD6301, which produces lipids at 47% of its

cell weight (Wilson et al. 2014), was found to increase the amount of lipids available to an animal and also altered the metabolic response of mice when challenged with *Escherichia coli* O157:H7. Animals provided JD6301 also had increased serum concentrations of interleukin-1α following exposure to *E. coli* ($p < 0.05$), a key cytokine in the innate immune response (Cybulsky et al. 1986). Moreover, JD6301 also demonstrated the ability to survive in the GI tract and potentially prevent successful colonization of *E. coli* O157:H7.

3.2 Materials and methods

3.2.1 Bacterial strain and growth conditions

Enterobacter cloacae strain JD6301 was routinely cultured in mineral salts medium (MSM) with 25 ug/ml novobiocin at 37°C. For supplementation of the bacteria to the water, samples were cultured for 24 hr, after which $1x10^{10}$ CFU (~25ml) were stored at -20°C in 20% glycerol. On the day of administration, frozen aliquots were thawed, centrifuged for 10 min at 8000 *x g* to remove glycerol, and resuspended in 150 ml sterile Ultrapure water (Millipore) supplemented with 0.3% xanthan gum. *Escherichia coli* O157:H7 (ATCC 43895) transformed with the plasmid pXEN-13 was cultured in nutrient broth (NB) supplemented with 100 µg/ml ampicillin (amp) at 37°C (Free et al. 2012).

3.2.2 Animals

Forty BALB/c mice 5-6 weeks of age were purchased from Harlan Laboratories and allowed to acclimate to the animal facility at Mississippi State University for 5 days. During this period mice were provided sterile water and irradiated chow *ad libitum*. Mice

were separated into two treatment groups (n=20 mice/treatment with 4 mice/cage). Group 1 received sterile water supplemented with 0.3% xanthan gum; group 2 received sterile water supplemented with *Enterobacter cloacae* JD6301 (10^9 CFU/mL) and 0.3% xanthan gum. Fresh water bottles were prepared every other day; bottles were weighed to measure rates of water disappearance. All animal studies were conducted in accordance with procedures approved by the Mississippi State University Institutional Animal Care and Use Committee (Protocol #12-066).

3.2.3 *E. coli* O157:H7 infection model

Mice were provided PBS or JD6301 for the duration of the study. Mice were provided JD6301for 6 days prior to *E. coli* challenge with continued supplementation until sacrifice. On d 0 all mice were challenged with *E. coli* O157:H7 at a concentration of $1x10^{10}$ CFU via oral gavage (Gagnon et al. 2006). One cage from each group (n=4 mice per group) was humanely sacrificed on d -1, 1, 2, 3, and 4. Prior to being sacrificed, blood was collected from the mice via retro orbital bleed and serum was separated using a StatSpin StatSampler blood collection system (TP5G, Iris Sample Processing, Inc., Westwood, MA) and stored at -20°C. Feed intake and body weights were collected on the day of sacrifice.

3.2.4 GI tract/fecal collection and contents enumeration

Sections of the GI tract (caecum and colon) were homogenized in 1.5 ml tubes with 2.3 mm zirconia/silica beads (11079125z; Biospec Products, Bartlesvile, OK) and 1 ml phosphate buffered saline (PBS) in a BeadBug micro tube homogenizer (Benchmark Scientific, Edison, NJ) for 30 sec at 4,000 rpm. Samples were diluted in PBS and plated

on nutrient agar (NA) supplemented with 75 µg/ml novobiocin to select for *Enterobacter cloacae* JD6301 and NA with 100 µg/ml ampicilin (NA amp) to select for *E. coli.* Colonies were enumerated following a 16 h incubation at 37°C.

Fecal samples were collected on d 1, 2, and 3. Single pellets were homogenized in 1 ml of PBS, serially diluted and plated on NA amp to select for growth of *E. coli*. Colonies were enumerated following a 16 h incubation at 37°C.

3.2.5 Serum analysis

Serum was collected and analyzed for concentrations of triglycerides (TAGs) using the serum triglyceride determination kit (TR100, Sigma Aldrich, St. Louis, MO). Triglycerides were analyzed per manufacturer's instructions using 10 µl of serum. After preparation, samples were analyzed with a Biotek Synergy HT plate reader immediately. Serum TAGs were compared between mice provided JD6301 supplemented water and PBS control animals.

Serum cytokine concentrations were analyzed using the Milliplex mouse cytokine/chemokine multiplex assay (MCYTOMAG-70K-PMX, EMD Millipore, Billerica, MA). The serum was prepared per manufacturer's instructions using 25 µl of sample. Following preparation, all samples were analyzed immediately using Luminex xMAP technology (Millipore). Cytokine concentrations were compared between JD6301 supplemented and non-supplemented groups to determine differences in cytokine production. Twenty-six cytokines/chemokines were analyzed in triplicate.

3.2.6 Duration of intestinal persistence of *Enterobacter cloacae* JD6301

Twenty 5-6 week old Balb/c mice acclimated to the facility were separated into five cages (n= 4 mice/cage). JD6301 was provided via water to mice in each cage for 6 days prior to removal. On d 0, JD6301 was removed and mice were provided sterile water. Serum and intestinal samples were collected from mice from one cage on d 0, 1, 2, 3, and 4 and analyzed as stated in *E. coli* challenge section with the exception of intestinal contents only being tested for the presence of JD6301. All animal studies were conducted in accordance with procedures approved by the Mississippi State University Institutional Animal Care and Use Committee (Protocol #12-066).

3.2.7 Statistical analysis

A Student's t test was performed to determine statistical significance. P values of less than 0.05 were considered statistically significant.

3.3 Results and discussion

3.3.1 Enumeration of GI tract contents and fecal samples

All mice that were provided *Enterobacter cloacae* JD6301 had populations present in the cecum and colon, confirming that JD6301 remains viable in the host environment. Prior to challenge, JD6301 was detected in nearly equivalent amounts in each section collected. However, after challenge with *E. coli* O157:H7, JD6301 concentrations fluctuated in all sections (Fig. 1). This suggests that the presence of *E. coli* O157:H7 may have some effect on the viability of JD6301. One potential explanation for fluctuations in JD6301 concentrations post-challenge could be interactions between JD6301 and *E. coli*. This potential interaction suggests JD6301 may act as a traditional

probiotic by preventing the successful binding of a pathogen to the host GI tract, thereby preventing establishment of infections.

E. coli O157:H7 was detected in the aforementioned sections of the GI tract in all animals post-challenge. Treated and control animals had comparable concentrations of *E. coli* in the colon and cecum d 1-3 (Fig. 3.1). However, by d 4, treated animals had, on average, reduced concentrations of *E. coli* in both the cecum and the colon. There was an increase ($p<0.05$) in *E. coli* populations in the cecum of untreated animals from d 1 to d 4. Conversely, there was no difference ($p=0.9$) of *E. coli* populations in treated animals from d 1 to d 4. These data could suggest that the presence of JD6301 prevents *E. coli* from colonizing within the cecum. This is of particular interest due to the fact that in both ruminants and non-ruminants, including humans, *E. coli* colonization often occurs at the cecum (Baines et al. 2008, Rhee et al. 2011). The increase in JD6301 populations in this section suggests JD6301 may be able to compete for resources in this key area of the GI tract, potentially preventing successful colonization of pathogens like *E. coli*.

Fecal shedding of *E. coli* O157:H7 was monitored post-challenge (Fig. 3.1). On d 1 comparable *E. coli* shedding was noted in control and treatment groups. By d 3, mice provided JD6301 had on average more shedding of *E. coli* than animals in the control group. Controls animals had a significant increase in *E. coli* populations in the cecum on d 4, and decreased fecal shedding of *E. coli* by d 3. Conversely, mice provided JD6301 had increased fecal shedding of *E. coli* by d 3 and decreased concentrations of *E. coli* in the cecum and colon by d 4. This suggests that JD6301 interfered with the successful adherence of *E. coli* to the intestinal lining.

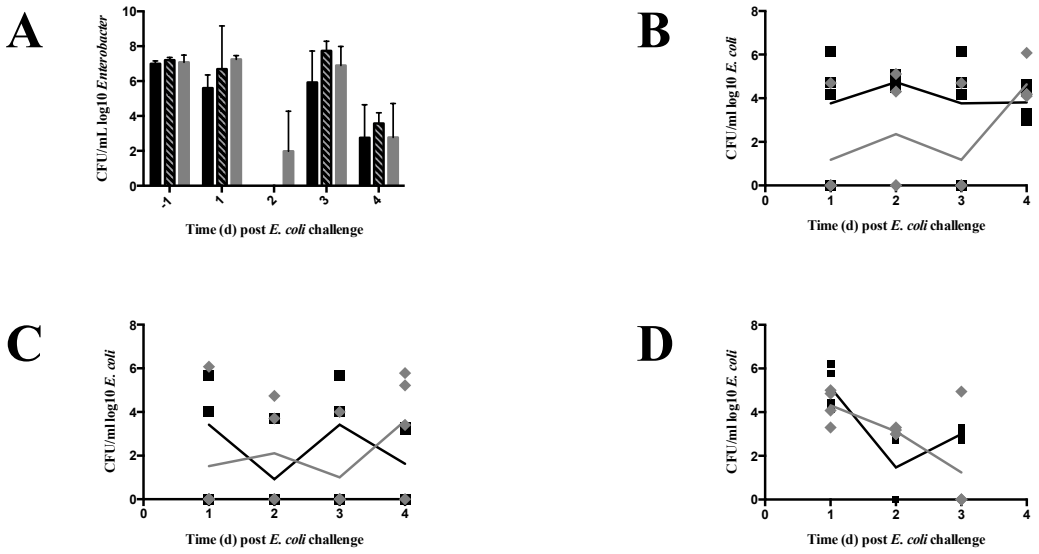

Figure 3.1 GI tract sections and fecal shedding

GI tract sections ileum (black), caecum (pattern), and colon (gray) were collected and contents were homogenized and plated to detect the presence of either JD6301 or *E. coli*. Presence of JD6301 was detected in all sections prior to *E. coli* challenge, with fluctuations post challenge (A). Cecum (B) and colon (C) sections of mice showed that by d 4, control animals (◆) had an overall higher burden of *E. coli* compared to animals provided JD6301 (■). Fecal shedding was detected via selective plating (D).

3.3.2 Serum TAGs and cytokine analysis

Serum was analyzed for both TAGs and cytokine/chemokine levels. Animals provided JD6301 had nearly double the concentration of serum TAGs on d -1 compared to non-treated groups. By d 1, control animals had an increase in concentrations of serum TAGs ($p<0.05$), while there was not a significant change in supplemented animals (Fig. 3.2). By d 4, the serum concentration of TAGs for all groups had returned to concentrations equivalent to those prior to *E. coli* challenge. Increased serum TAGs of treated animals on d -1 indicate that JD6301 may be providing additional usable energy to host animals upon administration. The significant increase of serum TAGs in control

animals on d 1 could be indicative of the metabolic burden placed on those animals in the presence of enteric pathogen challenge. Compared to control animals, mice provided JD6301 did not have a significant increase in serum TAGs on d 1, which suggests that these animals did not have to produce as many endogenous TAGs, but rather were able to utilize TAGs provided by JD6301. The decreased need to transport endogenous TAGs to the bloodstream during infections could demonstrate the overall lessened metabolic burden placed on animals provided JD6301.

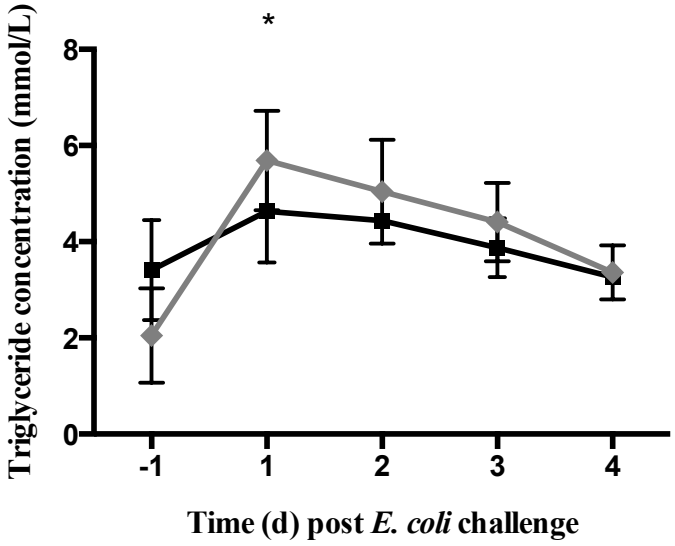

Figure 3.2 Serum triglyceride concentrations

Serum triglyercide concentrations were determined using a serum triglyceride kit using 10 µL of serum. One day prior to *E. coli* challenge, JD6301 supplemented animals (■) had a higher concentration of serum TAGs than control (◆) animals. By d 1 control animals had higher ($p < 0.05$) concentrations of serum TAGs.

Cytokine analyses indicated that by d 1, animals provided JD6301 had increased concentrations of the cytokine interleukin-1α (IL-1α) compared to control animals. Although JD6301 treated animals did not have a significant increase in serum TAGs

upon challenge with *E. coli*, those animals were still able to mount a response, as seen by the increase ($p<0.05$) of IL-1α one day after challenge compared to non-treated animals (Fig. 3.3). Additionally, JD6301 supplemented animals did not have an increase in IL-1α prior to challenge, confirming this was not a prolonged inflammatory response caused by JD6301, but rather an acute response prompted by challenge with *E. coli*. IL-1α is member of the Interleukin 1 family, which has 11 members total (Sims and Smith 2010). IL-1α is a cytokine that has widespread expression and induces an inflammatory response (Sims and Smith 2010). *In vivo* studies have shown that IL-1α also plays an important role in the recruitment of neutrophils (Barry et al. 2013). It will be important for future studies to examine the role JD6301 induced production of IL-1α may have in neutrophil recruitment.

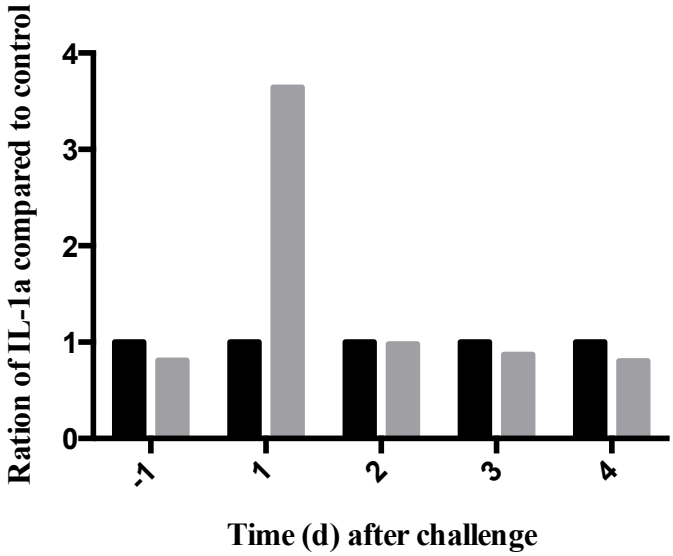

Figure 3.3 Ratio of IL-1α

Cytokine analysis was performed using Luminex technology. By d 1, JD6301 supplemented animals (gray) had a higher ratio of IL-1α present in the serum compared to control (black) animals.

3.3.3 Intestinal persistence of *Enterobacter cloacae* JD6301

To determine how often administration is required for JD6301 to remain present in the gut, the duration of intestinal viability was analyzed. *Enterobacter cloacae* JD6301 was detected in all intestinal sections collected from persistence study animals on d 0 and 1. However, by d 2, JD6301 was not detected in any of the sections collected (Fig. 3). This suggests that JD6301 can survive within the GI tract for no more than 48 hours. Serum TAG concentrations correlate with the increase and then decrease in JD6301 in the GI tract of persistence animals (Fig. 3.4). Persistence study animals sacrificed on d 0 had similar serum TAG concentrations as animals in the *E. coli* challenge on d -1 (Fig. 3.4). Within three days post-removal of JD6301, serum TAG concentrations had decreased below d 0 concentrations and continued to decrease on d 4, which was significantly less than d 0 concentrations ($p = 0.05$). Overall, these data suggest that while JD6301 is capable of providing benefits to the host, in order for JD6301 to confer these benefits, it must be repeatedly administered.

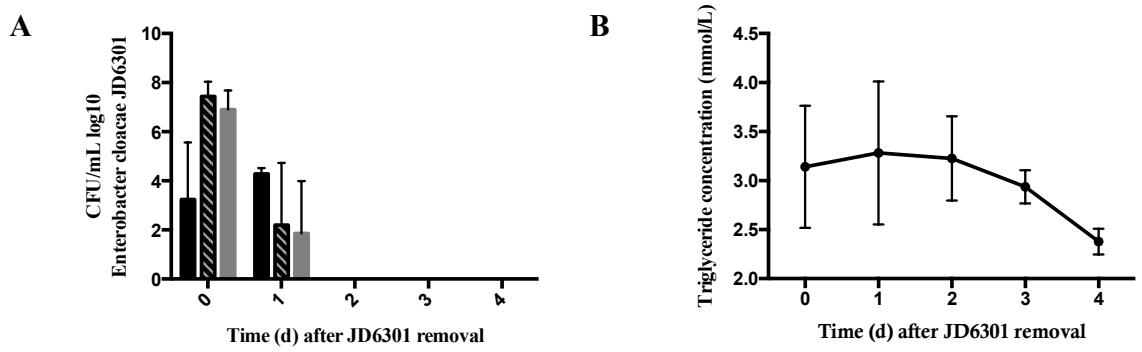

Figure 3.4 Intestinal presence of JD6301 and serum TAG concentrations

Persistence of *Enterobacter cloacae* JD6301 in the ileum (black), cecum (pattern), and colon (gray) sections of the GI tract. JD6301 was not detected in any section 48 hours after removal (A). Serum triglyceride concentrations of animals in the persistence study decreased after the removal of JD6301 (B).

3.4 Conclusion

The metabolic shift towards immunity animals undergo during periods of stress or infections leaves a deficiency in the energy available for other essential processes for survival. Our novel probiotic may be able to provide useable energy to animals during energy deficient states. Our data suggest that *Enterobacter cloacae* JD6301 may not only act as a novel source of useable energy, but also as more traditional probiotic by preventing the successful colonization of pathogens, like *E. coli* O157:H7. Providing JD6301 to animals was able to increase concentrations of serum TAGs, decrease overall colonization of *E. coli* in the caecum and colon, and promote increased levels of the cytokine IL-1α. While further study is needed to enumerate its mechanism of action, *Enterobacter cloacae* JD6301 may be useful as a tool to combat losses of livestock not only due to infection, but also starvation and energy depletion.

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CHAPTER IV

CONCLUSION

The gastrointestinal (GI) tract of mammals is an extremely complex environment and an integral part of health and immunity (Gaggia et al. 2010). As such, it is important to maintain the integrity and health of the GI tract, including the resident bacteria, in order to ensure its benefits are conferred to the host (Galdeano et al. 2007, Guarner and Malagelada 2003). Probiotics are often used to improve the health and immunity of the host (Gaggia et al. 2010, Hardy 2002). Probiotics are now widely used both in humans and animals and many benefits have been associated with their use (Reid and Friendship 2002). There are however gaps in knowledge of probiotic benefits, particularly related to their ability to provide useable energy to the host during metabolic shifts induced by stress or infection (Demas et al. 1997).

The literature reviewed in Chapter I examined the important role of the GI tract of mammals and how dysfunctions in this environment can lead to disease. Moreover, the role of probiotics and the current gaps in application of modern probiotics was also discussed. There are many different stressors that can lead to dysfunctions within the GI tract, including changes in diet, environment, and disease (Guarner and Malagelada 2003, Ng et al. 2009). These dysfunctions can affect the health of the host, and thus probiotics have often been used to balance the gut environment (Gaggia et al. 2010, Galdeano et al. 2007). Despite the benefits many probiotics offer, there are limitations in the ability of

current probiotics to offer additional energy to the host`. The benefits of additional energy to the host would be particularly useful during energy deficient periods when animals do not have available energy to continue normal metabolic processes (Chaucheyras-Durand and Durand 2010, Sheldon and Verhulst 1996). Through the use of lipids stored in inclusion bodies, *Enterobacter cloacae* JD6301 could supply the host with a potentially useable source of energy and provide a benefit to overall immunity (Barcia and Harris 2005, Im et al. 2011, Wilson et al. 2014).

The genomic sequence and data presented in Chapter II confirm that JD6301 has many traits that would make it an acceptable probiotic. It is able to survive in a range of temperatures, including those of potential hosts. Moreover, it has shown some resistance to acidic conditions. These are all traits that would be beneficial to JD6301 as a probiotic, as perhaps the most important consideration for potential probiotic strains are their ability to remain viable in the host (Ross et al. 2005). The inclusion bodies containing lipids contained by *Enterobacter cloacae* strain JD6301 are yet another feature that could make it an extremely useful tool as a novel probiotic. The lipids stored in these inclusion bodies could potentially act as useable energy when provided to a host in an energy-depleted state.

The *in vivo* study presented in Chapter III demonstrated the ability of *Enterobacter cloacae* strain JD6301 to act as a potential probiotic. Through supplementation via water, mice provided JD6301 had an overall more robust immune response and had lower amounts of pathogen present in the intestines when challenged with *E. coli* O157:H7. Animals in supplemented groups also had increased fecal shedding of *E. coli* compared to non-supplemented controls. Additionally, JD6301 was detected in

all sections tested throughout the study, with the exception of day 2, where concentrations varied. Altogether, these data suggest that JD6301 has the potential to act via a more traditional mechanism of action and inhibit the successful binding and subsequent colonization of a pathogen in the host GI tract.

In addition to its ability to affect pathogen colonization, JD6301 also seemed to induce a more robust immune response in animals supplemented. Mice provided JD6301 had an increased concentration of serum TAGs 1 day prior to challenge with *E. coli*. This increase in serum TAG concentration could be associated with the presence of JD6301 and the lipids potentially released by this bacterium to be used by the host. This could be particularly useful, as lipids play an important role in immune modulation (de Pablo and Alvarez de Cienfuegos 2000). JD6301 was also shown to affect cytokine production, as mice provided this bacterium had higher concentrations ($p<0.05$) of IL-1α detected 1 day after challenge. This supports other data that showed that certain fatty acids were able to modulate cytokine response, suggesting lipids could play a role in overall cytokine regulation (de Pablo and Alvarez de Cienfuegos 2000). Together, these data suggest that *Enterobacter cloacae* strain JD6301 has the potential to function as both a traditional probiotic, by inhibiting pathogens, and novel probiotic by providing energy to the host via lipids stored in lipids inclusion bodies. While more research is needed to elucidate the mechanism of action of JD6301, the development of this bacterium as a probiotic could initiate the development of many other products that could be beneficial to both human and animal health.

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APPENDIX A

FEED INTAKE, WATER DISAPPEARENCE AND MOUSE BODY WEIGHTS

A.1 Feed intake throughout study

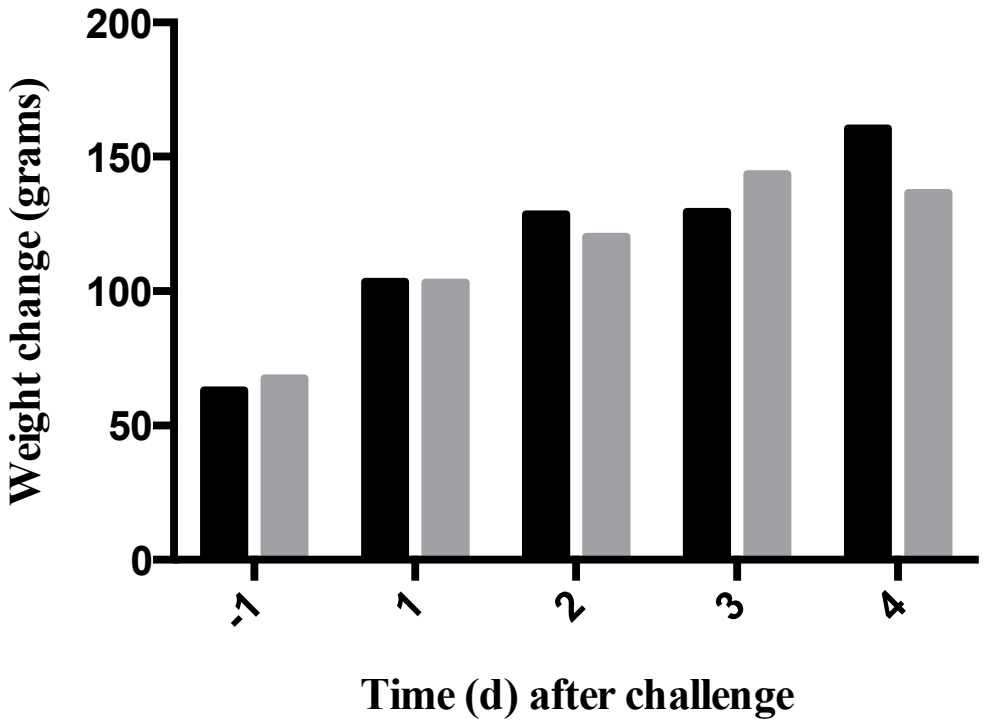

Figure A.1 Feed intake

Feed intake was monitored during the study. Prior to being added to cages, feed was weighed and recorded. On the day of sacrifice feed weight was measured again to determine feed intake throughout study. There was no difference in feed intake between control (black) and JD6301 supplemented (gray) groups.

A.2 Water disappearance throughout study

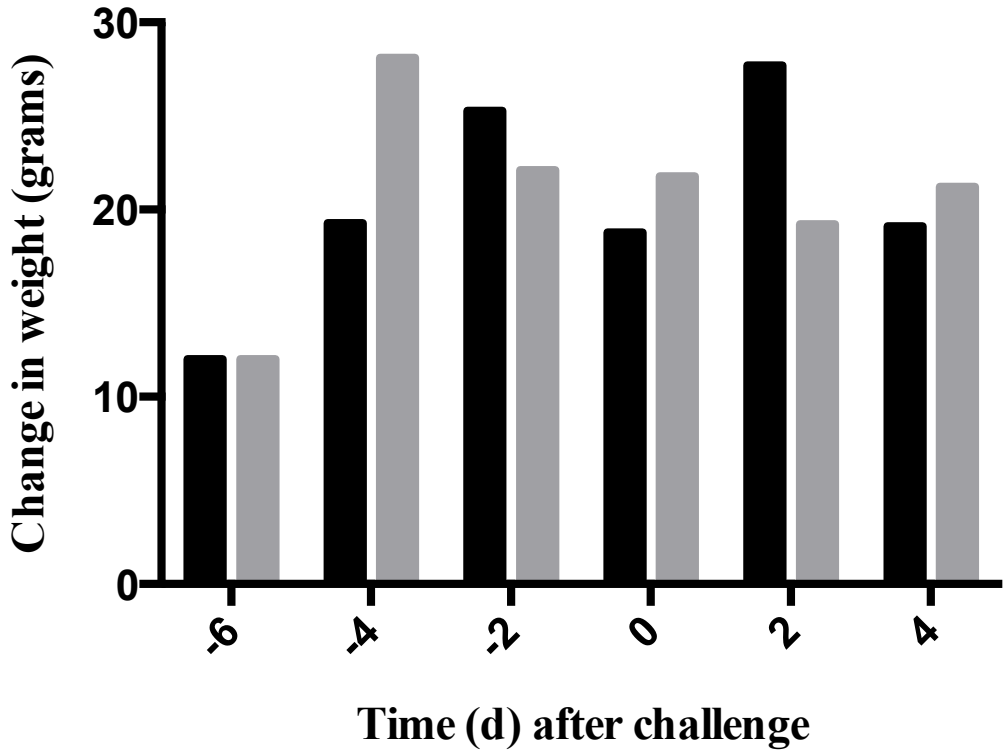

Figure A.2 Water disappearance

Water disappearance was measured every other day as water bottles were replaced. Presented data represents rates of water disappearance for the same group for control (black) and JD6301 supplemented (gray) throughout entire study.

A.3 Mouse body weights

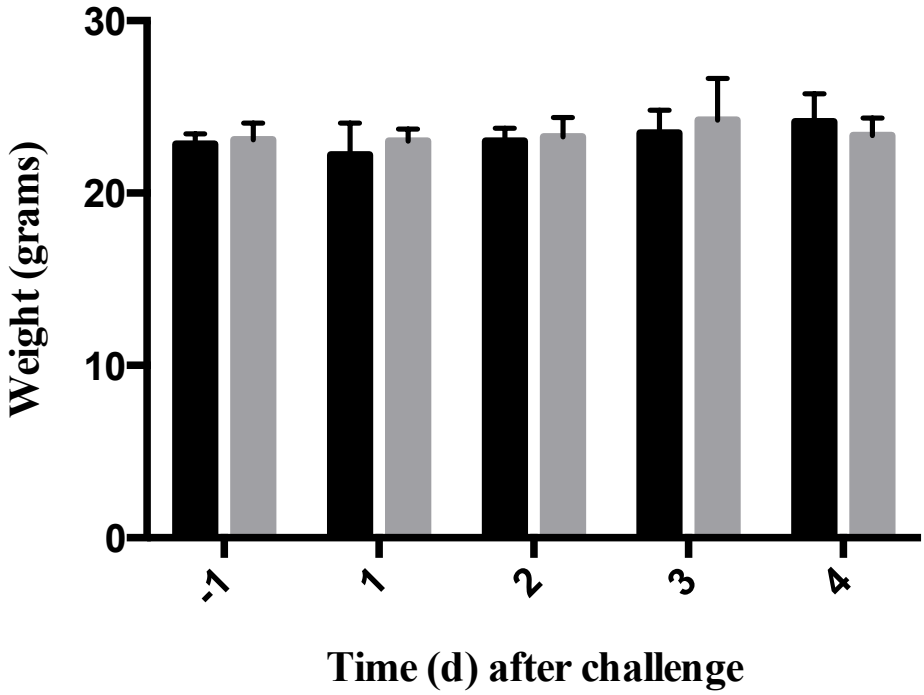

Figure A.3 Mouse body weights

Weights of each mouse were taken at time of sacrifice. No difference was seen in weights of mice in control (black) groups compared to JD6301 supplemented (gray) groups on any day throughout study.

APPENDIX B

CYTOKINE ANALYSIS

B.1 Supplemental cytokine analysis

Table B.1 Analyzed cytokines that showed variations in immune response between control and JD6301 supplemented animals

Cytokine	IL-1α		RANTES		G-CSF		IP-10	
Day -1	**Control**	**JD6301**	**Control**	**JD6301**	**Control**	**JD6301**	**Control**	**JD6301**
Mouse 1	67.47	166.62	25	25.47	104.4	168.47	98.54	243.01
Mouse 2	107.5	144.25	12.27	21.64	72.11	171.8	79.84	126.4
Mouse 3	107.5	3.59	9.77	not detected	57.93	not detected	111.05	5.53
Mouse 4	114.08	7.06	11.1	not detected	41.67	not detected	103.25	6.42
Day 1								
Mouse 1	10.45	7.06	2.49	not detected	3.58	5.97	27.63	25.78
Mouse 2	10.45	54.03	not detected	6.41	71.75	9.95	33.61	46.28
Mouse 3	not detected	62.86	1.67	5.59	7.92	21.15	19.24	38.97
Mouse 4	not detected	28.4	not detected	1.67	51.31	14.19	22.53	26.96
Day 2								
Mouse 1	55.05	40.92	4.61	3.08	37.2	20.04	16.61	18.72
Mouse 2	36.12	19.9	not detected	1.96	17.46	3.03	32.46	12.88
Mouse 3	5.33	1.86	not detected	not detected	9.95	not detected	28.14	10.36
Mouse 4	24.26	56.05	1.82	3.67	6.61	50.2	34.1	29.48
Day 3								
Mouse 1	69.26	36.12	3.15	4.61	6.93	22.26	35.74	32.62
Mouse 2	13.72	63.79	2.34	1.59	10.65	not detected	26.96	27.46
Mouse 3	40.92	21.38	2.56	not detected	9.95	4.44	26.79	19.94
Mouse 4	N/A	22.83	N/A	not detected	N/A	3.03	N/A	31.47
Day 4								
Mouse 1	84.31	51.96	3.3	6.35	23	51.31	38.65	35.08
Mouse 2	44.37	3.59	not detected	1.96	5.97	not detected	31.96	30.06
Mouse 3	29.73	28.4	2.86	3.15	8.59	20.04	22.88	23.31
Mouse 4	45.49	80.31	4.61	10.74	30.47	49.46	35.25	119.51