USING PROBIOTICS TO INHIBIT *IN VIVO* AND *IN VITRO* GROWTH OF *LISTERIA MONOCYTOGENES* AND *CRONOBACTER SAKAZAKII* DURING FETAL AND NEONATAL INFECTION

by

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ABSTRACT

Cronobacter sakazakii and *Listeria monocytogenes* are two pathogens that are known to cause wide range of diseases in infants, elderly people and immune-compromised people. Some of the major infections that both these pathogens can cause include necrotizing enterocolitis, bacteremia, meningitis, fever, headache and pneumonia. Both these pathogens are prevalent in infant food formulas, contaminated food items and surroundings. *Lactobacillus rhamnosus* GG has become one of the widely used microorganisms, to treat infections caused by *C. sakazakii* and *L. monocytogenes* because of its probiotic uses. Thus, LGG was tested on reducing the pathogen effects of *L. monocytogenes* and *C. sakazakii* during fetal and neonatal development. The primary aim of this study was to investigate the ability LGG to reduce growth and invasion of two pathogenic bacteria, *L. monocytogenes* and *C. sakazakii*, *in vivo* and *in vitro*. To carry out this research study, CD-1 pups were examined by gavaging with both10^6 LGG or LGG soluble material on gestation days (gd) 1.5, 3.5 and 4.5 and administered by giving single oral dose of

($10^{8, 9 \text{ or } 12}$) CFU *C. sakazakii* or vehicle reconstituted powdered infant formula (RPIF) at (gd) 2.5. This study revealed that the brain is the primary tissue, where higher percentage of *C. sakazakii* was isolated when compared to other tissues such as liver and spleen. To elucidate the mechanism of probiotic *Lactobacillus* rhamnosus GG (LGG) inhibition of *L. monocytogenes* and *C. sakazakii*, both *in vivo* and *in vitro* models were used. Importantly, stillbirths were reduced in guinea pigs receiving yogurt and *L. monocytogenes* as compared to *L. monocytogenes* alone (14% v. 75%). The data suggests under the conditions tested, LGG and LGG supernatant significantly reduce *C. sakazakii* growth and viability and the anti-microbial activity of LGG supernatant is due to (a) heat-stable, non-proteinaceous compound(s). Further, a dose dependent relationship was found with lactic acid treated LGG supernatant and reduced *C. sakazakii* growth. In conclusion, both an acidic pH environments and LGG supernatant treatments affected *L. monocytogenes* and *C. sakazakii* growth and viability *in vitro* thus, further studies will be needed to investigate the mechanisms involved.

INDEX WORDS: supernatant, infant formula, LGG, *in vitro*

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

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DEDICATION

This dissertation is dedicated to all of my predecessors before me who forged this path and to all of who may follow in my path, that they may see all things are possible through God.

ACKNOWLEDGEMENTS

I would sincerely like to acknowledge God for giving me the persistence to complete this terminal degree. My time at UGA has been grand. I will cherish my experiences here forever and I will always consider UGA home. I want to thank my committee for teaching me how to think critically while doing my research. I would also like to thank my parents, Dr. and Mrs. Stephen and Victoria Agyekum, my sisters, Dr. Georgina Agyekum and Dr. Melinda Agyekum-Taylor, and all of my friends for their support, encouragement and leadership along the way. To my wife, Kerryn: Sweetpea, I am forever grateful that you are my life partner. I would not have been able to do this without you. Lastly, to Dr. Mary Alice Smith: your commitment to my educational and personal development has been relentless throughout my entire academic tenure and for that I will always be in your gratitude.

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CHAPTER 1

INTRODUCTION

Human infants and neonates are often inadvertently exposed to food-borne pathogens which can result in severe disease and/or death. Developmental and reproductive pathogens such as *L. monocytogenes* and *C. sakazakii* are often the causes of infant and neonatal infections. Due to the incidences of infection, research investigating the use of probiotics in protecting infants and neonates has been conducted in recent years. A probiotic substance is a microorganism introduced into the body for its beneficial qualities. The term probiotic is related to products which contain adequate concentrations of live microbes. Probiotics are defined as viable microorganisms which when administered in adequate amounts provide health benefits on the host beyond intrinsic basic nutrition *(49)*. Health benefits of probiotics include enrichment of the immune system, lessening of symptoms of lactose intolerance, treating childhood diarrhea, treating ulcerative colitis, preventing antibiotic-associated diarrhea, preventing and treating Crohn's disease *(64, 86, 106)*. Prevention of recurrence of atopic diseases with breast, prostate and colorectal cancer has also been attributed to probiotic activity *(64, 86, 106)*. Probiotics can be found commercially mainly as food supplements most often in dairy products *(75)*.

The usage of probiotics represents an expanding research area, however much still remains to be done to standardize the meaning of the term probiotic and which microbial strains act as probiotics. Further, probiotic origins remain largely unclear, and *in vivo* and *in vitro* identification of the precise mechanistic base of these advantageous effects remains an important goal in research. Thus, we conducted an efficacy assessment of the probiotic *Lactobacillus rhamnosus* GG (LGG) in the presence of *Listeria monocytogenes* (*L. monocytogenes*) and

Cronobacter sakazakii (C. sakazakii). The major reason behind choosing these pathogenic microorganisms for the study is that both these organisms are known to cause high case fatality rates in fetuses (*L. monocytogenes*) and neonates (*C. sakazakii*). The two unique (gram positive *L. monocytogenes* and gram negative *C. sakazakii)* microorganisms have been researched both *in vitro* and *in vivo* by our lab due to their documented history of causing adverse effects to human populations. Unless a bacterium's probiotic usage is tested in the presence of microorganisms that cause fatality to living beings, one may not be able to understand its effectiveness in treating impending diseases.

C. sakazakii is a gram-negative opportunistic bacterial pathogen that can cause illness and death in at-risk infants via sepsis and severe disease manifestations *(122).* *C. sakazakii* is occasionally isolated from both food and non-food items. Some of the food products where *C. sakazakii* is prevalent are powdered infant food, pediatric infant formula food and products that are made with milk and herbal teas. In the same way, some of the non-food items where *C. Sakazakii* is prevalent are air, vacuum cleaners, floor, and room heaters *(65, 122).* As it is prevalent in both food and non-food items, it can easily infect human populations, especially newborn infants. Infants who are considered at-risk are those born prematurely and/or have low birth weights. Premature and low birth weight infants are often solely fed reconstituted powdered infant formula (RPIF) or are breast-fed supplemented with RPIF in order to receive proper nutrition.

The contamination of powdered infant formula with *C. sakazakii* has been linked to neonatal outbreaks resulting in morbidity and death *(15, 83, 127).* Reported cases of *C. sakazakii* infection in human infants have led to powdered infant formula recalls. In cases of *C. sakazakii* infection in neonates, powdered infant formula has been implicated as the source of the

bacterium, being cultured from both opened and unopened cans of powdered infant formula used to feed infected infants and the surfaces on which the formulas were mixed and prepared for feedings *(15, 84, 88, 113, 127).*

C. sakazakii is an intracellular pathogen, allowing it to evade host immune responses *(122)*. The pathogenesis of *C. sakazakii* is not completely understood. Oral route formula feeding is said to be the primary route by which *C. sakazakii* can infect humans. After ingestion, *C. sakazakii* passes through the gastrointestinal tract, colonizes the intestine and translocates across the intestinal epithelium and enters systemic circulation. Via the bloodstream, *C. sakazakii* can travel systemically and invade host tissues, including the liver, and cross the blood-brain barrier to enter the central nervous system. Lastly, there is also a strong association between some cases of necrotizing enterocolitis and the colonization of *C. sakazakii (16)*.

L. monocytogenes, is a gram positive, Category B bacterium associated with the illness listeriosis. When compared to other foodborne pathogens, *L. monocytogenes* is second only to Salmonella in the number of deaths that result annually from exposure *(126).* Due to its pathogenic nature, *L. monocytogenes* has become one of the highly discussed pathogens in the healthcare sector. In between January and June 1985, approximately 86 cases were recognized as infected by *L. monocytogenes* in California and Orange City. As per the records, nearly 2500 people are infected every year. It is also estimated that *L. monocytogenes* causes approximately 500 deaths every year *(126).*

The primary route of exposure to *L. monocytogenes* is through ingestion of contaminated foods. As contaminated food is the major source of infection in both epidemic and sporadic cases *(35, 46)*, the gastrointestinal tract is considered as a primary source from where pathogenic *Listeria* organisms enter into the host. *L. monocytogenes* is also found in the environment which

makes it difficult to eliminate from the food supply; thus potentially exposing many humans to this microorganism. While the specific mechanism of *L. monocytogenes* pathogenicity has not been elucidated, *L. monocytogenes* has the ability to induce its internalization into non-phagocytic cells and cross the intestinal, the placental and the blood–brain barriers *(51, 73)*.

Of further concern, certain subpopulations are more susceptible to *L. monocytogenes* infection. Fetuses and neonates are among these susceptible subpopulations. The fetal effect of *Listeria* primarily depends on the gestation time when infection occurs *(13, 35, 43)*. During the third trimester of pregnancy, cell mediated immunity is suppressed *(101)*. The third trimester is also the time in which listeriosis most commonly occurs *(101)*. Adverse effects can result in premature delivery, miscarriages and stillbirths *(13, 35, 43)*. The severe outcomes and the difficulty of diagnosis during pregnancy results in a need to develop preventive therapies. If a probiotic could be identified that decreased the likelihood of the occurrence of listeriosis during pregnancy or *C. sakazakii* infection in neonates, it would result in an easy preventive treatment without the concern of toxicity of drugs.

Within the literature there exist multiple hypotheses on the protective mechanisms of LGG affecting endogenous and exogenous pathogens (Table 1.1). Studies conducted over the past few decades have investigated the antimicrobial activity of LGG which has been attributed to proteins and proteinaceous substances, pH and or lactic acid produced substances. These mechanisms may or not be mutually exclusive. The current study hypothesis investigates pH, lactic acid and non-lactic acid substances involved in LGG antimicrobial effects on enteric pathogens; specifically *L. monocytogenes* and *C. sakazakii*.

Table 1.1 Mechanistic hypotheses of LGG effecting *L. monocytogenes* and *C. sakazakii*

Hypothesis	Author
Bacteriocin production for the anti-infective activity of *Lactobacillus salivaras* mechanism	Corr et al. 2007
Strong antimicrobial activity of *Lactobacillus rhamnosus GG* against salmonella typhimurium is due to lactic acid	DeKeersmaecker et al. 2006
Lactobacillus sakei 1 and its bacteriocin influence adhesion of stainless steel surface	Winkelstroder et al. 2011
Protein-mediated adhesion of *Lactobacillus acidophilus* BG2F04 on human enterocyte and mucus-secreting cell lines in culture.	Coconnier et al. 1992
pH, lactic acid and non-lactic acid dependent activities of probiotic *Lactobacilli* against *Salmonella enterica*	Fayol-Messaoudi et al. 2005

Thus, the overall goal of this study was to investigate the ability of LGG to reduce growth and invasion of two pathogenic bacteria, *L. monocytogenes* and *C. sakazakii*. Thus, the specific aims of this study were:

1) To test LGG in the prevention of *C. sakazakii* infection in a neonatal mouse model and further to research the mechanism of protection of probiotics *in vitro*

2) To determine whether probiotics can inhibit *L. monocytogenes* infection in time pregnant guinea pigs and to determine any mechanisms of inhibition through *in vitro* studies with probiotics. Determining the potential protective effect of LGG against microorganisms will

provide further information leading to therapeutic regimens that help in treating unique pathogenic infections.

CHAPTER 2

LITERATURE REVIEW

C. sakazakii and *L. monocytogenes* are two widely discussed pathogens in today's healthcare industry majorly because of their ability to cause severe infections to infants, elderly people and immune-compromised people *(9, 58)*. While the number people getting infected by these two pathogens are not significantly high, the mortality rates among infected people have become a major concern. Most of the previous studies have revealed that infant food feed formulas, contaminated manufacturing facilities, and poorly maintained hospital environments are major sources from which *C. sakazakii* is isolated *(65, 133)*. Whilst *C. sakazakii* causes diseases such as meningitis *(122)* and bacteremia *(122)*, *L. monocytogenes* can cause infections such as diarrhea and meningitis *(131)*. *L. monocytogenes* has also been isolated from pediatric powdered foods, beef and pork related products *(35, 62, 107)*.

Today, *Lactobacillus rhamnosus* LGG, due to its probiotic activity, has been widely used to treat various infections. Several studies have also revealed that infections caused by *C. sakazakii* and *L. monocytogenes* can also be treated with the help of LGG *(21, 57)*. To understand the ability of LGG to treat the infections of *C. sakazakii* and *L. monocytogenes*, thus, the following literature review will focus on the origins, prevalence, virulence factors, and pathogenicity of both these pathogens. Thus, the following literature review will focus on elaborating origins, prevalence levels, virulence factors and pathogenicity of *C. sakazakii* and *L. monocytogenes.* In addition, the following section will provide details on origin and probiotic usages of LGG.

LGG. LGG, a gram positive bacterium, is known for its lack of pathogenicity and usage in yogurt *(137),* probiotics and other dairy products such as fermented milk and pasteurized milk *(92)*. In most cases, LGG presents in digestive system and urinary tracts of human body *(11)*. Due to its lack of pathogenicity, this gram positive bacterium would help human beings by preventing the growth of harmful bacteria in the digestive system and urinary tracts.

Origin of LGG. LGG was initially considered as a subspecies of *Lactobacillus casei*, which is a species of genus *Lactobacillus* and exists in the mouth and intestines of human beings. Nonetheless, LGG has been used in various commercial purposes *(27)*. LGG has also been used in the production of alcoholic beverages and fermented meat products *(4, 108)*.

Virulence of LGG. LGG bacterium is known for its benefits to human beings, the virulence levels low. To understand the virulence levels of LGG, Vankerckhoven et al. *(130)* have conducted a study by taking 10 isolates of LGG isolates from the PROSAFE strain collection and compared the virulence and ability to cause experimental endocarditis between LGG and *L. Paracasei*. In this study, Vankerckhoven et al. *(130)* have found that nutritional isolates of LGG are less likely to cause experimental endocarditis when compared to the natural LGG isolates that grow in human body. Another study conducted by Moreillon et al. *(82)* has described that the probiotic isolates are less infective than clinical isolates of LGG.

Prevalence of LGG. LGG bacterium is prevalent in human stomach and intestine due to its ability to survive in the acid and bile *(44, 66, 72)*. The major sources of LGG are dairy products, fermented meat and fermented alcohol beverages *(72, 109)*. In addition, LGG bacterium is helpful in probiotics because of its nature to colonize the intestine and adhere to cells *(47)*. This bacterium is prevalent in the human digestive system and urinary tracts, it inhibits growth of other harmful bacteria and resists vaginal microbicides *(69, 111)*.

Probiotic Applications. Due to its properties, LGG could be used in a wide-range of medical applications. LGG has been successfully used to treat and prevent gastrointestinal diseases and dermatitis *(5, 94, 111, 128)*. Though LGG is widely used in prevention and treatment of various diseases, the most common use of LGG would be in treatment of diarrhea in infants and children *(63, 128)*. LGG is used to treat three types of diarrhea symptoms which include acute diarrhea, antibiotic-associated diarrhea and traveler's diarrhea *(55, 63)*.

Traveler's diarrhea is one of the common diseases in travelers in underdeveloped areas due to sudden climate changes and warmer living conditions. LGG has become one of the most sought after medicines to treat and control traveler's diarrhea *(55)*. According to the study conducted by Hilton et al. *(68)*, travelers who don't take LGG medicine will be more vulnerable to traveler's diarrhea.

Many studies have analyzed the ability of LGG to treat and prevent the disease, atopic dermatitis *(40, 48, 102)*. One such study conducted by Nermes et al. *(89)* took fecal and blood samples from 39 infected children for three months to determine the ability of LGG to reduce the proportion of immunoglobulin cells that cause atopic dermatitis. Nermes et al. revealed that the number of immunoglobulin cells was drastically reduced in children who have been receiving LGG regularly.

LGG is said to be safe to use for immune-compromised populations such as pregnant women, premature neonates, elderly individuals while also being used to treat rheumatoid arthritis, Crohn's disease and helocobacter pylori infection and adults, who suffer from Clostridium difficile-associated diarrhea *(25, 39, 45, 117)*. Apart from these above mentioned diseases, LGG medicine is extensively used to treat respiratory tract infections in children, urogenital tract infections, and vancomycin-resistant enterococcus and other infections *(7) (56)*.

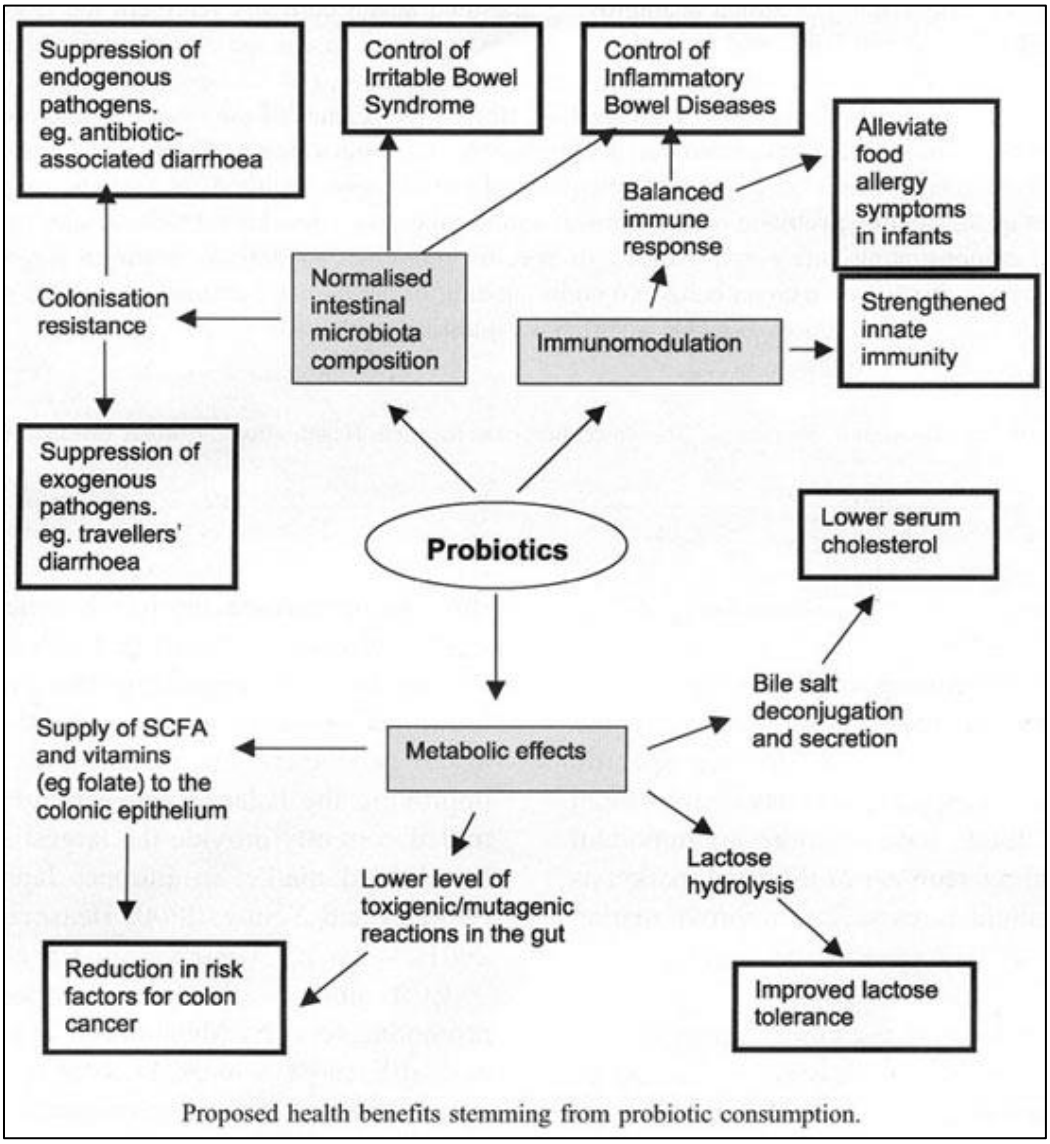
Nevertheless, LGG can be consumed as a supplement with milk, food and alcoholic products *(53)*. Table 2.1 shows the potential therapeutic uses of probiotics. The current study uses of probiotics to suppress endogenous and exogenous pathogens.

Mechanisms of Probiotic Activity. Probiotics have been proposed to affect enteric pathogens through several independent mechanisms. *In vitro*, LGG is believed to act through competitive exclusion of pathogenic bacteria *(21)*. Bacterial adhesion of LGG onto epithelial cells has been proposed as the protective mechanism. Further, Collado et al. suggest combinations of probiotic strains may complement one another's properties in protection against pathogenic bacteria *(21)*. The study further suggests the probiotic combination could be used in fermented milk as a therapeutic regimen *(21)*.

Bacteriocins, small proteinaceous substances are also proposed as the activity component involved during probiotic protection against pathogens. Bacteriocins are produced by many bacterial species and are currently used in the food industry in order to reduce the chemical preservatives in food *(23)*. *Lactobacillus salivarius* produces the bacteriocin Abp 118, which is active against *L. monocytogenes*. Production of Abp 118 is increased during early stationary bacterial phases *(23)*. In mice, the peaking of the Abp 118 offers *Lactobacillus salivarius* protection against *L. monocytogenes* infection *(23)*.

In contrast to the independent mechanisms hypotheses, a multifactorial mechanistic approach to probiotic antibacterial activity has been researched *(38, 110)*. The multifactorial hypothesis attributes the antibacterial activity to pH, lactic acid and non-lactic acid substances. *Lactobacillus* bacteria produces acidic metabolites such as acetic and lactic acid which lowers the pH thus inhibiting the growth of the pathogenic bacteria *(110)*.

Table 2.1 Proposed therapeutic uses of probiotics.



Proposed health benefits stemming from probiotic consumption.

Pathology of LGG. Though LGG bacterium is known for its low pathogenicity, some of the probiotic strains of LGG still cause infections and diseases in human beings *(18) (114)*. As humans are highly exposed to fermented milk, meat and alcohol products, the infections of LGG may be seen in human gastrointestinal systems. However, the study conducted by Salminen et al. *(105)* revealed that increased consumption of LGG would not lead to any bloodstream infection because there were no trends that suggest an increase in *Lactobacillus bacteremia* among all isolated cells from blood.

In summary, LGG is a gram positive bacterium that is primarily found in gastrointestinal and urinal tracts of human body. In addition to gastrointestinal tracts, LGG is also found in fermented milk, meat and alcoholic beverages. This bacterium is known for its low risk of pathogenicity, it helps to prevent and control wide-range of diseases such as acute diarrhea, traveler's diarrhea, anxiety, respiratory tract infections in children, urogenital tract infections, vancomycin-resistant enterococcus and atopic dermatitis *(25, 39, 45, 117) (64)*. Though the LGG bacterium is used for treatment purposes, it is also responsible for some clinical infections such as pneumonia, septic arthritics, meningitis and bacteremia *(105)*.

C. sakazakii. *Cronobacter* is a genus, which belongs to the family of Enterobacteriaceae and was also known as *Enterobacter sakazakii* before 2007 *(60)*. *Cronobacter* is a rod shaped and pathogenic bacterium, which has become famous for its ability to cause bacteremia and meningitis in infants *(83, 123)*. Though *Cronobacter* is also known to cause invasive infection in adults, the fatality rates are nearly 60 - 80 % higher in infants than adults *(52, 80, 127)*.

Origins of *C. sakazakii*. The origin of *C. sakazakii* was first recognized as yellow pigmented *Enterobacter cloacae*. Later in 1980, was assigned with a name, *Enterobacter sakazakii*, as it was isolated from the family of Enterobacteriaceae. *E. sakazakii* bacteria are

capable of motility, are non-sporeforming, and are Gram-negative facultative anaerobes *(59)*. *Cronobacter* is now no longer called *Enterobacter sakazakii* due to a change in taxonomy *(61)*.

C. sakazakii can be found in powdered infant formulas and which become contaminated during the manufacturing process; which most likely occurs after pasteurization. Around 50% of all nonsocomial infections can be attributed to *C. sakazakii* organisms *(59)*. *C. sakazakii* causes necrotizing enterocolitis (NEC) once consumed *(83)*. *C. sakazakii* can be found in contaminated fruits and vegetables ranging from 12°C-25°C *(12)*. Lastly *C. sakazakii* produces D-Lactic acid and is mucate negative *(59)*.

Virulence factors of *C. sakazakii*. The literature on virulence factors of *C. sakazakii* is limited because research on understanding major virulence factors and pathogenicity mechanisms has just started. Though the information is limited, the existing studies of Pagotto et al. *(90)* and Kline et al. *(67)* have given significant information on gram negative bacteria and their putative virulence factors that can cause fatal infections such as enterocolitis and meningitis.

As a gram negative pathogenic bacterium, there are virulence factors associated with *C. sakazakii*. The major virulence comes from lipopolysaccharide, which is the major component of the outer membrane of gram negative bacterium. Lipopolysaccharide is a combination of three major parts. They are O antigen, Lipid –A and Core Oligosaccharide. As the component Lipid A is known for its toxic properties, it has the capability to stimulate intense host immune response *(124)*. Once these immune responses are released, one can see symptoms such as fever during disease. If a high amount of lipopolysaccharide is present in the body, it may result in death.

Pagotto et al. *(90)* are said to be the first group of people to recognize and study the virulence factors of *C. sakazakii*. They focused on testing the enterotoxin production by gram negative bacteria. Using a mouse assay, they tested enterotoxin production. With this study,

Pagotto et al. *(90)* mentioned that some of the *C. sakazakii* strains are non-pathogenic and there is a difference between virulence factors of one strain of *C. sakazakii* to another. Further, this study mentions the potential risks involved in the infant milk formula, which can be contaminated with the *C. sakazakii.*

Prevalence of *C. sakazakii.* According to Krieg and Holt *(70)*, *C. Sakazakii* is prevalent in food items and other environment and clinical sources. Some of the major sources in which *C. sakazakii* bacterium has been isolated are food grains, air, vacuum, floors and room heaters *(6, 9). C. sakazakii* has also been isolated from hospital environments and materials used in clinics. Thus, apart from the natural sources, clinical sources also considered to be the major reasons behind prevalence of *C. sakazakii*. For example, Farmer et al linked the prevalence of *C sakazakii* in hospitals to admitted patients that were carriers of the bacterium *(36).*

From infected patients, *C. sakazakii* can be isolated from skin, gut, nose, throat, and blood *(31, 122)*. In another study, it was reported that newborns were exposed to *C. sakazakii* during passage through the birth canal *(83).*

C. sakazakii is prevalent in dry powdered foods; most of the unopened dried foods such as cheese, rice and herbs are also known to contain gram negative bacteria. In a survey conducted by Krieg and Holt *(70)* the authors reported that nearly 27% of powdered foods have been contaminated with *C. sakazakii*; Krieg and Holt *(70)* have conducted this study with the sample of 140 powdered food products.

This review has identified three major types of products that can serve as major sources of *C. sakazakii* contamination; these products include powdered infant food, pediatric infant formula food and products that are made with milk. Pediatric infant formula food can be contaminated through two routes. Whilst the first route by which *C. sakazakii* can enter into

pediatric infant formula food is unknowing addition of contaminated food ingredients, the second route is through unsanitary handling of food products *(12)*.

The prevalence of *C. sakazakii* is increased if the pediatric infant formula food is stored in a bottle for maximum number of days. According to Iversen et al. *(61)*, *C. sakazakii* bacterium is prevalent in food items that are stored in between 37° to 43°C. In another study, Kandhai et al. *(54)* has mentioned that the temperature between 8° to 47°C is perfectly suitable for *C. sakazakii* bacterium to flourish. Most households keep their refrigerators at 10° which are also prominent places, where *C. sakazakii* is prevalent.

The mechanism of *C. sakazakii* infection is not completely understood. The primary route of exposure is oral. Subsequently, *C. sakazakii* moves through the gastrointestinal tract. Once in the GI tract, *C. sakazakii* colonizes in the intestines and trans locates through the intestinal epithelium and enters the blood stream. Onset of *C. sakazakii* infection is generally acute.

L. monocytogenes. *Listeria Monocytogenes* (*L. monocytogenes*), a gram positive pathogenic bacterium, causes the infection listeriosis. *L. monocytogenes* has become one of the highly discussed pathogens in the healthcare sector these days because, in the United States alone, nearly 2500 people are getting infected due to this bacterium every year. The latest reports of World Health Organization also have confirmed that *L. monocytogenes* is responsible to nearly 500 deaths in the United States for every year.

Origin of *L. monocytogenes.* *L. monocytogenes* was first recognized and elaborated by Mr. Murray in 1926 when six young rabbits died due to unknown infections. Though the literature on the existence of *L. monocytogenes* bacterium was published in 1920, it was not recognized as an important pathogen until 1952 when studies in East Germany that the bacterium was harmful to both humans and animals *(29).* Whilst Murray named the organism that infected six rabbits, Bacterium *Monocytogenes,* Harvey Pirie has named the bacterium as *L. monocytogenes* in honor of Brother Arthur and Father Joseph Lister. Whilst most of the cases of *L. monocytogenes* were recorded in animals and infants, later in 1970s, *Listeria* was reported in adults with compromised immune systems. *Listeria* infections were also reported in both drug users and AIDS patients *(103).*

Until 1981, *L. monocytogenes* was known as a bacterium that causes illness to animals and neonatal sepsis. But in later half of 1981, it was recognized that *L. monocytogenes* caused foodborne illness. Since then, *L. monocytogenes* has been considered as one of the most dangerous gram positive bacterium in the food industry. Select human cohorts that are more susceptible to infection by *L. monocytogenes* are organ transplant recipients, AIDS patients, HIV infected people, pregnant women, cancer patients and old people, who are above 65 years of age *(29).*

Virulence of *L. monocytogenes.* Despite high presence of *L. monocytogenes* in the environment, few people are infected. Most studies have suggested that only few people are infected because not all serotypes of are bacterium are virulent. According to Lammerding et al. *(71)* on serotypes of *L. monocytogenes* isolated from 1363 patients, there are several types of strains or strains that cause severe infection among humans. Among 1363 patients who were tested, the strain 4b was present in nearly 64 percent of cases and was frequently isolated from pregnant women *(71)*. The other strains such as 1/2a, 1/2b and 1/2c were also detected in among the set of patients who have been tested. Whilst strain 4b was frequently isolated in pregnant women, strains 1/2a, 1/2b and 1/2c were present in non-pregnant women. The study has concluded that the strains 4b 4ab and 1/2a were the virulent strains *(71)*.

Similarly, a study conducted by Franciosa et al. *(41)* revealed that *L. monocytogenes* has strains that may cause both invasive and non-invasive listeriosis outbreaks. The differences in listeriosis outbreaks suggests that there may be differences between DNA sequences and virulent factors in any two strains of *L. monocytogenes(41)*. With these results, it can be concluded that not every strain of *L. monocytogenes*, which exists in outer environment and food particles, will pose same the threat to human health; even if they infect, the level of threats would not be the same.

Some of the major virulent factors of *L. monocytogenes* include internalins, surface protein p104, listeriolysin O, Act A protein, phospholipases and metallo-proteases *(79)*. Studies have identified Internalin-A as a listerial surface protein that helps *L. monocytogenes* enter into non-pathocytic cells. Similarly, Internalin-B helps *L. monocytogenes* to enter liver cells and plays a crucial role in invasion of hepatocytic cells located in liver. Apart from internalins,

another surface protein, p104, has been recognized very recently *(29)*. According to Pandiripally et al. *(91)*, the surface protein, p104, plays a crucial role in adhesion to intestinal cells.

Studies conducted by McKay and Lu *(77, 79)* have shown that listeriolysin O, a bacterial protecting toxin is required by *L. monocytogenes* for survival in the human body. *L. monocytogenes* bacterium with the help of listeriolysin will infect cells of the body because listeriolysin assists *L. monocytogenes* to readily escape from vacuoles. Once the *L. monocytogenes* bacterium escapes from vacuoles, Act-A protein mediates entrance into other cells of the human body and thus acts as one of the most important virulence factors. There are two distinct types of phospholipases that helps *L. monocytogenes* bacterium to escape from vacuoles and spread from one cell to another cell in the human body*(79)*. These phospholipases include phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PCPLC). Both these phospholipases play a crucial role in spreading the listeriae bacterium in brain cells *(29)*. In addition to these, metallopretese, protein P60 and Clp proteases and ATPases act as virulence factors for *L. monocytogenes (79).*

Prevalence of *L. monocytogenes*. According to Navratilova et al. *(87)*, *L. monocytogenes* is highly prevalent in raw milk. The major sources of *L. monocytogenes* bacterium in raw milk are gastrointestinal tracts of animals and environmental factors. Navratilova et al. *(87)* collected 15 milk samples to understand prevalence levels of *L. monocytogenes* bacterium in milk and its related products. The reported prevalence levels (in percentages) of *L. monocytogenes* bacterium in raw bulk milk, raw bulk milk with tanker trailer, raw bulk milk from silo prior to pasteurization, pasteurized milk and final milk products were 2.1%, 5.1%, 15%, 5% and 0%, respectively *(87)*.

Meat is also said to be a major source of *L. monocytogenes (87)*. As *L. monocytogenes* bacterium presents in intestinal tracts of animals, the meat processing procedure play a major role in prevalence of the bacterium in meat. The contamination at the time of slaughtering and cutting the meat would increase the possibility of occurrence in *L. monocytogenes* in both raw meat and processed meat. Among all meat products, beef is also the major source of *L. monocytogenes (87, 134)*.

Wendlandt and Bergann *(134)* analyzed prevalence levels of *L. monocytogenes* in two types of processing plants. The first processing plant produced smoked and cooked meat products, the second processing plant produced fermented dry meat products. Wendlandt and Bergann *(134)* observed that the prevalence levels of *L. monocytogenes* were higher in fermented dry meat products than smoked and cooked meat. Thus, the study concluded that contamination of meat happens majorly due to poor processing environment and secondary soiling from equipment of meat-processing plants.

In another study, Snapir *(116)* analyzed 169 samples of pork meat for prevalence levels of *L. monocytogenes*. The study revealed that out of 169 samples, *L. monocytogenes* was isolated

from 13 samples with 7.6% of prevalence levels. Thus, it can be concluded that *L. monocytogenes* is a foodborne pathogen, which is prevalent in food items such as milk related products and meat related products such as beef and pork *(116)*. The ubiquitous nature of the *L. monocytogenes* is the primary reason behind its dissemination in the environment and accession to milk and meat processing plants.

Pathology of *L. monocytogenes.* *L. monocytogenes* can cause localized or systemic infection. Whilst the invasive infection caused by *L. monocytogenes* would lead to listeriosis, the non-invasive infection would lead to febrile gastroenteritis. Some of the known manifestations of listeriosis are pneumonia, corneal ulcer, meningitis and cervical infections in pregnant women. Febrile gastroenteritis, which is caused by non-invasive infection, will show signs such as nausea, vomiting and diarrhea. The clinical onset of symptoms for both listeriosis and febrile gastroenteritis studies have stated that symptoms will be visible in a couple of weeks for listeriosis and in 12 hours for febrile gastroenteritis.

The infective dose of *L. monocytogenes* would change from one person to another based on his or her susceptibility to react to the bacterium and source of bacterium. In most of the cases, if the source of bacterium is milk related products, susceptible persons will suffer from diseases when the infective dose is approximately 1000 organisms.

When it comes to the pathogenicity of *L. monocytogenes,* there are three distinct lineages; all these three lineages will have potential to cause diseases to susceptible persons and animals. Whilst the first lineage, Lineage-1, are overrepresented in human clinical cases and underrepresented in animal clinical cases, the second lineage, Lineage-II, are underrepresented in human clinical cases and overrepresented in human clinical cases. Most of the Linage-II will be presented in environment and food particles. When compared to Lineage-I and Lineage –II, the

pathogens of Lineage-III are said to be rare isolates but are more represented in animal clinical cases than human clinical cases.

The number of reported cases of *L. monocytogenes* has doubled from early 1990s to early 2000s; whilst the number of reported cases was just 9, the number has increased to 20 in 2001. Though the number makes it clear that *L. monocytogenes* does not infect people frequently, the bacterium has been kept under surveillance because it is a rare genus, which are known for high mortality rates. Treatments are available to take care of the victims. A bacteriophage named as "Listeria Phage P100" will be supplied as food addictive to reduce the infection of *L. monocytogenes*. The Food and Drug Administration of the United States of America has approved a cocktail of 6 bacteriophages that could effectively control the infections of *L. monocytogenes*.

Prevention of *L. monocytogenes.* *L. monocytogenes* infects through food related products, to prevent the infections from *L. monocytogenes* bacterium, organizations that operate in food industry should follow milk and meat processing guidelines issued by Food Development Authority and other healthcare regulatory bodies. Effective sanitation of food contact surfaces, following impeccable packaging techniques and employing quality resources to carry out processing of food related products would certainly help in prevention of infections. In addition to that, practices such as following environmental sampling guidelines, testing food contact surfaces regularly, following general plant sanitation techniques and cleaning reservoirs in regular intervals would also help.

Listeriosis and Pregnancy. Almost one-third of the cases involving listeriosis occurs in pregnant women and carry the risk of feto-placental infection that often result in severe disease *(10) (85).* In humans, *L. monocytogenes* has the tendency to act within the feto-placental tissue

(17, 95). Commonly occurring during the third trimester, *(14, 17)*, the belief is that it is probably related to the major decline in cell-mediated immunity that occurs at 26-30 weeks of gestation *(132)*. With most listeriosis cases, the pregnant woman is asymptomatic. Severe listeriosis in pregnant women is rare *(115)*. Lastly, listeriosis in the pregnant female is usually self-limiting with the delivery of an infected baby; though some animal studies indicate that both mother and fetus are at risk for serious illness if the mother is infected by *L. monocytogenes* during pregnancy.

Pregnancy-related listeriosis in humans primarily affects the fetus or neonate. The effect of fetal *Listeria* infection is dependent on the point in gestation time when infection occurs. Susceptibility factors affecting fetal development are the changes in immunological parameters that take place during pregnancy. Increases and decreases of cytokine production are necessary for regulating homeostasis in the body during infection *(78)*.

Throughout pregnancy, cytokine production is regulated to avoid deleterious effects to the fetus. T helper 1-type (Th1) cytokines provoke an inflammatory response when expressed, and T helper 2-type (Th2) cytokines have an anti-inflammatory response when activated *(124)*. These Th1 cytokines have been shown to have deleterious effects on the placenta and fetus *(78)*. Infection from pathogens during pregnancy show that the normal relative ratio of Th1 and Th2 cytokines might be disrupted leading to deleterious effects to the fetus *(74, 78, 124)*.

In summary, *L. monocytogenes* is a gram positive pathogen that can cause listeriosis and febrile gastroenteritis. Some of the major sources of this bacterium are milk and meat, which are processed in contaminated environments. While contaminated food is the primary reason associated with *Listeriosis*, patient's susceptibility and poor immune systems would also help bacterium to easily spread and survive.

Treatments, in the form of bacteriophage or listeria phage P100 are available to potentially control the infections of *L. monocytogenes*. Further, to prevent infections from *L. monocytogenes*, food processing organizations should ensure the staff members follow guidelines related to sanitation, refrigeration and packaging.

CHAPTER 3

LACTOBACILLUS RHANMNOSUS GG AND ITS EXCRETED FACTORS REDUCES CRONOBACTER SAKAZAKII INVASION AND MORTALITY IN A NEONATAL MOUSE[1]

[1] Augustine Kwaku Agyekum, Gabriel Gross, Karin Sheehan, Mary Alice Smith, 2013. To be submitted to The Journal of Food Protection

ABSTRACT

Cronobacter sakazakii (*C. sakazakii*) is an opportunistic pathogenic bacterium that has been associated with outbreaks in infants, especially those in neonatal intensive care units. Although *Cronobacter* outbreaks in human infants are rare, when they occur the case fatality rate is high (40-80%). The current study investigated whether the probiotic *Lactobacillus rhamnosus* GG (LGG) or soluble excreted products of LGG can prevent or lessen the degree of invasion by *C. sakazakii* in CD-1 neonatal mouse pups. Specifically, the goals of this study were to 1) determine the role of the probiotic LGG or its soluble excreted products, administered in reconstituted powdered infant formula (RPIF), on the ability of *C. sakazakii* to invade target tissue, and 2) determine whether RPIF containing LGG or LGG excreted products prevents or reduces mortality. To obtain LGG excreted products, 10^6 CFU LGG was separated by filtration from the growth media in which it was grown and both LGG cells and the LGG excreted products were tested. CD1 pups were orally gavaged with either 10^6 CFU LGG or LGG excreted products on post-natal days (PNDs) 1.5, 3.5 and 4.5. A single oral dose of ($10^{8, 9 \text{ or } 12}$) CFU *C. sakazakii* or vehicle (RPIF) was administered at PND 2.5. LGG treatments reduced

C. sakazakii invasion compared to *C. sakazakii* treatment alone. When evaluating *C. sakazakii* invasion, both viable LGG bacteria as well as LGG excreted products significantly reduced the number of animals with *Cronobacter* isolated from any tissue as compared to the infected positive control group. Both LGG and LGG excreted product significantly reduced the *Cronobacter* invasion of brain tissue. Mortality in *C. sakazakii* exposed animals was reduced when animals were treated with LGG excreted product.

Cronobacter sakazakii (*C. sakazakii)* is a gram negative, opportunistic bacterium that has been associated with illness in neonatal intensive care units *(6, 9, 125, 127).* The pathogen has been isolated from reconstituted powdered infant formula (RPIF), and most commonly affects at-risk infants, particularly premature and immune compromised individuals *(30).* Infection may result in necrotizing enterocolitis (NEC), meningitis, bacteremia, septicemia, and death. Infants who recover from *C. sakazakii* infection may suffer morbidities such as hydrocephaly, developmental delays, mental retardation, or other permanent neurological disorders *(42).*

Probiotic bacteria are live microbial supplements that colonize the gastrointestinal tract and potentially provide benefits to host organisms *(92).* Probiotics may provide benefits to the host through their ability to 1) remain viable and stable after culture, 2) survive gastric and pancreatic digestion and 3) induce a host response once they enter the intestinal ecosystem *(21).* Furthermore, probiotic supplementation has resulted in the reduction of NEC-like intestinal lesions in various animal models *(21).*

The most frequently used probiotics are *Lactobacillus* and *Bifidobacterium*. *Lactobacillus rhamnosus* GG (LGG) was isolated from human intestinal flora and has widely been tested in human adults *(23)*. The mechanism by which probiotics protect against infection is not fully understood, but it has been proposed that anti-bacterial compounds produced by probiotics are one mechanism that protect against infection *(23)*. For example, Corr *et al.* found the production of bacteriocin, an anti-bacterial peptide produced by LGG, protected against *C. sakazakii* infection *(23)*. While previous studies have shown that probiotics can prevent attachment of *C. sakazakii* to intestinal cells *in vitro (21),* no previous work has looked at the potential of LGG to prevent or reduce invasion by *C. sakazakii in vivo* in neonatal mice. The

objectives of our study were to use our neonatal mouse model *(100)* 1) to determine the effects of the probiotic LGG and its soluble excreted products on the ability of *C. sakazakii* to invade brain, liver, and spleen tissues, and 2) to determine whether reconstituted powdered infant formula (RPIF) containing LGG or LGG excreted products reduces mortality in the *C. sakazakii* infected neonatal mouse.

MATERIALS AND METHODS

Animals. Timed-pregnant CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) at gestation day (gd) 17. Animals were maintained in an isolated room with a 12 h: 12 h light/dark cycle and provided rodent chow and drinking water *ad libitum*. Dams were housed individually and allowed to give birth naturally at gd 19 or 20. Neonatal mice were sexed and randomly assigned to foster mothers.

Preparation of LGG, LGG excreted products, and *C. sakazakii*. The probiotic LGG (provided by Mead Johnson Nutrition) was activated through three successive transfers into de Man, Rogosa and Sharpe (MRS) (Oxoid, LTD, Basingstoke, England) broth and incubated at 37°C for 24 hrs. The cells were isolated via centrifugation (8,000 x g at 4°C for 15min), washed twice with phosphate buffered saline (PBS), and re-suspended in vehicle (RPIF) at a concentration of 10^6 CFU/ml LGG. The LGG concentration was determined by measuring the optical density (OD) of the culture and comparing to a standard curve developed through serial dilutions of the culture. The dose was then confirmed by plating LGG on tryptic soy agar (TSA) (Oxoid) for 24 hrs, and calculating CFU/ml. A concentration of 10^6 CFU/ml LGG was used for treatment, and was added to 5 ml RPIF. To determine whether LGG produces a substance that interferes with *C. sakazakii* infection, LGG soluble excreted products (hereafter referred to as

LGG supernatant) was tested in our animal model. The LGG supernatant was provided by Mead Johnson Nutrition in lyophilized form after growing 10^6 CFU/ml LGG in media and removing LGG. For treatments with LGG supernatant, lyophilized LGG supernatant was reconstituted by diluting 0.562g of lyophilized supernatant into 60.5mls PBS. The supernatant was filtered, cooled and stored at 4ºC.

Stock cultures of *C. sakazakii* (strain 3290) frozen on ceramic beads at -80°C were grown to test concentrations in tryptic soy broth (TSB) (Oxoid, LTD, Basingstoke, England). *C. sakazakii* strain 3290 was chosen due to its high invasion rate in CD1 pups *(99)*. *C. sakazakii* 3290 was isolated from a clinical sample. The *C. sakazakii* culture was prepared and dose confirmed as described for LGG, except the cells were activated through two successive transfers in TSB.

Treatment of pups. Treatment methods for this study have been previously described by Richardson et al.. *(99).* Briefly, pups were treated with LGG or LGG supernatant in RPIF on postnatal days (PND) 1.5, 3.5 and 4.5, and with *C. sakazakii* on PND 2.5 via oral gavage using a 24 x 1'' (25.4 mm) W/1-1¼ stainless steel animal feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.) attached to a 1 ml syringe. RPIF was mixed with sterile deionized water for reconstitution per manufacturer's instructions.

Prior to litter assignment, pups were randomized by sex and dam. Vanilla flavoring (The Kroger Co., Cincinnati, OH.) was applied to the nose (snout) of each dam and pup to mask animal scents and create olfactory confusion. This was done to increase acceptance of the pups by the foster mothers. Serial dilutions of RPIF inoculated with various concentrations of *C. sakazakii* strain 3290 were prepared.

Each pup received a volume of 0.1 ml of *C. sakazakii* treatment in RPIF or vehicle control. The positive control group received *C. sakazakii* confirmed doses of 10^8, 10^9, or 10^{12} CFU/ml in RPIF and de Man, Rogosa and Sharpe MRS unconditioned media.

Pups were observed for morbidity or mortality at least twice per day during the post-treatment period. Any neonatal deaths before 7 days post treatment were recorded. Any neonates appearing sick or moribund were humanely euthanized and tissues collected and the day of death recorded. All remaining pups were euthanized at post-treatment day (PTD) 7 via CO_2 asphyxiation. Mortality data are presented as total mortality (Table 1A) over the course of the entire study period and as adjusted mortality (Table 1B) counting only those deaths occurring 24 hrs after the last gavage treatment. The adjusted mortality was calculated to remove any deaths that might have been related to the gavage procedure or stress of repeated gavage exposures.

All animal work was done in full compliance with federal regulations including the Animal Welfare Act and was approved by the Institutional Animal Care and Use Committee. The University of Georgia is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Culture of *Cronobacter species* from tissue samples. Liver, spleen, and brain were harvested from each neonatal mouse and stored in a Whirl Pack (Nasco, Fort Atkinson, WI) ™ filter bag on ice until processed for culture within 8 hrs of collection. *Enterobacteriaceae* enrichment (EE) broth (Oxoid) was added to the sample at a ratio of 10 ml EE to 1 g sample. The samples were placed in a stomacher machine (Brinkmann laboratories, Westbury New York) for 2min at 260 rpm, and then incubated for 24hrs at 37°C. Following, the samples were streaked onto plates of violet red bile glucose (VRBG) agar in duplicate for selective growth of

Enterobacter spp, and then incubated at 37°C for 24 hrs. Growths were sub-cultured onto TSA plates and incubated for 48 hrs at 25°C. RapID ONE Identification System (Remel, Inc., Lenexa, K.S., USA) was used for positive biochemical confirmation of *C. species*.

Statistical analyses. Statistical analyses for *C. sakazakii* infectivity and mortality data were done using SAS version 9.1 (SAS Institute, Cary, N.C.) and Microsoft Excel (Microsoft Corporation, Redmond, W.A.). Significant differences ($p \leq 0.05$) in values when comparing treatments were determined using Scheffe's test and Excel *t*-test. One-way analysis of variance (ANOVA) tests were done using Dunnett's *t*-test and Excel *t*-test to determine significant differences between treatment groups and the control group ($p \leq 0.05$). Because there were no significant differences in mortality or invasion in the different dose groups (*C. sakazakii* $10^{8, 9, 12}$ CFU), the data from these dose groups were combined to increase statistical power. This is in agreement with our previous work where there was not a dose dependent difference in invasion or mortality *(99, 100)* in PND 3.5 challenged neonates.

RESULTS

Previous work in our laboratory described the CD-1 neonatal mouse as a model for *C. sakazakii* infection *(99).* Additionally, Richardson et al.. examined whether there were virulence differences between three *C. sakazakii* strains (one food isolate and two clinical isolates) in CD-1 pups treated via the oral route *(100).* The study revealed that *C. sakazakii* strain 3290 (a clinical isolate) was the most invasive in neonatal mouse tissues. Thus, this study examined whether or not oral administration of the probiotic LGG or LGG supernatant in RPIF could prevent or decrease the effect of *C. sakazakii* strain 3290 in the CD-1 neonatal mouse.

For any group receiving *C. sakazakii*, the overall mortality rate was 32% (Table 3.1A). This was in contrast to the two vehicle control groups that did not receive *C. sakazakii* that had 7% mortality rate. When the data were adjusted according to our definition of *C. sakazakii*-related deaths (deaths occurring 24 hrs or more after last gavage treatment), the mortality decreased in all groups including the *C. sakazakii* and *C. sakazakii* plus LGG groups (Table 3.1B). No mortality was seen in the group receiving *C. sakazakii* and LGG supernatant (Table 3.1B). The LGG supernatant and RPIF control groups had only one death from a total of 116 animals.

The number of animals with at least one tissue invaded by *Cronobacter* was significantly reduced when pups received co-treatments with either LGG or LGG supernatant (Table 3.2). *Cronobacter* was not isolated from either LGG supernatant or RPIF-treated control groups. Pup weights were not correlated with infection (data not shown). While previous data from 1.5 day old CD-1 pups indicated a dose-response effect of *C. sakazakii* exposure *(100)*, data from the current study of 2.5 day old treated mice did not show significance among different *C. sakazakii* concentrations and effect, but did show a *C. sakazakii* treatment-related effect.

When examining individual tissues from the animals treated with *C. sakazakii* only, the brain tended to have *Cronobacter* isolated at a higher percentage than either liver or spleen; however, co-treatment with LGG or LGG supernatant reduced invasion in the brain by about 65% (Table 3.3A). Although the overall invasion rate of the liver was only 16%, it is noteworthy that in animals receiving LGG as co-treatment, we never isolated *Cronobacter* from liver tissues in any experiment and co-treatment with LGG supernatant reduced isolation of *Cronobacter* from liver by about one-half (Table 3.3B). Whereas both LGG and LGG supernatant treatments

significantly reduced isolation of *Cronobacter* from brain and liver tissues, only LGG treatment significantly reduced that seen in spleen tissues (Table 3.3C).

DISCUSSION

The objectives of this study were to determine whether LGG or LGG supernatant could reduce or prevent the degree of invasion or mortality after *C. sakazakii* exposure in CD-1 neonatal mice. Other animal studies have shown that probiotics are effective in reducing mortality. For example, *Lactobacillus bulgaricus* improves survival of newborn rats exposed to *E. sakazakii in a* necrotizing enterocolitis model *(57).* In our study, a protective effect was provided by administration of LGG before and after exposure to *C. sakazakii* providing additional evidence that probiotics can reduce invasion of *C. sakazakii*. LGG consistently reduced isolation of *Cronobacter* in neonatal mouse tissues (Table 3.3). Animals that received the LGG had fewer tissues invaded by *Cronobacter,* indicated by the lower percentage of animals from which *Cronobacter* was isolated (Table 3.2). No dose-dependent relationship was found between concentration of *C. sakazakii* and its invasion rate at the age of the neonates treated in this study; however, invasion rate was reduced in animals treated with LGG regardless of dose. The reduced invasion of *C. sakazakii* in brain tissue in groups receiving LGG treatment is important, because meningitis is the leading cause of morbidity and mortality in *C. sakazakii* infections *(57, 81).* Because the brain is a target tissue of *C. sakazakii* in humans, this could be an important finding for developing therapies and/or preventing adverse effects to the brain.

Overall, the total percentage of tissues invaded by *Cronobacter* was decreased in groups receiving both *C. sakazakii* and LGG or LGG supernatant (Table 3.2). The current study indicates that LGG, and/or its supernatant, limits the degree of invasion by *C. sakazakii* in

neonatal mice. Further research is needed to examine the mechanisms of protection by LGG and its supernatant against *C. sakazakii* infection.

The high mortality rate in any group receiving *C. sakazakii*, including LGG and LGG supernatant groups, is somewhat surprising. In general, the cause of death could not be verified because sufficient tissues could not be recovered for analysis because cannibalization by the dam is common after neonatal death. However, to be conservative in attributing mortality to *C. sakazakii* infection, we defined *C. sakazakii*-related deaths as those occurring 24 hrs after the last gavage treatment. It is interesting that groups receiving *C. sakazakii* and *C. sakazakii* with LGG had a similar adjusted mortality rate (20% and 17%, respectively) and was higher than *C. sakazakii* with LGG supernatant (Table 3.1). We observed that LGG was much more viscous than LGG supernatant, and this might be a contributing factor possibly affecting the gavage of the pups. The low mortality rate in the vehicle control groups suggests that most deaths in *C. sakazakii* treated groups were, in fact, the result of *C. sakazakii* exposure and not due to any procedure or stress.

CONCLUSIONS

The probiotic LGG and its excreted products collected during the fermentative process (LGG supernatant) reduced the overall invasion of *Cronobacter* in neonatal mice orally exposed to RPIF with *C. sakazakii*. Of tissues examined, the brain was most often invaded by *Cronobacter*, but also received the most protection from treatment with LGG or LGG supernatant. For the brain and liver, both LGG and LGG supernatant were equally protective against *Cronobacter* invasion. However, for spleen, LGG was more effective in preventing the invasion than LGG supernatant. Based on adjusted mortality rates, LGG supernatant was most

effective in protecting the neonatal mice from *C. sakazakii*–related death. The varying results found when comparing LGG and LGG supernatant treatments suggest there are properties of each treatment that allow for protection from *C. sakazakii* infection mechanistically. This study contributes to the growing literature suggesting that probiotics can be effective in preventing certain food borne infections.

TABLES AND FIGURES

Table 3.1. Mortality of CD-1 pups after treatment with *C. sakazakii* with or without LGG or LGG supernatant.

A. Total Mortality	
Treatment Group (Doses)	**Mortality (#Dead/total treated)**
***C. sakazakii* (10^{8-12} CFU)**	**34% (24/71)**
***C. sakazakii* (10^{8-12} CFU) plus LGG supernatant**	**29% (17/58)**
***C. sakazakii* (10^{8-12} CFU) plus LGG**	**33% (20/60)**
Supernatant control	**7% (4/58)**
Powdered Infant Formula only	**7% (4/61)**

B. Adjusted Mortality**	
Treatment Group (Doses)	**Mortality (#Dead/total treated)**
***C. sakazakii* (10^{8-12} CFU)**	**20% (12/59)**
***C. sakazakii* (10^{8-12} CFU) plus LGG supernatant**	**0% (0/41)**
***C. sakazakii* (10^{8-12} CFU) plus LGG**	**17% (8/48)**
Supernatant control	**2% (1/55)**
Powdered Infant Formula only	**0% (0/57)**

***C. sakazakii* doses represent a combination of three independent experiments conducted with concentrations of *C. sakazakii* at 10^8, 10^9, or 10^{12} CFU/ml. **Adjusted mortality = deaths of neonates occurring more than 24 hrs after the last gavage treatment.**

Table 3.2. Percentage of animals with at least one invaded tissue sample after exposure to *C. sakazakii* with or without LGG or LGG supernatant.

Combined Experiments	Animals positive for *Cronobacter*			
Treatment Group	**# positive**	**total treated**	**% positive***	
***C. sakazakii* (10^{8-12} CFU/ml)**	**15**	**58**	**26%**	**A**
***C. sakazakii* (10^{8-12} CFU/ml) with LGG supernatant**	**10**	**49**	**20%**	**B**
***C. sakazakii* (10^{8-12} CFU/ml) plus LGG**	**6**	**36**	**17%**	**B**
LGG Supernatant Control	**0**	**55**	**0%**	**C**
Powdered Infant Formula only	**0**	**49**	**0%**	**C**

***C. sakazakii* doses represent a combination of three independent experiments conducted with concentrations of *C. sakazakii* at 10^8, 10^9, or 10^{12} CFU/ml.**

****Treatment groups with the same letter are not statistically different. ($p \leq 0.05$).**

Table 3.3. Isolation of *Cronobacter* from brain (A), liver (B) and spleen (C) tissues following oral exposure of neonatal mice to *C. sakazakii* with or without LGG or LGG supernatant.

A. Brain

Treatment Group	Brains			
	# positive	total treated	% positive*	
C. sakazakii (10^{8-12} CFU/ml)	11	59	19%	A
C. sakazakii (10^{8-12} CFU/ml) with LGG supernatant	4	60	7%	B
C. sakazakii (10^{8-12} CFU/ml) plus LGG	3	46	8%	B
LGG Supernatant Control	0	60	0%	B
Powdered Infant Formula only	0	48	0%	B

B. Liver

Treatment Group	Livers			
	# positive	total treated	% positive*	
C. sakazakii (10^{8-12} CFU/ml)	9	59	16%	A
C. sakazakii (10^{8-12} CFU/ml) with LGG supernatant	5	60	9%	B
C. sakazakii (10^{8-12} CFU/ml) plus LGG	0	46	0%	B
LGG Supernatant Control	0	60	0%	B
Powdered Infant Formula only	0	48	0%	B

C. Spleen

Treatment Group	Spleen			
	# positive	total treated	% positive*	
C. sakazakii (10^{8-12} CFU/ml)	6	59	13%	A
C. sakazakii (10^{8-12} CFU/ml) with LGG supernatant	7	60	10%	A,B
C. sakazakii (10^{8-12} CFU/ml) plus LGG	1	46	3%	B,C
LGG Supernatant Control	0	60	0%	C
Powdered Infant Formula only	0	48	0%	C

*** Treatment groups with the same letter are not statistically different ($p \leq 0.05$).**

CHAPTER 4

LACTOBACILLUS RHAMNOSUS GG INHIBITION OF *LISTERIA MONOCYTOGENES IN VITRO* AND *IN VIVO*[2]

[2] A. Kwaku Agyekum, Rahat Wadwha Desai, Kendra Edwards, Denita Williams and Mary Alice Smith, 2013. Submitted to The Journal of Food Protection 11/4/2013.

ABSTRACT

To elucidate the mechanism of probiotic *Lactobacillus* rhamnosus GG (LGG) inhibition of *L. monocytogenes, in vivo* and *in vitro* models were used. The objectives of the current study were 1) to study the effects of LGG on *L. monocytogenes* invasion in pregnant guinea pigs, 2) to determine whether LGG or a soluble product of LGG (LGG supernatant) inhibits *L. monocytogenes* growth *in vitro*, and 3) to characterize any protective mechanisms of effect by LGG or LGG supernatant via changes in pH or protein activity. The effects of yogurt consumption on host susceptibility to *Listeria monocytogenes* infection were evaluated using a pregnant guinea pig model. A commercially available yogurt was orally fed to pregnant guinea pigs (gd 33-36) followed by a single oral challenge 10^9 CFU *L. monocytogenes.* Host susceptibility was determined by isolation of *L. monocytogenes* from maternal and fetal tissues, along with fecal shedding and number of stillbirths. Pregnant guinea pig dams treated with yogurt containing probiotics reduced *L. monocytogenes* that was isolated from tissues compared to *L. monocytogenes* treatment alone in liver (56% v 100%, respectively) and spleen (14% v 75%, respectively). Fetal tissues from dams receiving yogurt had fewer positive samples when compared to *L. monocytogenes* treatment alone in fetal liver (17% v 71%, respectively) and fetal brain (21% v 71% respectively). Importantly, stillbirths were reduced in guinea pigs receiving yogurt and *L. monocytogenes* as compared to *L. monocytogenes* alone (14% v. 75%). *L. monocytogenes* was not isolated from any viable fetus in dams receiving yogurt and *L. monocytogenes*. *In vitro*, *L. monocytogenes* ranging from $10^{1\text{-}7}$ colony forming units (CFU)/ml PBS were prepared and added to 10^6 CFU/ml LGG or LGG supernatant. LGG supernatant's ability to inhibit *L. monocytogenes* growth was dependent upon *L. monocytogenes* concentration up to the highest dose tested, 10^7 CFU/ml. LGG supernatant treatments inhibited *L.*

monocytogenes growth in a dose dependent manner at concentrations up to 10^5 CFU/ml *L. monocytogenes*. Neither LGG supernatant nor LGG treatments yielded any consistent pattern on *L. monocytogenes* growth at 10^7 CFU/ml. Heat treated and enzymatic time course experiments explored the protein dependent activity of LGG supernatant. Heat treating LGG supernatant did not restore *L. monocytogenes* growth. Changes in pH environment of *L. monocytogenes* resulting from the addition of LGG supernatant significantly reduced *L. monocytogenes* growth. In response to exposure to acidic environments both LGG supernatant or lowering of pH significantly reduced *L. monocytogenes* growth at all *L. monocytogenes* test concentrations when compared to controls. Determining the protective effects of LGG against *L. monocytogenes* growth *in vitro* may provide further information necessary for therapeutic and preventive strategies against listeriosis**.**

INTRODUCTION

Probiotic bacteria are microorganisms that colonize the gastrointestinal tract and potentially provide benefits to host organisms *(125).* Probiotics may provide protection to the host against infection by their characteristics such as their ability to 1) remain viable and stable after culture, 2) survive gastric digestion and 3) induce a host response once they enter the intestinal ecosystem *(3, 16).* Furthermore, probiotics supplementation has resulted in the reduction of necrotizing enterocolitis (NEC) like intestinal lesions in various animal models *(16).* Specifically, *Lactobacillus rhamnosus* GG (LGG) was isolated from human intestinal flora and has been tested widely in humans *(6).*

Lactobacillus rhamnosus strain GG is a gram-positive, lactic acid-producing bacterium that was first isolated from the stools of a healthy human *(32).* LGG has been employed experimentally as a supplement to the human neonatal intestinal microflora and has been studied to determine its effects on the enhancement of immunoglobulin (Igg) secretion *(16).* This probiotic LGG also prevents rotavirus-induced diarrhea, *Salmonella* infection *in vitro,* and the recurrence of colitis *(26, 120).* Lastly, LGG adheres to epithelial cells in tissue culture and displace intestinal pathogens, including *E. coli (63).*

Listeria monocytogenes (*L. monocytogenes*) is a foodborne pathogen that also poses a threat to fetuses and neonates. *L. monocytogenes* can cause stillbirths and abortions while the mother is typically asymptomatic. First-trimester infection can lead to spontaneous abortion, while second-and third-trimester infections lead to pre-term birth followed by neonatal illness or fetal death with preterm delivery *(24) (34) (35).* *L. monocytogenes* is also a facultative intracellular pathogen that is dependent on invading host cells in order to grow and survive. While the specific mechanism on *L. monocytogenes* has yet to be determined; *L. monocytogenes*

gains entry into the systemic circulation by binding to E-cadherin in the intestine, entering the epithelial cells, and moving into the macrophages and other cells *(104)*.

Humans display hemochorial placentation; because guinea pigs are the most similar of any rodent model in this type of placentation *(96)*, they serve as a representative model for placental infection. In addition, *L. monocytogenes* binds to the E-cadherin receptor in the intestine and enters epithelial cells. The E-cadherin receptor in the guinea pig has the same amino acid sequence in the active binding site as humans, while mice and rats do not *(73)*.

Guinea pigs have the same pregnancy outcome as do humans following exposure to *L. monocytogenes* at a dose of 10^6 CFUs or greater *(76, 136)*. Additionally previous work in our laboratory have shown that the guinea pig model is effective in assessing *L. monocytogenes* based invasion and infection *(136) (58)*.

We propose that commercially prepared yogurt containing *Lactobacillus* and *Bifidobacterium* may interfere with this transmission and offer protection against *L. monocytogenes* infection. Previous studies performed with rats pre-treated with *Lactobacillus* and inoculated with *L. monocytogenes* have shown reduced numbers of *L. monocytogenes* in the liver and spleen two days following exposure *(28)*. Thus, our first objective was to evaluate the effect of yogurt treatment on host susceptibility to *L. monocytogenes* infection.

The mechanism in which probiotics protect against enteric pathogens remains largely uncharacterized; however few *in vitro* studies investigating the mechanisms underlying the antibacterial activity of *Lactobacillus rhamnosus* GG against *L. monocytogenes* have been performed *(37)*. Against *Salmonella enterica*, studies suggest the mechanism of antibacterial activity used by *Lactobacillus GG* is multifactorial. The factors involved in antibacterial activity of *Lactobacillus* GG against *Salmonella enterica* are metabolites produced by lactic acid and

acetic acid as well as a lowering of pH *(37)*. Bacteriocins were not identified as factors in antibacterial activity against *Salmonella enterica*. In contrast to the previous hypotheses, *in vivo* studies have identified bacteriocins of *Lactobacillus spp.* (*salivarius* UCC118) as mediators of antimicrobial activity against *L. monocytogenes (23).*

Thus, our overall goal was to determine whether LGG or a secreted factor of LGG could reduce *L. monocytogenes* growth and offer protection during pregnancy. The objectives of the current study were 1) to study the effects of LGG on *L. monocytogenes in vivo* 2) to determine whether LGG or a soluble product of LGG (LGG supernatant) inhibits *L. monocytogenes* growth *in vitro,* and 3) to characterize any protective mechanisms of effect by LGG or LGG supernatant via changes in pH or protein activity.

MATERIALS AND METHODS

Preparation of *L. monocytogenes* inocula. As previously described in Williams et al. *(135)***,** *L. monocytogenes* (strain 12443) was originally isolated from a non-human primate stillbirth and this strain was used in previous guinea pig studies demonstrating its virulence to fetuses *(135)*. *L. monocytogenes* cells were activated by two successive transfers into 10ml TSB (tryptic soy broth) and incubated for 24h at 35°C. The cells were obtained by centrifugation (8,000 x g at 4°C for 15min), washed twice and re-suspended in 10ml sterile phosphate buffered saline. To obtain the target concentration, the optical density (OD) of a sample of the *L. monocytogenes* suspension was determined using a standard curve developed by serially diluting the *Listeria* sample, plating on LSA, and calculating colony forming units (CFUs) at each dilution. A dose of 10^9 CFU/ml was used for treatments. The inoculum was added to 5ml sterile whipping cream with 0.5g Splenda™ added for palatability.

Animal Treatments. Animal methods were adapted from Williams et al. *(135)*. Twelve timed-pregnant guinea pigs were purchased from Elm Hill Labs on gestation day 29 and were housed at the University of Georgia (UGA) animal facility. The guinea pigs were housed individually in cages equipped with air filters and the control animals were housed in a separate room from *L. monocytogenes* treated animals. Guinea pigs were fed 5ml of commercially available yogurt containing *Lactobacillus* and *Bifidobacterium* on gestation days (gd 33 - gd 36). On gestation day 35, after receiving yogurt in the morning; food was withheld for four hours and guinea pigs were fed sweetened whipping cream inoculated with *L. monocytogenes* in the afternoon. Controls were treated first and received yogurt treatment and sterile whipping cream without *Listeria*. Fecal samples were collected three times a week for the duration of the study (beginning one week prior to *L. monocytogenes* inoculation) and *L. monocytogenes* was isolated by culture to determine fecal shedding. The guinea pigs were sacrificed on gestation day 56; tissues were collected including placenta, maternal liver and spleen, and fetal liver and brain.

***L. monocytogenes* culture from fecal and tissue sample.** Culture methods from fecal and tissue sample were adapted from Williams et al. *(135)*. Either fecal or tissue samples were weighed and placed in a whirl pak filter bag (Nasco). UVM broth was added to the sample at a ratio of 10mls UVM to 1g sample. The sample was placed in a stomacher machine for 2min at 260 rpm. The UVM diluted sample was incubated for 24h at 35°C. 100ul of incubated UVM sample was then transferred to 9.9ml Fraser broth for *Listeria* enrichment and incubated at 35°C for 24h. At 24h, the sample was streaked onto Listeria Selective Agar (LSA) plates for colony isolation and incubated at 35°C for 24h. Samples positive for *Listeria* were then confirmed using Rapid' L Mono (Remel) culture plates. Rapid' L Mono plates are differential for species of *Listeria.*

Culture preparation. As previously described in Agyekum et al. 2013, the probiotic LGG (obtained from Department of Food Science and Technology-UGA) was frozen on ceramic beads and activated through three successive transfers and incubated. Briefly, cells were isolated via centrifugation, washed twice with phosphate buffered saline (PBS), and re-suspended. Doses were confirmed by calculating colony forming units CFU/ml. A concentration of 10^6 CFU /ml LGG was used for treatment and was added to 5mL of vehicle. *L. monocytogenes* (strain 12443) cells were activated by two successive transfers into 10ml tryptic soy broth (TSB) and incubated for 24h at 37°C. The cells were obtained by centrifugation (8,000 x g at 4°C for 15min), washed twice and re-suspended in 10ml sterile PBS. Target concentration and the optical density (OD) of a sample of the *L. monocytogenes* suspension was determined using a standard curve developed by serially diluting the *Listeria* sample, plating on LSA (Listeria Selective Agar) (Oxoid), and calculating CFU/ml at each dilution.

Preparation of LGG supernatant. Upon centrifugation, the LGG supernatant was removed from the LGG pellet. For the heat shock treatments, LGG supernatant was then placed in a water bath at 60°C for 10 min. For LGG supernatant treatments with an added centrifugation step, the LGG supernatant was centrifuged an additional 15 min at 4g. For filtered supernatant treatments, the LGG supernatant was filtered through a 0.22µm pore filter (Millipore, MA).

Protein Activity of LGG supernatant after heat shock, filtration and centrifugation. Bradford protein assay (Bio Rad, Hercules CA) was conducted to determine the protein content of LGG supernatant. Protein concentration of supernatant samples was determined and PBS used as a control. LGG supernatant was treated for 25min with trypsin (200mg/ml) and neutralized with fetal bovine serum (FBS; ATCC). Once treated, the LGG supernatant was used as a treatment against *L. monocytogenes* for its effects on growth. Following treatment, the

cultures were then plated on LSA and enumerated for CFU/ml after incubation for 37°C for 24hrs.

***In vitro* growth assays of *L. monocytogenes* and LGG or LGG supernatant.** For growth inhibition studies, 1ml volumes of *L. monocytogenes* concentrations 10^3, 10^2, and 10^1 CFU/ml were each separately incubated with equal volumes of 10^6 CFU/ml LGG or LGG supernatant. LGG supernatant was filtered through a 0.22µm pore size membrane filter (Millipore, MA). After 1h incubation at 37°C, 0.1 ml of each inoculum was plated on LSA and incubated for 24h at 37°C. Following incubation, the plates were analyzed for their respective CFU.

Responses of *L. monocytogenes* to acidic environment. Measurements of pH were made using a digital pH Meter (Accumet 15, Fisher Scientific)**.** Upon culture, *L. monocytogenes* pelleted bacteria was serially diluted into its respective treatment diluent (PBS or MRS), which had been titrated with 2M HCl or LGG supernatant to pH 4.37. NaOH was added drop wise to the diluent to achieve pH 6.7 for basic environments. Following, addition of the appropriate medium/treatments were added to the *L. monocytogenes* cultures.

Responses to LGG supernatant after exposure to heat and proteolytic enzymes. The following methods were adapted from de Keersmaecker et al. *(26)*. Briefly, the LGG supernatant (24 hr; pH 4.5) was heated at 65°C and 110°C for 1 h. To test the sensitivity to proteases (all purchased from Sigma), the LGG supernatant was incubated at 37 °C for 1 hr with and without 200 mg/ml trypsin, 100mg/ml proteinase K, 2M HCL, 200 mg/ml pronase, 150mM lactic acid and sterile De Man–Rogosa–Sharpe medium (MRS) at pH 4.5 (adjusted) were included. Heat treatment temperatures were 60°C and 110°C. *L. monocytogenes* concentrations ranged from $10^{1\text{-}3}$CFU/ml. Low concentrations were chosen for enumeration when analyzing *L.*

monocytogenes viability. Heat was used to denature any potential heat-sensitive protein activity of LGG supernatant.

Statistical analyses. Statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, N.C.) and Microsoft Excel (Microsoft Corporation, Redmond, W.A.) Significant differences ($P \leq 0.05$) in values comparing the treatments of probiotic were determined using Scheffe's test and Excel *t*-test. One-way analysis of variance (ANOVA) tests were done using Dunnett's *t*-test and Excel *t*-test to determine significant differences between treatment groups and the control groups ($P \leq 0.05$) for *L. monocytogenes*.

RESULTS

Probiotic Inhibition of *L. monocytogenes* growth *in vivo*. Data collected on fecal shedding from treated animals is highly correlated to previous studies *(135)* indicating an average shedding during the three-week collection period of 52% for those with viable pregnancies and 100% for the one pregnancy ending in stillbirths (Figure 4.1). All control guinea pigs were negative for *L. monocytogenes* for all fecal and tissue samples examined.

Data collected during our study shows that yogurt, containing LGG and Bifidobacterium, consumed prior to and during exposure to *L. monocytogenes* reduces the number of stillbirths in pregnant guinea pigs (Table 4.1). Of 7 pregnant guinea pigs receiving yogurt and *L. monocytogenes*, only one dam went into premature labor and delivered stillbirths while all others maintained their pregnancy until the date of sacrifice (gd 56). Guinea pigs receiving a high dose of *L. monocytogenes* give birth at a significantly earlier time than controls *(136)*. In the current study, one guinea pig had stillbirths, and she delivered prematurely at gd 50 compared to an average of gd 45 for animals having stillbirths after receiving *L. monocytogenes* alone (Table 4.1). Tissues cultured from the dam with stillbirths revealed *L. monocytogenes* present in the

maternal spleen but not in the maternal liver. In comparison, dams receiving *L. monocytogenes* alone at 10^8 CFU were much more likely to have *L. monocytogenes* isolated from their liver or spleen than those receiving yogurt (Table 4.2).

When compared to previous studies *(136)*, pregnant guinea pigs exposed to 10^9 CFU *L. monocytogenes* and yogurt had a reduction in fetal invasion by *L. monocytogenes* as compared to guinea pigs treated with 10^8 CFU *L. monocytogenes* alone (Table 3). Similarly, the placentas from animals receiving *L. monocytogenes* alone were more likely to be invaded when compared to those receiving yogurt and *L. monocytogenes* (Table 4.3).

Tissue samples collected from viable fetuses all tested negative for *L. monocytogenes*; however, *L. monocytogenes* was isolated from the stillborn fetuses at a high rate of 71% from brain and liver tissues (Table 4.3). The overall rate of isolation of *L. monocytogenes* from fetuses of dams receiving yogurt was lower than in dams receiving *L. monocytogenes* alone.

Tissue invasion of the fetus or of the placenta appears to be required for stillbirth based on our findings that *L. monocytogenes* was isolated from stillbirths but not from viable fetuses. There was a reduction in stillbirths in guinea pigs treated with yogurt (14%) as compared to treatments with *L. monocytogenes* alone (75%). Fetal tissue samples collected from viable fetuses all tested negative for *L. monocytogenes*, while *L. monocytogenes* was isolated from tissues of stillbirths (Table 4.3).

Based on our *in vivo* findings, we wanted to investigate the mechanisms for how LGG might be protective against *L. monocytogenes* infection. There are several possible methods by which this could occur including protein interactions, changes in pH, and activation of proteolytic enzymes.

Removal of live LGG from supernatant and quantification of protein in LGG supernatant. Three methods were tested to determine their efficacy in removing viable LGG from LGG supernatant. Protein analysis and quantification of LGG in supernatant after filtration, heating, or centrifugation resulted in no significant differences in the quantity of protein amongst treatments (Table 4). Quantification of total protein was necessary in order to examine the effects of these conditions on LGG supernatant activity. To verify that viable LGG was removed from the supernatant, all treatments were plated and enumerated, showing that filtration treatment was the only treatment that successfully removed viable LGG from the supernatant (Table 4.4).

Effects of LGG and LGG supernatant on *L. monocytogenes* growth *in vitro*. In the current study, we examined whether LGG or LGG supernatant inhibits the growth of *L. monocytogenes*. We found treatments with LGG inhibited *L. monocytogenes* growth at 10^3, 10^5, and 10^7 CFU/ml concentrations (Figure 2). *L. monocytogenes* growth was reduced most at 10^5 CFU/ml (Figure 2). *L. monocytogenes* treatments with LGG supernatant reduced *L. monocytogenes* growth in a concentration dependent manner at 10, 10^3, and 10^5 CFU/ml (Figure 4.2).

Responses to LGG after exposure to heat. To determine whether a heat-sensitive protein in LGG supernatant was responsible for the reduction in growth of *L. monocytogenes* at low concentrations, the supernatant was heat-shocked (HSS) before incubating with *L. monocytogenes*. Heat shock treatments at every test concentration were statistically significant from control (*L. monocytogenes* alone) (Figure 4.3). However, no statistical difference was found when comparing heat shocked supernatant treatments with *L. monocytogenes* plus untreated LGG supernatant.

Analysis of pH environment on *L. monocytogenes* growth. When examining the effects of LGG supernatant on *L. monocytogenes* growth, we found LGG supernatant treatments

significantly reduced the pH of *L. monocytogenes* inoculum when compared to control (neutral pH). The number of viable *L. monocytogenes* cells was measured in order to assess the effects of an acidic environment from probiotic exposure (Figure 4.1). To determine the effect of the acidic environment on *L. monocytogenes* growth, LGG supernatant (pH 4.37) was titrated to a slightly acidic environment (pH 6.7) drop wise with 2M NaOH. 24 hrs showed the greatest contrast between acidic and basic treatments (Figure 4.1). However, by 120 hrs significance was found when comparing 10^7 CFU *L. monocytogenes* titrated to 6.7 and all other treatments (Figure 4.5). This may be due to the presence of undissociated organic acids in the LGG supernatant which is influenced by pH. pH influences the ratio of dissociated to undissociated acid, as explained by the Henderson–Hasselbach equation. Both forms of acid can inhibit bacterial growth, the undissociated form of organic acids was reported to be more inhibitory, per mole, than its corresponding dissociated form *(33, 93)*.

Responses to LGG supernatant after exposure to heat and proteolytic enzymes. The growth inhibitory effect of LGG supernatant was not changed with treatment of proteases or heat (Table 4.3, Fig. 4.3 and 4.6). When the LGG supernatant was treated with 200 mg/ml trypsin, pH to 4.6, 200 mg/ml proteinase K, 200 mg/ml pronase or 200 mg/ml pepsin, no significant differences were found when comparing the effects of the un-treated LGG supernatant on *L. monocytogenes* growth. However, when lactic acid was added to LGG supernatant, *L. monocytogenes* growth was decreased when compared to controls (Figure 4.6).

DISCUSSION

The current study investigated whether yogurt containing probiotics LGG and Bifidobacterium reduced the effects of *L. monocytogenes* on pregnant guinea pigs. There was a reduction in stillbirths in guinea pigs treated with yogurt (14%) as compared to treatments with

L. monocytogenes alone (75%). There are several possible mechanisms by which probiotics might help prevent *L. monocytogenes*-induced stillbirths including competitive growth in the intestinal tract. One such mechanism may be from the probiotics reducing the number of *L. monocytogenes* that invade the maternal tissues, competitively binding to receptor sites such as the E. cadherin in epithelial cells, or producing a toxin that kills *L. monocytogenes* before it invades the fetus. Additional testing is necessary in support of the aforementioned hypothesis.

The results found in the current study are in accordance with Rautava et al. *(97)* who found when given to pregnant pigs during pregnancy and breast feeding, probiotics reduced atopic eczema for the infant. However, the proposed mechanism of protection offered by Rautava et al. was the elevated TGF-β2 in breast milk caused by daily probiotic supplementation. Further, their data suggests that probiotics exert their effect on early immunologic mechanisms involved in the development of atopic disease *(97)*. Both the current study and Rautava et al. suggest that administration of probiotics to pregnant mothers during pregnancy and breast-feeding appears to be a safe and effective mode of enhancing immune protective properties of the mother.

This study further investigated whether LGG protects against *L. monocytogenes* induced stillbirths and whether LGG or a LGG supernatant inhibits *L. monocytogenes* growth. Changes in pH and protein activity were investigated as protective mechanisms used by LGG or LGG

supernatant. The complete mechanism of protection provided by LGG or LGG supernatant has not been identified. However, our study indicates that LGG and LGG supernatant treatments were not significantly different from one another; suggesting LGG supernatant treatments are as effective as the bacteria itself. Antimicrobial antimicrobial activity of LGG supernatant protection against *L. monocytogenes* was found to be pH dependent and

were not (heat sensitive) or sensitive to enzyme treatment (Figures 4.4-4.6). Lastly, yogurt containing *Lactobacillus* and *Bifidobacterium* provides a protective effect to fetuses when the dam is exposed to *L. monocytogenes*.

The findings in this study support our hypothesis and other studies that found LGG and LGG supernatant inhibit *L. monocytogenes* viability and growth *in vitro* (Figure 4.2). Antimicrobial activity of Lactobacillus had been previously investigated using an *in vitro* method in which activity was measured in the presence of various growth media or PBS *(11, 20)*. Thus, it has been suggested that *Lactobacilli* species' antimicrobial activity against pathogenic bacteria may result mainly from strain-specific lactic acid molecules within their respective supernatants *(37)*. Further, past studies have shown the Lactobacillus secretes a low-molecular mass, heat stable, inhibitory substance *(112)*. For the current study, it is important to note the mechanisms of protection provided by LGG and LGG supernatant affecting pathogenic bacteria are non-exclusive, given the effects from both treatments on *L. monocytogenes* growth and viability *in vitro* and *in vivo*.

During this experiment we found environmental changes in pH caused by treatments with LGG supernatant affected viability and growth of *L. monocytogenes* (Fig. 4.5). When *L. monocytogenes* cultures were treated with LGG supernatant, the pH environment of the culture (pH 4.6) was significantly different from the neutral pH environment of the control cultures (pH 6.7). Within the literature, LGG supernatant's ability to produce acetic and lactic acids, thus lowering pH, has also been observed as mechanisms of antimicrobial activity activity *(129)*.

Our data suggest heat sensitive proteins are not the major contributing component to the inhibitory effects of LGG (Fig. 4.3). This is also in agreement with previous studies *(8, 118)*. Thus, under the conditions tested, the anti-microbial activity of LGG is likely due to a heat-

stable, non-proteinaceous low molecular weight compound(s). Further, these results can be attributed to a change in pH; such as may be expected with lactic acid production.

Based on our previous findings *(136)(1a)* and the findings of our current study; information on effective therapeutic regimens, including yogurt containing probiotics for listeriosis during pregnancy, may help reduce adverse outcomes. New data supporting successful therapies on the prevention and or reduction of *L. monocytogenes* infection will aide in the decrease of listeriosis related mortalities.

ACKNOWLEDGEMENTS

Funding for this research project was provided by the University of Georgia (UGA) Center for Food Safety. The Alfred P. Sloan Foundation and the UGA Interdisciplinary Toxicology Program are acknowledged for providing graduate stipends (KA) and the UGA College of Public Health for salary support (MAS). We would also like to thank Yerkes National Primate Research Center, Atlanta, GA for providing the *L. monocytogenes* strain. Lastly, thank you to Dr. Joe Frank for providing the LGG strain.

TABLES AND FIGURES

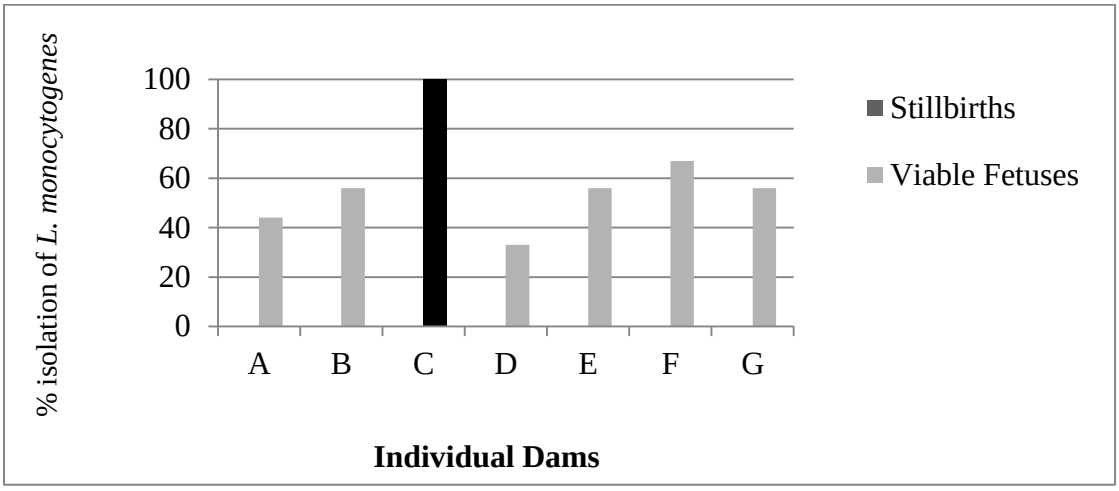


Figure 4.1. Percentage of fecal samples positive for *L. monocytogenes* following oral exposure to yogurt and 10^9 CFU *L. monocytogenes* to pregnant guinea pigs. Animals were fed 0.05 ml commercial yogurt prior to and after challenge with *L. monocytogenes*. n=10 days of fecal collection.

Table 4.1. Pregnancy outcomes and stillbirths following exposure to *L. monocytogenes* with or without co-treatment of yogurt.

Treatment groups	Number of dams	Number of dams with Stillbirths	Post-treatment Day of Stillbirths
Control	9	0	-
***L. monocytogenes** 10^8 CFU/ml**	4	3	10*
***L. monocytogenes* 10^9 CFU/ml + yogurt**	7	1	15

*** Data from Williams et al.. 2007**

Table 4.2. Comparison of *L. monocytogenes* isolated from maternal tissues after oral exposure of pregnant guinea pigs to *L. monocytogenes* with or without yogurt co-treatment.

Maternal Dose	No. of dams with infected fetuses (% Positive)	Maternal Liver (% Positive)	Maternal Spleen (% Positive)	No. of dams with infected placenta (% Positive)
Control	0/9 (0)	0/9 (0)	0/9 (0)	0/9 (0)
10^8 CFU *L. monocytogenes**	3/4 (75)	4/4 (100)	3/4 (75)	3/4 (75)
10^9 CFU *L. monocytogenes* CFU + yogurt	1/7 (14)	4/7 (56)	1/7 (14)	1/7 (14)

*** Data from Williams et al.. 2007**

Table 4.3: Isolation of *L. monocytogenes* from fetal tissues after maternal exposure to *L. monocytogenes* with or without co-treatment with yogurt.

Maternal Dose	Number of Fetuses	Placenta (% Positive)	Fetal Liver (% Positive)	Fetal Brain (% Positive)
Control	9	0(0)	0 (0)	0 (0)
***L. monocytogenes* 10^8 CFU/ml**	17[a]	9(64)	12 (71)	12 (71)
***L. monocytogenes* 10^9 CFU/ml + yogurt**	36	6 (17)[b]	6 (17)[b]	8 (22)[b]

[a]Tissue samples collected from only 14 fetuses
[b]Positive fetal tissue samples originating from a single dam with 9 stillbirths
*** Data adapted from Williams et al.. 2007**

Table 4.4. Comparison of heat, filtration, and centrifugation for removing viable LGG from LGG supernatant.

Supernatant sample	Total Protein Quantification (mg/ml)	CFU/ml LGG
MRS Media (LGG Selective)	2.77	ND ≤ 10 CFU/ml
Filtered LGG supernatant (0.22µm)	2.78	ND ≤ 10 CFU/ml
Heated Supernatant 60ºC for 15min	2.79	TNTC
Supernatant centrifuged 4xG for 15 min	2.86	TNTC

TNTC = too numerous to count.
ND = not detectable at lowest detectable limit.

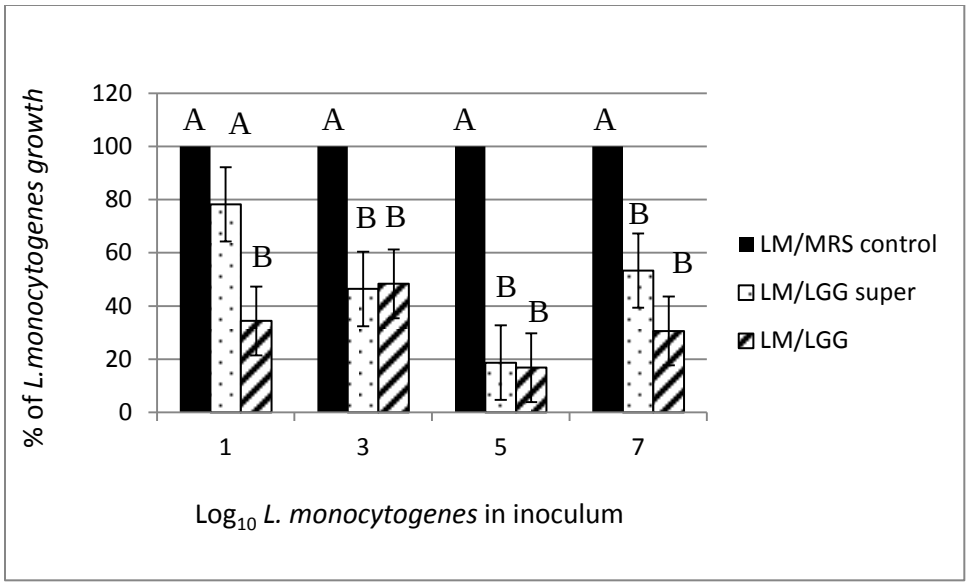


Figure 4.2. The effect of LGG and LGG supernatant on *L. monocytogenes* growth in an *in vitro* system. Either 10^6 CFU/ml LGG or LGG supernatant was added to 10^{1-7} CFU/ml of *L. monocytogenes* in PBS. After 1 hr incubation, *L. monocytogenes* was cultured to determine the growth and compared to *L. monocytogenes* cultures grown without either LGG or LGG supernatant. Data is shown as the % of *L. monocytogenes* growth when incubated in presence of either LGG or LGG supernatant. The activities are shown in comparison with that of untreated *L. monocytogenes* (100%). Bars represent the standard deviation of the mean. Bars with the same letter are not significantly different from each other within each dose group Both LGG and LGG supernatant were effective in reducing the growth of *L. monocytogenes.* CFU/ml confirmed on LSA.

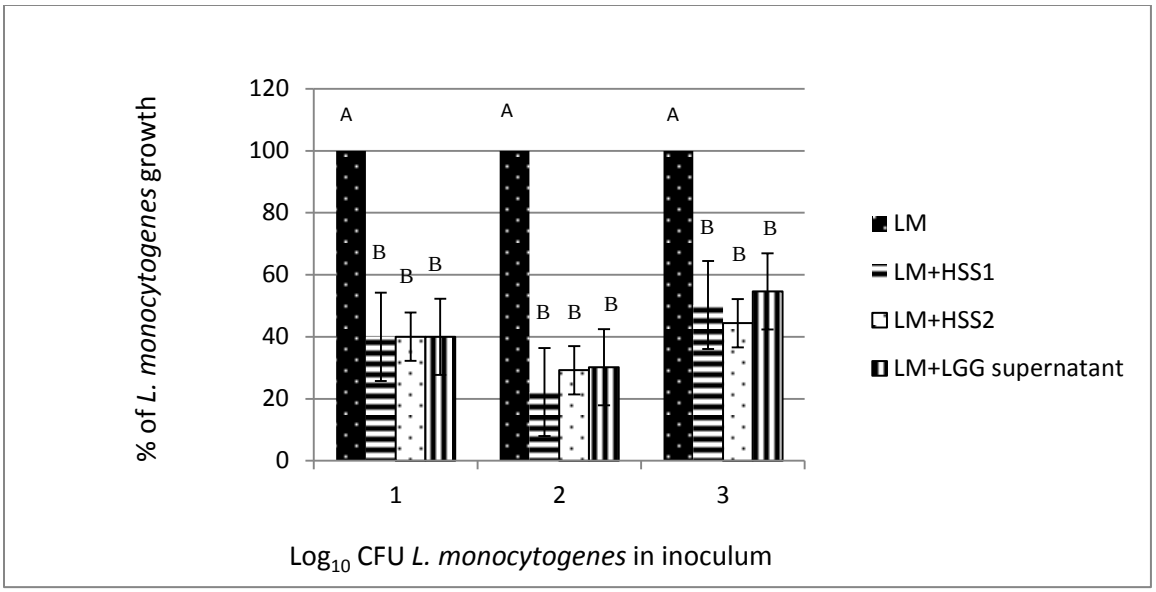


Figure 4.3. To determine whether a heat-sensitive protein in LGG supernatant was responsible for the reduction in growth of *L. monocytogenes* at low concentrations, the supernatant was heat-shocked (HSS) before incubating with *L. monocytogenes*. HSS = heat shocked treated LGG supernatant at 60°C for 1hr. HSS2 = heat-shocked treated LGG supernatant at 110°C. There was not a significant difference when comparing supernatant that had been heat shocked with supernatant that had not been heat shocked (LM +LGG supernatant). This suggests that the component of LGG supernatant responsible for reduction in growth of *L. monocytogenes* is not a heat sensitive protein because heat shock did not significantly re-store *L. monocytogenes* growth. The activities are shown in comparison with that of untreated *L. monocytogenes* (100%). However, it cannot be ruled out that a protein might act in combination with another mechanism to reduce the growth. Bars represent the standard deviation of the mean. Each value shown is the mean of three independent experiments. *Indicates significance ($p \leq 0.05$). CFU/ml confirmed on LSA.

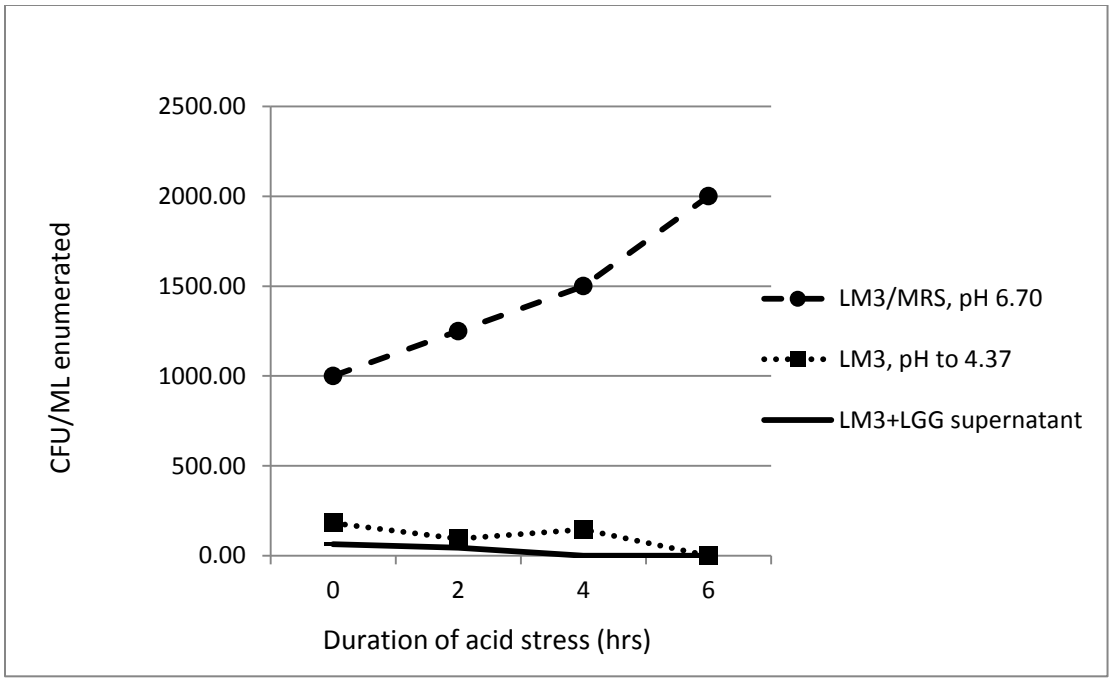


Figure 4.4 Enumeration of viable *L. monocytogenes* after exposure to an acidic environment for six hours. Relationship between LGG supernatant and culture medium adjusted to pH 4.37affecting *L. monocytogenes* growth at 10^3 CFU/ml. *L. monocytogenes* growth affected by change of pH caused from LGG supernatant (solid line). *L. monocytogenes* inoculum was adjusted to pH with acid and incubated for the above times (small dotted line). N=3. Each value shown is the mean of three experiments. *L. monocytogenes* + MRS (large dotted line) was used as the control group.

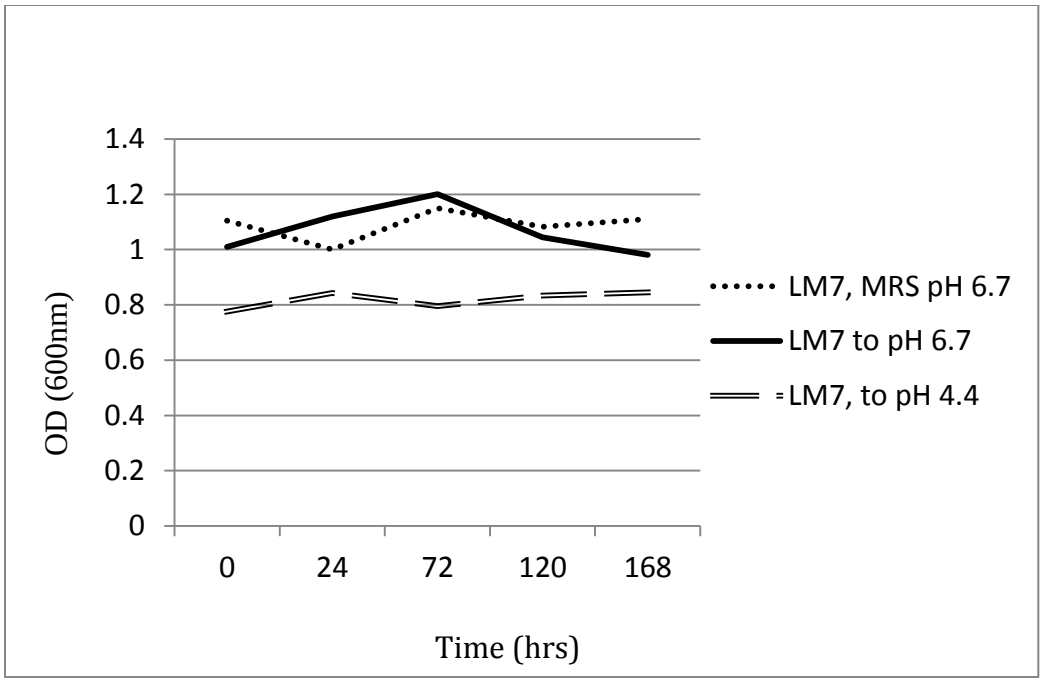


Figure 4.5. Responses of *L. monocytogenes* growth measured in total particles after change in pH environment after 168 hrs. *L. monocytogenes* growth affected by change of pH caused from titration pH 4.4 (double line). *L. monocytogenes* inoculum was treated to pH 6.7 dropwise and incubated for the above times (solid line). Each value shown is the mean of three experiments. N=3. *L. monocytogenes* + MRS (pH 6.7) was used as the control (dotted line).

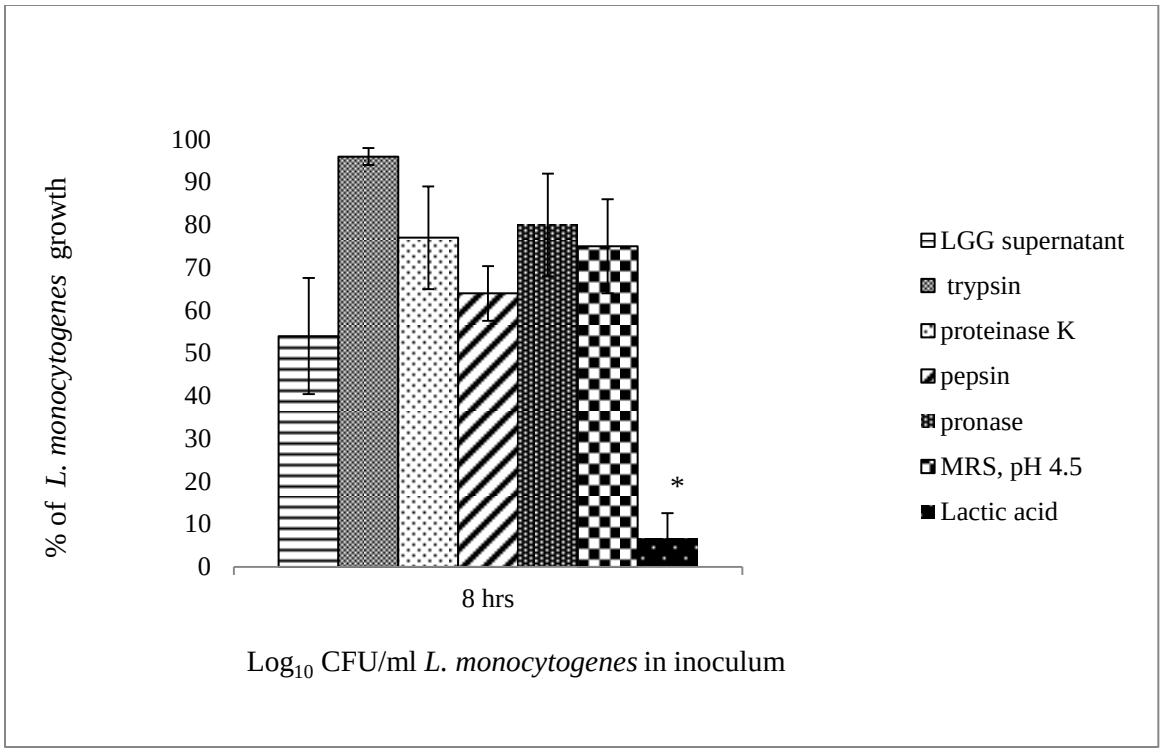


Figure 4.6. Effects of physical or chemical treatments on the antimicrobial activity activity of LGG supernatant against *L. monocytogenes*. LGG supernatant of 24 h old MRS cultures was prepared and treated with different enzymes after 8hr exposure. For comparison, 200 mg/ml trypsin, 100mg/ml proteinase K, 2M HCL, 200mg/ml pepsin, 150mM lactic acid and sterile De Man–Rogosa–Sharpe medium (MRS) 4.5 (adjusted) were included. The activities are shown in comparison with that of untreated LGG supernatant. Each value shown is the mean of three experiments. Bars represent the standard deviation of the mean. *Indicates significance ($p \leq 0.05$). CFU/ml confirmed on LSA.

CHAPTER 5

pH, LACTIC ACID AND NON-LACTIC ACID DEPEENDENT MECHANISMS OF *LACTOBACILLUS RHAMNOSUS* GG INHIBITION OF *CRONOBACTER SAKAZAKII* GROWTH INVASION [3]

[3] A. Kwaku Agyekum, Mary Alice Smith, 2013. To be submitted to The Journal of Food Protection

ABSTRACT

Contaminants in infant formula have been associated with disease and death in infants, particularly pre-mature and high risk infants. *Cronobacter sakazakii* (*C. sakazakii*) is an opportunistic pathogenic bacterium that is sometimes associated with these cases. The overall goal of our research study is to investigate *C. sakazakii* growth and invasion resulting in illness. The objectives of the current *in vitro* study were 1) to determine whether and to what extent LGG or a product of LGG (LGG supernatant) inhibits *C. sakazakii* growth, 2) to identify and characterize protective mechanisms of LGG or LGG supernatant against *C. sakazakii* growth such as via pH changes and protein activity. To test our objectives C. sakazakii was exposed to LGG, LGG supernatant for 1 hr and enumerated. For effects of pH environment on *C. sakazakii*, the culture medium was titrated to an acidic (pH 4.37) and neutral pH (pH 7). To test whether a protein was involved affecting the activity of LGG supernatant, LGG supernatant was treated with proteolytic enzymes. The data suggests under the conditions tested, LGG and LGG supernatant significantly reduce *C. sakazakii* growth and viability and the anti-microbial activity of LGG supernatant is due to (a) heat-stable, non-proteinaceous compound(s). Further, a dose dependent relationship was found with lactic acid treated LGG supernatant and reduced *C. sakazakii* growth. In conclusion, both an acidic pH environments and LGG supernatant treatments affected *C. sakazakii* growth and viability *in vitro* thus, further studies will be needed to investigate the mechanisms involved.

INTRODUCTION

Probiotics are live microorganisms that confer health benefits on the host. Probiotics exert a protective effect against pathogens by modulating immune responses, safe guarding intestinal barrier functions, or an inhibition of bacterial toxins *(3, 64, 94, 137)*. The most commonly studied strains of probiotics are *Lactobacillus* spp and *Bifidobacteria*. *Lactobacillus* strains have been used *in vitro* to treat infectious disease *(63)*. However, the mechanism of antibacterial probiotic *Lactobacillus* strains may be multifactorial.

A wide-range of probiotic strains are in usage today and each strain claims to have different health benefits. For example, *Bacillus coagulans* GBI-30, 6086 improves the immune response to a viral challenge and *Bifidobacterium animalis* subsp. *lactis* BB-12 has positive effects on the gastrointestinal system. *Bifidobacterium longum* subsp. *infantis* 35624 and *Lactobacillus acidophilus* NCFM reduce abdominal pain and side effects of antibiotic therapy, respectively. *Lactobacillus rhamnosus* GG (LGG) was isolated from human intestinal flora and has been tested widely in humans *(22)*. The current study will investigate the ability of LGG or LGG supernatant to affect *C. sakazakii* activity *in vitro*.

Cronobacter sakazakii (*C. sakazakii*; formerly classified as *Enterobacter sakazakii*) is an opportunistic pathogenic bacterium that is associated with cases, although rare, of illness in neonatal intensive care units, where it primarily infects premature and very-low-birth-weight (VLBW) infants *(45)*. Affected infants may develop various illnesses such as necrotizing enterocolitis (NEC), bacteremia, sepsis and meningitis *(59)*. *C. sakazakii* infection in these at-risk infants may also result in death *(59)*. Infants who recover from *C. sakazakii* infection may develop subsequent morbidities including hydrocephaly, mental retardation and developmental delays *(59)*.

Within the literature there have been few studies reporting the effects of *C. sakazakii* infection *(99, 100)*. Additionally, there exists limited data on the use of probiotics preventing *C. sakazakii* growth and invasion. However, in recent years due to the sporadic cases of *C. sakazakii* related infection resulting in neonatal death, certain studies have tested various compounds affecting *C. sakazakii* in powdered infant formula *(1, 2, 30, 121)*. Thus, the current study is novel in its *in vitro* approach using LGG of LGG supernatant to reduce *C. sakazakii*.

C. sakazakii is also considered as a pathogen, which affects elderly people and immune-compromised people in addition to infants *(50)*. Whilst the major reason behind affecting infants is its prevalent nature in powdered food formulas, the major reason behind infecting elderly people and immune-compromised people is its prevalent nature in contaminated food and contaminated surroundings. However, the complete mechanism in which probiotics protect against enteric pathogens remains largely uncharacterized. Few *in vitro* studies investigating the mechanisms underlying the antibacterial activity of *Lactobacillus* GG against enteric pathogens have been performed. Hunter et al. *(57)* demonstrated that *E. sakazakii* induces significant production of nitric oxide (NO) in rat intestinal epithelial cells (IEC-6) upon infection. The elevated production of NO, which is due to increased expression of inducible NO synthase, is responsible for apoptosis of IEC-6 cells. Notably, pretreatment of IEC-6 cells with *Lactobacillus bulgaricus* (ATCC 12278) attenuated the up-regulation of NO production and thereby protected the cells from *E. sakazakii-induced* apoptosis. Lastly, pretreatment with *L. bulgaricus* promoted the integrity of enterocytes both *in vitro* and in the infant rat model of NEC, even after challenge with *E. sakazakii*.

In vitro investigations of LGG's protection against pathogenic bacteria within CaCo-2 cell lines have also been conducted; Reid et al. *(98)* found Lactobacilli strains express

adhesiveness properties that enable them to inhibit the adhesion of bacterial pathogen to host cells. However, using a CaCo-2 cell line, Chauvière et al. *(19)* suggest that properties enabling *Lactobacilli* to protect host cells against bacterial pathogens are strain specific.

The overall goal of our research is to investigate the mechanisms involved with LGG or LGG supernatant affecting *C. sakazakii*. The objectives of the current *in vitro* study are 1) to determine whether and to what extent LGG or a product of LGG (LGG supernatant) inhibits *C. sakazakii* growth, 2) to identify and characterize protective mechanisms of LGG or LGG supernatant against *C. sakazakii* growth such as via pH changes and protein activity.

MATERIALS AND METHODS

Culture preparation of LGG. As previously described in Agyekum et al., 2013 *(1a)*, the probiotic LGG (obtained from Department of Food Science and Technology-UGA) was frozen on ceramic beads at -80°C and activated through three successive transfers into de Man, Rogosa and Sharpe (MRS) broth (Oxiod, LTD, Basingstoke, Hampshire, England). Briefly, the cells were incubated at 37°C for 24hr, isolated via centrifugation (8,000 x g at 4°C for 15min), and washed twice with PBS (phosphate buffered saline). Following, the cells were re-suspended in a PBS vehicle at a concentration of 10^6 CFU/ml. The target concentration was obtained by measuring optical density (OD 600nm) of a sample of the *C. sakazakii* suspension and comparing to a standard curve developed by serially diluting the *C. sakazakii* sample, plating on CSA (*Cronobacter* Selective Agar) (Oxoid), and calculating CFU/ml at each dilution.

Preparation of LGG supernatant for centrifugation, heating and filtration. Upon centrifugation of the 10^6 LGG bacteria, the LGG supernatant (soluble product of LGG) was removed from the LGG pellet and used during experimentation. For heat shock treatments, LGG supernatant was placed in a water bath at 60°C or 110°C for 10 min. For filtered supernatant

treatments, the LGG supernatant was filtered through a sterile 0.22µ-pore-size (Millipore, MA). Protein was analyzed by Bradford (Bio Rad, Hercules CA) and un-treated MRS used as a control. OD was read at 600nm. Enumeration was done by CFU/ml on MRS selective agar.

Preparation of *C. sakazakii.* *C. sakazakii* (strain 3290) cells were activated by two successive transfers into 10ml TSB (tryptic soy broth) and incubated for 24h at 37°C. The cells were obtained by centrifugation (8,000 x g at 4°C for 15min), washed twice and re-suspended in 10ml sterile PBS. Target concentration and the optical density (OD) of a sample of the *C. sakazakii* suspension was determined using a standard curve developed by serially diluting the *C. sakazakii* sample, plating on CSA (*Cronobacter* Selective Agar)(Oxoid), and calculating CFU/ml at each dilution.

***In vitro* growth and viability of *C. sakazakii* after exposure to LGG or LGG supernatant.** *In vitro* growth and viability methods were adapted from Agyekum et al. 2013 *(1a).* Briefly, for growth inhibition studies, 1ml volumes of *C. sakazakii* of concentrations 10^3, 10^2, and 10^1 CFU/ml were each separately incubated with equal volumes of 10^6 CFU/ml LGG or LGG supernatant. After 1h incubation at 37°C, 100ul of each inoculum was plated on CSA and incubated for 24h at 37°C. Following incubation, the plates were analyzed for their respective CFU. For viability assays, OD 600nm was measured.

Analysis of *C. sakazakii* growth in acidic environments caused by pH or LGG supernatant.

The following methods were adapted from De Keersmaecker *(26).* *C. sakazakii* inoculum was harvested at different growth phases up to 168 hrs and its MRS media treated with 2M HCl or LGG supernatant to achieve a pH of 4.37. 2M NaOH was added dropwise to the MRS media to achieve an inoculum pH of 6.7 for neutral to basic environments. Since pH and the presence of metabolites such as lactic acid have been shown to inhibit the growth of

pathogens and even kill them, two controls (*C. sakazakii* + MRS, *C. sakazakii* + supernatant) were used in these experiments. It is noteworthy that the LGG produces L-lactic acid while MRS broth containing D, L-lactic acid (60mM; MRS-LA).

LGG supernatant after exposure to heat and proteolytic enzymes. As previously described, from Fayol-Messaoudi et al. *(38)*, Agyekum et al. and De keersmaecker et al. *(26)*; to determine whether the effects found from LGG supernatant could be attributed to any protein, proteolytic enzymes were tested (all purchased from Sigma). The LGG supernatant was incubated at 37 °C for 1 hr with and without proteinase K (100 mg/ml), trypsin (200 mg/mL), pepsin (200 µg/mL), 150mM D,L lactic acid and sterile De Man–Rogosa–Sharpe medium (MRS) at pH 4.36. Each LGG supernatant treatment was then used as a treatment on 10^7 CFU/ml *C. sakazakii* concentrations and plated on CSA for enumeration.

Responses to Catalase from Co-culture Inocula with *C. sakazakii.* All catalase test methods were adapted from Taylor et al.. *(119)*. To determine whether LGG's effects on *C. sakazakii* were caused due to oxidative stress we examined treatment co-cultures with *C. sakazakii* using the drop catalase method *(119)*. Fresh dilutions of 3% H_20_2 100mM were made aliquoted. The catalase method entailed placing one drop of H_20_2 onto a well-developed, isolated colony of *C. sakazakii* so that the reaction of one such clone could be observed *(119)*. The frosted appearance of the bubbles elaborated under the cover slip indicated the speed of reaction. Control treatments involved dropping H_20_2 into a catalase positive solution and observing the vigor of the reaction.

Statistical analyses. Statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, N.C.) and Microsoft Excel (Microsoft Corporation, Redmond, W.A.) Significant differences ($P \leq 0.05$) in values comparing the treatments of probiotic were determined using Scheffe's test and

Excel *t*-test. One-way analysis of variance (ANOVA) tests were done using Dunnett's *t*-test and Excel *t*-test to determine significant differences between treatment groups and the control groups ($P \leq 0.05$) for *C. sakazakii*.

RESULTS AND DISCUSSION

The objectives of the current *in vitro* study were 1) to determine whether and to what extent LGG or a product of LGG (LGG supernatant) inhibits *C. sakazakii* growth, 2) to identify and characterize protective mechanisms of LGG or LGG supernatant against *C. sakazakii* growth such as via pH changes and protein activity. LGG and LGG supernatant were both significant in reducing *C. sakazakii* growth and viability *in vitro*. The reduction in *C. sakazakii* growth following LGG supernatant treatment cannot be solely attributed to pH environment because titrating the supernatant to pH 6.7 did not fully restore *C. sakazakii* growth. Further, the changes in pH environment caused by LGG supernatant cannot be solely attributed to the effects found reducing *C. sakazakii*.

Quantification of protein from treatment variables. To determine the best method for removing viable LGG from supernatant three treatments were performed. Protein quantification of LGG supernatant after filtration, heating, or centrifugation resulted in no significant differences in protein content amongst treatments Agyekum et al. 2013. When plated and enumerated, the filtration treatment was the only treatment that successfully removed all viable LGG remaining in the supernatant. Thus subsequent testing of LGG supernatant was done with filtered LGG supernatant.

Effects of LGG and LGG supernatant on *C. sakazakii* growth. In the current study, we examined whether LGG or LGG supernatant inhibits the growth of *C. sakazakii*. We found

treatments with LGG resulted in 36%, 52% and 23% of *C. sakazakii* growth at concentrations 10, 100, and 1000 CFU/ml. No significant difference was found in when comparing treatments; however both LGG and LGG supernatant treatments were significantly different from controls (Figure 5.1). *C. sakazakii* treatments with LGG supernatant resulted in 18%, 28% and 29% of growth of *C. sakazakii* at concentrations at concentrations 10, 100, and 1000 CFU/ml when compared to control. The current *in vitro* findings support the data found in our *in vivo* experiment which showed treatments of LGG and LGG supernatant significantly reduced *C. sakazakii* infected neonatal effects in mice; however, no significant difference was found between *in vivo* LGG and *in vivo* LGG supernatant treatments Agyekum et al. 2013.

Analysis of pH environment on *C. sakazakii* growth. The physical and enzymatic treatment of the supernatant was based on methods by De Keersmaeker et al. *(26)*. To test the possibility that the antimicrobial activity activity of LGG supernatant was only due to the low pH (4.37), the growth of *C. sakazakii* in culture with LGG supernatant (adjusted to pH 7) was tested (Figure 5.1). Results show significance between LGG supernatant adjusted to pH 7 and LGG supernatant treatments when expressed as % of *C. sakazakii* control (Figure 5.1). Thus, the data suggest that the effects found reducing *C. sakazakii* viability are non-exclusive to pH. Supported by other studies the ability of LGG supernatant to produce acetic and lactic acids, thus lowering pH, has also been observed as mechanisms of antimicrobial activity activity *(38, 112, 129)*.

Responses of *C. sakazakii* to acidic environment. The results show pH affecting the total particles of *C. sakazakii* when compared to control (Figure 5.2). At each time point recorded, treatments adjusted to acidic environments (pH 4.4) yielded less *C. sakazakii* than control treatments.

Responses lactic acid and proteolytic enzymes from LGG supernatant. The antimicrobial activity of LGG supernatant affecting 10^7CFU/ml *C. sakazakii* growth was examined after 8 hrs (Figure 5.3). Results show no significant difference in LGG activity affecting *C. sakazakii* growth when comparing LGG supernatant control to LGG supernatant treated with trypsin, proteinase K, pronase and MRS (pH 4.35). In contrast, treatments with LGG supernatant treated with lactic acid significantly reduced *C. sakazakii* growth when compared to controls (5.3).

Dose-response between Lactic Acid and LGG supernatant. We conducted a dose-response experiment analyzing the effects of lactic acid on LGG supernatant. The concentrations of lactic acid ranged from 60mM to 150mM. 60mM is the concentration of lactic acid found in MRS medium. Similar to previous studies conducted by Al-Holy et al. *(2)*, lactic acid reduced the growth of *C. sakazakii in vitro* (Figure 5.4). Although lactic acid is a weak organic acid, its undissociated form can penetrate the cytoplasmic membrane of bacterial pathogens resulting in a reduced pH *(1, 2)*. The aforementioned mechanism is suggested to account for the anti-bacterial action.

The findings of this study suggest the antibacterial activity of LGG supernatant is dependent upon pH. These conclusions are based on the effectiveness of pH on *C. sakazakii* activity against i*n vitro* (Figure5.2). However, previous experiments conducted by Corr et al. *(23)* suggest that proteinaceous toxins produced bacteria are involved in the antibacterial mechanism of LGG. While they may play a role *in vivo*, proteinaceous toxins have been ruled out as necessary components of growth inhibitory effects of LGG supernatant against *C. sakazakii in vitro*. This data is in line with data found with Stevens et al.. 1991 and (Baba and Schneewind, 1998). Thus, under the conditions tested, the anti-microbial activity of LGG

supernatant is due to (a) heat-stable, non-proteinaceous low molecular weight compound(s). Significance and Impact of the Study: The use of probiotics containing lactic acid could be beneficial to control *Cronobacter* in the infant formula industry.

ACKNOWLEDGEMENTS

Funding for this research project was provided by the University of Georgia (UGA) Center for Food Safety. The Alfred P. Sloan Foundation and the UGA Interdisciplinary Toxicology Program are acknowledged for providing graduate stipends (KA) and the UGA College of Public Health for salary support (MAS). We would also like to thank Dr. Joe Frank for providing the *C. sakazakii* and LGG strain.

TABLES AND FIGURES

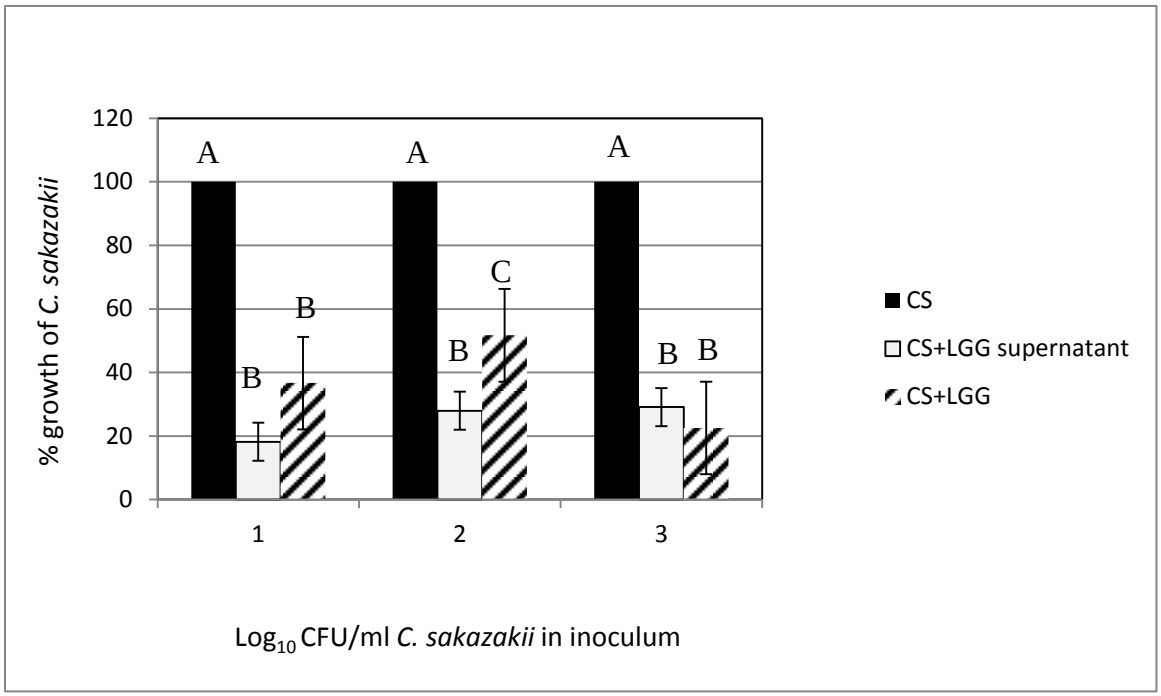


Figure 5.1. The effect of LGG and LGG supernatant on *C. sakazakii* viability within an *in vitro* system. Either LGG or LGG supernatant was added to 10^{1-3} CFU/ml of *C. sakazakii* in PBS. After 1 hr incubation, *C. sakazakii* was cultured to determine the growth and compared to *C. sakazakii* cultures grown without either LGG or LGG supernatant. Data is shown as the % growth of *C. sakazakii* when incubated in presence of either LGG or LGG supernatant. Bars with the same letter are not significantly different from each other within each dose group. Both LGG and LGG supernatant were effective in reducing the growth of *C. sakazakii.* CFU/ml confirmed on CSA.

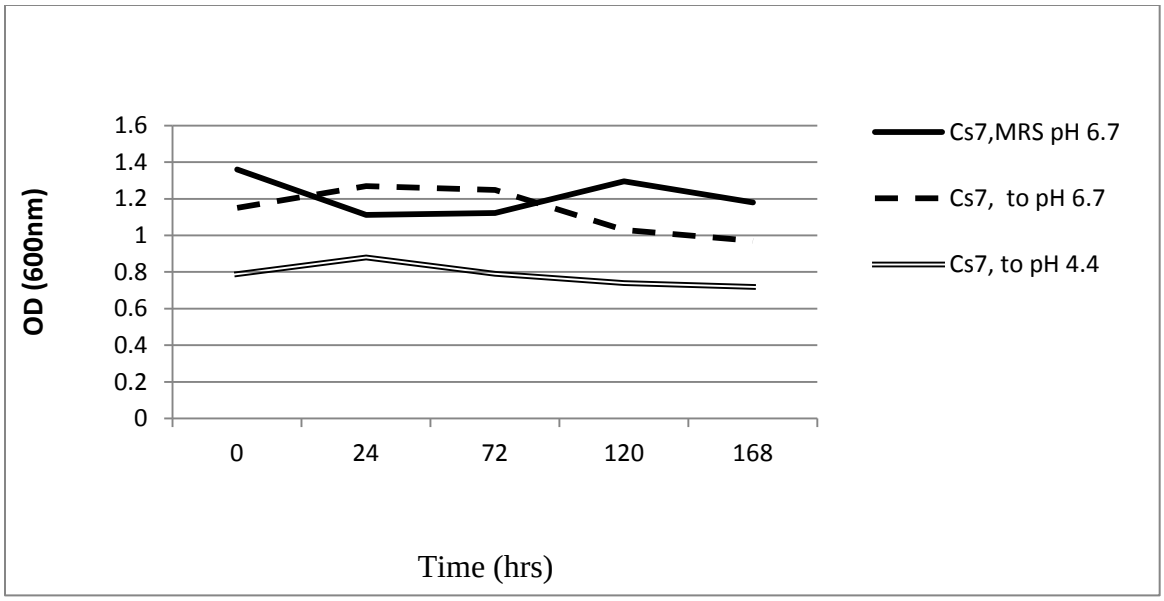


Figure 5.2. Effects of pH on *C. sakazakii* after exposure to an acidic environment for 168 hours. *C. sakazakii* inoculum was adjusted to a neutral pH and incubated for the above times (small dotted line). *C. sakazakii* growth affected by change of pH caused from titration (double line). N=3. Each value shown is the mean of three experiments. *C. sakazakii* + MRS (solid line) was used as the control group.

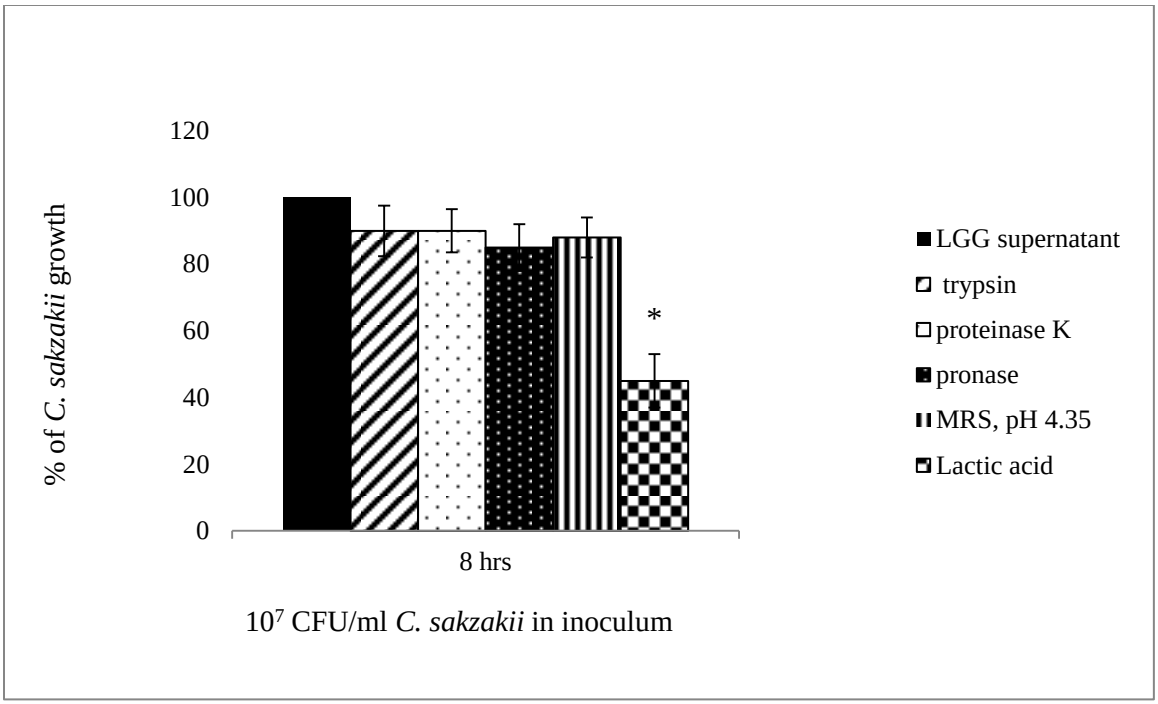


Figure 5.3. LGG supernatant affecting 10^7 *C. sakazakii* growth after exposure to proteolytic enzymes. LGG supernatant of 24 h old MRS cultures was prepared and treated with 200 mg/ml trypsin, 250 mM proteinase K, 200 mg/ml pronase sterile De Man–Rogosa–Sharpe medium (MRS) at pH 4.35, 150mM lactic acid were included. The activities are shown in comparison with that of untreated LGG supernatant. Each value shown is the mean of three experiments. Data is shown as the % growth of *C. sakazakii*. Bars represent the standard deviation of the mean. *Indicates significance ($p \leq 0.05$). CFU/ml confirmed on CSA. N=3. Replicates = 3.

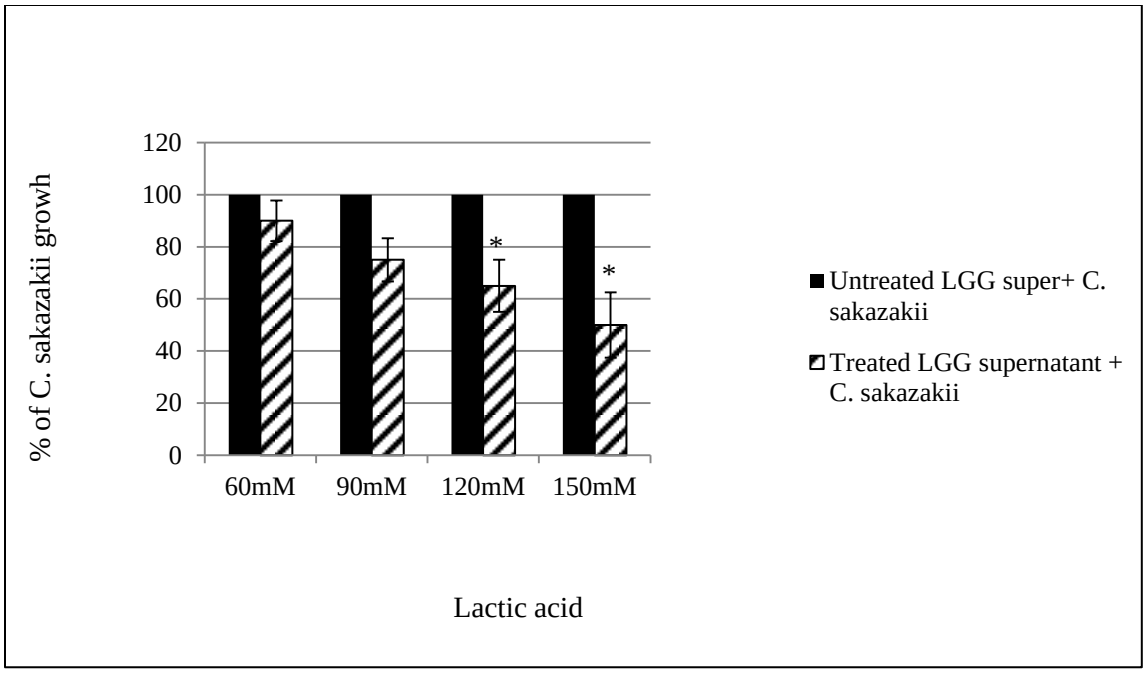


Figure 5.4. Dose-response relationship between lactic acid treated LGG supernatant affecting *C. sakazakii* growth. Dose dependent relationship is found between lactic acid treated LGG supernatant and *C. sakazakii* growth. LGG supernatant was treated with 60mM, 90mM, 120mM, and 150mM lactic acid (striped bars). Untreated LGG supernatant + *C. sakazakii* was used as control (solid bars). Cultures were then incubated for 1hr. Data is shown as the % growth of *C. sakazakii* when incubated in presence of either LGG or LGG supernatant. Each value shown is the mean of three experiments. Bars represent the standard deviation of the mean. *Indicates significance ($p \leq 0.05$). CFU/ml confirmed on CSA. N=3. Replicates = 3.

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CONCLUSION

The overall goal of this study was to investigate the ability of LGG to reduce growth and invasion of two pathogenic bacteria, *L. monocytogenes* and *C. sakazakii*. Specifically, we investigated LGG in the prevention of *C. sakazakii* infection in a neonatal mouse model and to illustrate the probiotic inhibition of *L. monocytogenes* infection in time pregnant guinea pigs. To determine any mechanisms of inhibition by LGG or an excreted factor of LGG (LGG supernatant) affecting *L. monocytogenes* and *C. sakazakii*; *in vitro* studies using probiotics were performed.

C. sakazakii was studied due its adverse effects on neonates. Both LGG and LGG supernatant reduced the overall invasion of *Cronobacter* in neonatal mice orally exposed to reconstituted powdered infant formula (RPIF) with *C. sakazakii*. The brain was most often invaded by *Cronobacter*, but also received the most protection from treatment with LGG or LGG supernatant. For the brain and liver, both LGG and LGG supernatant were equally protective against *Cronobacter* invasion. Mortality data showed LGG supernatant was most effective in protecting the neonatal mice from *C. sakazakii*–related death.

L. monocytogenes was studied due to its adverse effects on the developing fetus during pregnancy. Thus, we investigated whether yogurt containing probiotics LGG and Bifidobacterium reduced the effects of *L. monocytogenes* on pregnant guinea pigs. There was a reduction in stillbirths in guinea pigs treated with yogurt (14%) as compared to treatments with *L. monocytogenes* alone (75%). Stillbirths resulting with the probiotic treated pregnant guinea pig dam occurred 5days later than any stillbirths found with our positive control dams.

This study further investigated whether LGG protects against *L. monocytogenes* induced stillbirths and whether LGG or a LGG supernatant inhibits *L. monocytogenes* growth *in vitro*.

Our study indicates that LGG and LGG supernatant treatments were not significantly different from one another; which suggests LGG supernatant treatments are as effective as the bacteria itself. Lastly, our *in vivo* study shows yogurt containing *Lactobacillus* and *Bifidobacterium* provides a protective effect to fetuses when the dam is exposed to *L. monocytogenes*.

Environmental changes in pH caused by treatments with LGG supernatant affected viability and growth of *L. monocytogenes in vitro*. When *L. monocytogenes* cultures were treated with LGG supernatant, the pH environment of the culture (pH 4.6) was significantly different from the neutral pH environment of the control cultures (pH 6.7). Our data suggest heat sensitive proteins are not the major contributing component to the inhibitory effects of LGG (Fig. 4.3).

Further, *in vitro* tests were performed in order to determine whether and to what extent LGG or a product of LGG (LGG supernatant) inhibits *C. sakazakii* growth, 2) to identify and characterize protective mechanisms of LGG or LGG supernatant against *C. sakazakii* growth such as via pH changes and protein activity. We found LGG and LGG supernatant were both significant in reducing *C. sakazakii* growth and viability *in vitro*.

The reduction in *C. sakazakii* growth following LGG supernatant treatment cannot be solely attributed to pH environment. *In vivo* testing of LGG and LGG supernatant show that the treatments were not significantly different from one another in protecting from *C. sakazakii.* We found LGG and LGG supernatant were both significant in reducing *L .monocytogenes* and *C. sakazakii* growth and viability *in vitro*.

In summary, this study suggests the antibacterial activity of LGG supernatant is independent of protein activity. These effects may result from lactic acid production by LGG. These conclusions are based on the ineffectiveness of heat and proteolytic enzymes on LGG

supernatant's activity against *C. sakazakii in vitro*. While they may play a role *in vivo*, proteinaceous toxins have been ruled out as necessary components of growth inhibitory effects of LGG supernatant against *C. sakazakii in vitro*. Thus, under the conditions tested, the anti-microbial activity of LGG supernatant is due to (a) heat-stable, non-proteinaceous low molecular weight compound(s). Significance and Impact of the Study: The use of probiotics containing lactic acid could be beneficial to control *Cronobacter* and listeriosis infections during neonatal and fetal development.