DEVELOPMENT OF AN IN VITRO MODEL FOR INHIBITION OF *SALMONELLA* ADHESION TO EPITHELIAL CELLS BY *LACTOBACILLUS*

A Thesis

by

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ABSTRACT

Lactobacillus species are widely used as probiotics because of their health promoting properties and are potentially an important alternative to the sub-therapeutic use of antibiotics in poultry production. Administration of probiotic *Lactobacillus* cultures to poultry has been demonstrated to improve pre-harvest microbial food safety by reducing gastrointestinal colonization of poultry by human foodborne pathogens. Although competition for adhesion sites on gastrointestinal tissues is thought to contribute to the competitive exclusion of pathogens by *Lactobacillus,* the mechanisms responsible for this functionality are not well understood. The goal of this study was to develop a series of assays to investigate competitive exclusion of *Salmonella* by *Lactobacillus* cultures *in vitro* using the LMH chicken epithelial cell line.

We evaluated the effect of several factors including survival of bacteria in cell culture medium, sequence of bacterial addition to the LMH cell line, co-incubation times, and the number of post-incubation washes needed to remove non-adherent bacteria from the LMH cells. These results were used to develop a set of standardized experimental conditions to evaluate the ability of *Lactobacillus* cultures to inhibit binding of *Salmonella* to the chicken LMH cell line. Additionally, by varying the sequence in which probiotic and pathogenic bacteria were added to the chicken LMH cell line, we developed assays to characterize the exclusion (*Lactobacillus* first), displacement (pathogen first), and competition (simultaneous addition) of pathogens from epithelial cells by *Lactobacillus* cultures. Exclusion of *Salmonella* from chicken epithelial cells by *Lactobacillus crispatus* ST1, *Lactobacillus crispatus* JCM 5810, *Lactobacillus gallinarum* JCM 8782, and *Lactobacillus gallinarum* ATCC 33199 was evaluated. *L. crispatus* JCM 5810 and *L. crispatus* ST1 significantly reduced adhesion of *Salmonella* by 80 and 50 %, respectively;

L. gallinarum ATCC 33199 and *L. gallinarum* JCM 8782 significantly reduced adhesion of *Salmonella* by 70 and 50 %, respectively ($P < 0.05$).

The model we have developed is expected to be applicable to other human foodborne (e.g., *Campylobacter*) and poultry pathogens (e.g., avian pathogenic *E. coli*, *Clostridium perfringens*) and candidate probiotic cultures from other genera (e.g., *Bacillus*, *Bifidobacterium*). Further development and validation of this model in live poultry and the use of isogenic adhesion mutants of model *Lactobacillus* species including *L. crispatus* and *L. gallinarum* will contribute to a mechanistic understanding of probiotic functionality in poultry.

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TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

CONTRIBUTORS AND FUNDING SOURCES v

TABLE OF CONTENTS vi

LIST OF FIGURES viii

CHAPTER I INTRODUCTION AND LITERATURE REVIEW 1

- 1.1 Introduction 1
- 1.2 *Lactobacillus* Species as Probiotics 3
- 1.3 *In vitro* Characterization Factors 3
  - 1.3.1 Cell Adhesion 4
  - 1.3.2 Survival of Bacteria in Gastrointestinal Tract 7
  - 1.3.3 Antimicrobial Compounds 9
  - 1.3.4 Probiotics and Their Immunologic Effects 9
- 1.4 Competitive Exclusion 11
- 1.5 *In vivo* Application 12
  - 1.5.1 Reduction of Pathogens in Live Poultry Models 12
- 1.6 Conclusion 13

Chapter II EVALUATION OF INHIBITION OF *SALMONELLA* ADHESION TO CHICKEN LMH EPITHELIAL CELLS BY *LACTOBACILLUS IN VITRO* 14

- 2.1 Introduction 14
- 2.2 Materials and Methods 17
  - 2.2.1 Chicken LMH Epithelial Cell Line 17
  - 2.2.2 Bacterial Strains 18
  - 2.2.3 Adhesion Inhibition Assays 18
  - 2.2.4 Statistical Analysis 19
- 2.3 Results 20
  - 2.3.1 Evaluation of Experimental Conditions 20
  - 2.3.2 Exclusion of *Salmonella* from LMH cells by *Lactobacillus* 20
  - 2.3.3 Displacement of *Salmonella* from LMH cells by *Lactobacillus* 21
  - 2.3.4 Competition between Salmonella and Lactobacillus for adhesion to LMH cells 22
- 2.4 Discussion 22

Chapter III CONCLUSION 33

References 34

LIST OF FIGURES

FIGURE Page

1 Evaluation of Assay Conditions.. 29

2 Exclusion of *Salmonella* from chicken LMH epithelial cells by *Lactobacillus* 30

3 Displacement of *Salmonella* from chicken LMH epithelial cells by *Lactobacillus*.. 31

4 Competition between *Salmonella* and *Lactobacillus* for adhesion to chicken LMH epithelial cells 32

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Antibiotics have been used widely in the production of poultry and other livestock animals to prevent animal disease, promote growth, and improve feed efficiency. However, concern over the development of antibiotic resistant bacteria and increasing consumer demand for antibiotic free production has led to opposition of the use of antibiotic growth promoters (AGP) (Dibner and Richards, 2005). Additionally, contamination of foods of animal origin by foodborne pathogens poses a significant risk to public health. Thus, the development of alternatives to AGPs and interventions to reduce microbial pathogen carriage pre-harvest is of growing importance in animal agriculture.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill, 2014). When administered to livestock, they have been shown to improve growth performance (Awad et al., 2009), enhance immunity (Cunningham-Rundles et al., 2000; Nayebpor et al., 2007; Haghighi et al., 2008), and reduce gastrointestinal colonization by pathogenic bacteria (Morishita et al., 1997; Pascual et al., 1999; Ghareeb et al., 2012). Because of these benefits, probiotics are potentially important alternatives to the use of antibiotics in poultry production and are useful as pre-harvest food safety interventions (Yang et al., 2009; Huyghebaert et al., 2011).

Nurmi and Rantella (1973) demonstrated that oral administration of fecal contents from adult hens protected newly hatched chicks from *Salmonella* infection and suggested

gastrointestinal colonization by beneficial bacteria present in fecal contents inhibited intestinal colonization by *Salmonella*. Because modern production systems present a relatively low exposure to bacteria, newly hatched chicks are no longer exposed to beneficial bacteria during their first days of life, increasing their susceptibility to enteric diseases (Schneitz and Mead, 2000). Blanchfield et al. (1984) demonstrated administration of an inoculum containing adult intestinal fecal/cecal contents to chicks reared in a controlled modern production environment protected against *Salmonella* infection. Administration of probiotics has also demonstrated to young chicks when their immune systems have yet to mature. It has been shown that as birds age, by sampling of the environment, birds can ingest beneficial bacteria and develop resistance to infections caused by avian pathogenic agents such as *Salmonella*, and other enteric microorganisms (Milner and Shaffer, 1952). The reduction of colonization by pathogenic bacteria by probiotic or other resident bacteria has come to be referred to as competitive exclusion. Adhesion to epithelial tissues has been suggested to be important to the beneficial functionality of probiotics, and has been associated with inhibition of pathogens. However, the factors that contribute to adhesion are not well understood.

Although numerous researchers have demonstrated the ability of probiotics to reduce pathogens colonization *in vivo* (Santini et al., 2010), this benefit appears to be to be strain dependent (Soerjadi et al., 1982; La Ragione et al., 2004). Strain dependency is not well understood and several factors are thought to contribute to survival and effectiveness of probiotics to persist and provide protection against pathogens including: adherence to epithelial cells (Spivey et al., 2014b), tolerance to intestinal environment (Ouwehand et al., 2005), exhibition of immunological effects (Galdeano and Perdigon, 2006), and well as

production of antimicrobial compounds. Understanding these criteria *in vitro* could help create criterion for evaluating and screening potential probiotics for use *in vivo*.

1.2 *Lactobacillus* Species as Probiotics

Lactobacillus species are generally recognized as safe (GRAS) for use in production and are non-spore forming, Gram-positive Lactic Acid Bacteria (LAB). These species ferment glucose to produce lactic acid as the major product of their primary metabolism using either the EMP or 6-phosphogluconate pathways. Production of lactic acid is believed to give them a competitive advantage in allowing them to alter their environment and make it more favorable for *Lactobacillus* and less favorable to other bacteria. Production of organic acids makes *Lactobacillus* a favorable choice for use as a probiotic in poultry production (Collado, 2007). Other components that make *Lactobacillus* a favored choice for use as a probiotic include specialized structures that are specific to certain strains such as pili and specific binding proteins that promotes adherence to the epithelium of poultry. *Lactobacillus crispatus* and *Lactobacillus gallinarum* are both gastrointestinal isolates of poultry, have been demonstrated to effectively adhere to the epithelial cells *in vitro* and persist in GI tract of poultry *in vivo,* and have been demonstrated to inhibit growth of poultry intestinal pathogens (Neal-McKinney et al., 2012).

1.3 *In vitro* Characterization Factors

The ability of probiotics to competitively exclude pathogens has been suggested to be strain dependent. Several factors are thought to contribute to both the survival and

effectiveness of probiotics in the gastrointestinal tract and their ability to colonize and provide protection against pathogens. These factors include: adherence, which can be influenced by the possession of specific binding proteins, tolerance to the harsh intestinal environment (Ouwehand et al., 2005), the exhibition of immunological effects such as the production of antibacterial compounds (Galdeano and Perdigon, 2006), as well of the possession of specific binding proteins. Such factors are thought to contribute to the efficacy of probiotics as pathogen inhibitors in the GI tract.

1.3.1 Cell Adhesion

Adhesion is the ability of a microorganism to attach to epithelial tissues. It is thought to be critical to the functionality of probiotics by promoting persistence and colonization in the gastrointestinal tract and has been suggested to contribute to the inhibition of gastrointestinal colonization by pathogens by blocking binding sites (Chichlowski et al., 2007). Host specificity was originally thought to contribute to adhesion (Fuller, 1977). Host specificity is the concept that only bacteria isolated from a host of the same species will be effective in providing protection against pathogens. However, this is not always the case. Strains isolated from dairy cows have been shown to adhere to human cell lines (Tuomola and Salminen, 1998). This theory was tested and it was found that in the investigation of adhesion within various hosts (human, canine, possum, emu, ostrich, and salmon) Lactic Acid Bacteria (LAB) were not host specific but rather, depended on strain specificity (Rinkinen et al., 2003). This has also been shown in a study testing LAB from mucous collected from adult human and infant fecal samples, in which adhesion was described to be strain dependent *in vitro* (Kirjavainen et al., 1998). It has also been observed that LAB isolates from pig origin did not adhere to pig epithelial cells

(Mäyrä-Mäukinen et al., 1983). This shows that while probiotic bacteria from the host can have an effect on adherence, strain specific traits are also contributing factors in the effectiveness of adhesion and perpetual colonization in the GI tract. The mechanisms responsible for strain specificity of probiotic species in terms of adherence and competitive exclusion abilities are not well understood. Co-aggregation, surface determinant proteins, and possession of specialized structures are all factors, which can contribute to increased adhesion. Aggregation is seen as an important factor contributing to adherence. Aggregation increases the mass of bacteria and allows persistence in the gastrointestinal tract. Non-co-aggregating bacteria can be easily flushed out of the gastrointestinal system (Collado et al., 2007a). Co-aggregation and interactions between *Lactobacillus* and uropathogens in the urogenital tract has been demonstrated to lead to competitive exclusion of those pathogens (Reid et al., 1988). One study showed that some strains of *Lactobacillus* isolated from the chicken crop adhered to specific pathogens while others did not. This showed that this effect seems to be strain dependent (Vandevoorde et al., 1991). While chemical factors such as low pH and high electrolyte concentrations can increase this effect, bacterial cells that were subjected to heat (thus losing proteolytic effects) loss their ability to co-aggregate. This indicates that specific proteins might be involved in co-aggregation of specific strains to specific bacteria. Further identification of these proteins can therefore confirm this theory.

Specific structural proteins are thought to increase the ability of probiotics to not only bind to the epithelial lining of the gastrointestinal tract, but to other pathogens as well. Special extracellular appendages called adhesins serve as an anchor for bacteria, and they can increase adherence. These adhesins are in the form of a pilus or fimbriae. These appendages can attach to cell walls, and help beneficial bacteria remain in the gastrointestinal tract during digestion. A

host-specific factor that can increase adherence of bacteria to the lining of the gastrointestinal tract is the production of mucus. Mucous not only provides a layer of protection for mucosal cells from pathogenic microorganisms, but it also serves as an initial binding site and nutrient source for beneficial bacteria. Through extracellular adhesins, adherence of microorganisms to mucosal cells is possible (Collado et al., 2007b).

Specific adhesins are thought to contribute to the ability of probiotics to bind to epithelial cells and exclude binding of pathogens. It was found that *Lactobacillus casei* TMC 0409 highly adhered to Caco-2 and human mucosal cells, while *Lactobacillus rhamnosus* LA-2 showed lower adherence. While *L. casei* successfully inhibited *Salmonella* Typhimurium ATCC 29631, *L. rhamnosus* did not. However, *L. rhamnosus* showed greater inhibition of *Listeria monocytogenes* ATCC 15313 (Gueimonde, 2006). This suggests that strain specificity via their adhesion proteins and subsequent mucosal receptors at binding sites is more likely the responsible factor for increased inhibition of pathogens (Tuomola et al., 1999). Differentiation of surface layer proteins could also be a factor in the varied levels of adhesion and inhibition. Surface layer proteins form a crystallized two-dimensional structure around the outer cell surface of bacterial cell. This layer is often described as a protective layer that is anywhere from 40 to 200 kDa in mass (Sara and Sleytr, 2000). This layer is thought to be important in the initial binding of probiotic bacteria to mucosal surfaces. Genes encoding surface layer proteins have been sequenced for *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus helveticus*, and *Lactobacillus crispatus*. By the removal of surface-bound proteins in species of Lactobacilli, adherence decreases. This was shown when after the removal of the surface bound proteins, adherence of *L. crispatus* ZJ001 to HeLa cells was decreased (Chen, et al., 2007). This was also seen in the removal of surface proteins by treatment with guanidine-CHl, in which adherence of

Lactobacillus planatarum 423 to Caco-2 cells was significantly decreased. While removal of the surface proteins of *L. planatarum* 423 decreased adherence, adherence was not completely prevented suggesting that extracellular proteins could also play a role in adhesion. In analysis of *L. planatarum* 423, surface-bound proteins elongation factor Tu and the glycolytic glyceraldehyde-3-phosphate dehydrogenase and triosephosphate isomerase were found. These proteins are released from bacteria when there are environmental changes such as stress. While not well understood, this was suggested to be another factor contributing to adhesion (Ramiah et al., 2008).

Another contributing factor in adhesion of probiotic bacteria is their outer appendages such as pili. The pilus of *Lactobacillus rhamnosus* GG (LGG) are composed of three subunits SpaA, SpaB, and SpaC. SpaC is the key component of the pilus allowing for adherence to mucous and intestinal cells. The pilus of LGG also contains cross-linked covalent bonds between the side chains of Lys and Asn residues that are thought to help with strength in circulation and resistance to shear stresses within the host (Coulibaly et al., 2007). These are similar to the covalent bonds that are seen in bacteriophages (Wikoff et al., 2000). Improving the strength of bacteria within circulation, as well as the structure of the pilus is another theory for increasing adhesive capacity.

1.3.2 Survival of Bacteria in Gastrointestinal Tract

Resistance to the harsh conditions present in a host and survival in the gastrointestinal tract is an important factor for screening bacteria for potential use as a probiotic. If a bacterium can survive under the harsh conditions of the gastrointestinal tract it can have a chance at adherence and can begin colonization so that it can then exert beneficial effects on the host.

In order for bacteria to successfully adhere to the gut epithelial cells to begin colonization they must be able to survive in the acidic environment of the stomach tract. Potential candidates for use as probiotics need to be screened first to determine their resistance to the acidic environment of the host GI tract (Conway et al., 1987). Using hydrochloric acid to increase the acidity of media, researchers found *Lactobacillus* strains isolated from ceca of chickens that were high adherers showed better survival than those that were high adherers isolated from the ileum. Isolates from environments with a low pH are suggested to be better adherers and better candidates for use as probiotics.

Potential candidates for use as a probiotic must also be able to tolerate bile in the small intestines. In selection of probiotics, bile tolerance can be tested *in vitro* to mimic the host environment so that the potential survival of the probiotic within the host can be determined. A successful probiotic must be able to resist bile salts in the lower intestine. Bile salts are known to reduce the survival of bacteria due to the destruction of bacterial cell membranes. Selection of probiotics that are specialized and resistant to bile salt concentrations similar to those seen in the host is important for competitive exclusion purposes. There are several components that contribute to bile tolerance including: BSH, genes for cell membrane biosynthesis, bile exporters, and oxidative and acid stress responders (Min-Zhe Liong, 2011). *Lactobacillus* and *Bifidobacterium* that are isolated from the gastrointestinal tract possess bile salt hydrolases (Begley et al., 2006). Bile salt hydrolases cleave the glycine or taurine moiety from the steroid core of the conjugated bile salt, causing physico-chemical changes including decrease in solubility, making them poorer detergents (Hofmann and Mysels, 1988). This leaves a free amino acid and an un-conjugated bile acid molecule. While it has been reported that *Lactobacillus buchneri* JCM1069 showed some hydrolysis of tauro-conjugated bile salts (Moser

and Savage, 2001), most probiotic species are better at hydrolyzing glyco-conjugated bile salts (Kim and Lee., 2004). Probiotics are more resistant to hydrolyzed bile salts, making survival in the lower intestines much greater.

1.3.3 Antimicrobial Compounds

Lactic Acid Bacteria (LAB) have been studied extensively and have been reported to produce antimicrobial compounds that are inhibitory to other bacteria, these compounds being organic acids, bacteriocins, and other bacteriocidal substances. Bacteriocins are proteinaceous compounds that have an inhibitory effect on other bacteria (Zacharof et al., 2012). One study found that a heat stable, acid tolerant, and bile resistant strain of *Lactobacillus acidophilus* from the human intestine produced a bacteriocin that had an inhibitory effect against *Listeria* and *Bacillus* strains. While this antimicrobial compound was heat stable, some other antimicrobial compounds are not (Oh, 2000). In another study, it was found that a strain of *Lactobacillus reuteri* converted glycerol into a bactericidal compound called reuterin. It was shown that during co-incubation of *L. reuteri* with *Escherichia coli* K12, as temperature increased, production of the compound increased. However, past 45°C, production of the compound slowed, showing that production of antimicrobial compounds can be temperature dependent (Talarico and Dobrogosz, 1989).

1.3.4 Probiotics and Their Immunologic Effects

Probiotics are being researched for their ability to provide protection against infections caused by bacteria and viruses. Some probiotic species are thought to stimulate and enhance immune response in both local and systemic infections. Applications of probiotics include

prevention and treatment of gastrointestinal diseases. In poultry, adherence of probiotics to intestinal cells is important because it leads to colonization. After colonization, probiotics can exert immune factors which up-regulate humoral response to infections. Gram-positive lactic-acid bacteria comprise most of the probiotics, which are studied today (Leeber, et al., 2010). The two major classes of lactic-acid bacteria are *Lactobacillus* and *Bifidobacterium*. Metchnikoff (1907), alongside many recent reports, suggests that *Lactobacillus* are important for maintaining balanced microflora in the intestinal tract. *Lactobacillus* have been isolated from the crop (Smith, 1965), intestine (Beasley, 2004), and caecum (Barnes, 1979) of chickens. Protection from the hosts immune system, allows for probiotics to adhere and begin exerting beneficial effects. An example of this would be the peptidoglycan layer (MurNAc) in *Lactobacillus fermentum* (Logart and Neujahr, 1975). This layer protects the bacteria from being lysed by lysozyme while in circulation (Venema and Carmo, p.30). Bacteria, which are protected in circulation, can adhere and colonize intestinal cells and up-regulate the immune response. In a study by Fuller (1978), *Lactobacillus* strains 59 and 74/1 were isolated from the crop of chickens. They were then administered *in vivo* to broilers. Administration of these strains of *Lactobacilli* to broiler chickens decreased the incidence of *E. coli* colonization. This could be because, once colonized the *Lactobacili* strains fermented and lowered the pH, making the environment unfavorable to many other pathogenic microorganisms. *Lactobacillus* administration has also inhibited colonization of *Campylobacter* (Ghareed et al., 2012), *Salmonella* (Chen et al., 2012), and *Clostridium* (La Ragione et al., 2004). It has been shown that feeding broilers a direct fed microbial at 0.1% and 0.15% resulted in an increase of antibody titers to Infectious Bursal Disease Virus compared to the control treatment (Nayebpor et al., 2007). Similarly, antibody production was increased compared to the control against Newcastle

disease virus in broilers supplemented with probiotics (Khaksefidi et al., 2006). The reason for why the humoral response is up-regulated is not well understood. However, some probiotics produce certain biologic molecules that can help communicate with the immune system when it is under attack. *Lactobacillus* species produce lipoteichoic acid. This molecule is anchored to the cytoplasmic membrane of *Lactobacillus*. It is thought to bind to toll-like receptors 2 and 6 (Wells et al., 2011). Toll-like receptors recognized pattern associated molecular patterns put off by an immune system under attack. Once lipoteichoic acid from *Lactobacilli* binds to these toll-like receptors it can enhance their effectiveness in recognizing these patterns (Ganguly et al., 2010). Another specialization of *Lactobacilli* is that their DNA can be recognized by toll-like receptor 9. This receptor is important for signaling monocytes and Dendritic cells to produce Interleukin-12, which can signals T helper 1 cells, which then produce Interferron-γ, Interleukin-2, and Tumor Necrosis Factor β which all activate macrophages to engulf and kill targeted bacteria.

1.4 Competitive Exclusion

Selection of beneficial bacteria for use of probiotics is a process that evaluates several components. Effective probiotic cultures must have the ability to survive and adhere to the gastrointestinal tract, and exclude pathogens from binding to mucosal surfaces (Collins, Thornton, & Sullivan, 1998; Salminen, & Isolauri, 2002a). To effectively evaluate the efficacy of potential probiotics for use in live production, *in vitro* models of competitive exclusion must be applied. To develop an effective assay, several components of competitive binding must be evaluated. Considering the survival of probiotic bacteria in the GI tract and mimicking that environment is important for development of an *in vitro* assay. Also under consideration is the

ratio of probiotic to pathogen, and if higher amounts of probiotic bacteria affect success of exclusion. Another component being evaluated is the method of addition of probiotic and pathogen; this could dictate when the probiotic needs to be administered *in vivo*. The last component includes how probiotics interact with various pathogens. In poultry, probiotic bacteria should prevent colonization of pathogens such as avian nonpathogenic *E. coli*, *Salmonella* Typhimurium, *Campylobacter jejuni*, and *Clostridium perfringens*. For example, in investigation of *L. salivarius* ability to exclude *S.* Typhimurium and *S.* Enteritidis no significant difference in salmonellae shedding or caecal adherence was seen. However, there were differences between the untreated and treated groups in the crop (Soerjadi, et al., 1981). Ragione and Narbad (2003) showed that *L. johnsonii* could reduce colonization of *C. perfringens* but have no effect on *S.* Enteritidis or *E. coli*. Schneitz and Mead (2000) suggest that competitive exclusion is based off of four principle modes of action including creation of a restrictive physiological environment, competition for receptor sites, depletion of substrates, and secretion of antibiotic like substances.

1.5 *In vivo* Application

1.5.1 Reduction of Pathogens in Live Poultry Models

The chicken LMH cell line was derived from a hepatocellular carcinoma induced in a male leghorn chicken (Kawaguchi et al., 1987). Previous studies have demonstrated the chicken LMH epithelial cell line to serve as an important in *vitro* model for poultry epithelium. A recent study demonstrated competition between *Campylobacter jejuni* strains for adherence to epithelial cells using the LMH cell line (Garriga et al., 1998). This model was further validated using live

broiler chicks (Konkel et al., 2007). A study by Spivey et al. (2014b) adapted the LMH cell line for use as an adhesion model to investigate the binding capabilities of strains of *Lactobacillus*. Adhesion of *Lactobacillus crispatus* and *Lactobacillus gallinarum* to the LMH cell line was evaluated using *Salmonella* Typhimurium and *Bacillus subtilis* as high and low-adherent controls, respectively, and determined the *L. crispatus* and *L. gallinarum* cultures to adhere effectively to the cell line. Additionally, results of the *in vitro* assay were found to be indicative of in vivo persistence of *Lactobacillus* in live broiler chicks

1.6 Conclusion

Because of probiotic strains' ability to competitively exclude pathogens in live models (Morishita et al., 1997; Pascual et al., 1999; Ghareeb et al., 2012), understanding their functionality is important to developing *in vitro* models for screening potential candidates for use in feed/water products. While screening of beneficial strains in consideration for their use as a probiotic has previously included the evaluation of several factors such as acid tolerance (Conway et al., 1987), heat tolerance, and resistance to bile, there has been recent attention on adhesion as a mechanism for probiotic functionality. Several components such as extracellular appendages, and possession of specific binding proteins have been researched as a factor for increased adhesion. A model has recently been developed to investigate adherence of two poultry *Lactobacillus* strains, in comparison to adherence of *Salmonella*. While this model can show the adherence capability of bacterial strains, there is still little understanding as to how these strains inhibit binding of poultry pathogens such as *Salmonella* in the gastrointestinal tract.

CHAPTER II

EVALUATION OF INHIBITION OF *SALMONELLA* ADHESION TO CHICKEN LMH EPITHELIAL CELLS BY *LACTOBACILLUS IN VITRO*

2.1 Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill, 2014). They have been shown to improve growth performance (Awad et al., 2009), enhance immune responses (Cunningham-Rundles et al., 2000; Nayebpor et al., 2007; Haghighi et al., 2008), and reduce colonization of the intestinal epithelial cells by pathogenic bacteria (Morishita et al., 1997; Pascual et al., 1999; Ghareeb et al., 2012). Nurmi and Rantala (1973) suggested that administration of probiotics could reduce infection by pathogens using a method described as competitive exclusion. It has been shown that as birds age, sampling of the environment allows for the introduction and colonization of probiotic bacteria, which helps the birds develop resistance to infections caused by pathogenic agents such as *Salmonella* (Milner and Shaffer, 1952). However, this phenomenon is limited in modern production systems. It has been demonstrated that the development of new technology and better biosecurity practices has led to a decrease in the exposure of young chicks to beneficial bacteria. This leads to chicks having increasingly less protection against enteric diseases provided by environmental probiotic colonization (Schneitz and Mead, 2000); thus, protection must be re-established. One study demonstrated that by feeding young chicks adult intestinal microflora by oral gavage, protection against *Salmonella* was increased (Blanchfield et al., 1984). Multiple studies have reported the ability of probiotics to exclude pathogens, such as *Salmonella*. While numerous researchers

have had success in demonstrating the ability of probiotics to reduce colonization of pathogens *in vivo (*Pascual et al., 1999; Santini et al., 2010), success of competitive exclusion seems to be strain dependent (Soerjadi et al., 1982; La Ragione et al., 2004).

Lactobacillus species are non-spore forming Gram-positive bacteria that ferment glucose using both the Embden-Meyerhof-Parnas (EMP) pathway for metabolism resulting in the end product of lactic acid, and the 6-phosphogluconate pathway resulting in the end products: acetate, formate, succinate, and lactic acid. Production of these compounds allows them to alter their environment making it more favorable for *Lactobacillus* growth, and less favorable to other bacteria giving them a competitive advantage. Production of these compounds makes *Lactobacillus* a favorable choice for use as a probiotic in poultry production (Perdigon, 2000). Lactobacillus are generally recognized as safe (GRAS), and have other components that make *Lactobacillus* a favored choice for use as a probiotic. This includes specialized structures that are specific to certain strains such as pili, and specific binding proteins that make them better adherers to the epithelium of poultry. *Lactobacillus gallinarum*, and *Lactobacillus crispatus* are both isolated from the poultry intestine. Both have been shown to inhibit growth of poultry intestinal pathogens (Neal-McKinney et al., 2012). Strain dependency is not well understood and several factors are thought to contribute to both the survival and effectiveness of probiotics, as well as their ability to colonize and provide protection against pathogens. These factors include: tolerance to the harsh intestinal environment (Ouwehand et al., 2005), the exhibition of immunological effects such as the production of antibacterial compounds (Galdeano and Perdigon, 2006), as well as the possession of specific binding proteins. The most recent factor believed to contribute to the efficacy of probiotics is their ability to adhere to epithelial cells (Tuomola and Salminen,

1998). Increased adherence is thought to lead to better colonization. The reasons for this are not well understood. To better understand why adherence plays such a key role, we first need to understand how probiotics prevent the binding of pathogens. Understanding these criteria *in vitro* could help researchers create more specific criterion for evaluating and screening potential probiotics for use *in vivo*.

Salmonella, a human pathogen, is often found in the gastrointestinal tract of poultry making its incidence in poultry products common. Salmonellosis is one of several bacterial human foodborne illnesses, making it a public health concern throughout many production systems (Van Immerseel et al., 2006). Previous applications for control of *Salmonella* in the gastrointestinal tract of poultry included the therapeutic use of antibiotics. Recent studies indicate that the use of therapeutic antimicrobials in animal production has led to the incidental development of multidrug resistant bacteria (MDR). MDR strains pose a threat when infections in humans become difficult to treat with human antibiotics due to drug resistance (Glynn et al., 1998). In 2015, the United States published the final rule revising the Veterinary Feed Directive in 21 CFR Part 558, limiting the use of antibiotics in poultry production. Many companies have since switched to antibiotic-free production. Without the use of antibiotics, alternatives are being researched for their ability to reduce the incidence of pathogens such as *Salmonella* in the gastrointestinal tract of poultry. Probiotic cultures are being researched as an alternative to the use of antibiotics in poultry production (Yang et al., 2009; Huyghebaert et al., 2011). Evaluation of probiotics that are heat stable, resistant to acid/bile, good adherers, and are able to exclude pathogens is important for their survival and efficacy in poultry production.

The chicken LMH cell line was derived from a hepatocellular carcinoma induced in a male leghorn chicken (Kawaguchi et al., 1987). Previous studies have identified the Chicken LMH epithelial cell line of importance for *in vitro* assays for poultry. A recent study demonstrated competition between *Campylobacter jejuni* strains for adherence to epithelial cells using the LMH cell line (Garriga et al., 1998). This model was further validated using live broiler chicks (Konkel et al., 2007). A study by Spivey et al. (2014b) developed an adhesion model to investigate the binding capabilities of strains of *Lactobacillus crispatus*, and *Lactobacillus gallinarum*. In this study, we evaluated conditions for an *in vitro* model of competitive binding of *Salmonella* by *Lactobacillus* probiotic cultures. The *in vitro* adhesion inhibition assay evaluated three modes of probiotic: pathogen addition to chicken LMH epithelial cells (exclusion, displacement, and competition). This *in vitro* assay can help with evaluation of novel *Lactobacillus* probiotics prior to their use in live models.

2.2 Materials and Methods

2.2.1 Chicken LMH Epithelial Cell Line

Chicken LMH hepatocellular carcinoma epithelial cells (ATCC CRL-2117) were cultured using Waymouth's MB 752/1 medium (ThermoFisher Scientific, Waltham, MA) supplemented with 10 % fetal bovine serum (**FBS**) (ThermoFisher) in 0.1 % gelatin (MilliporeSigma, Burlington, MA) coated flasks. LMH epithelial cells were maintained at 37 ºC in a humidified 5 % CO_2 incubator.

2.2.2 Bacterial Strains

The bacterial strains used in this study are listed in **Table 1**. *Lactobacillus* strains were cultured using deMan, Rogosa, and Sharpe (**MRS**) medium (Difco, Franklin Lakes, NJ) in 10 % CO_2 at 37 ºC. *Salmonella* Typhimurium was cultured aerobically at 37 ºC using tryptic soy medium (**TSB/TSA**; Difco). For adhesion inhibition assays, 18 h cultures of bacteria were harvested by centrifugation, and washed 3× and re-suspended using Waymouth's + 1 % FBS. *Salmonella* was resuspended by absorbance ($O.D._{600\ nm}$) to a multiplicity of infection (**MOI**) of 100:1 with LMH cells, while *Lactobacillus* cultures were resuspended to ratios of approximately 1:1 and 10:1 with *Salmonella*. Counts of bacterial suspensions were confirmed by enumeration using TSA and MRS for *Salmonella* and *Lactobacillus*, respectively.

2.2.3 Adhesion Inhibition Assays

The ability of *Lactobacillus* to inhibit adhesion of *Salmonella* to chicken LMH epithelial cells when co-cultured together was evaluated using methods adapted from Spivey et al. (2014b) and Horie et al. (2002). Epithelial cells were prepared by seeding gelatin coated 24-well plates with LMH cells (3×10^5 cells $well^{-1}$) and incubating for 18 hours. Non-adherent LMH cells were removed by washing 3 × with Waymouth's + 1 % FBS prior to inoculation with bacteria.

Inhibition of *Salmonella* adhesion by *Lactobacillus* was evaluated using three assay types where the order in which bacteria were added to the chicken epithelial cells was varied. To evaluate exclusion of *Salmonella*, suspensions of the *Lactobacillus* were added to the chicken LMH cells, centrifuged at 600 × g for 5 min to promote bacterium-host cell contact, and incubated for 30 min at 37 ºC in a humidified 5 % CO_2 incubator. Following incubation, non-

adherent bacteria were removed by rinsing the wells 3× with assay medium, *Salmonella* was added to the plates, centrifuged at 600 × g for 5 min, and incubated for an additional 30 min at 37 ^{o}C in 5 % CO_2. The wells were rinsed 5× with sterile PBS to removing remaining non-adherent bacteria, and the LMH cells were lysed with 0.1 % Triton X-100 (MilliporeSigma) in PBS. Bacterial suspensions from each well were diluted serially in PBS for enumeration of *Salmonella* using XLT-4 agar (Difco). Displacement of *Salmonella* by *Lactobacillus* was evaluated by reversing the order in which the bacteria were added to the LMH cells, while competition between *Salmonella* and *Lactobacillus* was evaluated by adding the bacteria concurrently.

2.2.4 Statistical Analysis

Bacterial counts were $\log_{10}$ transformed for statistical analysis. Bacterial counts and % *Salmonella* reductions were analyzed using ANOVA with $\alpha = 0.05$. Results from independent assays were pooled for analysis and blocked by independent assay when appropriate. Significantly different means were determined using Duncan's multiple range test *post-hoc.*

2.3 Results

2.3.1 Evaluation of Experimental Conditions

Lactobacillus gallinarum ATCC33199 and *Salmonella* Typhimurium were used to evaluate survival of bacteria and number of post-incubation washes on the number of adherent bacteria. *L. gallinarum* and *Salmonella* were incubated in Assay Medium (Waymouths + 1 % FBS) at 37 ºC for up to 90 min (**Figure 1A**). Counts of *L. gallinarum* were not significantly different after 30, 60, or 90 min of incubation when compared to counts pre-incubation. Counts of *Salmonella* were not significantly different at 30 or 60 min incubation when compared to counts pre-incubation but were significantly different after 90 min incubation ($P<0.001$). Thus, *Lactobacillus* and *Salmonella* may be co-incubated in assay medium for up to 60 min without a decrease in viable counts. After incubating *L. gallinarum* and *Salmonella* individually with LMH cells for 30 min, individual wells were washed 0, 1, 3, 5, and 8 times prior to enumeration of adherent bacteria (**Figure 1B**). The number of adherent *L. gallinarum* and *Salmonella* did not significantly decrease through 8 washes. Thus, LMH cells may be washed up to 8 times using assay medium without decreasing the number of adherent bacteria. These represent a standard set of conditions for use in assays to evaluate competition between *Lactobacillus* and *Salmonella* for adhesion to chicken LMH epithelial cells *in vitro.*

2.3.2 Exclusion of *Salmonella* from LMH cells by *Lactobacillus*

In order to evaluate their ability to exclude *Salmonella* from adhesion to epithelial cells, *Lactobacillus* cultures were added to the chicken LMH epithelial cells and given the

opportunity to adhere to the cells prior to the addition of *Salmonella*. All four *Lactobacillus* cultures that were evaluated significantly reduced the number of adherent *Salmonella* when added to the epithelial cells at 10 × the number of *Salmonella* (**Figure 2A**) with, the greatest reduction at over 1.0 $\log_{10}$ CFU being observed with *L. crispatus* JCM 5810 and *L. gallinarum* ATCC 33199. However, when *Lactobacillus* were added at 1× the number of *Salmonella* only *L. crispatus* ST1 and *L. crispatus* JCM 5810 significantly reduced the number of adherent *Salmonella* (**Figure 2B**), with both cultures reducing adherent *Salmonella* by approximately 0.5 $\log_{10}$ CFU. Although they did not significantly reduce the number of adherent *Salmonella* when compared to the untreated control, *L. gallinarum* ATCC 33199 and *L. gallinarum* JCM 8782 did reduce adherent *Salmonella* to a level similar to *L. crispatus* JCM 5810. Additionally, *Salmonella* was excluded by more strains and the reduction of adherent *Salmonella* was greater at the higher concentration than for the lower concentration.

2.3.3 Displacement of *Salmonella* from LMH cells by *Lactobacillus*

In order to evaluate their ability to exclude *Salmonella* from adhesion to epithelial cells, *Salmonella* were added first and given the opportunity to adhere to the chicken LMH cells prior to the addition of *Lactobacillus*. All four *Lactobacillus* cultures that were evaluated significantly reduced the number of adherent *Salmonella* when added to the epithelial cells at 10 × the number of *Salmonella* (**Figure 3A**) with the greatest reduction at over 1.0 $\log_{10}$ CFU being observed with *L. crispatus* ST1. However, when *Lactobacillus* were added at 1× the number of *Salmonella* only *L. crispatus* ST1 and *L. crispatus* JCM 5810 significantly reduced the number of adherent *Salmonella* (**Figure 3B**) with, the greatest reduction being

with *L. crispatus* JCM 5810 reducing adherent *Salmonella* by approximately 0.5 log_{10} CFU. Although they did not significantly reduce the number of adherent *Salmonella* when compared to the untreated control, *L. gallinarum* ATCC 33199 and *L. gallinarum* JCM 8782 did reduce adherent *Salmonella* to a level similar to *L. crispatus* ST1. Additionally, *Salmonella* was excluded by more strains and the reduction of adherent *Salmonella* was greater at the higher concentration than for the lower concentration.

2.3.4 Competition between *Salmonella* and *Lactobacillus* for adhesion to LMH cells

In order to evaluate their ability to compete with *Salmonella* for adhesion to epithelial sites, *Lactobacillus* cultures and *Salmonella* were added concurrently for co-incubation with the chicken LMH cells. All four *Lactobacillus* cultures that were evaluated significantly reduced the number of adherent *Salmonella* when added to the epithelial cells at 10 × the number of *Salmonella* (**Figure 4A**) with the greatest reduction at over 0.5 log_{10} being observed with *L. gallinarum* ATCC 33199 and *L. crispatus* ST1. In addition, all four *Lactobacillus* cultures that were evaluated significantly reduced the number of adherent *Salmonella* when added at 1× the number of *Salmonella* (**Figure 4B**) with, the greatest reduction being with *L. crispatus* JCM 5810 reducing adherent *Salmonella* by approximately 1.0 log_{10} CFU. In this case, *Salmonella* was excluded by more strains and the reduction of adherent *Salmonella* was greater at the lower concentration than for the higher concentration.

2.4 Discussion

Due to the decreased use of antibiotic growth promoters (AGP) in the poultry industry, there has been increased interest for the use of probiotics to control *Salmonella* colonization in the gastrointestinal tract pre-harvest. Administration of probiotics has been demonstrated to promote weight gain in poultry, reduce mortality, and reduce infection of poultry by pathogen bacteria such as *Salmonella* when applied in live models (Higgins et al., 2007). Research has shown that administering probiotics in poultry feed/water reduces the colonization of pathogens such as *Salmonella* by competitive exclusion. *Lactobacillus* has been seen as a favored candidate for use as a probiotic in poultry, as it is a natural inhabitant of the poultry gut, and has many components that provide a competitive advantage over other strains. While competitive exclusion has been shown to reduce pathogens in live bird models, the mechanisms responsible for this have not been well understood. Adhesion has been researched as a factor that contributes to reduction of pathogens *in vitro*. While in other species *in vitro* models have been developed to help better understand how probiotics reduce colonization of pathogens, no *in vitro* models have been developed for poultry.

Using an adhesion assay developed in our laboratory previously by Spivey et al. (2014a), we developed an inhibition adhesion assay to characterize the ability of model *Lactobacillus crispatus* and *Lactobacillus gallinarum* strains to inhibit adhesion of *Salmonella* to chicken epithelial cells *in vitro.* We chose these strains because they were previously used in our laboratory in an adhesion assay, which showed them to successfully adhere to chicken LMH epithelial cells *in vitro* when compared to the adherence of *Salmonella*. They have also been used for *in vivo* models evaluating phytate degradation from recombinant *Lactobacillus* cultures and

it's subsequent effect on growth parameters (Askelson et al., 2014); thus, we are prepared to use these cultures in future live models.

Previous studies have investigated competitive exclusion of poultry pathogens by *Lactobacillus* species *in vitro* using both the human intestinal Caco-2 cells (Servin et al., 1992; Lee et al., 2003) and the human uro-epithelial cells (Chan et al., 1985). Furthermore, several *in vitro* studies using animal derived cell lines have demonstrated the ability of *Lactobacillus* species to inhibit adhesion of pathogens. However, no poultry specific cell lines have been used to evaluate *Lactobacillus* species. The chicken LMH cell line was derived from a hepatocellular carcinoma induced in a male leghorn chicken (Kawaguchi et al., 1987). Previous studies have identified the chicken LMH epithelial cell line for use in *in vitro* assays investigating probiotic functionality for poultry. *In vitro* studies using the LMH cell line have demonstrated competition for adhesion sites between *Campylobacter jejuni* strains (Garriga et al., 1998), and also identified genes important for invasion of epithelial cells by *Salmonella* Enteritidis (Shah et al., 2012). A recent study in our laboratory developed an adhesion model to investigate the binding capabilities of strains of *Lactobacillus crispatus* and *Lactobacillus gallinarum*, and compared it to the adhesion of *Salmonella* to LMH cells (Spivey et al., 2014b). This adhesion assay was used to develop our adhesion inhibition model using the same *Lactobacillus* probiotic cultures and evaluating their ability to inhibit binding of *Salmonella* to chicken LMH epithelial cells. The *in vitro* adhesion inhibition assay developed evaluated three modes of probiotic: pathogen addition to chicken LMH epithelial cells (exclusion, displacement, and competition). The adherence assay previously developed in our laboratory by Spivey et al., (2013) characterized experimental conditions important to adherence of *Lactobacillus* species. This included co-incubation of *Lactobacillus* cultures with chicken LMH cells in PBS, number of post-incubation washes, and

ratio of CFU bacteria per cell for use in the adhesion assay. In addition to the experimental parameters decided from development of the adherence assay we experimentally determined the survival of bacteria in Assay Medium, number of post-incubation washes, and differing ratios of probiotic to pathogens, which mimic what concentration of probiotic might be used at the commercial level.

In the adhesion assay by Spivey et al. (2013), it was reported that 30 min was an appropriate time to evaluate the adhesion of *Lactobacillus* cultures to chicken LMH cells in PBS. However, viability of microorganisms decreased after prolonged incubation in PBS due to the lack of nutrients. Thus, using Waymouth's medium supplemented with 1% FBS was evaluated. An adherence assay completed by Konkel et al. (2007) showed the ability of *Campylobacter jejuni* cultures, suspended in media supplemented with 1% FBS, to adhere to chicken LMH cells. We used *L. gallinarum* ATCC 33199 and *Salmonella* to evaluate the experimental conditions for use in our assay. We found that survival of our cultures in Assay Medium using 1% FBS, unlike PBS, remained stable up to 60 minutes during co-incubation. After 60 minutes, while not statistically significant, slight decreases in viability were notable (Figure 1). Therefore, we used Waymouth's + 1% FBS for suspension of our bacterial cultures for use in the inhibition adhesion assay.

In the aforementioned adhesion study, it was found that when using PBS 5 washes was optimal for removing non-adherent *Lactobacillus* before the disruption of LMH cells for bacterial enumeration. In this study, to remain consistent, we used Waymouth's + 1% FBS to rinse cells after the assay. Therefore, we wanted to determine the number of washes suitable to remove non-adherent *Lactobacillus*. We evaluated recovery of bacterial cells at 0, 1, 3, 5, and 8 washes. We found 3 washes to be optimal for rinsing off all non-adherent bacteria prior to

disruption of cells for enumeration of bacteria for evaluating competitive exclusion of *Salmonella* by *Lactobacillus* strains using the chicken LMH cell line. While not significant, a slight decrease of viability was seen after 3 washes (Figure 2).

The multiplicity of infection used was 100:1 as recommended by Spivey et al. (2013). As dose dependency is an important factor in the evaluation of probiotics and their efficacy, two different concentrations of probiotic:pathogen were evaluated. Probiotics were investigated at equal concentrations of probiotic:pathogen (1:1), as well as a dosage of 10^8 and ten times more than *Salmonella* (10:1). This is comparable to what is used in feed/water products. Our results for 10:1 showed less variability than our results for 1:1. This could be associated with different cell surface factors, which are responsible for higher adherence when there are more cells of probiotics per cells of pathogen.

Our assay showed that all methods of addition: Exclusion, Displacement, and Competition inhibited *Salmonella* on LMH cells. However, the effects were more significant for Exclusion and Displacement. This result was different from a previous study by Lee et al. (2003) that evaluated the ability of *Lactobacillus casei* and *Lactobacillus rhamnosus* to inhibit binding of *Salmonella* and pathogenic *Escherichia coli* on Caco-2 human epithelial cells by Exclusion, Displacement, or Competition. Their results showed reduction of *Salmonella* and pathogenic *Escherichia coli* to be strain dependent, indicative that other factors could be involved with this ability. They also determined that inhibition of pathogens by Displacement was not as successful as Competition and Exclusion. They attributed this to Displacement being time sensitive, as with more time to adhere, inhibition was increased. Our assay showed that all four strains of *Lactobacillus* significantly reduced binding of *Salmonella* to LMH cells for all methods of addition, even though the effects were greater for Exclusion and Displacement. We also

evaluated the effect of two different concentrations of probiotic: pathogen. While both concentrations 1:1 and 10:1 reduced *Salmonella*, greater effects and less variability was seen at a concentration level of 10:1. This could be indicative that some probiotic strains ability to inhibit binding of a pathogen *in vitro* depends on their relative concentration to that pathogen. Although our research demonstrated this effect, too much statistical variability was seen at 1:1 concentrations. Further validation would be needed to ensure that 10:1 is the best application for this model.

Table 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source or Reference
Lactobacillus crispatus		
ST1	Chicken crop isolate	(Ojala et al., 2010)
JCM 5810	Chicken fecal isolate	JCM[1]
Lactobacillus gallinarum		
ATCC 33199^T	Chicken crop isolate, type strain	ATCC[2]
JCM 8782	Chicken fecal isolate	JCM
Salmonella Typhimurium		
TDC XX	USDA-ARS[3], primary poultry isolate	(Byrd et al., 1998)

[1]JCM = Japan Collection of Microorganisms

[2]ATCC = American Type Culture Collection

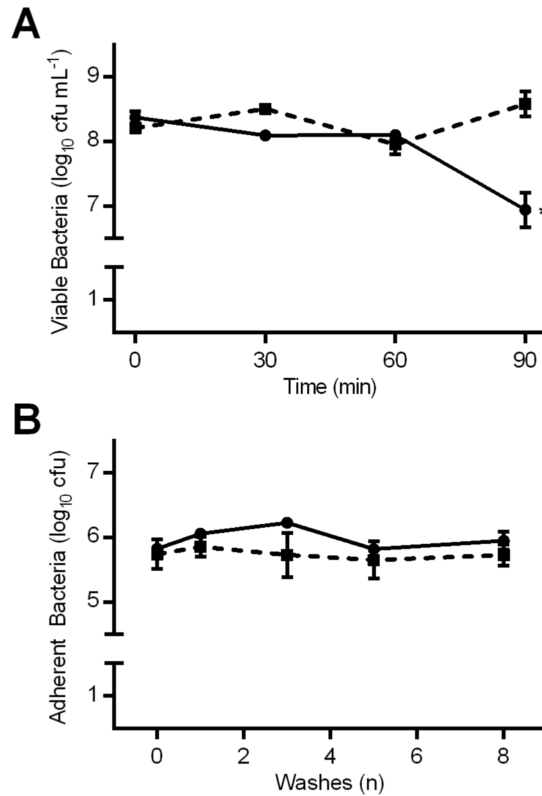

Figure 1. Evaluation of assay conditions. (A) *L. gallinarum* (squares) and *Salmonella* (circles) were resuspended in Waymouths Medium + 1% FBS, incubated in 5 % CO_2 at 37 °C, and enumerated after 0, 30, 60, and 90 min. The mean $\log_{10}$ ± SEM cfu mL^{-1} viable bacteria from 3 independent suspensions for each time point are reported. *indicates mean is significantly different than 0 min ($P<0.05$). **(B)** *L. gallinarum* (diamonds) and *Salmonella* (circles) cultures were co-incubated with LMH cells (100 cfu per LMH cell) and washed 0, 1, 3, 5, and 8 times using Waymouths Medium + 1% FBS prior to enumeration. The mean $\log_{10}$ ± SEM cfu adherent bacteria from four independent wells from two independent assays are reported.

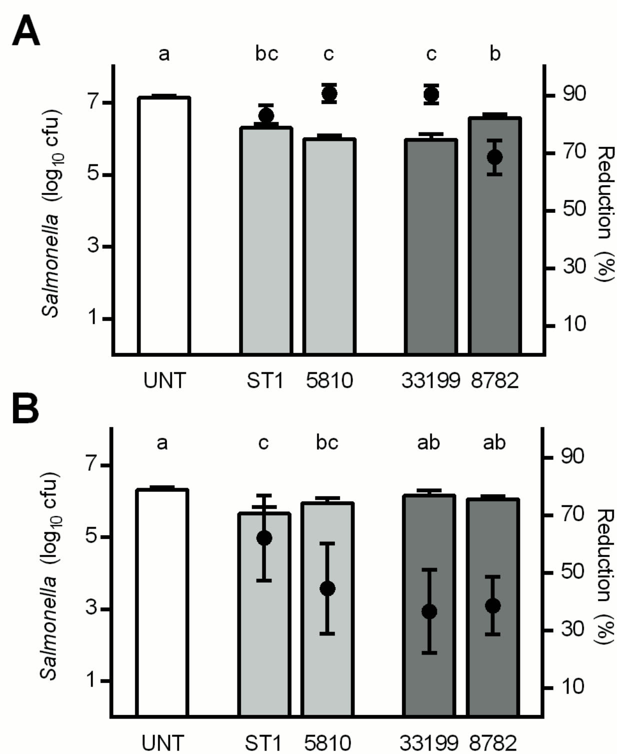

Figure 2. Exclusion of *Salmonella* from chicken LMH epithelial cells by *Lactobacillus.* Adherent *Salmonella* were enumerated from LMH cells which were untreated (UNT) or treated with *L. crispatus* (ST1 and JCM 5810) and *L. gallinarum* (ATCC 33199 and JCM 8782). *Lactobacillus* were added to LMH cells at **(A)** 10 × and **(B)** 1 × the number of *Salmonella* subsequently added to the LMH cells. The mean $\log_{10}$ ± SEM cfu adherent *Salmonella* (bars) and mean ± SEM % *Salmonella* Reduction (circles) as compared untreated cells of 4 independent wells from 2 independent assays is reported. Different letters above the bars indicates the mean adherent *Salmonella* differs significantly ($P < 0.05$).

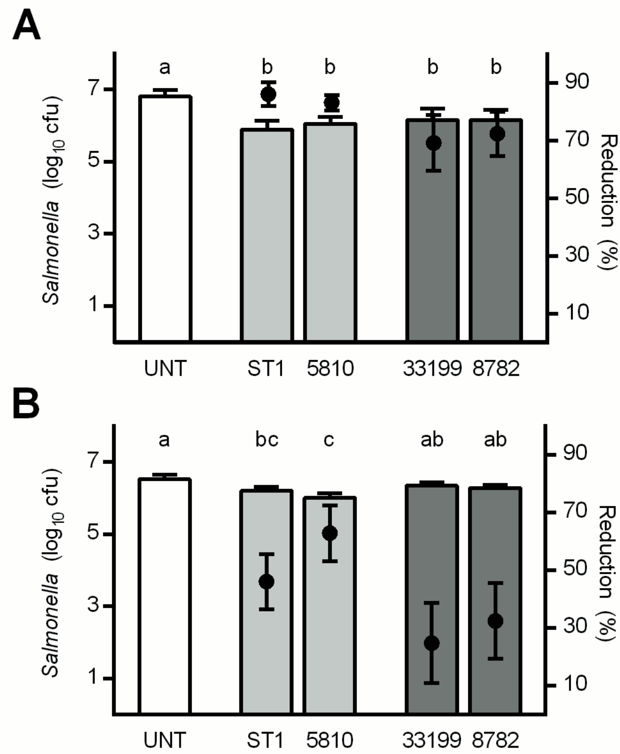

Figure 3. Displacement of *Salmonella* from chicken LMH cells by *Lactobacillus.* Adherent *Salmonella* were enumerated from LMH cells which were untreated (UNT) or treated with *L. crispatus* (ST1 and JCM 5810) and *L. gallinarum* (ATCC 33199 and JCM 8782). After *Salmonella* were added to LMH cells, *Lactobacillus* were added at **(A)** 10 × and **(B)** 1 × the number of *Salmonella*. The mean $\log_{10}$ ± SEM cfu adherent *Salmonella* (bars) and mean ± SEM % *Salmonella* Reduction (circles) as compared untreated cells of 4 independent wells from 2 independent assays is reported. Different letters above the bars indicates the mean adherent *Salmonella* differs significantly ($P < 0.05$).

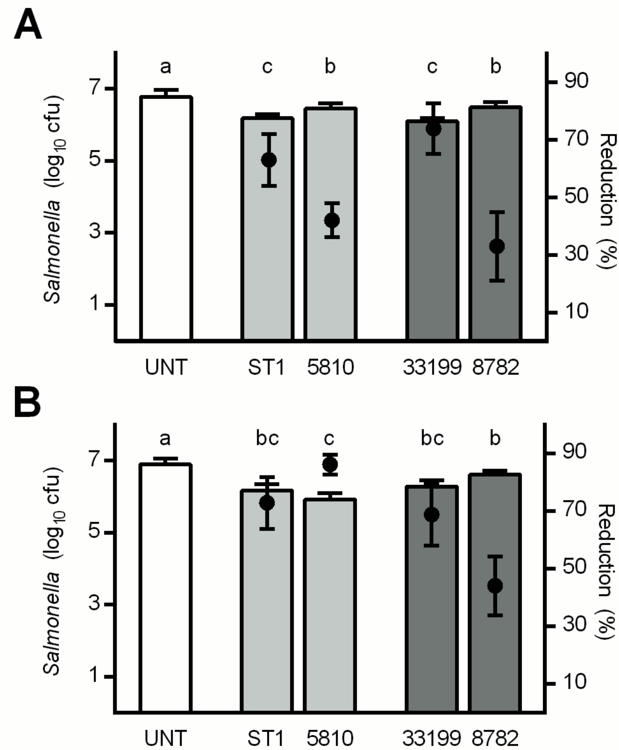

Figure 4. Competition between *Salmonella* and *Lactobacillus* for adhesion to chicken LMH cells. Adherent *Salmonella* were enumerated from LMH cells which were untreated (UNT) or treated with *L. crispatus* (ST1 and JCM 5810) and *L. gallinarum* (ATCC 33199 and JCM 8782). *Lactobacillus* were added concurrently with *Salmonella* to LMH cells at **(A)** 10 × and **(B)** 1 × the number of *Salmonella*. The mean $\log_{10}$ ± SEM cfu adherent *Salmonella* (bars) and mean ± SEM % *Salmonella* Reduction (circles) as compared untreated cells of 4 independent wells from 2 independent assays is reported. Different letters above the bars indicates the mean adherent *Salmonella* differs significantly ($P < 0.05$).

CHAPTER III:

CONCLUSION

The goal of this study was to investigate how probiotic cultures in the gastrointestinal tract of poultry can competitively exclude pathogens by evaluating the ability of *Lactobacillus* cultures to adhere to epithelial cells and inhibit adhesion of *Salmonella in vitro*. In this study, we used four *Lactobacillus* strains which were all either crop or fecal isolates from poultry (**Table 1**). These were all used previously in an adhesion assay that was developed in our laboratory, and were all successful adherers to the chicken LMH cell line (Spivey et al., 2013). Therefore, using these strains, we developed an assay to evaluate three methods of addition of *Lactobacillus* cultures and *Salmonella* onto chicken epithelial LMH cells: Exclusion, Displacement, and Competition, and we have shown the effect of the three addition methods on the inhibition of *Salmonella* by *Lactobacillus*.

We have determined the conditions (washes, survival of bacteria in assay medium) that were important to standardize this assay and ensure that it could be replicable. Standardization of this assay allowed us to have conclusive results. Our results indicated that this model could be used to show which strains were effective in reducing *Salmonella*, and which method of addition showed greater results. The methods we have adapted will allow us to determine the inhibitory effect of a specific probiotic strain, and which addition method proves more successful for that strain. Knowing the functionality of a probiotic strain will allow us to take a closer look at the factors that might be responsible for differences in reduction for the three methods of addition. Further testing may prove to be useful in identifying which specific factors (i.e. adherence protiens, immunological factors) make a probiotic strain more effective, so that those factors can be amplified.

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