LACTOBACILLUS STRATEGIES TO REDUCE CAMPYLOBACTER JEJUNI COLONIZATION OF BROILER CHICKENS

BY

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THESIS

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CHAPTER 1 INTRODUCTION

1.1 Motivation

*Campylobacter jejuni* is one of the leading causes of human bacterial illness in the world. The majority of cases of campylobacteriosis are associated with the consumption of raw or undercooked poultry meat or cross-contamination with other food products. Fewer than 500 cells are estimated to cause illness symptoms of diarrhea, abdominal pain, and in rare cases Guillain-Barré syndrome (Young *et al.*, 2007). Strategies to reduce the presence of *Campylobacter* in the poultry supply are underdeveloped.

Maternal antibodies protect broiler chickens from colonization by *C. jejuni* up until three weeks of age. Subsequently, *C. jejuni* colonizes the cecum of broiler chickens at $10^8$ colony-forming units per gram of cecal contents. Coprophagy, consumption of feces, spreads *C. jejuni* throughout the broiler chicken flock. *C. jejuni* outer membrane proteins are recognized by the host immune system in response to colonization. Particularly important is the surface-displayed protein, Fibronectin-like protein A (FlpA), responsible for *C. jejuni* attachment to host epithelial cells (Konkel *et al.*, 2010). Vaccine strategies may exploit expression of FlpA for eliciting a broiler chicken immune response within a critical window of time.

*Lactobacillus* is considered Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration, with an extensive history in human food products. Poultry producers have previously adopted probiotic feeding and have benefited from improved broiler weight gain and control of intestinal human pathogens (Kalavathy *et al.*, 2003, Schneitz., 2005). Probiotics function under several proposed mechanisms, with numerous studies conducted to evaluate the efficacy of lactobacilli (see Chapter 2.2 Review of Literature for more information on
probiotics). Select lactobacilli strains produce a proteinaceous, paracrystalline surface-layer (S-layer) structure on their exterior, with potential significance in attachment to host epithelial cells.

In addition to their role as probiotics, recombinant lactobacilli offer a viable vaccine candidate and a novel approach for expression of select proteins to elicit host immune responses (Wells, 2011). At an early age, dosage of broiler chickens with recombinant lactobacilli elicits a host immune response prior to exposure to *C. jejuni*. Limited research evaluates the efficacy of these strains when orally administered to broiler chickens.

The literature indicates current intervention techniques incompletely eliminate pathogen colonization, though a minor reduction may significantly reduce the impact on human illness. Future analysis must focus on the efficacy of commercialized lactobacilli strains, intended for administration in the oral tract of broiler chickens. Treatment at the farm reduces the introduction of *Campylobacter* into the retail market and decreases the incidence of campylobacteriosis.
1.2 Objectives

The overall objectives of this research were to 1) colonize broiler chickens with *Campylobacter jejuni*; 2) construct recombinant *Lactobacillus* for oral delivery of vaccines against *C. jejuni*; and 3) evaluate the immunogenicity of the recombinant *Lactobacillus* strains in broiler chickens. To meet these objectives, *Lactobacillus* strains were engineered to express *C. jejuni* FlpA Domain II under a promoter fused to a secretion signal with or without an anchoring signal from the mucus-binding protein of *L. acidophilus* NCFM. Additional work must be conducted to evaluate the exterior protein expression and *in vivo* antigen-immune response within broiler chickens. If successful, the recombinant *Lactobacillus* strain could be an attractive candidate as a food-grade live vaccine against *Campylobacter* colonization. In addition, a non-genetically modified delivery approach was proposed utilizing three cell wall binding domains.

1.3 References


CHAPTER 2 REVIEW OF LITERATURE

As a leading foodborne pathogen in the United States, *Campylobacter jejuni* has a high incidence in retail poultry meat products, with 845,000 illnesses estimated annually (Batz *et al*., 2011). Several studies have found that greater than 60% of raw retail chicken tested positively for this organism (Nannapaneni *et al*., 2005, Wong *et al*., 2007, Zhao *et al*., 2001). In the United States, reports estimate the annual financial burden of *Campylobacter*-poultry associated health care costs at $1.2 billion. Additionally, it is estimated that 9,000 Quality Adjusted Life Years are lost annually (Batz *et al*., 2011). Therefore, reduction of *C. jejuni* presence in chicken products is a public health concern.

This review will first provide an introduction to *Campylobacter* spp., particularly *C. jejuni* and *Lactobacillus* spp. The review will then focus upon understanding the role of lactobacilli as probiotics and vaccines, particularly the expression and localization of proteins. It will also address how the poultry industry could administer recombinant lactobacilli as a potential solution for the reduction of *C. jejuni* colonization in broiler chickens. It will conclude with discussion about the selection of the optimal lactobacilli strain, promoter, protein presentation, adjuvant, and study parameters to increase the probability of experimental *in vivo* success.

2.1 *Campylobacter*

*Campylobacter* spp. are characterized as gram-negative, curved-shaped, microaerophilic bacteria with flagellar motility. Of the various species, *Campylobacter coli*, *Campylobacter fetus*, and *C. jejuni* cause foodborne diseases in humans; with the latter being more heavily
studied. Parkhill, et al. (2000) published the C. jejuni NCTC 11168 genome, increasing knowledge of the relatively small genome (1.6 million bp) and demonstrated the prevalence of hypervariable sequences. Subsequent research has elucidated a great deal of diversity and variation amongst strains, which may be critical for survival.

Environmental Reservoirs of *Campylobacter*

Interestingly, *C. jejuni* does not survive outside the host for an extended period and is susceptible to low pH environments and is sensitive to high oxygen-containing environments. Common environmental reservoirs of *Campylobacter* spp. are agricultural commodities (poultry, cattle, sheep, and pigs), wild animals (birds, rabbits, and rodents), and domestic animals. Additionally, *Campylobacter* spp. are often isolated from environmental samples including sewage, surface and coastal waters (Humphrey et al., 2007, Jones, 2001).

*Campylobacter* is especially pervasive through poultry farms and is the target agricultural commodity for intervention. Typically, a broiler chicken becomes colonized with *Campylobacter* in the ceca. *Campylobacter* spp. are shed through the excreta. Widespread colonization occurs within a few days due to the practice of coprophagy. Broiler flocks are often colonized with *C. jejuni* by three weeks of age, perhaps due to age-related mechanisms including the declining presence of maternal antibodies (Sahin et al., 2003, Wigley, 2013). Within the GIT of chickens, colonization is highest in the mucosal layer of cecal crypts (between $10^6$ and $10^8$ colony-forming unit (CFU)/g cecal contents) (Allen et al., 2008, Beery et al., 1988, Hermans et al., 2012, Meade et al., 2009). During defeathering and evisceration of these contaminated carcasses, processing steps spread *Campylobacter* strains between flocks and further exacerbate the incidence of *C. jejuni* in retail chicken meat (Hermans et al., 2011b).
Campylobacteriosis Pathology

It is estimated that as few as 500-800 Campylobacter cells may result in infection and development of campylobacteriosis in humans (Young et al., 2007). Patients of all ages experience a range of symptoms from asymptomatic to severe illness. Symptoms of the self-limiting gastroenteritis may include fever, abdominal cramping, diarrhea, and malaise, which may last between several days to one week (Godschalk et al., 2004, Jay et al., 2005, Nyati & Nyati., 2013). A strong correlation exists between individuals with prior episodes of C. jejuni infections and Guillain-Barré syndrome (GBS); these mechanisms have been extensively reviewed (Hughes & Cornblath, 2005, Kuwabara & Yuki, 2013, Nachamkin et al., 1998, Yuki & Odaka, 2005). In the rare case of GBS, the lipooligosaccharide present on the surface of C. jejuni molecularly mimics the human neuronal gangliosides (Young et al., 2007). As host elicited antibodies react with peripheral nerve targets, this auto-immune response attacks the myelin sheath of the central nervous system and may result in ascending paralysis. While most patients with GBS are able to recover with mild side effects, total paralysis and death may result for patients that do not receive sufficient treatment.

Mechanisms of Campylobacter Colonization, Attachment, and Invasion

The mechanisms responsible for campylobacteriosis in humans are not well understood. A great deal can be learned from C. jejuni colonization of broiler chickens. In the gastrointestinal tract (GIT), Campylobacter survival, colonization, and virulence is impacted by both host and Campylobacter produced factors. Host defenses include frequent turnover of the mucosal layer and epithelial cells, an innate immune system, and a commensal microbial community (Ivanov & Honda, 2012, Kim et al., 2010). Conversely, chemotaxis, flagellar
motility, exterior protein display, and iron regulation promote *Campylobacter* passage through the GI lumen and colonization of the basolateral surface of the host epithelial cell (Hermans et al., 2011a, Montville et al., 2012, Young et al., 2007). Motility is critical for *C. jejuni* colonization, as non-motile strains colonize broiler chickens at considerably lower levels and have greater difficulty in reaching the deep cecal crypts and resisting host defense mechanisms.

In addition, great variation of the lipooligosaccharide structure is present amongst *Campylobacter* spp. (Karlyshev et al., 2005). As this organism is found predominantly in the GIT, this structure may be integral for colonization of a wide assortment of hosts and intestinal niches. Surface-displayed proteins interact with host binding sites and influence colonization (Flanagan et al., 2009). Though lacking visible surface pili, *C. jejuni* attach to host epithelial cells through binding of the *Campylobacter* adhesion to fibronectin (CadF) outer membrane protein and the fibronectin-like protein A (FlpA) (Konkel et al., 1997, Konkel et al., 2010). These adhesins are required for maximal *C. jejuni* binding and invasion, though additional adhesins are also present that promote attachment. Secretion of the virulence proteins, termed *Campylobacter* invasion antigens (CiA), from the bacterium’s flagellar Type III Secretion System (T3SS) are required for maximal cell invasion (Christensen et al., 2009, Konkel et al., 1999, Konkel et al., 2004, Malik-Kale et al., 2008). The promotion of the *C. jejuni* invasion has been attributed to the ability of the CiA proteins to modify the host cell regulation pathways. Further research on *C. jejuni* surface proteins and colonization of host epithelial cells will provide clarification of the mechanisms for pathogenicity.
**Intervention Strategies**

To reduce the incidence of campylobacteriosis, present strategies focus on the reduction of *Campylobacter* spp. at the farm (Hermans *et al*., 2011b). Previously, antibiotics were supplemented in chicken feed to prevent acquisition of harmful bacterial species including *C. jejuni*. However, antibiotics negatively impact the composition and function of the microbiome as well as physiology of the chicken, resulting in antibiotic-resistant pathogens (Luangtongkum *et al*., 2009, Miles *et al*., 2006, Smith *et al*., 1999). Alternative research strategies have included development of a live-attenuated *Salmonella* vaccine which expresses *Campylobacter* antigens, a formalin inactivated *C. jejuni* whole cell vaccine, and a recombinant *Escherichia coli* clone which expresses fusion proteins from the *C. jejuni* flagellin gene (Buckley *et al*., 2010, Rice *et al*., 1997, Widders *et al*., 1998). Annamalai, *et al.* (2013) demonstrated that vaccinated groups with encapsulated *C. jejuni* outer membrane proteins (OMP) or solely OMP vaccinated subcutaneously induce a protective antibody response and prevent *C. jejuni* colonization in the chicken. However, the subcutaneous vaccination method is not feasible for large-scale application in broiler farms. Presently, there are no commercially available vaccines which fully eradicate *C. jejuni* colonization in poultry. A key emphasis of treatment methods should be placed upon reduction of colonization within the ceca and conservation amongst *C. jejuni* isolates.

Our limited understanding of the mechanisms responsible for *C. jejuni* colonization and invasion make it difficult to develop approaches to prevent campylobacteriosis. Both researchers and feed companies seek a strategy to control *C. jejuni* colonization of broiler chickens, to promote the GI health of the animal while reducing or eliminating the presence of *C. jejuni*. In poultry, the GIT serves as the only site of amplification for these pathogens through the food
chain (Wagenaar et al., 2006). *Lactobacillus* has been successfully isolated and characterized from the intestinal contents of broiler chickens (Ham et al., 2011, Ojala et al., 2010). At the farm, probiotics given to broiler chickens offer the benefit of reducing human exposure to these pathogens. Oral gavage of lactobacilli-based treatments prior to inoculation of *C. jejuni* resulted in decreased *C. jejuni* colonization in cecum samples (Neal-McKinney et al., 2012). Under this probiotic method, the researchers orally dosed broiler chicks with 10⁷ CFU/mL *Lactobacillus* spp. and followed four days later with a booster shot. At fourteen days post hatch, the broiler chicks were then orally inoculated with *C. jejuni* at 10⁹ CFU/mL. Several days after *C. jejuni* inoculation and subsequent colonization, the broiler chicks were euthanized. Upon analyzing the culture-based methods, the *Lactobacillus* spp. differed in their ability to reduce *C. jejuni* colonization. While *L. crispatus* was found to effectively reduce *C. jejuni*, *L. gallinarum* did not reduce colonization in any of the inoculated chickens. Further research must be conducted to evaluate these strain variations.

Attenuated *Salmonella* vectors expressing linear peptides of *Campylobacter* have decreased the *C. jejuni* colonization of challenged chickens to undetectable levels. Vaccination with Cj0113 (Omp18) in the *Salmonella* vector resulted in a strong humoral response with sIgA concentrations measured from the ileum mucosa (Layton et al., 2011). Alternative research studies may consider *C. jejuni* colonization-associated proteins expressed on the surface of GRAS organisms. This two-part approach exploits probiotic and vaccine mechanisms for reduction of *C. jejuni* colonization in the gastrointestinal tract of broiler chickens.
2.2 Lactobacillus

Lactic Acid Bacteria (LAB) are a taxonomically-related group of microorganisms, characterized as Gram-positive, non-pathogenic microorganisms that produce lactic acid as the primary end-product of carbohydrate metabolism. The genus Lactobacillus, a member of the LAB, contains non-spore forming, anaerobic or microaerophilic, fermentative, and catalase-negative microorganisms with complex nutritional requirements (Jay et al., 2005).

Environmental Reservoirs for Lactobacillus spp.

These organisms have been isolated from numerous environments, including fermented food, plants, fecal material, sewage, silage, as well as the healthy GIT of humans and animals (Magnusson et al., 2003, Siezen et al., 2010). Lactobacillus spp. have been found in fermented foods including kimchi, cheese, sausage, sourdough, as well as pickles. Plant isolates have been found in lilac flowers, grass, dandelion flowers, rowan leaves, and coltsfoot flower. Lactobacillus spp. are considered commensal microorganisms that colonize the GIT of humans and animals.

Regulatory Designation for Lactobacillus spp.

The Food and Drug Administration (FDA) recognizes the historical use of Lactobacillus spp. in the food industry and classifies the food additive as Generally Recognized As Safe (GRAS). This genus has been widely used as probiotics and vaccines, owing to the reported beneficial health effects (Avall-Jaaskelainen & Palva, 2005, Bernardeau et al., 2008, Borchers et al., 2009). Lactobacillus species also have promise for use as therapeutics and prophylactics.
While the poultry industry has accepted the oral administration of *Lactobacillus* spp., federal regulations for probiotics in humans and animal feed remain to be clearly defined (Anadon *et al*., 2006, Degnan, 2008). In the United States, the Food and Drug Administration (FDA) requires GRAS status for microorganisms intended for human consumption. In Europe, the European Food Safety Authority (EFSA) serves as the European Union (EU) risk assessment body for food and feed safety. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) provides independent scientific advice regarding the safety and/or efficacy of additives and products or substances used in animal feed. EFSA proposed a list of microorganisms for QPS status, based upon historical safety (EFSA, 2007). The Qualified Presumption of Safety (QPS) system evaluates the safety of microorganisms in the food chain through an ongoing dialog, as the literature evolves.

*Lactobacillus* as Probiotics

Upon adhesion to the mucosal layer, probiotic functionality involves several proposed mechanisms. These include, but are not limited to, coaggregation with pathogens, competitive exclusion, decreased pH of the intestinal lumen, secretion of specific compounds such as bacteriocins, improved mucosal barrier function, and modulation of the immune response (Bron *et al*., 2011, Fooks & Gibson, 2002, Klaenhammer, 1995). Inhibition of pathogenic bacteria adhesion to epithelial cell surface receptors occurs via numerous mechanisms, including competitive exclusion (lactobacilli bind before pathogens) and competition (lactobacilli and pathogens presented simultaneously). Even transient colonization of the GIT allows the probiotics to provide these beneficial effects to the host.
Lactobacillus strains stimulate or modulate the immune system by increasing defenses against infection, increasing IgA production, regulating of T-helper 1 cells, altering cytokine profiles, and inducing maturation of dendritic cells (Christensen et al., 2002, Duong, 2011, Havenith et al., 2002). In addition, antimicrobial activities are associated with the S-layer proteins of several Lactobacillus species, including Lactobacillus helveticus, Lactobacillus crispatus, and Lactobacillus kefir (Jakava-Viljanen & Palva, 2007, Wang et al., 2008). These species have shown inhibition of pathogen colonization, such as Salmonella enterica serovar Enteritidis (Golowczyc et al., 2007, Pascual et al., 1999) and Salmonella enterica serovar Typhimurium (Chen et al., 2012). Though, this requires further insight into the fundamental antimicrobial mechanisms.

Lactobacillus’ Vaccine Potential

While previous research has focused on administration of live vaccines and live attenuated strains for bacterial delivery of pathogenic bacteria, including Salmonella, Bordetella, and Listeria (Detmer & Glenting, 2006), the pathogenicity of these vaccines is of great concern. Researchers are cognizant of the potential reversion of attenuated pathogenic strains that may colonize and replicate within the host’s GIT. The use of non-pathogenic microorganisms such as recombinant lactobacilli may overcome these concerns and offer a strategy of a sound delivery vector. At this time, lactobacilli have been utilized for vaccine development utilizing genes from Streptococcus pneumoniae, Helicobacter pylori, Enterotoxigenic E. coli, Listeria monocytogenes, as well as Human immunodeficiency virus (HIV) (Tarahomjoo, 2012). The ability of lactobacilli to survive the harsh environmental conditions of the GIT allows the
organism to reach the intended luminal location intact. Additionally, the recombinant microorganism may be shed with minimal concern of further propagation in the environment.

2.3 *Lactobacillus* Characteristics

**Surface Layer**

The majority of Bacteria and Archaea may be characterized with an exterior cell wall which allows the organism to respond to environmental fluctuations. The surface-layer (S-layer) consists of a paracrystalline structure with large proteins and is synthesized through self-assembly (Avall-Jaaskelainen & Palva, 2005). The intended function of the S-layer remains largely unknown, as even bacteria within the same species do not exhibit the same structure or the same function. Predicted functions of the S-layer include a protective barrier against environmental hazards, regulation of nutrient and metabolite passage, preservation of cellular structure, and oversight of exterior surface recognition and cell adhesion (Beganovic *et al*., 2011). Understanding the mechanisms of the S-layer offers great insight to the functionality of lactobacilli.

The most common electron microscopy techniques for studying the S-layer proteins of lactobacilli include negative staining, thin-sectioning, and freeze-etching (Avall-Jaaskelainen & Palva, 2005). Under atomic force microscopy, a smooth layer may be seen with a globular layer of proteins on the outer surface of *L. crispatus* DSM20584 while a rough surface of polymeric surface conformations may be seen with *L. helveticus* ATCC 12046 and *L. helveticus* ATCC 15009 (Schar-Zammaretti & Ubbink, 2003). These exterior structural variations indicate the complexity and the variety of the potential S-layer functions.
The S-layer consists of a single, homogeneous protein species with a molecular weight between 40 to 71 kDa (Avall-Jaaskelainen & Palva, 2005, Engelhardt & Peters, 1998). These oftentimes acidic proteins are composed of 40-60% hydrophobic amino acids with little to no sulfur-containing amino acids (Sleytr & Beveridge, 1999). Unlike most bacterial species, the highly basic S-layer of lactobacilli has an isoelectric point ranging 9.35 to 10.40 (Avall-Jaaskelainen & Palva, 2005, Smit et al., 2001, Ventura et al., 2002). In lactobacilli, the S-layer may consist of 10-15% of the total cellular proteins (Boot et al., 1996b). At this time, S-layers have been found in several Lactobacillus species (Table 2.1), though are not present in all species as L. casei and L. rhamnosus are void of this protein layer (Table 2.2). Controversy surrounds L. fermentum, as no S-layer protein gene sequence exists in the public database yet electron microscopy revealed the presence of an S-layer (Hynonen & Palva, 2013). This same phenomenon is also true for L. gasseri and L. johnsonii. Previous reviews diverge on the classification of particular species (Avall-Jaaskelainen & Palva, 2005, Hollmann et al., 2010, Hynonen & Palva, 2013). Improvements in genomic sequencing have expanded the capabilities of mining for genetic sequences associated with S-layer formation.

The three primarily characterized S-layer proteins are SlpA, SlpB, and SlpC (Sun et al., 2013). In the majority of Lactobacillus cells, SlpA is highly expressed while SlpB and SlpC are oftentimes silent (Delcour et al., 1999). The exposed proteins must be in the correct conformation in order to elicit the intended activities. The most well understood anchoring region, LPXTG box, covalently binds these exterior proteins to the cell wall (Leenhouts et al., 1999). The C-terminal region oversees cell-wall anchoring, while the N-terminal region coordinates the self-assembly domain (Claus et al., 2005, Hu et al., 2011); though studies have also found numerous N-terminally anchored proteins (Kleerebezem et al., 2010).
coordination amongst these anchoring domains remains critical for display of the proteins on the cell surface.

Though the function of the S-layer remains largely unknown, lactobacilli that require the crystalline protein layer do not function properly without it present. *L. acidophilus* M92 exhibited reduced adhesion to mouse ileal epithelial cells upon removal of S-layer proteins (Frece *et al.*, 2005). Attempted knock-out of *L. acidophilus* slpA proved unsuccessful, indicating necessity of the gene (Boot *et al.*, 1996a). In addition, attempted deletion of the ApuA gene N-terminal repeat region was unsuccessful (Kim *et al.*, 2009). Removal of the S-layer in *L. helveticus* MIMlh5 resulted in decreased pro-inflammatory factors COX-2 and tumor necrosis factor alpha (TNF-α) induction levels in human U937 macrophages (Taverniti *et al.*, 2013). Without the S-layer, cells may be more susceptible to environmental hazards due to the incomplete surface layer protection and inability to regulate passage of key metabolites.

The natural self-assembly domain of the S-layer into regular arrays promotes the display of foreign antigens along with an immune-stimulating effect. The potential applications of S-layers for biotechnology include vaccine development, protein surface display, heterologous protein display, as well as secretion signals of S-layer protein genes (Bermudez-Humaran *et al.*, 2011, Hu *et al.*, 2010, Kajikawa *et al.*, 2012, Mercenier *et al.*, 2000). Foreign antigens anchored to the cell wall induce immune responses in animal models. As long as a sufficient concentration of the foreign antigen is secreted or anchored on the cell surface, the exterior placement location does not impact immunogenicity.
Promoters

Promoters initiate and regulate transcription of genes, through specification of a binding site for a RNA polymerase holoenzyme and a transcriptional start point. *Lactobacillus* species vary in their promoter strengths, with the -35 and -10 hexamers and UP element as important factors in promoter selection (McCracken *et al.*, 2000). Constitutive promoters allow continuous transcription of the associated gene, while inducible promoters require stimulus by a substrate to begin transcription. For genes under the control of the lac operator, the molecule Isopropyl β-ᴅ-ᴅ-thiogalactopyranoside (IPTG) regulates initiation of gene expression. The Nisin Controlled gene Expression system (NICE) functions by induction of the gene with sub-inhibitory levels of nisin added to the culture medium. A linear dose-response curve occurs with increasing amounts of nisin using β-glucuronidase as the reporter gene (Mierau & Kleerebezem, 2005). Selection of a native host promoter ensures recognition by the host strain.

The strong promoters involved with S-layer synthesis are very efficient. The constitutive promoter of the *L. acidophilus* S-layer gene is twice as efficient as the lactate dehydrogenase gene – one of the strongest promoters described in bacteria (Boot *et al.*, 1996b). Some lactobacilli species have multiple promoters in front of the S-layer protein gene, including *Lactobacillus brevis* which has two active adjacent promoters (Hynonen *et al.*, 2010, Kahala & Palva, 1999). For recombinant lactobacilli expressing foreign antigens, the promoter selected must maintain a balance between sufficient protein expression and maintenance of the exterior surface structure.

Microarray analysis provides a valuable tool for identifying the expression levels of lactobacilli genes dependent upon carbohydrate sources. Duong, *et al.* (2011) utilized microarray analysis to identify operons involved in the transport and catabolism of
fructooligosaccharides (FOS), lactose \((lac)\), trehalose \((tre)\), and genes directing glycolysis to select promoters for lactobacilli expression vectors. The putative promoters FOS \((P_{FOS})\), \(lac\) \((P_{lac})\), and \(tre\) \((P_{tre})\) were implemented into the construction of carbohydrate-induced vectors, while the promoter \(pgm\) \((P_{pgm})\) was selected for construction into a constitutive expression vector. Results indicate that the expression systems maintained activity comparable to the native genes positioned in the original \textit{Lactobacillus} genome, supporting the use of these promoters in vector systems. Selection of the promoters greatly influences the expression level of the gene of interest.

**Protein Expression**

The manner in which the bacterial vector delivers the antigen via cellular location (cytoplasmic, secreted, or anchored in or on the cell wall) significantly impacts the immunogenicity. Cytoplasmic localization of a protein protects it from the harsh external environment, though requires cell lysis for protein delivery. Secretion of produced proteins into the external environment leads to direct contact with the food source, GIT, etc. Anchoring of the protein (surface display) to the cell wall promotes simultaneous interaction with the environment and protection from proteolytic degradation. Anchoring of the antigen to the surface of the cell wall produces the largest antigen immunogenicity (Bermudez-Humaran \textit{et al}., 2004, Norton \textit{et al}., 1996). Therefore, the majority of vaccination studies should focus upon cell-wall anchored protein delivery, rather than cytoplasmic or secreted delivery locations.

There are numerous options for anchoring of proteins to the lactobacilli extracellular surface. Previous studies include lipid-mediated N-terminal anchoring to the cell membrane, N-terminal anchoring to the cell membrane facilitated by a non-cleaved N-terminal signal peptide
(SP), C-terminal sortase-mediated covalent anchoring to the cell wall, and noncovalent anchoring through additional domains that directly interact with the cell wall, such as the LysM domain (Fredriksen et al., 2012, Hu et al., 2010). The sortase-mediated route has received the greatest amount of attention (Marraffini et al., 2006, Ton-That et al., 1999). In the case of two recombinant L. acidophilus strains displaying the same Salmonella flagellin (FliC) in different anchoring motifs, the cell surface structures displayed divergent dendritic cell maturation and cytokine production (Kajikawa et al., 2011). FliC was first fused to the C-terminal region of the cell envelope proteinase (PrtP) and then bound to the cell wall via electrostatic bonds. In the second method, FliC was conjugated to the mucus binding protein (Mub) anchoring region and covalently linked to the LPXTG motif of the cell wall (Kajikawa et al., 2011). The surface-displayed antigens were highly sensitive to gastric and small intestine secretions, diminishing the likelihood of oral administration of this recombinant strain as it may not survive the harsh environment of the stomach and small intestine.

Strain variations amongst lactobacilli impact the quantity of expressed antigen. The method of antigen presentation on the cellular surface, especially between species, may elicit different immunogenic responses between lactobacilli. Thus, when selecting lactobacilli strains the expressed antigen concentration must be evaluated in vitro before inoculation in vivo with subsequent analysis of the host serum. Western Blotting results indicate a clear visual if the antigen is present, though do require a specifically designed antibody for detection. Flow-cytometric analysis offers an additional form of recognition, utilizing fluorescent proteins which pass through an electronic detector (Kajikawa et al., 2011). As previously stated, the promoter expression and secretion of the protein across the cell membrane influence the level of antigen expression.
Adjuvants

As gram-positive bacteria, lactobacilli have intrinsic adjuvant properties to induce either a proinflammatory or regulatory immune response both *in vitro* and *in vivo*. *Lactobacillus* strains present microbe-associated molecular patterns (MAMPs) which include lipoproteins, lipoteichoic acids (LTA), peptidoglycan (PG), and muramyl dipeptide (MDP) which in turn are recognized by Toll-like receptor (TLR) 2, TLR 6, and nucleotide-binding oligomerization domain 2 (NOD2) (Lebeer *et al.*, 2010, Stoeker *et al.*, 2011). Due to the absence of flagella these strains lack the ability to elicit a TLR5 response.

Within a host, adjuvants enhance the ability of the immune system to respond to an antigen. While intrinsic adjuvants exist, vaccines co-administered with an adjuvant are characterized by increasing uptake by or stimulating DCs and macrophages. Bacterial flagellin proteins fused with other antigens act as an adjuvant, potentially due to the activation of NF-κB via TLR5 and caspase-1 inflammasome through Nod-like receptor Caspase recruitment domain 4 (NLRC4) (Fredriksen *et al.*, 2012). Intragastric immunization of mice with *L. acidophilus* displaying Gag and *S. enterica* ser. Typhimurium flagellin (FliC) resulted in an increase of Gag-specific IgA-secreting cells; indicating the presence of FliC resulted in an adjuvant effect on local IgA production (Kajikawa *et al.*, 2012). The intended *in vivo* immune response must be confirmed before selection of an adjuvant, as the immune systems of various animal species differ in their ability to detect particular stimulations.

2.4 Selection of Probiotic Strains in the Poultry Industry

To qualify as a probiotic for use in the poultry industry, the strain must be a natural inhabitant of the GIT, survive low pH conditions and the presence of bile acids, adhere to the
intestinal epithelium, and compete with autochthonous and allochthonous organisms present (Ehrmann et al., 2002, Koenen et al., 2004a, Lutful Kabir, 2009, Patterson & Burkholder, 2003). For the potential probiotic to be endorsed in the poultry industry, it must achieve the intended beneficial effects while withstanding industrial processes and maintaining viability under prolonged storage conditions. Upon commercialization, the probiotic may be administered via drinking water, feed routes, or sprayed directly onto the birds. The probiotic would then enter the GIT through the oral cavity; a realistic strategy for large scale facilities that cannot administer hundreds of birds through oral gavage. Poultry producers must be knowledgeable of the probiotic functionality and the appropriate handling requirements to ensure effective treatment.

As probiotic and vaccine strategies continue to undergo rigorous in vivo testing, the efficacy of live oral administration relies upon three key factors – dosing, timing, and frequency. Animal studies require a certain degree of finesse and experience, to optimize these three factors for the intended strain. The key time to administer a lactobacilli treatment-based method involves the lag period between the descending maternal antibodies (<3 weeks of age) and the increasing probability of C. jejuni colonization. Lactobacilli require sufficient time to replicate within the lumen and to colonize the cecum; boosters enhance the effect of the lactobacilli. A practical strategy must balance treatment timing, dosing, and frequency with the natural colonization of the GIT.

Lactobacillus spp. Probiotics for Broiler Chickens

Human pathogens associated with raw and undercooked poultry products include S. enterica ser. Enteritidis and C. jejuni. Current practices (eg. antibiotics, vaccination,
modification of diet, feed additives, prebiotics, and control of biosecurity) are not sufficient for total eradication of these pathogens from the poultry facility and outbreaks persist throughout the United States. The mucosal layer of the GI epithelium inhibits invasion of pathogenic bacteria and foreign compounds through a chemical and physical barrier, while allowing normal activity of commensal bacteria (Bron et al., 2011). To successfully invade this layer, pathogenic bacteria adhere and penetrate the protective layer to invade enterocytes which may result in clinical infection (Koutsos & Arias, 2006). Through prevention of pathogenic bacteria adhesion and penetration of the mucosal layer, the rate of infection may be reduced.

Upon survival through the gastric environment of the stomach and escaping from bile salts, probiotics now face the challenge of the epithelial mucosal layer. The intestinal mucosa is composed of a nutrient rich, one-cell-thick layer, which rests on a sub-layer of enterocytes that sample the environment with follicles (Bron et al., 2011). The peristaltic motion of the intestine moves bacteria, nutrients, and waste through the lumen (Abreu, 2010). To maintain a presence, probiotics must adhere to glycans on the mucosal layer long enough to impart the predicted beneficial effects. Adherence to the epithelial lining of the GIT and an inability to be easily removed promotes colonization.

As previously discussed, dosage of probiotics into the GIT of broiler chickens offers numerous mechanistic advantages for control of pathogens and benefits to host health. Farmers have already adopted the usage of probiotics, which reduce abdominal fat deposition, improve body weight gain, improve feed conversion rate, and control intestinal human pathogens (Kalavathy et al., 2003, Schneitz, 2005). Probiotics and prebiotics fed to broiler chickens decrease E. coli and total aerobic populations while increasing cecal volatile fatty acids and non-VFA concentrations (Mookiah et al., 2014). Probiotics have been shown to be growth promoters
in broiler chickens (Kalavathy et al., 2008, Mountzouris et al., 2007, Mountzouris et al., 2010). The most commonly used probiotics for livestock and poultry include *Lactobacillus*, *Enterococcus*, *Bacillus*, and *Saccharomyces* (Gaggia et al., 2010). As there remains great debate on the efficacy of these probiotics, the results vary greatly by probiotic strain, concentration of dose, timing, host breed, and age of the host (Koenen et al., 2004b, La Ragione et al., 2004). Further investigations are needed to explore the appropriate timing of probiotic dosage along with application in a commercial setting, rather than a controlled experimental model.

Animal studies indicate the ability of lactobacilli to prevent colonization of human pathogens. When co-administered with *S. enterica* serovar Enteritidis C-114 by oral gavage in broiler chickens, *L. salivarius* was found to prevent pathogen colonization. A follow-up feed study indicated a secondary dose would be needed to ensure the presence of the probiotic at the time of poultry harvest (Pascual et al., 1999). A combination of *L. acidophilus* and *L. fermentum* inhibited the adhesion of pathogenic *Salmonella* to the chicken mucosal layer *in vitro*, a stronger effect than administering each strain individually (Ma et al., 2006). Multistrain (or multispecies) probiotics may be more successful in reducing pathogen adhesion than monostrain treatments (Timmerman et al., 2004). Further studies are investigating the efficacy of these lactobacilli strains in preventing *C. jejuni* colonization.

For *in vivo* studies, the primary source of the lactobacilli strain impacts the colonization within the GIT. Strains isolated from the equivalent animal model increase the likelihood of colonization and proliferation in the intended luminal location (Chen et al., 2012). Ma, et al. (2006) found that *Lactobacillus* strains from the cecum indicated greater adhesion *in vitro* to the mucosal layer of the ileum, cecum, and colon than to that of the duodenum and jejunum. The composition of the mucosal layer impacts the specific bacteria that adhere. Initially,
identification of potential strains occurs in vitro with selection by survival factors such as
withstanding low pH, bile acids, host enzymatic and hydrolytic reactions, low generation time, as
well as genetic stability (Gaggia et al., 2010). While these factors do not guarantee the
prolonged colonization of the probiotic, there exists an increased probability of survival through
the GIT and production of subsequent beneficial effects.

*Lactobacillus* spp. Vaccines for Broiler Chickens

A promising application of lactobacilli is their use as a live vaccine, for presentation of
dNA or proteins to the GI mucosal surfaces (Wells, 2011). In this approach, synthesis of
antigens by the host machinery overcomes problems such as incorrect protein folding and
glycosylation. The VP2 and VP3 antigens of infectious bursal disease virus (IBDV) were
produced by recombinant *Lactococcus lactis* strains (Dieye et al., 2003). The lactococcal strains
producing staphylococcal nuclease Nuc fused to VP2 (Nuc-VP2) and VP3 were orally
administered to chickens. Those bacteria producing the Nuc-VP2 complex induced a systemic
and specific immune response against Nuc but not against VP2. Vaccinated chickens did not
indicate a detectable immune response against VP2 and VP3. Further research must explore the
role of the cell-wall anchoring site and expression level required for a detectable immune
response.

An alternate study expressed the foreign highly pathogenic avian influenza (HPAI)
protein, hemagglutinin 1 (HA1), with recombinant *Lactobacillus delbrueckii* ssp. Lactis D17
(LDL17-pH) orally administered to broiler chickens (Wang et al., 2013). The LDL17-pH
triggered a mucosal and systemic immune response, indicated by increased specific anti-HA1
IgA antibody levels in the mucosa and the anti-HA1 IgG level in sera. Vaccinated chickens had a
higher survival rate, compared to the control group, when challenged with a lethal dose of H5N1 virus. This study demonstrated the feasibility of recombinant Lactobacillus as a candidate for oral administration of avian influenza virus vaccine.

Vaccine applications may also exploit the noncovalent binding domain of LysM with the benefit of producing non-genetically modified organisms (GMO). The AcmA binding domains of L. lactis displayed the VP1 protein of chicken anemia virus (CAV) on the surface of L. acidophilus (Moeini et al., 2011). After verification of the binding of the CAV VP1 protein on the bacterial cell surface, broiler chickens were orally gavaged with the lactobacilli cells carrying the VP1 protein. Evaluation of vaccinated birds indicated increased levels of Th1 cytokines interleukin (IL)-2, IL-12, and IFN-γ. This strategy indicates the potential of L. acidophilus and the LysM domain to orally delivery vaccines to broiler chickens.

A positive correlation may exist between the ability of bacteria to adhere to the chicken mucosal lining and the presence of an S-layer in avian isolates, such as L. acidophilus. Recombinant lactobacilli that express heterologous proteins manipulate this immunogenic recognition and prepare the host for future challenge by pathogenic organisms. Limited research has evaluated the outcome of recombinant lactobacilli orally administered to broiler chickens. Broiler chickens orally administered Lactobacillus spp. along with microencapsulated recombinant FliC protein and the subunit B of cholera toxin elicited host immune responses (Baptista et al., 2014). More specifically, this strategy stimulated the humoral and cellular immune responses, in combination with altering the population of CD8+ T lymphocytes in the cecum. Existing vaccine strategies are capable of eliciting a cell-mediated immune response and systemic antibody-immune response (Brisbin et al., 2011); though require a booster or multiple doses to effectively evoke an adaptive immune response in the host.
Challenging a broiler chicken with a recombinant vaccine prepares the immune system for future interactions with a specific infectious organism. Thus, the host responds sooner, with a large repertoire of antibody molecules to recognize the infectious antibodies. While the host-produced antibodies target the foreign antigen, these antibodies also interact with host receptors to eliminate the antigen and to promote the activation of the immune response. A vaccination approach must overcome the antigen variances between strains, an incomplete knowledge of inducing a protective immune response, and provide protection against pathogens early in the physical development of the bird (Wagenaar et al., 2006). Utilization of both a probiotic and vaccination approach combines the relative mechanisms for administration in vivo.

Molecular biology techniques utilize cloning vectors for heterologous protein expression. These tools improve the ease of studying genes of interest from prokaryotes and eukaryotes. Typically an engineered plasmid contains a (i) multiple cloning site (MCS), (ii) antibiotic resistance marker, and (iii) an origin of replication (ori). When selecting a vector, important factors to consider include copy number, size of the insert, and the type of promoter (constitutive or inducible).

2.5 Future Direction

Present research indicates that treatment with lactobacilli probiotics and vaccines results in incomplete elimination of pathogen colonization, though the reduction in pathogens will presumably decrease the probability of human health consequences. The future implementation of lactobacilli probiotic and vaccine strategies largely depends upon commercialization and administration costs, dosage operation in production facilities, acceptance of GMOs by regulatory standards, and efficacy of strains. To achieve success in the poultry industry, these
novel protection strategies must offer greater benefits than the present routes of administration and achieve a higher degree of efficacy to compensate for the increased cost of animal care.

2.6 References


_Curr Opin Neurol_ **18**, 557-561.


## 2.7 Tables

**Table 2.1 Lactobacillus species with S-layer protein genes present**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Method of Identification</th>
<th>Identified Proteins</th>
<th>Reference</th>
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<td><em>L. acidophilus</em></td>
<td>ATCC 4356, LMG 11469, NCFM ATCC 4796</td>
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<td>slpA, slpB</td>
<td>(Boot et al., 1995)</td>
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<td></td>
<td>Bioinformatics</td>
<td>slpA, slpB</td>
<td>(Hynonen &amp; Palva, 2013)</td>
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<td><em>L. amylolyticus</em></td>
<td>DSM 11664</td>
<td>Bioinformatics</td>
<td>slp</td>
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<td>(Masuda &amp; Kawata, 1983)</td>
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<td></td>
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<td>(Masuda &amp; Kawata, 1981)</td>
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<td>Experimental*</td>
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<td><em>L. gigeriorum</em></td>
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<td><em>L. hilgardii</em></td>
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<td><em>L. johnsonii</em></td>
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* Experimental methods included sequencing as component of protein identification.
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<th>Absent Proteins**</th>
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<tr>
<td><em>L. casei</em></td>
<td></td>
<td>Bioinformatics</td>
<td></td>
<td>(Hynonen &amp; Palva, 2013)</td>
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<td><em>L. delbrueckii ssp. bulgaricus</em></td>
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<td><em>slpA</em></td>
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<td><em>L. delbrueckii ssp. delbrueckii</em></td>
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**Genomic databases were queried only for the proteins SlpA and SlpB.**
<sup>1</sup>Blast Query conducted on December 19, 2012
<sup>2</sup>NCBI Search conducted on February 4, 2013
<sup>3</sup>NCBI Search conducted on May 5, 2014
CHAPTER 3 LACTOBACILLUS DISPLAY OF HETEROLOGOUS PROTEIN EXPRESSION

3.1 Abstract

As a leading foodborne pathogen in the United States, Campylobacter jejuni in retail poultry meat products results in a $1.3 billion annual cost of illness with over 845,000 illnesses estimated annually. In humans, campylobacteriosis is characterized by undesirable abdominal pain, cramping, diarrhea, and in rare circumstances Guillain-Barré Syndrome. Current prevention strategies focus on the reduction of commensal C. jejuni colonization of chickens at the farm. The Food and Drug Administration recognizes Lactobacillus spp. as a Generally Recognized as Safe food additive, with a plethora of food products formulated with probiotics. An interesting area of research involves recombinant lactobacilli that express foreign proteins on the exterior cell surface. Within the host gastrointestinal tract, C. jejuni FlpA binds fibronectin and is required for bacterial adherence to host intestinal cells. The aim of this study was to construct recombinant Lactobacillus strains for oral delivery of vaccines against Campylobacter spp. and to evaluate the immunogenicity in chickens. Lactobacillus strains were engineered to express C. jejuni FlpA Domain II under a promoter fused to a secretion signal with or without an anchoring signal from L. acidophilus NCFM. While L. acidophilus and L. gallinarum yielded successful transformants, L. crispatus and L. helveticus were not easily transformed, suggesting strain-to-strain variations. A non-genetically modified alternative proposes the display of foreign antigens on the surface of Lactobacillus through cell wall binding domains. Additional work must be conducted to evaluate the exterior protein expression and in vivo antigen-immune response within broiler chickens. If successful, recombinant Lactobacillus strains could be an
attractive candidate as a food-grade live vaccine against *Campylobacter* colonization. Furthermore, treatment at the farm reduces the introduction of *Campylobacter* into the retail market and decreases the incidence of campylobacteriosis.

### 3.2 Introduction

*Campylobacter jejuni* is the third-leading foodborne pathogen in the United States, with campylobacteriosis most prevalent amongst consumers exposed to raw or undercooked poultry products or those that handled cross-contaminated surfaces. Consumers exposed to *C. jejuni* may experience cramping, abdominal pain, diarrhea, and in rare cases the autoimmune disease termed Guillain-Barré Syndrome (GBS) (Godschalk *et al.*, 2004, Jay *et al.*, 2005, Nyati & Nyati, 2013). These mechanisms have been extensively reviewed, though prevention methods for campylobacteriosis remain limited (Hughes & Cornblath, 2005, Kuwabara & Yuki, 2013, Nachamkin *et al.*, 1998, Yuki & Odaka, 2005).

In order to develop adequate strategies to control the colonization of this human pathogen, a fundamental understanding of the mechanisms responsible for colonization, attachment, and invasion is needed. Several studies have outlined how chemotaxis, flagellar motility, exterior protein display, and iron regulation promote *Campylobacter* spp. passage through the GI lumen and colonization of the basolateral surface of the host epithelial layer (Hermans *et al.*, 2011, Montville *et al.*, 2012, Young *et al.*, 2007). *C. jejuni* attachment to the host mucus and epithelium is required for the initial stages of colonization. Present on the surface of *C. jejuni* are proteins responsible for attachment to host fibronectin-binding sites, including Fibronectin-like protein A (FlpA) (Flanagan *et al.*, 2009, Konkel *et al.*, 2010). Translocation of *C. jejuni* to the basolateral surface of human epithelial cells is critical for
subsequent colonization and disease onset, as this prevents the host clearance of the organism through peristalsis and fluid flow.

Lactic Acid Bacteria (LAB) are a taxonomically-related group of organisms, known for the fermentation of sugars into the primary metabolic end-product, lactic acid. LAB are commensal organisms found in the gastrointestinal tract (GIT) and mucosal surfaces of animals and humans, as well as environmental sources (Magnusson et al., 2003, Siezen et al., 2010). The *Lactobacillus* genera have been recognized by the Food and Drug Administration (FDA) as Generally Recognized As Safe (GRAS), noting the historical safety of this organism in food products. In addition, *Lactobacillus* is a suitable probiotic and vaccine candidate due to the ability to survive passage of the GIT and to exert beneficial attributes to the health of the host (Avall-Jaaskelainen & Palva, 2005, Bernardeau et al., 2008, Borchers et al., 2009). The reported benefits include production of bacteriocins and modulation of the immune system, as well as prevention of tissue inflammation, gastrointestinal disorders, urinary tract infections, and allergies.

Lactobacilli have been genetically manipulated to express a plethora of foreign antigens. Expression of the recombinant proteins may result in either secretion into the extracellular matrix or anchoring onto the exterior bacterial cell wall. Antigens anchored to the cell wall stimulate the greatest antigen immunogenic response (Bermudez-Humaran et al., 2004, Norton et al., 1996). Examples of protein anchoring mechanisms include lipid-mediated N-terminal anchoring to the cell membrane, N-terminal anchoring to the cell membrane facilitated by a non-cleaved N-terminal signal peptide (SP), C-terminal sortase-mediated covalent anchoring to the cell wall, and noncovalent anchoring through domains that directly interact with the cell wall, such as the
LysM domain (Fredriksen et al., 2012). Lactobacilli expression vectors that produce foreign antigens for exposure in the GIT are capable of stimulating the immune system.

Previous literature has demonstrated recombinant Lactobacillus spp. as a delivery vector for foreign antigens. Orally-delivered Lactobacillus vaccine strains have been developed against the infective bacteria Helicobacter pylori, Enterotoxigenic Escherichia coli (ETEC), Salmonella enterica serovar Enteritidis, Bacillus anthracis, Borrelia burgdorferi, as well as Human papillomavirus (HPV-16) and Rotavirus (Tarahomjoo, 2012). Lactobacillus spp. are GRAS organisms and natural isolates that are thought to colonize the GIT, to persist within the host and impart natural adjuvant traits. In a recent study, mice were orally immunized with Lactobacillus casei expressing a fragment of β-intimin (L. casei Intcv) and challenged with Citrobacter rodentium (Ferreira et al., 2011). All vaccines were able to reduce C. rodentium recovered from feces and fecal samples indicated host production of anti-Intcv IgA. An additional study demonstrated the effectiveness of recombinant Lactobacillus delbrueckii subsp. lactis D17 expressing the highly pathogenic avian influenza (HPAI) protein, hemagglutinin 1 (HA1) (Wang et al., 2013). Broiler chickens orally inoculated with a lethal dose of H5N1 virus had a higher survival rate when vaccinated with the HPAI. Additionally, increased mucosal and systemic immune responses were measured in terms of anti-HA1 IgA antibody levels in the mucosa and anti-HA1 IgG levels in sera.

Anchoring of purified proteins onto the surface of Lactobacillus through a cell wall binding domain offers the benefit of a non-genetically modified organism that may elicit a host immune response. Several surface-associated proteins include covalently anchored proteins (N- or C-terminally anchored proteins, lipoproteins, and LPxTG-anchored proteins) and noncovalent cell wall binding domains (LysM, choline, putative peptidoglycan, S-layer proteins with SLH
domains, WxL, and SH3) (Kleerebezem et al., 2010). Of great interest are the latter group, non-covalent cell wall binding domains, which may be exploited as a non-genetically modified surface display system for heterologous proteins on *Lactobacillus*. LysM-containing fusion proteins have been deeply summarized in the literature (Visweswaran et al., 2014). This system has been demonstrated with the *L. casei* A2 bacteriophage lysin gene as a cell wall anchor (Ribelles et al., 2012, Ribelles et al., 2013), the *L. fermentum* bacteriophage LysM domain (Hu et al., 2010), and the cell wall binding domain of the AcmA protein (Raha et al., 2005).

Strategies to reduce *C. jejuni* colonization in broiler chickens remain limited. Our objective is to either anchor or secrete *C. jejuni* FlpA on the surface of *Lactobacillus* species and to evaluate the protein expression *in vitro*. Exposure of the broiler chicken GIT to *C. jejuni* colonization-associated proteins in the GIT should stimulate the immune response of the chicken. Recombinant *Lactobacillus* strains expressing *C. jejuni* FlpA will be evaluated for future oral gavage of broiler chickens.

### 3.3 Materials and Methods

**Bacterial Strains and Growth Conditions**

*Lactobacillus* strains were either procured from the American Type Culture Collection or gifted by collaborators (Table 3.1). All bacterial stock cultures were stored at -80°C in 12.5% glycerol (v/v). *Lactobacillus* strains were grown in de Mann, Rogosa, Sharpe (MRS) broth (Difco, Sparks, MD) or on MRS supplemented with 1.5% agar (Fisher, Fair Lawn, NJ). Cultures were incubated at 37°C anaerobically (Coy, Grass Lake, MI) with a gas composition of 90% nitrogen, 5% hydrogen, and 5% carbon dioxide. *Lactobacillus* strains were grown in the
presence of Erythromycin (Em, 5 µg/mL) (Fisher Scientific), when needed, unless otherwise noted.

*Escherichia coli* strains were grown aerobically at 37°C with shaking in Luria-Bertani (LB) broth (Difco) or on LB supplemented with 1.5% agar. Em was supplied at a concentration of 150 µg/mL and Ampicillin (Fisher Scientific) was supplied at a concentration of 100 µg/mL.

**DNA Preparation and Manipulations**

*E. coli* plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA, USA). Electrocompetent *E. coli* MC1061 cells, *E. coli* Top10 cells, and *E. coli* BL21 (DE3) cells were prepared and transformed according to standard protocols (Appendix A, Appendix B). Additional DNA manipulations were performed according to manufacturer procedures. Restriction enzymes and T4 DNA Ligase were purchased from New England Biolabs (NEB, Ipswich, MA). The ligation procedure followed the NEB Ligation Protocol with T4 DNA Ligase (M0202). EconoTaq PLUS 2X Master Mix was purchased from Lucigen (Middleton, WI). PCR primers were created using Clone Manager 9 (Sci-Ed Software, Raleigh, NC) and synthesized by IDT (Coralville, IA). PCR reactions were performed according to standard procedures using EconoTaq PLUS 2X Master Mix (Lucigen). The DNA Clean and Concentrator Kit (Zymo Research) was used to purify PCR products and the concentration of the DNA measured (NanoDrop™ 1000 Spectrophotometer, Thermo Scientific). Gel Loading Dye (Blue, 6X), Supercoiled DNA Ladder, 1 kb ladder, and a 100 bp ladder were purchased from NEB. The Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA) was used to isolate DNA fragments from agarose gels. DNA sequencing was performed by the W. M. Keck Center (Urbana, IL).
Plasmid Construction

Plasmids constructed or used for this study are listed in Table 3.2. The *C. jejuni* fibronectin-like protein A (FlpA; Cj1279c) harbors the fibronectin type II domain, which was identified in the GenBank database (AL111168.1). The cassette design was structured after Kajikawa, *et al.* (2012) and utilized a lab developed vector, pMJM-8 (Figure 3.1). Sequences of interest were identified from the NCBI Conserved Domains Database by previously designed primers (Kajikawa *et al.*, 2012). These primers identified the promoter region of the *pgm* gene from *L. acidophilus* NCFM, the region encoding the ribosome binding site of mucin-binding protein (Mub) from *L. acidophilus* NCFM (LBA 1709 and LBA 1392), the signal sequence of Mub (LBA 1709 and LBA 1392), the region encoding the anchor region of Mub (LBA 1709 and 1392), and the *fliC* gene from *S. enterica* serovar Typhimurium (Figure 3.2). *C. jejuni flpA* Domain II was selected based on fibronectin-binding activity and the sequence identified through the NCBI Conserved Domains Database.

Sequences were inputted and managed utilizing the software program, Clone Manager (Sci-Ed Software, Cary, NC). The cassette places *C. jejuni flpA* Domain II under constitutive promotion of the phosphoglycerate mutase promoter (*Ppgm*) fused to a signal peptide with or without an anchor motif of Mub from *L. acidophilus* NCFM (Figure 3.2). The cassette contains *Ppgm*, one of the strongest promoters in lactobacilli (Duong, T., *et al.*, 2010). All cassette sequences were codon optimized based on the codon usage table of *L. acidophilus* ATCC 4356 (http://www.kazusa.or.jp/codon/).

In order to select the restriction endonucleases, the enzyme site was limited to one location within the multi-cloning site of the vector (enzyme site not found in insert), mandatory buffer activity of 100%, and affordability. Care was taken to not select restriction endonucleases
with compatible ends, in order to avoid self-ligation. In the case a restriction enzyme site was identified more than once between the vector and the insert and a suitable restriction enzyme could not be identified, codon optimization was performed. New England Biolabs (NEB) restriction endonucleases were chosen to be compatible within Buffer System 4. Restriction enzyme sites were manually added to the 5’ and 3’ ends of the synthesized sequences for ease of cloning (Figure 3.4, Figure 3.6, and Figure 3.8 – underlined).

The pMJM-13 and pMJM-14 cassettes were synthesized and cloned via the *SacII/BamHI* sites into pMJM-8 by Genewiz, Inc. (South Plainfield, NJ). The pMJM-15 cassette was synthesized and cloned via the *BamHI/Apal* sites into pMJM-8 by Genewiz, Inc. The pMJM-8 plasmid was derived from pGK12, with a multi-cloning site (MCS) from pBluescript and an Em resistance gene (Figure 3.1). The resulting vector pMJM-13 (Figure 3.3) contains the secretion (S1709) and anchor (A1392) signal sequences of Mub from *L. acidophilus* NCFM (Figure 3.4), while the vector pMJM-14 (Figure 3.5) contains only the secretion signal sequence of Mub (S1709) (Figure 3.6). The vector pMJM-15 (Figure 3.7) contains the secretion (S1392) and the anchor (A1709) signal sequences of Mub along with the *S. enterica* serovar Typhimurium *fliC* gene (Figure 3.8).

**Transformation and Verification**

Lyophilized plasmid samples (4 µg) received from Genewiz, Inc. were spun down, resuspended in 20 µL molecular grade water, allowed to stand for 5-10 min on ice, vortexed, and stored at -20°C. From the resuspended stock solution (0.2 µg/µL), 2 µL of working solution (200 pg/µL) was used in the electroporation procedures.
The commercially synthesized vectors were transformed into either *E. coli* MC1061 or *E. coli* Top10. Transformants were plated on LB agar supplemented with the appropriate antibiotic, Em (150 µg/mL), Am (50 µg/mL), or Km (50 µg/mL). Colony PCR was conducted to identify successful transformants.

Purified plasmids pMJM-8, pMJM-13, and pMJM-14 were introduced into *L. acidophilus* NCFM, *L. acidophilus* ATCC 4356, *L. crispatus* CC1-1, *L. crispatus* JCM5810, *L. gallinarum* ATCC 33199, and *L. helveticus* CNRZ32 by electroporation. Lactobacilli transformations were conducted as described by Luchansky, *et al.* (1988; Appendix C). Briefly, lactobacilli cells were made electrocompetent using 3.5 × SMEB buffer (1 M sucrose and 3.5 mM magnesium chloride, pH 7) and electroporation was conducted at 2.5 kV using an Electroporator 2510 (Eppendorf, Westbury, NY, USA). After 16 h incubation in MRS broth, the electroporated samples were spread plated on MRS agar supplemented with Em (5 µg/mL). Large, cream-colored colonies grew within two days and were transferred three times in MRS broth containing Em (5 µg/mL).

Recombinant *Lactobacillus* strains were confirmed through the plasmid isolation protocol (O’Sullivan and Klaenhammer, 1993; Appendix D) and subsequent PCR. The PCR reaction conditions used are as follows. The 50 µL reaction mixture began with an initial denaturation cycle of 94°C for 5 minutes; denaturation at 95°C for 30 seconds, annealing at 50°C for 1 minute, elongation at 72°C for 2 minutes for 35 cycles; heated to 72°C for 10 minutes; and stored at 4°C (Biorad MyCycler™, Hercules, CA). The primers (pMJM13&14A and pMJM14R - BamHI) were designed using Clone Manager 9 (Sci-Ed Software) and were used to amplify a 1,434 bp internal region of pMJM-13 and a 951 bp internal region of pMJM-14 (Table 3.3). A series of forward primers were designed to amplify the internal region of pMJM-15 (Table 3.3). DNA fragments were purified from agarose gels using the Zymoclean Gel DNA Recovery Kit.
(Zymo Research, Orange, CA) and submitted for sequencing at the UIUC Core Sequencing Facility (Urbana, IL).

**Protein Preparation and Western Blotting**

Protein expression of the successful transformants was evaluated using a Western Blot Assay. Mid-log phase (OD$_{600}$=0.5-0.8) cultures were harvested by centrifugation at room temperature, and sent for evaluation by collaborators at Washington State University. Broth grown cultures were prepared in three manners, broth culture supernatant, sonicated supernatant, and resuspended pellet. For the sonicated supernatant, the initial bacterial pellets were resuspended in PBS and sonicated for 10 min. The resuspended pellet in PBS was recovered from the post-sonicated sample, due to difficulty in centrifuging the sample. All three samples were mixed with 1× laemelli buffer and boiled 10 min (>95°C) before running the samples on polyacrylamide gels for blotting (Figure 3.9, Figure 3.10). The protein sequence of *C. jejuni* flpA Domain II is predicted to have a size of 11 kD.

Supernatant of recombinant *Lactobacillus* strains in MRS (MJM272, MJM273, and MJM275) and MRS (negative control) was lyophilized to evaluate the presence of the secreted protein. Overnight *Lactobacillus* cultures were passed into 100 mL of MRS and grown to 0.5 OD$_{600 \text{nm}}$. Samples of 25 mL were aliquoted into 50 mL conical tubes and centrifuged at 3,200 × g at 4°C for 10 min. The supernatant was transferred into a new 50 mL conical tube and the centrifugation steps repeated. The final supernatant sample was lyophilized over 72 h in a vacuum freeze-dry system (Labconco, Kansas City, MO). The sample was frozen at -20°C until further analysis. The lyophilized sample was re-constituted in 1 mL PBS and centrifuged at 3,220 × g at 4°C for 30 min. The cell pellet and supernatant were then separated for analysis,
with the cell pellet re-suspended in 50 μL PBS. The two samples were mixed with 1× laemelli buffer and boiled 10 min (>95°C) before analysis by SDS-Page (results not shown).

**Plasmid Construction Verification**

B-glucuronidase (GUS) is widely used as a reporter gene to evaluate promoter activity and subsequent gene expression. The *Lactobacillus gasseri* gusA is more active at acidic pH than its *E. coli* counterpart (Russell & Klaenhammer, 2001). The mutant *L. gasseri* gusA3 produces β-glucuronidase with increased activity in neutral pH ranges (Callanan et al., 2007). The *L. gasseri* β-glucuronidase (GusA3) reporter gene GenBank (AF305888) was synthesized (Genewiz, Inc.) and cloned via the XbaI/BamHI sites into the cloning vector pUC57 to construct pMJM-27 (Figure 3.11). The vector pMJM-27 was transformed into *E. coli* MC1061 and the plasmid isolated from successful transformants plated on LB supplemented with Em (150 μg/mL).

The vectors pMJM-14 and pMJM-27 were enzyme digested at the XbaI/BamHI sites according to standard procedures. The enzyme digested plasmids underwent gel electrophoresis and were excised from the agarose gel, followed by gel extraction and purification steps. GusA3 was cloned via the XbaI/BamHI sites into pMJM-14 to construct pMJM-28 (Figure 3.12) to evaluate expression of *Ppgm*. As previously described, the vector (pMJM-28) was transformed into *E. coli* MC1061, grown overnight, and the plasmid isolated then sequence verified with primers GusA3_4681, GusA3_5052, GusA3_5470, and GusA3_5860 (Table 3.3). The vector (pMJM-28) was transformed into *Lactobacillus* strains (MJM 7, MJM 39, MJM 207, and MJM 208) and transformants were plated on MRS agar supplemented with Em (5 μg/mL) and 5-Bromo-4-chloro-1H-indol-3-yl β-D-glucopyranosiduronic acid (X-gluc). The blue colonies were
recovered and the plasmids isolated for sequence verification. An overnight culture (50µL) was centrifuged for 5 min at 8,327 × g and reconstituted with PBS, then mixed with 1× laemelli buffer and boiled 10 min (>95°C) before running the samples on polyacrylamide gels for blotting. The protein sequence of *L. gasseri gusA3* is predicted to have a size of 70 kDa.

**Cell Wall Binding Domain**

To evaluate the cell wall binding of a heterologous protein to the surface of lactobacilli, additional cassettes were constructed that employ three cell wall binding domains (Figure 3.13). The cell wall binding domains selected include *L. gasseri* ATCC 33323 LysM domain-containing protein (*L. gasseri* LysM; NCBI Ref. YP_814716.1); *L. gasseri* ATCC 33323 Lysozyme M1, Bacterial SH3 domain (*L. gasseri* Lysozyme SH3; NCBI Ref. YP_814010.0); and *L. lactis* subsp. *cremoris* MG1363 AcmA protein (*L. gasseri* AcmA; GenBank CAL96887.1). The *L. gasseri* LysM domain and *L. gasseri* Lysozyme SH3 domains were predicted in the literature (Kleerebezem et al., 2010) based on identification in the LocateP Database (www.cmbi.ru.nl/). The *L. lactis* subsp. *cremoris* cell wall binding protein region of the AcmA protein was identified in separate literature (Raha et al., 2005). Proteins of interest were then queried under the NCBI Conserved Domain Search and evaluated for gene product, family, and relevant description. The sequences of the genes of interest (*flpA* DII and enhanced green fluorescent protein (EGFP)) and the cell wall binding domains (*L. gasseri* LysM, *L. gasseri* Lysozyme SH3, and *L. lactis* subsp. *cremoris* AcmA) were codon optimized based on the codon usage table of *E. coli* (www.idtdna.com/).

The cassettes were synthesized as gBlocks® Gene Fragments by IDT and cloned with blunt ends into pUC19 digested with *SmaI*. This ligation formed the following plasmids, pMJM-
30 (pUC19 with \textit{C. jejuni flpA} DII), pMJM-31 (pUC19 with \textit{L. gasseri} LysM), pMJM-32 (pUC19 with \textit{L. gasseri} Lysozyme SH3), and pMJM-33 (pUC19 with \textit{L. lactis} subsp. \textit{cremoris} AcmA) (Table 3.2). Electrocompetent \textit{E. coli} Top10 cells were transformed and successful transformants identified using blue-white screening of cells on LB agar supplemented with Am (50 µg/mL), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and IPTG. The isolated and purified plasmids were enzyme digested with the appropriate restriction enzymes then underwent gel electrophoresis and agarose gel purification.

Enzyme digested samples underwent a three-piece ligation reaction including the cell wall binding domain cut via \textit{XhoI}/\textit{HindIII}, the \textit{C. jejuni FlpA} cut via \textit{BamHII/XhoI}, and the vector cut via \textit{BamHII/HindIII} (pRSET-B, Invitrogen, Carlsbad, CA, USA). These ligation procedures constructed six vectors that contain either \textit{flpA} DII or EGFP, with one of three cell wall binding domains (\textit{L. gasseri} LysM, \textit{L. gasseri} Lysozyme SH3, or \textit{L. lactis} subsp. \textit{cremoris} AcmA). The vectors are represented schematically along with the expression cassette detailed for pMJM-37 (Figure 3.14, Figure 3.15), pMJM-38 (Figure 3.16, Figure 3.17), pMJM-39 (Figure 3.18, Figure 3.19), pMJM-40 (Figure 3.20, Figure 3.21), pMJM-41 (Figure 3.22, Figure 3.23), and pMJM-42 (Figure 3.24, Figure 3.25). Following transformation of electrocompetent \textit{E. coli} BL21 (DE3) cells, successful transformants were selected on LB agar supplemented with ampicillin (50 µg/mL) and the isolated plasmid then purified for sequence verification.

The pRSET-B expression vector was chosen for high-level recombinant protein expression in \textit{E. coli}. Integral features of the vector include T7 promoter, ribosome binding site, N-terminal (6xHis) tag, T7 gene 10 sequence, N-terminal Xpress\textsuperscript{TM} epitope tag, enterokinase cleavage site, multiple cloning site, T7 reverse priming site, and an ampicillin resistance gene (β-lactamase). This cassette utilized the inducible T7 promoter to express the \textit{C. jejuni FlpA}
Domain II. Induction was achieved by the addition of isopropyl β-D-thiogalactoside (IPTG). The cassette contains a polyhistidine (6xHis) tag region and Xpress™ epitope for ease of identification. Several of the constructed plasmids are derivatives of pRSET-B (Table 3.2).

The green fluorescent protein (GFP) serves as a tool for evaluation of protein expression. In particular, pEGFP-N1 contains a mutant of the wild-type GFP allowing for greater expression in mammalian cells and brighter red fluorescence (Invitrogen). For verification, an additional cassette was created with EGFP and the three cell wall binding domains (Figure 3.13). The primers LZ_EGFP F and LZ_EGFP R-XhoI (Table 3.3) were used to amplify a 719 bp internal region of pEGFP-N1. The primers AB_FlpA F and AB_FlpA R (Table 3.3) were used to amplify a 295 bp internal region of pMJM-37, pMJM-38, and pMJM-39. The primers pRSETB_F and pRSETB_R (Table 3.3) were used to amplify a 286 bp internal region of pRSETB.

_E. coli_ BL21 (DE3) containing pMJM-37 – pMJM-42 were evaluated for protein expression with a Western Blot Assay. Culture samples were mixed with 1× laemelli buffer and boiled 10 min (>95°C) before running the samples on polyacrylamide gels for blotting. The protein sequence of EGFP is predicted to have a size of 27.3 kDa. The protein sequence of LysM is predicted to have a size of 7.2 kDa. The protein sequence of Lysozyme SH3 is predicted to have a size of 27.2 kDa. The protein sequence of AcmA is predicted to have a size of 22.3 kDa.

For purification of the recombinant protein fragment and binding to _Lactobacillus_ spp., the cell culture was harvested 2-3 h following induction and resuspended in 1×PBS (Raha et al., 2005). The cells were then lysed and centrifuged at 10,000 × g for 15 min. The supernatant was applied to a Ni²⁺ affinity column and the recombinant protein eluted to calculate the final
concentration. Exponentially growing *Lactobacillus* spp. were centrifuged and resuspended in 600 µl of MRS broth. Then 200 µl of purified *flipA* DII was added to the cells and incubated at 30°C for 2 h. Following incubation, the mixture underwent centrifugation at 2,000 × g for 10 min and washing of the cell pellet three times with 1 mL 1×PBS. Binding of the purified protein and attached domains to *Lactobacillus* spp. was evaluated by ELISA and fluorescence microscopy.

### 3.4 Results

Previous research indicated the ability for *L. acidophilus* NCFM, *L. crispatus* JCM5810, and *L. helveticus* CNRZ32 to competitively exclude *C. jejuni* in the GIT of broiler chickens (Neal-McKinney *et al*., 2012). Additionally, *Lactobacillus* spp. have been shown to impart beneficial effects which include the reduction of pathogenic colonization. Lactobacilli produce organic acids and bacteriocins, modulate the host immune response, and manipulate the host microbiome. Therefore, the four selected lactobacilli strains were chosen as host strains for recombinant protein expression.

An important consideration when designing recombinant protein expression vectors is codon optimization. This additional step advantageously alters the codons to those preferred by the targeted bacteria, while maintaining the amino acid sequence of the synthesized protein. Attention must be spent verifying the codon optimized sequence in order to confirm the presence of the correct amino acid sequence as well as restriction enzymes. Unfortunately, the first synthesis of the cassette design was not properly verified before synthesis. The restriction enzyme sites were found codon optimized out of the sequence. Therefore, the plasmid was unable to be digested at the desired restriction enzyme sites and was deemed unusable.
Fortunately, Genewiz, Inc. was contacted willing to assist with resolving this error and the restriction enzyme sites were corrected and shipped following verification of sequence analysis.

Re-growth of recombinant lactobacilli strains, particularly MJM270 and MJM271, from 12.5% glycerol stock stored at -80°C was difficult at times. Therefore, the Em concentration was reduced from 5 µg/mL to 2.5 µg/mL. Previously 2.5 µg/mL Em was sufficient to prevent background growth of untransformed lactobacilli. In order to confirm recombinant protein expression *in vitro*, a Western Blot Assay was performed. The estimated molecular mass of the recombinant protein expressed on the cell surface is 11 kDa. Unfortunately, the Western Blot Assay did not indicate the presence of the FlpA DII protein from all transformed lactobacilli grown in MRS broth supplemented with 2.5 µg/mL Er (Figure 3.9, Figure 3.10).

While this protein has a low molecular weight (>15 kDa), it should still be able to be visualized using a Western Blot Assay. Therefore, systemically each strain was tested for the presence of the FlpA DII cassette. It was discovered that the *L. crispatus* strains (MJM 270 and MJM 271) found to be lacking the plasmids pMJM13 and pMJM14, respectively. This explains the difficulty of the strains to grow in the Em conditions originally intended (5 µg/mL). All other transformed lactobacilli strains that were confirmed to contain pMJM-13 and pMJM-14; however, it is not clear why the recombinant protein was not expressed or detected. Additional steps were then taken to evaluate the plasmid construction and promoter expression.

Supernatant of the recombinant *Lactobacillus* strains in MRS (MJM272, MJM273, and MJM275) and MRS (negative control) were lyophilized and resuspended in PBS to evaluate with SDS-Page. Strains that contained pMJM-13 and pMJM-14 did not indicate expression of a protein in the predicted protein size. This is not surprising, as secreted proteins are difficult to...
express in media in sufficient concentrations for detection. Additional steps will be taken to precipitate the protein following an acetone protocol.

Further lactobacilli transformations had isolates present on MRS agar with Em (5 µg/mL), yet plasmid isolation protocols and gel electrophoresis of PCR product did not indicate the presence of a plasmid. The concentration of Em in the MRS agar plates was verified to select for only recombinant lactobacilli. This approach was postponed until further evaluation of the plasmid construct.

To evaluate the construction of the cassette, *L. gasseri gusA3* was ligated via the *XbaI/BamHI* sites into pMJM-14. Then pMJM-28 was transformed into *E. coli* MC1061 and an isolate was obtained. Upon amplification of pMJM-28 in *E. coli* MC101, the plasmid was purified and remains to be transformed into *Lactobacilli* strains. Expression of GusA3 by lactobacilli strains will indicate the functionality of the vector construction.

### 3.5 Discussion

In the poultry industry, strategies to reduce *C. jejuni* colonization in broiler chickens remain limited. *Lactobacillus* spp. displaying heterologous proteins function as both a probiotic and as a vaccine for stimulation of the host GIT. Anchoring of a foreign antigen to the cell wall produces a larger antigen response, than secreted or cytoplasmic protein expression (Bermudez-Humaran *et al.*, 2004, Norton *et al.*, 1996). A vector for *C. jejuni FlpA DII* protein expression anchored (pMJM-13) or secreted (pMJM-14) onto the wall of several *Lactobacillus* spp. was constructed. Stimulation of the broiler chicken immune system prior to *C. jejuni* challenge would contribute to the reduction of pathogen colonization and incidence of human illness.
The cassette design was modeled after a successful dual cell surface display of HIV-1 gag and Salmonella enterica serovar Typhimurium in Lactobacillus acidophilus NCFM intended for vaccine delivery (Kajikawa et al., 2012). Production of the Gag protein was confirmed by Western Blotting. BALB/c mice were immunized intragastrically with the recombinant Lactobacillus strains. Following collection of lymphoid tissues and organs for detection of IgA-producing cells, it was demonstrated that induction of Gag-specific IgA-producing cells was present at the local intestinal mucosa. Only recombinant bacterial strains displaying Gag and FliC elicited this immune response, as recombinant strains displaying Gag only did not promote this effect. Based on the success of the in vitro and in vivo experiments with the dual display of Gag and FliC, we constructed a similar cassette that displayed C. jejuni FlpA Domain II rather than HIV-1 Gag. Electing to exclude the FliC adjuvant, we focused on the expression of FlpA. Even without the FliC adjuvant present, the authors were able to confirm production of the Gag protein by Western Blotting. However, our similarly modeled construct was unable to express FlpA by Western Blotting. Whether due to error in the sequence or a frameshift mutation, this issue has not yet been fully resolved.

To evaluate the cassette construct, gusA3 was ligated into pMJM14 and remains to be transformed into Lactobacillus strains and evaluated for protein production by Western Blotting. The GusA3 reporter gene was previously cloned for characterization of operons implicated in transport and catabolism of fructooligosaccharides (FOS), lactose (lac), trehalose (tre) and genes directing glycosis (Duong et al., 2011). The construct based on phosphoglycerate mutase (pgm) promoter was constitutively highly expressed. Thus, Ppgm has previously expressed GusA3 in Lactobacillus.
The strategy of recombinant *Lactobacillus* expressing FlpA through either secreted or anchored mechanisms was suspended in favor of attaching FlpA to *Lactobacillus* through various cell wall binding domains. While still in its infancy, the use of non-genetically modified organisms has great potential for use in the food industry. First, these strains cannot propagate the protein into subsequent generations, thus limiting concerns of horizontal gene transfer. Second, the attachment to the cell surface ensures the protein is present upon delivery, rather than assuming the protein is expressed while in the GIT. However, there are limitations as *in vivo* models remain limited and there exist concerns of structural degradation.

For the future, additional approaches include evaluation of the promoter strength. For recombinant bacterial cells, it’s imperative that a balance exists between basal metabolic functions and expression of the foreign antigen. In the case of anchoring proteins, too strong of a promoter may result in cell surface attachment of foreign antigens that inhibit the functionality of basic cell processes. In turn, the cell cannot balance environmental stresses with protein expression and may lyse. Selection of the appropriate promoter strength maintains this delicate balance. While *Ppgm* has been noted as one of the strongest promoters in *Lactobacillus*, alternative promoters may be considered to limit protein expression. Inducible promoters offer more flexibility for protein expression because they must be activated by either chemical or physical factors for protein expression to occur. Therefore, the selected lactobacilli strains may benefit from an inducible promoter over a constitutive promoter.

This project outlines two different mechanisms as an alternative strategy to current methods targeting the reduction of *C. jejuni* colonization in broiler chickens. The first includes the proven stimulation of GI host immune responses through exposure to probiotics (Avall-Jaaskelainen & Palva, 2005, Bernardeau *et al*., 2008, Borchers *et al*., 2009). The second focuses
on the cell wall binding of foreign antigens to the exterior surface of *Lactobacillus* cells to elicit a specific immune response. In both cases, the clear aim is to target and to stimulate host receptors for prevention of *C. jejuni* colonization. A mucosal live vaccine in mice utilized *L. casei* and *Lactococcus lactis* displayed E7 antigen (cell-binding domain from *L. casei* A2 phage lysine) as the cell wall anchor (Ribelles et al., 2013). This non-genetically modified lactic acid bacteria was vaccinated intranasally in mice and prevented HPV-16-induced tumor formation. We propose the novel approach of inoculating non-genetically modified lactobacilli strains displaying the *C. jejuni* FlpA DII protein for the express purpose to reduce *C. jejuni* colonization in broiler chickens.

### 3.6 Conclusion

While other researchers have successfully secreted and anchored foreign antigens on the surface of lactobacilli, we were unable to isolate and to verify successful transformants in this study. Additional work will be conducted to evaluate the plasmid construction and component sequences. For the future, we propose a non-genetically modified lactic acid bacteria for vaccination and reduction of *C. jejuni* colonization of broiler chickens. Upon optimization of this strategy, conducting *in vivo* experimental procedures will evaluate the efficacy of *Lactobacillus* strains as vaccine vectors.
3.7 References


### 3.8 Tables and Figures

#### Table 3.1 List of bacterial strains

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant Characteristics</th>
<th>Source or reference</th>
</tr>
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<td></td>
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<tr>
<td>MJM7</td>
<td><em>L. acidophilus</em> NCFM</td>
<td>Klaenhammer Lab</td>
</tr>
<tr>
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<td><em>L. acidophilus</em> ATCC 4356</td>
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<td>Konkel Lab</td>
</tr>
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* In progress, remains to be constructed.
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<td>pMJM-15</td>
<td>Em&lt;sup&gt;R&lt;/sup&gt;, derivative of pMJM-8, <em>S. enterica</em> serovar Typhimurium</td>
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<td>pMJM-27</td>
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<td>pMJM-32*</td>
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<td>pMJM-33*</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, pUC19 with blunt-end ligation of <em>L. lactis</em> subsp. cremonis AcmA</td>
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<td>pMJM-37*</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, pRSET-B with ligation of <em>C. jejuni flpA</em> DII and <em>L. gasseri</em> LysM</td>
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<td>pMJM-38*</td>
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<td>pRSET-B</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, expression vector</td>
<td>BD Biosciences</td>
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<td>pEGFP-N1</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, encodes the GFPmut1 variant</td>
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* In progress, remains to be constructed.
Table 3.3 Primer sequences for plasmid gene amplification

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<td>pRSETB_R (R)</td>
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Figure 3.1 Schematic of plasmid pMJM-8. Multi-cloning site indicated by cluster of restriction enzyme sites.
**Figure 3.2.** Gene map of an expression cassette for *C. jejuni* *flpA* and *S. enterica* serovar Typhimurium *fliC*. Figure A indicates cassette in pMJM-13, Figure B indicates cassette in pMJM-14, and Figure C indicates cassette in pMJM-15. *P*<sub>pgm</sub>, promoter region of *pgm* gene from *L. acidophilus* NCFM; R, region encoding ribosome binding site of Mub from *L. acidophilus* NCFM (LBA1709 or LBA1392); S<sub>1709</sub>/S<sub>1392</sub>, region encoding the signal sequence of Mub (LBA1709 or LBA 1392); *flpA*, *flpA* gene from *C. jejuni*; A<sub>1709</sub>/A<sub>1392</sub>, region encoding the anchor region of Mub (LBA1709 or LBA 1392); *fliC*, *fliC* gene from *S. enterica* serovar Typhimurium.
Figure 3.3 Schematic of plasmid pMJM-13. Secreted and anchored *C. jejuni* FlpA DII. Insert represented as ORF-1.
**Figure 3.4** Sequence of expression cassette in pMJM-13. $P_{pgm}$, promoter region of $pgm$ gene from *L. acidophilus* NCFM; RBS, region encoding ribosome binding site of Mub from *L. acidophilus* NCFM (LBA1709); $S_{1709}$, region encoding the signal sequence of Mub (LBA1709); $flpA$ DII, $flpA$ gene from *C. jejuni*, domain II; $A_{1392}$, region encoding the anchor region of Mub (LBA1392). Restriction sites are underlined.
**Figure 3.5** Schematic of plasmid pMJM-14. Secreted *C. jejuni* FlpA DII. Insert represented as ORF-1.
Figure 3.6 Sequence of expression cassette in pMJM-14. $P_{pgm}$, promoter region of $pgm$ gene from *L. acidophilus* NCFM; RBS, region encoding ribosome binding site of Mub from *L. acidophilus* NCFM (LBA1709); $S_{1709}$, region encoding the signal sequence of Mub (LBA1709); flpA DII, flpA gene from *C. jejuni*, domain II. Restriction sites are underlined.

```
CCGCGGTGCGGACAAGTAGAAATAACACTAAACAAAAAATACAAAAATTTCTTTTTTG
| SacI     | Pgm   |
TTTTTCATGATTTTTACACTTCTCTTAGTAAGCTTTTGTTATAAGTTAGCACAACAAA
|          |       |
AGCAGAAAATAAAAAGTAGAAAATAAAAAAAAAAGATTTTTTGCCCATATCTCTAT
|          |       |
GAAAAAAAACTGTGAAATGTGAAATATGGATGAACATTGAATTAAAAGGAGA
|          |       |
RBS
AAATTATGGGACAAGGAAGGAGTAAAAAATAGATTCTCTCATCAGAAAGTTATCAA
|          |       |
S1709
CTGGTTTGGCTACAGTATTTCTTGGTTCAATTTTTCTTCTTTGGACAACGGACAGAC
TGTACAGGCAGACTCAGTTGAGCCATCTAGACGTGGAGGGGCTTTTCTTTGTT
| XbaI  C. jejuni flpA DII |
AGGGCAGTAACTAATTTTGCTAACCCTATCAAGTTGATCTGGCTCTACACCTCG
ACTTCCGTGTGACTACATCATGTAGCTACAAAGGGTGATGACAAAGGAGTT
CAAGAAATTTGCAAGAGGTAAAGAAACGTTAACAACGCTGAGTACATCGATTCAAGAT
TTGAAACAAAAGAAATTCAATCATATAGAATTATCGCTGTAAGTTTTATGAAT
CAAGTCAGGTAGTTCACAAGTTGATCTCAACTATAAGGATCC
| BamHI    |
```
Figure 3.7 Schematic of plasmid pMJM-15. *fliC* gene from *Salmonella enterica* serovar Typhimurium. Insert represented as ORF-1.
**Figure 3.8** Sequence of expression cassette in pMJM-15. RBS, region encoding ribosome binding site of Mub from *L. acidophilus* NCFM (LBA1392); S1392, region encoding the signal sequence of Mub (LBA 1392); *fliC*, *fliC* gene from *S. enterica* serovar Typhimurium; A1709, region encoding the anchor region of Mub (LBA1709). Restriction sites are underlined.
Figure 3.8 Continued.

```
GCTGCTTTGACTGTGCAGGTTACTGGAAAATGTTAGTACAGCTCTACTGAAGCTGGAGGAACTCTAGGTTAGTTTGCTGTAAGGTGGAAGCTGACGCTATGCAAGCTAGCTGACGCTATAGCAAGCTGGAGGAGG
TACACTGCTGACGACTGTCTACTCAACCAAGCTGAAACCTATTACGAGCTGGTAAACACTACGCAGCTGTACGCTGCAACAA
CTACAGAAAAACCCATGCAGAAGATTGACGCTGCATTGGCCTCAGGAGTACACATT
GCCTAGTGATTGGTGCGACTTTAAAAACGGTTCTCCTCAGCTATCAACAAACTTG
GGAAACACTGTAACCAACTTAACCTAGTGCAAGAGTCTGATCGAAGATGACTTGCTACTGAGGTTTCAAAATAGTCACGACGTCGACTGCTCAACAGGAGCGTTCCTTA
```

**XhoI** A1709

```
TCGAGGTTTCCAACTGTAACCTCAAACCTGACCCTGGAAAAAGGACACACAAAGTTTCTTA
```

**Apal**

```
TAAGGGGCC
```

STOP
Figure 3.9 Protein Verification of *C. jejuni* FlpA DII. Figure A indicates samples from the sonication supernatant. Figure B indicates sonicated cell pellet resuspended in PBS. Samples are as follows (1) *Campylobacter* whole cell lysate (WCL) 1:1000, (2) *L. crispatus* MJM206, (3) *L. crispatus* MJM270, (4) *L. crispatus* MJM271, (5) *L. gallinarum* MJM208, (6) *L. gallinarum* MJM272, (7) *L. gallinarum* MJM273, (8) Blank, and (9) *Campylobacter* WCL 1:1000.
Figure 3.10 Protein Verification of *C. jejuni* FlpA DII. Samples are as follows (1) *L. acidophilus* ATCC 4356 (MJM 114), (2) *L. gallinarum* ATCC 33199 (MJM 272), (3) *L. gallinarum* ATCC 33199 (MJM 273), (4) *L. gallinarum* ATCC 33199 (MJM 275), (5) *L. acidophilus* NCFM (MJM330), (6) *L. acidophilus* ATCC 4356 (MJM322), (7) *L. acidophilus* NCFM (MJM332), and (8) *Campylobacter* WCL 1:1000.
Figure 3.11 Schematic of plasmid pMJM-27. Derivative of pUC57 with *L. gasseri* GusA3 (Callanan *et al.*, 2007). Insert represented as ORF-1.
Figure 3.12 Schematic of plasmid pMJM-28. Derivative of pMJM-14 with *L. gasseri* GusA3 (Callanan *et al.*, 2007). Insert represented as ORF-1.
**Figure 3.13** Gene map of an expression cassette for cell wall binding domains and genes of interest ligated into pRSET-B. Figure A indicates the cassette with *C. jejuni* flpA Domain II and Figure B indicates the cassette with EGFP sequenced from pEGFP-N1 (GenBank U55762). *P*<sub>T7</sub>, promoter region of the T7 bacteriophage; R, region encoding ribosome binding site; ATG, translational initiation site for fusion protein; N-terminal 6xHis tag, allows for purification of fusion protein on metal-chelating resins and allows for antibody detection of fusion protein; N-terminal Xpress<sup>TM</sup> epitope tag, allows detection of fusion protein by the Xpress<sup>TM</sup> antibody; EK, enterokinase cleavage site to remove fusion tag; flpA, flpA gene from *C. jejuni*; EGFP, enhanced β-glucuronidase from pEGFP-N1; Cell wall BD, cell wall binding domains on C-terminal.

![Diagram A](image1)

![Diagram B](image2)
Figure 3.14 Schematic of plasmid pMJM-37. Derivative of pRSET-B with \(flpA\), \(flpA\) gene from \(C.\) jejuni; \(LysM\), LysM domain from \(L.\) gasseri. Insert represented as ORF-1.
Figure 3.15 Sequence of expression cassette in pMJM-37. Features found in pRSET-B (T7 promoter, RBS (Ribosome binding site), N-terminal Polyhistidine (6xHis) region, T7 gene 10 leader, N-terminal Xpress™ Epitope, EK recognition site (Enterokinase cleavage site), T7 reverse priming site; flpA, flpA gene from C. jejuni; LysM, LysM domain from L. gasseri. Restriction sites are underlined.

```
TAATACGACTCACTATAGGGAGACCACAACGCGTTTCCCTCTAGAAAATAATTGT
T7 promoter
TAACTTTAGAAGGAGATATACATATGCGGGTTCTCATCATCATCATCATCATGG
Polyhistidine (6xHis) region
TATGGCTAGCATGACTGGTGACAGCAAATGGGTCGGGATCTGTAAGACGATGAC
Xpress™ Epitope
GATAACGATCCGATGCGCTTTGGAGGCGGTTCGTCGTACTACAGGCCGTCAACAACT
BamHI flpA
TACGAAATCGCATTAAATTATCTGCGCTCCGATCCCGACTTTTCGTCTGACTCC
TATACTTGAGCGTACCAAGGGCGACGATAAAAGATTTCAAGAAAATTGCGGAAG
TGAAAAACCGCTGAAATGCAATATATGACAGCTCAAAACGAATGAGAA
TTCAGTACCGATGGTACTTTAATGCGCATTAAATCGGGAATCGAATCTCA
AGTTGTGCCTCTCTCTCGAGACCTGGCAGAACTATTTCTGTAATAGCAATTCA
XhoI LysM
CAAAGTCAGGGCACCAGTAGCTATACCGTGAGGGGCGTATAGCCGTGAGCAAG
ATTGCACCGAAACAACACATCCGTCCATCATTTGAAACAGCTGAATGGCTGG
AAAGTGCTGACAGACATTCTACGCTAGTCAATCAAAATAAGGAAGGCTTGATCC
HindIII
GGCTGCTAAGAACGCGAAAGGAGAATGAGTTGGTGCTGGCACCAGCTGAGCA
T7 reverse priming site
ATAACTAG
```
Figure 3.16 Schematic of plasmid pMJM-38. Derivative of pRSET-B with \( flpA \) gene from \( C. \) \( jejuni; \) Lysozyme SH3 domain from \( L. \) \( gasseri \) (LGAS0160). Insert represented as ORF-1.
Figure 3.17 Sequence of expression cassette in pMJM-38. Features found in pRSET-B (T7 promoter, RBS (Ribosome binding site), N-terminal Polyhistidine (6xHis) region, T7 gene 10 leader, N-terminal Xpress™ Epitope, EK recognition site (Enterokinase cleavage site), T7 reverse priming site; flpA, flpA gene from C. jejuni; SH3, Lysozyme SH3 domain from L. gasseri (LGAS0160). Restriction sites are underlined.
Figure 3.17 Continued.

CAACCTGTCCATGCTAATACCAATAATAATGTTAATAATAACTGGAACGAAACAAAA
TGGCGTCTTCAATTACAGGTGGTGTATGATCAATCTGCTACGCTTACGCTAGCAACAACT
CAAAGGTTATTGCACTTCTGCCGACAAATACTGAAATTAAGTATGATGCTCATATCGT
ACCACCGGCCATATACCTGGCGTCGGCCAACCCGCCGAGGAATGGTCAGTATGGCT
ATTTGTCGTCGCTGAAACAATCAGGCGTGGGTACCTATCGGAAAGCTTGATCC

GGCTGCTAAACAAGCCCGAAAGGAAGCTGAGTGGCTGCTGCGCACCAGCTGAGCA

ATAACTAG
Figure 3.18 Schematic of plasmid pMJM-39. Derivative of pRSET-B with \textit{flpA} gene from \textit{C. jejuni}; AcmA domain from \textit{L. lactis} subsp. cremoris. Insert represented as ORF-1.
Figure 3.19 Sequence of expression cassette in pMJJ-39. Features found in pRSET-B (T7 promoter, RBS (Ribosome binding site), N-terminal Polyhistidine (6xHis) region, T7 gene 10 leader, N-terminal Xpress™ Epitope, EK recognition site (Enterokinase cleavage site), T7 reverse priming site; flpA, flpA gene from C. jejuni; AcmA, AcmA domain from L. lactis subsp. cremoris. Restriction sites are underlined.
Figure 3.20 Schematic of plasmid pMJM-40. Derivative of pRSET-B with *EGFP* gene from pEGFP-N1; LysM domain from *L. gasseri*. Insert represented as ORF-1.
Figure 3.21 Sequence of expression cassette in pMJM-40. Features found in pRSET-B (T7 promoter, RBS (Ribosome binding site), N-terminal Polyhistidine (6xHis) region, T7 gene 10 leader, N-terminal Xpress™ Epitope, EK recognition site (Enterokinase cleavage site), T7 reverse priming site; EGFP, EGFP gene from pEGFP-N1; LysM, LysM domain from L. gasseri. Restriction sites are underlined.
**Figure 3.22** Schematic of plasmid pMJM-41. Derivative of pRSET-B with *EGFP* gene from pEGFP-N1; Lysozyme SH3 domain from *L. gasseri* (LGAS0160). Insert represented as ORF-1.
Figure 3.23 Sequence of expression cassette in pMJM-41. Features found in pRSET-B (T7 promoter, RBS (Ribosome binding site), N-terminal Polyhistidine (6xHis) region, T7 gene 10 leader, N-terminal Xpress™ Epitope, EK recognition site (Enterokinase cleavage site), T7 reverse priming site; *EGFP*, *EGFP* gene from pEGFP-N1; *SH3*, Lysozyme SH3 domain from *L. gasseri* (LGAS0160). Restriction sites are underlined.
Figure 3.23 Continued.

CGGTCTTAATACCTCGAACAAGTGGTGGATACCAACTGGTTAAACAAAGAAG
GCACGGTTACACCACAGCCGCCACATTAACCTGGTACCAGGTGCACTTACTAATC
TTCCCATATTGCCCCAGTTACCTGCAAATTCAAGAAGTTAATATGACCGATATAAGTAC
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CTGGTAGGCGCGTGACGCAAGGGCAGTTTGCCCACTTTCAAGGCTAGCCCGGTG
GCCAATCATGTAACCAAACCCTACACAGAACCAGCCAAGCCAGGCCCCAAACAGT
AACAAACCGTCCCAAACCTGTCCATGCTAAATAAATAATGTGTAATAATAACTG
GACGAAACAAAAATGGCGCTTTCTTATTACAGGTTGGTGCTATCAATCTCGTACCAGG
GCTAGCAACCAACTCAAAGTTATGGAACCTTCTGCACGACAAATACTGAAATTAGT
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GGTCAGATGCTATTGCTGTCGCTAGAACAATCAGCGTGGGTACCTATC
GGAAGGTTTGGATCGCGGCTCGTAACAAAGCCCGAAGGAAGCTGAGTGGCTGCTG

**HindIII**

CCACCGCTGAGCAATAACTAG

**T7 reverse priming site**
**Figure 3.24** Schematic of plasmid pMJM-42. Derivative of pRSET-B with *EGFP* gene from pEGFP-N1; AcmA domain from *L. lactis* subsp. *cremoris*. Insert represented as ORF-1.
Figure 3.25 Sequence of expression cassette in pMJM-42. Features found in pRSET-B (T7 promoter, RBS (Ribosome binding site), N-terminal Polyhistidine (6xHis) region, T7 gene 10 leader, N-terminal Xpress™ Epitope, EK recognition site (Enterokinase cleavage site), T7 reverse priming site; EGFP, EGFP gene from pEGFP-N1; AcmA, AcmA domain from L. lactis subsp. cremoris. Restriction sites are underlined.
Figure 3.25 Continued.

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TGTGGGGTATTTCACACGGCTACGGTATTTCACCTGAGCTGAGTCTGCTGCAAGATCCAGTGCTGC
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CTAGGTCCACAAACTCTGGTGCTCAAATAATAGTGCAICCAACAAACCCGACCCAC
ATCCGTTACCACGGCACAACCCACCTCACAAACCCACCGTCCTAAGCTACGG
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AAAAAGCTTGATCCCGGCTGCTAAACAAAGCCCGAAAGGAGTGAATGGGTGCT
HindIII
GCCACCGCTAGAAATACGTAG
T7 reverse priming site
```
4.1 Abstract

_Campylobacter_ spp. are commensal, zoonotic bacteria that colonize the gastrointestinal tract of broiler chickens. Additionally, _Campylobacter jejuni_ is a leading foodborne pathogen that causes the human illness, campylobacteriosis, characterized by diarrhea, abdominal pain, cramping, and in rare cases Guillain-Barré Syndrome. Human exposure to _C. jejuni_ occurs primarily via cross-contamination and inadequate heating of retail chicken meat. _C. jejuni_ is estimated to contaminate over 60% of retail poultry meat. Targeted reduction of _C. jejuni_ on the poultry farm could lessen the risk of consumer exposure to _C. jejuni_ from retail poultry meat. The goal of this study was to develop a model for _C. jejuni_ colonization of broiler chickens, to utilize for future treatment strategies. Day-old broiler chickens were co-housed in stainless steel batteries and fed standard diet without bacitracin for three weeks. On day 14, chickens (n=15 per treatment group) were orally administered with $10^9$ CFU/mL of either _C. jejuni_ NCTC 11168, F38011, or PBS sham inoculum. Chickens were euthanized and necropsied on d 21 for direct- plating of the cecal contents on Mueller-Hinton blood agar and subsequent enumeration of _C. jejuni_. While unsuccessful in the first study, the second study indicated the capability of _C. jejuni_ to replicate and colonize the broiler chicken cecal crypts. Critical components for colonization include handling of the inoculum to ensure the presence of flagella for chemotaxis and motility purposes. Future studies may utilize this _C. jejuni_ challenge model to impart treatment strategies.
4.2 Introduction

*Campylobacter* spp. are characterized as microaerophilic, non-spore-forming, Gram-negative, with either a curved, S-shaped, or spiral rod. *Campylobacter* is native to the gastrointestinal tract (GIT) of a wide range of animals. The zoonotic pathogen *Campylobacter jejuni* is the most researched member species of the genus *Campylobacter*. *C. jejuni* easily colonizes broiler chickens as a commensal organism and serves as the primary source of *C. jejuni* for human infections. In humans, *C. jejuni* infection results in campylobacteriosis, an acute gastroenteritis that causes an estimated 845,000 illnesses annually in the United States (Batz *et al.*, 2011). *Campylobacter* spp. have been identified as the third leading foodborne pathogen in annual burden of disease, including data from cost of illness and the number of illnesses, hospitalizations, and deaths (Batz *et al.*, 2011). Therefore, developing novel strategies for the control of *C. jejuni* in broiler chickens is of great public interest.

Interestingly, broiler chickens are protected from *C. jejuni* colonization early in life due to the presence of maternal antibodies. However, maternal antibodies slowly diminish after three weeks and no longer protect the broiler chicks from colonization of the GIT by *C. jejuni* (Cawthraw & Newell, 2010, Sahin *et al.*, 2003). *C. jejuni* exploit a number of survival and colonization mechanisms to adapt to the ever-changing conditions of the chicken GIT (Hermans *et al.*, 2011a). Most importantly, the *C. jejuni* flagellum propels the organism through the GIT, overcomes host protective mechanisms, and promotes internalization into the deep cecal crypts. Mutant strains lacking formation of a functional flagellum exhibit dramatically decreased levels of internalization (Grant *et al.*, 1993). Multiple animal studies have demonstrated the necessity of *C. jejuni* motility and chemotaxis for effective colonization (Chang & Miller, 2006, Morooka *et al.*, 1985, Nachamkin *et al.*, 1993). The highest concentration of *C. jejuni* in broiler chickens
are found within the mucosal layer of the cecal crypts (between $10^6$ and $10^8$ colony-forming units (CFU)/g of cecal contents) (Allen et al., 2008, Beery et al., 1988, Hermans et al., 2012, Meade et al., 2009). Following colonization of *C. jejuni* in the broiler chicken cecum, broiler chickens shed the bacterium in feces, resulting in colonization of the entire flock within a few days. Interestingly, *C. jejuni* is a commensal organism in chickens and colonization is not marked by observable clinical symptoms of infection (Newell & Fearnley, 2003).

*C. jejuni* is commonly transmitted to humans via contamination of retail poultry meat. Spread of the bacterium often occurs during the defeathering and evisceration steps, as the carcasses are readily contaminated by fecal material (Hermans et al., 2011b). Over 60% of retail poultry products test positively for the presence of *Campylobacter* (Nannapaneni et al., 2005, Wong et al., 2007, Zhao et al., 2001). As a leading foodborne pathogen in the United States, *C. jejuni* in retail poultry meat products results in a $1.3$ billion annual cost of illness (Batz et al., 2011). In humans, campylobacteriosis results in undesirable abdominal pain, cramping, diarrhea, and in rare circumstances Guillain-Barré Syndrome (GBS). At the poultry farm, strategies for the reduction of *C. jejuni* colonization in broiler chickens remain underdeveloped.

Prior to implementation of treatment strategies to reduce *C. jejuni* colonization, the laboratory must successfully colonize broiler chickens with *C. jejuni*. Strain-to-strain variations are present amongst *C. jejuni*, with human isolates less likely to colonize chickens than poultry isolates (Korolik et al., 1998, Ringoir & Korolik, 2003). Additionally, broiler chickens must be orally gavaged with a higher inoculum of *C. jejuni* ($10^4$-$10^6$ CFU/mL), as compared to cloacal challenge ($<10^2$-$10^4$ CFU/mL) (Shanker et al., 1988). Previous research studies have successfully colonized the chicken GIT following oral inoculation (Beery et al., 1988, Konkel et al., 2007, Line et al., 2008, Van Deun et al., 2008). Therefore, our objective was to develop lab
specific protocols and procedures for the colonization of broiler chickens with *C. jejuni* and the recovery of *C. jejuni* from the cecal crypts.

4.3 Materials and Methods

Bacterial Strains and Growth Conditions

*Campylobacter jejuni* NCTC 11168 and F38011 (kindly provided by Dr. Michael Konkel, Washington State University, Pullman, Washington) were grown on Mueller-Hinton (Difco) broth supplemented with 1.5% agar (Fisher) and 5% bovine citrate blood (Quad Five, Ryegate, MT; MH blood agar) (Figure 4.1). Microaerobic growth occurred in a VWR incubator (Radnor, PA) maintained at 37°C with a gas composition of 85% nitrogen, 10% carbon dioxide, and 5% oxygen. Glycerol stock (stored at -80°C) of *Campylobacter* was streaked on MH blood agar and passed every 24-48 h before use as a primary culture.

Broiler Chickens

Day-old chickens were randomly divided into study groups and placed into cohabitated stainless steel batteries. Broiler chickens inoculated with *C. jejuni* were housed separately from control broilers, with an established workflow to reduce risk of cross-contamination. Water and standard diet without bacitracin were provided ad libitum. Corn and soybean meal was procured from the University of Illinois Poultry Farm, which has previously used this feed for the upbringing of broiler chickens and layer hens. Fecal material was collected in a removable metal tray below the wire mesh floor of the cage. The studies were conducted at the University of Illinois with experiments and procedures approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC; protocol no. 13059).
Study One – *Campylobacter jejuni* Colonization and 2′FL Treatment of Broiler Chickens

This study sought to colonize Ross 708 broiler chickens with *C. jejuni* and to evaluate 2′-Fucosyllactose (2′FL) as a treatment for reduction of *C. jejuni* colonization. Broiler chicks were randomly assigned a treatment group. The six treatment groups consisted of a (1) control group, *C. jejuni* free and no 2′FL, (2) *C. jejuni* challenged with no 2′FL dosage, (3) *C. jejuni* challenged with low 2′FL dosage (1 mg), (4) *C. jejuni* challenged with medium 2′FL dosage (10 mg), (5) *C. jejuni* free with high 2′FL dosage (100 mg), and (6) *C. jejuni* challenged with high 2′FL dosage (100 mg). Each treatment group was assigned three cages of birds, which each held five birds, for a total of fifteen birds within each treatment group (Table 4.3). With the exception of Group 1 which had four cages of birds for twenty total birds and Group 5 which had two cages of birds for ten total birds.

**Inoculation of *Campylobacter jejuni*.** At 14 days of age, broiler chickens were inoculated with *C. jejuni* NCTC 11168 via oral gavage of a 0.2 mL bacterial suspension (~10^9 CFU/mL). The bacterial suspension was prepared as follows; a frozen stock was passed twice on MH blood agar at 37°C under microaerobic conditions. Phosphate Buffered Saline (PBS) was added to the MH blood agar and a sterile loop was utilized to bring the colonies into suspension. Inoculum was normalized by measuring the optical density at 600 nm wavelength (OD_{600 nm}, Spec 21, Bausch and Lomb). To confirm inoculum concentration, serial dilutions (10^3-10^8 CFU/mL) were made before plating on MH blood agar. Plates were incubated as previously described and colonies were enumerated to determine the concentration of the inoculum. All bacterial enumerations were performed in duplicate. Control birds received PBS without *C. jejuni*, on the same dosage timeline.
Preparation of 2’FL. The 2’FL was synthesized as previously described (Lee et al., 2012). Lyophilized 2’FL samples were solubilized in sterile PBS to final concentrations of 200, 20, and 2 mg/mL to dose birds with 0.5 mL of 100, 10, and 1 mg, respectively. For treatment groups receiving 2’FL, broiler chickens were orally gavaged with 2’FL suspended in PBS on days 19 and 20 of age. Control birds received PBS without 2’FL, on the same dosage timeline.

Broiler Chicken Sampling Procedures. At 21 days of age, all broiler chickens were removed from their batteries, weighed, and euthanized by carbon dioxide asphyxiation. Ceca were removed following an incision made below the ribcage. Cecal contents were collected aseptically and immediately prepared for enumeration of bacterial populations (as described below). Cecal contents (~200 mg) for analysis by quantitative PCR (qPCR) were flash frozen in liquid nitrogen and stored at -80°C until use. The handling of deceased experimental animals complied with University of Illinois IACUC and Division of Animal Resources welfare laws and guidelines. Necropsy followed a tiered order of selecting one cage from each treatment group at a time, to account for potential Campylobacter oxygen sensitivities.

Enumeration of Cecal Campylobacter Populations. Both ceca were cut open longitudinally, weighed, diluted 1:2 (wt/vol) in Mueller Hinton Broth, and stomached for two min on high. For enumeration, the stomached sample was diluted at 1:5 (wt/vol) in PBS followed by 10-fold serial dilutions. Upon brief vortexing of samples, 10 µL from dilutions $10^{-1}$ to $10^{-6}$ was spread plated on Campy-Cefex agar plates (Appendix E). The plates were incubated microaerobically (10% CO₂, 5% O₂) at 37°C for 72 h and colonies were counted and recorded. All bacterial enumerations were performed in duplicate. Contaminating growth was evident on lower
dilutions (10^3 CFU/mL), with *C. jejuni* colonies (round, with orange- or salmon-colored colonies) observable on higher dilutions.

**DNA Extraction and Quantitative Real-Time PCR (qPCR).** DNA was extracted from flash-frozen cecal content samples following established procedures (Barry *et al.*, 2009, Yu & Morrison, 2004). Step-by-step directions may be found in Appendix F. The two aliquots were combined into one tube and 40 µL of DNase-free RNase added. The samples were briefly vortexed and incubated at 37°C for 15 min. DNA was purified using a QIAGEN Stool Mini Kit (Qiagen, Valencia, CA) with a modified protocol. Briefly, 15 µL proteinase K were pipetted into the sample, according to the manufacturer’s instructions. A positive control of *C. jejuni* NCTC 11168 was used as an extraction control with each assay. Samples were stored at -20°C until further use.

A standard curve was prepared from extracted DNA of pure cultures of *C. jejuni* NCTC 11168 grown overnight, following the procedures previously described. The pure culture was normalized by optical density (OD_{600 nm}) and DNA concentration measured (NanoDrop™ 1000 Spectrophotometer, Thermo Scientific). Serial dilutions (10^1-10^9 CFU/mL) were performed to create the *Campylobacter* standard curve.

The internal control template was created from a set of primers specific for the 16S rRNA of *Campylobacter* (Bui *et al.*, 2012) and purchased from Integrated DNA Technology (Coralville, IA). The set of primers includes AB_Cjej1F (5’ – GCGTAGGCGGATTATCAAGT - 3’) and AB_Cjej1R (5’ – CGGATTTTACCCCTACACCA - 3’). Two microliters of extracted bacterial DNA were added to 8 µl of reaction mixture (5 µl of 2X Power SYBR Green PCF Master mix (Applied Biosystems, Carlsbad, CA), bovine serum albumin at a final concentration
of 1 µg BSA/µL (New England Bio Labs), 0.5 µM of each primer). The 10 µL qPCR reaction was quantified using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). The amplification followed an initial denaturation cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Following amplification, a dissociation step was included to analyze the melting profile of the amplified products. Data was analyzed using the SDS Standard Core 2.4.1 software package (Applied Biosystems) and samples were identified as either positive or negative for *C. jejuni* in comparison with the standard curve. Positive and negative controls were included in each assay to confirm the limit of detection.

**Study Two – Campylobacter jejuni Colonization of Broiler Chickens**

The primary objective of this study was to colonize Ross 308 broiler chickens with *C. jejuni* and to verify the presence of *C. jejuni* in inoculated broiler chickens. The study consisted of four randomly assigned groups, including (1) control group, *C. jejuni* free, (2) *C. jejuni* NCTC 11168 challenged on day ten, (3) *C. jejuni* NCTC 11168 challenged on day fourteen, and (4) *C. jejuni* F38011 challenged on day fourteen. Each treatment group was assigned three cages of birds, which each held five birds, for a total of fifteen birds within each treatment group (Table 4.4). If not receiving an inoculation, the birds received a control dose of PBS on day 10 and day 14. Necropsy of all birds occurred on day 21 of age.

**Inoculation of Campylobacter jejuni.** Care was taken when passing *C. jejuni* to select from the growth area of highest concentration, rather than selection of isolated colonies (Figure 4.1). Samples were concurrently passed on MH blood agar and MH medium supplemented with 0.4%
agar (motility agar). Broiler chickens were inoculated with *C. jejuni* as detailed in the protocols of the first broiler chicken study.

### 4.4 Results

In the first study, in order to confirm the ability of *C. jejuni* NCTC 11168 to colonize the cecum of broiler chickens and to evaluate the efficacy of 2’FL as a treatment strategy, 90 chickens were divided into six groups (Table 4.3). Group 1 was the uninoculated, no treatment control group, Group 2 was inoculated with *C. jejuni* and no 2’FL dosage, Group 3 was inoculated with *C. jejuni* and a low 2’FL dosage (1 mg), Group 4 was inoculated with *C. jejuni* and a medium 2’FL dosage (10 mg), Group 5 was an uninoculated, high 2’FL dosage (100 mg) group, and Group 6 was inoculated with *C. jejuni* and a high 2’FL dosage (100 mg). Birds were orally gavaged on 14 d of age and were necropsied on 21 d of age. Direct plating on Campy-Cefex agar was performed to determine the level of cecal colonization.

Enumeration of the cecal samples indicated an absence of *C. jejuni* in the inoculated groups (group 2, group 3, group 4, and group 6). Additionally, *C. jejuni* was not recovered from the uninoculated chickens (group 1 and group 5). While the higher plating dilutions (10^6-10^8) did not have bacterial growth, the lower plating dilutions (10^3-10^5) had visible bacterial growth that was inconsistent with typical *C. jejuni* colony morphology and was similar for all samples regardless of treatment group.

Therefore, qPCR was conducted to determine the level of colonization below the detectable plating threshold of 10^3 CFU/mL. *Campylobacter* spp. primers successfully amplified the *Campylobacter* genomic DNA used for the standard curve. However, the primer set didn’t amplify when using DNA extracted from cecal contents. Only one treatment replicate was found
to have *Campylobacter*, though at very low levels ($10^1$-$10^2$ CFU/g) and were amplified in an unreliable range (CT>35) outside the standard curve range. The intention of this study is to have definitive *C. jejuni* colonization of broiler chickens ($>10^5$ CFU/g of cecal contents) to evaluate novel treatment strategies. Results indicate the broiler chickens were not successfully colonized with *C. jejuni* NCTC 11168 at day 21 of age.

To verify the selection of the Campy-Cefex agar, chicken cecum samples were collected from the poultry farm and prepared according to the previous instructions (results not shown). One set of samples were inoculated with *C. jejuni* NCTC 11168, while the other group served as a negative control. Plates with the negative control samples (not spiked with *C. jejuni*) were not found to support growth of *Campylobacter*. This indicates the bird was not previously colonized with *Campylobacter*. For the samples inoculated with *Campylobacter*, the Campy-Cefex agar supported the growth of these bacteria. This isolation indicates the plating method with Campy-Cefex was successful and confirmed that the broiler chickens were not colonized with *C. jejuni* in Study One.

Further conversations with collaborators indicated that the *C. jejuni* NCTC 11168 strain used for the broiler chicken inoculum had attenuated motility. Whether due to the original frozen culture or the passage technique, the *C. jejuni* lacked the flagellar motility necessary for colonization of the cecal crypts. The collaborators shipped an ‘enhanced motility’ strain of *C. jejuni* NCTC 11168 and a passage technique was determined (Figure 4.1). In addition, the *C. jejuni* must be passed for several days prior to the inoculation, to allow the cells to recover from the harsh -80°C conditions. Along with growth on MH blood agar, the strains were also passed concurrently on MH supplemented with 0.4% agar to test for motility. The motility agar was stab inoculated and incubated in microaerobic conditions at 37°C overnight.
In the second study, motility assays using MH agar supplemented with 0.4% agar were performed to select a highly motile strain (before administration to broiler chickens). Two *C. jejuni* strains F38011 and NCTC 11168 were chosen for this study, based on their previously indicated ability to colonize broiler chickens (Jones *et al.*, 2004, Neal-McKinney *et al.*, 2012).

To confirm the ability of *C. jejuni* to colonize the cecum of broiler chickens, 60 chickens were divided into four groups, each consisting of 15 birds (Table 4.4). Group 1 was the uninoculated, control group, Group 2 was inoculated with *C. jejuni* NCTC 11168 on day 10, Group 3 was inoculated with *C. jejuni* NCTC 11168 on day 14, and Group 4 was inoculated with *C. jejuni* F38011 on day 14. *C. jejuni* enumeration by direct plating of cecal samples indicated successful colonization of the birds with *C. jejuni* NCTC 11168 and F38011. The number of bacteria recovered per gram of cecum contents varied between birds of the same group (10^3-10^9 CFU/mL). *C. jejuni* were not recovered from the uninoculated chickens (group 1), indicating the established workflow and sample handling prevented cross-contamination. The concentration of *Campylobacter* colonized within the cecum varied amongst the broiler chickens, with day 14 inoculated *C. jejuni* NCTC 11168 the most robust (Figure 4.2). *C. jejuni* were recovered from the cecal contents of 13 of 15 birds inoculated with *C. jejuni* NCTC 11168 on day 10 (group 2). *C. jejuni* were recovered from the cecal contents of 15 of 15 birds inoculated with *C. jejuni* NCTC 11168 on day 14 (group 3). *C. jejuni* were recovered from the cecal contents of 10 of 15 birds inoculated with *C. jejuni* F38011 on day 14 (group 4). Overall these results indicate an ability to colonize the chicken cecum with *C. jejuni*. The best strategy for colonization appeared to be inoculation with *C. jejuni* NCTC 11168 on day 14.
4.5 Discussion

The objective of this study was to colonize the cecum of broiler chickens with *C. jejuni*, in an effort to establish a model to test novel strategies for the control of *C. jejuni* colonization. *C. jejuni* isolates selected for this study have previously been shown to colonize chickens (Jones *et al.*, 2004, Neal-McKinney *et al.*, 2012). Of the two isolates inoculated in this study, both were able to colonize the cecum of broiler chickens. Based on direct plating results, *C. jejuni* NCTC 11168 was recovered from the cecum in greater numbers than *C. jejuni* F38011. In the cecum samples collected, concentrations of *C. jejuni* colonization varied between $10^3$-$10^9$ CFU/mL. This finding is in agreement with the existing literature, where *C. jejuni* colonization of the mucosal layer of the cecal crypts is between $10^6$-$10^9$ CFU/mL (Allen *et al.*, 2008, Beery *et al.*, 1988, Hermans *et al.*, 2012, Meade *et al.*, 2009).

Culturing techniques were determined to be critical in the passage of *C. jejuni* prior to administration to the broiler chickens, to maintain colonization potential of the isolates. It was determined that flagellar motility of the selected strains was critical to obtain colonization. Therefore, motility assays were developed and employed to ensure the presence of flagella prior to inoculation. Of the two strains inoculated (NCTC 11168 and F38011), both appeared to be equally motile therefore both were used for inoculation in the second study. Care was taken in the second study to maintain the motility and chemotaxis of the flagella, which was absent in the first study. It has been reported that the flagellum is critical in colonization of the intestinal mucus coating the cecal crypts (Beery *et al.*, 1988). Important in the flagella motility are the presence of two filament genes, *flaA* (encodes the major flagellin) and *flaB* (encodes the minor flagellin). While the impact of environmental conditions varies for the activity of these two genes, only *flaA* has been shown to be essential for chicken colonization (Hermans *et al.*, 2011a,
Particular *C. jejuni* mutants that lack flagella or have modified chemoreceptors, are unable to colonize the GIT of the chicken (Hermans *et al*., 2011a, Kanungpean *et al*., 2011). Additionally, deoxycholate, a bile acid, may be added to the MH agars during outgrowth from the -80°C to stimulate virulence and assist in colonization of the broiler chicken GIT.

Differential flagellum protein expression between a robust- (A74/C) and a poor-colonizing (11168) *C. jejuni* isolate in poultry indicates the importance of the flagellum in colonization (Hiett *et al*., 2008). The flagella-encoding genes differing between these two isolates were located in the hypervariable regions of the *C. jejuni* genome. This variability evidently extends into the protein level and may influence the survival of the organism in the environment and host selection. In spite of the poor-colonizer stigma, *C. jejuni* NCTC 11168 successfully colonized the cecum of 21 d broiler chickens and were enumerated from the cecal contents in concentrations up to $10^9$ CFU/mL. Unfortunately, research is rather limited regarding the paralysis of the *C. jejuni* flagella and subsequent colonization in broiler chickens.

### 4.6 Conclusion

In summary, we were able to successfully colonize broiler chickens with *C. jejuni*. Establishment of the sound study parameters allows us to explore a variety of novel strategies for the control of *C. jejuni* colonization. In the future, broiler chickens should be inoculated on day 14 with a highly motile *C. jejuni* NCTC 11168. Additionally, handling techniques of the *C. jejuni* isolates prior to administration are critical to the colonization ability of the strains. Prior to inoculation, passage on motility assays is highly encouraged, to verify the presence of the flagella which is critical for colonization of the mucosal layer overlaying the cecal crypts.
4.7 References


### 4.8 Tables and Figures

**Table 4.1** Proposed timeline for animal facility preparation.

<table>
<thead>
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<th>Task</th>
<th>6 weeks Prior</th>
<th>5 weeks prior</th>
<th>3 weeks prior</th>
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<th>Minus 2 days</th>
<th>Minus 1 day</th>
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<td>Verify Facility Availability</td>
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<td>Coordinate Feed Delivery</td>
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<td>Set-up Batteries and ERML Rooms</td>
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<tr>
<td>Add Feed and Water to batteries</td>
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<td>x</td>
<td>x</td>
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<tr>
<td>Chick Arrival</td>
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<tr>
<td>Study Clean-up</td>
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</tr>
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</table>
Table 4.2 Composition of standard diet 4 without bacitracin on percentage basis. Prepared by the University of Illinois Urbana-Champaign Poultry Farm. CP, crude protein.

<table>
<thead>
<tr>
<th>INGREDIENT</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine Ground Corn (1/8&quot; screen)</td>
<td>52.85</td>
</tr>
<tr>
<td>Dehulled SBM (47% CP)</td>
<td>37.50</td>
</tr>
<tr>
<td>Pork Meal (50% CP)</td>
<td>2.00</td>
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<tr>
<td>Dicalcium Phosphate</td>
<td>1.50</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.10</td>
</tr>
<tr>
<td>Salt</td>
<td>0.40</td>
</tr>
<tr>
<td>Poultry Trace-Mineral Premix</td>
<td>0.15</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.20</td>
</tr>
<tr>
<td>Choline Chloride (60%)</td>
<td>0.10</td>
</tr>
<tr>
<td>Poultry Vitamin Premix</td>
<td>0.20</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>4.00</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
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Table 4.3 Experimental design of first broiler chicken study. To assess the effect of 2’FL-inoculation on *C. jejuni* colonization of broiler chickens.

<table>
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<th>3</th>
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<th>5</th>
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<tbody>
<tr>
<td><strong>DAY 14</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>inoculation</td>
<td>PBS only</td>
<td><em>C. jejuni</em></td>
<td>NCTC 11168</td>
<td><em>C. jejuni</em></td>
<td>NCTC 11168</td>
<td>PBS only</td>
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<td><em>C. jejuni</em></td>
<td>NCTC 11168</td>
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<td></td>
<td><em>C. jejuni</em></td>
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<td><strong>DAY 19 &amp; 20</strong></td>
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<td></td>
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<tr>
<td>2’FL</td>
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<tr>
<td>inoculation</td>
<td>PBS only</td>
<td>PBS only</td>
<td>1 mg 2’FL-</td>
<td>10 mg 2’FL-</td>
<td>100 mg 2’FL-</td>
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<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Necropsy</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>SAMPLES=</td>
<td>1) Cecum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) Freeze cecum sample</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Table 4.4 Experimental design of second broiler chicken study. Assess the effect of timing on *C. jejuni* colonization of broiler chickens.

<table>
<thead>
<tr>
<th>Challenge Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em> inoculation</td>
<td>d 14</td>
<td>d 10</td>
<td>d 14</td>
<td>d 14</td>
</tr>
<tr>
<td>PBS only</td>
<td><em>C. jejuni</em> NCTC 11168</td>
<td><em>C. jejuni</em> NCTC 11168</td>
<td><em>C. jejuni</em> NCTC F38011</td>
<td></td>
</tr>
<tr>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
</tr>
</tbody>
</table>

**DAY 21**

Necropsy

SAMPLES= 1) Cecum  
2) Freeze cecum sample
Figure 4.1 Experimental *Campylobacter* handling procedures. *Campylobacter* passage on Mueller-Hinton (MH) blood agar and motility test on MH supplemented with 0.4% agar. A dense growth area is selected, over the traditional individual colony isolation.
Figure 4.2 *C. jejuni* colonization of cecum. Broiler chickens were colonized with *C. jejuni* NCTC 11168. Cecum samples were collected from broiler chickens on d 21. The materials and methods describe the enumeration of CFU per gram of cecal content. N indicates the number of chickens of each group of 15 that were colonized with *C. jejuni* (limit of detection, $10^3$ CFU/g of cecal contents). The bar indicates the median CFU for each group, which was determined using all birds within the group. The absence of a bar indicates the number of *C. jejuni* cells was below the limit of detection.
Strategies to reduce *Campylobacter jejuni* colonization of broiler chickens at the poultry farm remain limited. The literature indicates current intervention techniques incompletely prevent colonization of *C. jejuni*, though a minor reduction in colonized broiler chickens may significantly reduce the incidence of campylobacteriosis. A novel strategy builds on the innate probiotic mechanisms of lactobacilli. Herein, we described the construction of antigen-presenting vectors for lactobacilli vaccine delivery to the gastrointestinal tract of broiler chickens. The antigen-presenting vectors either anchored or secreted the *C. jejuni* cell surface adhesion, FlpA DII (antigen), to the exterior cell surface of recombinant *Lactobacillus* spp. An alternate strategy explored the non-covalent attachment of *C. jejuni* FlpA DII to the surface of *Lactobacillus* spp. through three cell wall binding domains, *L. gasseri* ATCC 33323 LysM protein, *L. gasseri* ATCC 33323 Lysozyme M1 – bacterial SH3 domain, and *L. lactis* susbp. *cremoris* MG1362 AcmA protein. In addition, establishment of study procedures to colonize broiler chickens with *C. jejuni* provided a foundation for exploring oral administration of *in vivo* treatment strategies.

Throughout the course of this project, we encountered several learning points to consider for future work.

First, verification of protein expression from the recombinant *Lactobacillus* spp. proved more challenging than anticipated. Both sonication and boiling of the supernatant and cell pellet were employed to evaluate protein expression. However, these procedures were inadequate to detect the *C. jejuni* FlpA DII expressed by recombinant *Lactobacillus* spp. Verifying the protein expression of the recombinant *Lactobacillus* spp. is imperative prior to oral administration of
broiler chickens. Treatment strategies will only be explored upon approval of full diagnostic analysis of the recombinant *Lactobacillus* spp.

Second, we will explore a non-genetically modified *Lactobacillus* strain that displays *C. jejuni* FlpA DII. Cell wall binding domains are effective in non-covalent attachment of heterologous proteins to the surface of lactobacilli. However, additional research remains to be conducted on the *in vivo* stimulation of the immune response and stability of the protein through the gastrointestinal tract.

Third, animal trials resulted in successful colonization of the broiler chicken cecum with *C. jejuni*. The flagellar motility of the *C. jejuni* strains was found to be integral to broiler chicken colonization. While *C. jejuni* NCTC 11168 is considered a poor colonizer, in these studies the strain achieved colonization of the cecum at a higher concentration than that of *C. jejuni* F38011. As the laboratory handles additional *C. jejuni* strains for experimental protocols and preparation for *in vivo* colonization, care will be taken to maintain flagellar motility.

From the animal studies and laboratory experiments, we gained distinct insights for future work to focus upon. This includes verifying the recombinant protein expression in the supernatant and the cell wall of lactobacilli, exploring a non-genetically modified *Lactobacillus* spp. for cell wall binding of a heterologous protein, and handling procedures of *C. jejuni* will be followed to ensure motility of strains and subsequent colonization. The work conducted in this thesis provides a foundation for further research on the reduction of *C. jejuni* colonization of broiler chickens.
APPENDIX A. ELECTROCOMPETENT *ESCHERICHIA COLI*

1. Inoculate 200mL LB with E. coli MC1061 (from frozen stock). Use 1-Liter flask, incubate at 37 C, and shake at 250 rpm.

2. Monitor OD. When it reaches 0.5 to 0.6, transfer cells to a 250mL centrifuge bottle (pre-chilled) and centrifuge the cells at 3,200 × g, 4° C for 10 minutes.

3. Wash the cells 3X with pre-chilled sterile water

4. Wash with sterile 10% glycerol (prechilled)

5. Resuspend cells to a final volume of 1-1.5mL in 10% glycerol

6. Dispense cells to Eppendorf tubes on ice (85uL per tube is good)

7. Store tubes at –80°C until use.
APPENDIX B. **ESCHERICHIA COLI TRANSFORMATION**

1. One tube per transformation, chill tube on ice.
   — 40mL electrocompetent cells per tube
   — 2mL lig. Mixture or up to 16mL if clean DNA

2. Transfer cells/DNA to a 0.2 micron cuvette (pre-chilled)

3. Zap at 2.5 V, 200 ohms, and 25 uFD (capacitance). Time constant should be near 1.0

4. Add 960mL of pre-warmed (37C) LB to tube and transfer contents to small culture tube

5. Incubate at 37C for 1 hour with shaking

6. Plate cells on appropriate plates (10mL and 100mL)
APPENDIX C. LACTOBACILLUS TRANSFORMATION

Electroporation Buffer: 1M Sucrose 171.1g or 342.2g  
(3.5X SMEB) 3.5mM MgCl$_2$ 0.356g 0.712g  
ddH$_2$O up to 500mL 1L

- pH should be around 7.0
- Filter sterilize through 0.2μm membrane

1. Inoculate 100mL MRS with 1mL of an overnight culture and incubate at 37°C until it reaches an OD$_{600} = 0.5 – 0.8$.  
   a. Pre-warm the MRS for faster growth  
   b. Take 3.5-5.5 hours to get to 0.5  
   c. All liquid cultures can be done outside the anaerobic chamber; however, do not use a shaker

2. Centrifuge culture at 3,200 × g for 10min at 4°C and wash with cold 3.5X SMEB.  
   Centrifuge in 2, 50 ml conical tubes  
   a. Resuspend each pellet in 20 ml 3.5X SMEB (40 ml total)  
   b. Can combine into one tube if desired but use 40 ml per wash

3. Repeat step 2 at least twice for three total washes.

4. Resuspend cells in 1.0mL 3.5X SMEB (concentrate 100 fold).  
   a. Mix well using a pipettor

5. Transfer 0.2mL cells to a microfuge tube and add DNA; mix and transfer to a cold 0.2cm cuvette.

6. Electroporate at 2.45kV, 25μFD, 200Ω.  
   a. We now use an eppendorf electoporator that has only a voltage setting (we use 2.5 kV; typical time constant is ~3.3)

7. Transfer cells (gently) to 3.0mL MRS and incubate at 37°C.  
   a. ~16 hours is sufficient  
   b. Does not need to be in anaerobic shaker but no shaking  
   c. We use small culture tubes but you could use falcon tubes

8. Dilute cells accordingly and plate on MRS plus appropriate antibiotic.  
   a. L. gasseri ATCC 33323 – 5.0 mg/ml erythromycin  
   b. Make sure you plate a negative control  
   c. I usually use add 40ml and 200ml on two plates each

9. Incubate 2-3 days in anaerobic chamber at 37°C
APPENDIX D. LACTOBACILLUS PLASMID ISOLATION PROTOCOLS

RAPID LACTOBACILLUS MINI-PREP PLASMID ISOLATION PROCEDURE


Procedure is as follows:

A. Make 5 or 10 ml overnight broth culture

B. Pellet entire amount in 15 ml plastic conical tube: 3,200 x g/10 min at 4°C

C. Wash cell pellet with 10 ml dd H20, re-centrifuge (same conditions).

D. Resuspend pellet with 200 ul 25% sucrose containing 30 mg/ml Lysozyme (10 mg/ml, stored at 4°C) and Transfer cell suspension to 1.5 mL eppendorf tube, incubate 15 min at 37°C

E. Add 400 ul of alkaline SDS solution, mix immediately and incubate 7 min at RT

F. Add 300 ul ice cold 3M sodium acetate (PH 4.8), mix well and spin max speed 15 min at 4°C

G. Transfer supernatant to new 1.5 mL eppendorf tube and 650 ul of isopropanol (RT), mix well and spin max speed 15 min at 4°C

H. Remove all liquid and resuspend pellet in 320 ul dd H20.

I. Add 200 ul of 7.5M ammonium acetate containing 0.5 mg/ml ethidium bromide

J. Add 350 ul chloroform and mix well and spin max speed 5 min at RT

K. Transfer upper phase to new epdi and add 1 ml ethanol (-20°C) mix well and spin max speed 15 min at 4°C

L. Rinse pellet with 500 ul 70% ETOH; recentrifuge for 1 min (conditions of Step J); dry pellet for 30 minutes (modified – 2-3h in Biosafety Cabinet with fan on).

M. Add 40 ul Nanopure, RNAse-free H20 containing RNase (0.1 mg/ml)
APPENDIX D. CONTINUED

LACTOBACILLUS MINI-PREP PLASMID ISOLATION PROCEDURE

I. Procedure is as follows:
A. Inoculate 10 ml MRS broth with 2 ml of overnight culture. Incubate at 37°C for 2 hours.
B. Pellet entire amount in 15 ml plastic conical tube: 3,200 × g/10 min at Room Temperature.
C. Wash cell pellet with 5 mls dd H₂O, re-centrifuge (same conditions).
D. Resuspend pellet with 1.0 ml cell suspension buffer (Soln A, stored at 4°C) containing Lysozyme (10 mg/ml, stored at 4°C) and hold on ice 1.0 hour. Resuspend by pipetting on ice. Check to ensure that water bath is at 65°C (for step H).
E. Transfer cell suspension to 2.0 mL eppendorf tube with screw cap and pellet in microfuge for 1.0 minute at 8,327 × g. Pour off supernatent.
F. Add 500 ul pH adjusted Lysis solution (Soln B) to cell pellet. Add NaOH to Lysis solution just before use. Test pH with paper.
G. Disrupt pellet by aspiration with pipetman and vortex vigorously for 20-30 seconds.
H. Heat eppendorf tubes at 65°C for 30 min. Cool 10 min. at RT (room temp).
I. Add 250 ul of High Salt Solution; mix well by tapping (if the target plasmid is larger than 10 KB) or invert vortexing (if smaller than 10 KB).
J. Add 400 ul of Tris Saturated Phenol (bottom layer); invert to mix.
K. Add 400 ul of Chloroform; invert to mix.
L. Centrifuge at 6,118 × g in microfuge for 5 minutes.
M. Transfer aqueous layer (on top) to a new 2.0 mL eppendorf tube with screw cap (upper layer; approx. 750 ul); add 750 ul (Chloroform: isoamyl alcohol (24:1)) and mix.
N. Centrifuge at 6,118 × g in microfuge for 5 minutes.
O. Transfer aqueous layer to a new 1.5 mL eppendorf tube with flip cap and add 750-800 ul 100% isopropanol to fill the tube, mix well by inversion.
P. Chill isopropanol preps at -20°C for at least 30 minutes. Turn on microfuge to 4°C.
Q. Spin in 4°C microfuge at 6,118 × g for 15 min.
APPENDIX D. CONTINUED

R. Pour off isopropanol and rinse pellet with 500 ul 70% ETOH; recentrifuge for 2 min (cond. of Step Q); dry pellet for 15 minutes (modified – 2-3h in Biosafety Cabinet with fan on).

S. Add 50 ul Nanopure, RNase-free H2O containing RNase (20 ug/ml) (2 ul 500 ug/ml). Leave in refrigerator overnight, store in -20°C.

Notes:
— For Lactococcal plasmid isolation, phase-shift (Step A and B) may not be required; just use 5-10 ml of overnight culture.
— In most cases, adjustment of pH of lysis solution to 12.4 is essential. If you don't observe good lysis, check pH.
— Disruption of cell pellet by pipetman at step G is boring but essential. Do it until you observe clearance of soln.
— Heating at 65°C and slow cooling to room temperature is helpful to remove chromosomal DNA; however, plasmid larger than 25 KB may be removed together.
— For restriction digestion, washing the DNA pellet with 70% ethanol at room temp is critical.
— For very low copy number DNA, or larger plasmid DNA, TE-saturated phenol (pH 7.0-8.0) is helpful.

II. Prepare the following solutions:

A. Cell Suspension Buffer (100 ml). Filter sterilize and store at 4 degrees.
   - 50 mM Tris-Cl
   - 5 ml 1.0 M Tris-Cl (pH 8.0)
   - 0.2 ml 0.5 M EDTA (pH 8.0)
   - 8% Sucrose 8.0 g

B. Lysis Solution (100 ml).
   - 50 mM Tris-Cl
   - 5 ml 1.0 M Tris-Cl (pH 8.0)
   - 5 mM EDTA 1 ml 0.5M EDTA (pH 8.0)
   - 3% SDS 30 ml 10% SDS Soln. (Made in dd H2O)

   Add approximately 35 ul of 3.0 N NaOH/ml lysis solution until the pH is 12.4, just before use at Step F. Measure 10 ml water into tube, add 1.2g NaOH. Filter Sterilize and store at room temperature.

C. High Salt Solution (100 ml).
   - 3.0 M Potassium Acetate 29.4 g
   - 1.8 M Formic Acid 5.0 ml (90%)

   Filter Sterilize if there is a brown precipitate and store at room temperature.

D. STE
   - 0.1 M NaCl 2 ml 5.0 M Stock Solution
   - 10 mM Tris-Cl 1 ml 1.0 M Tris-Cl (pH 8.0)
   - 1 mM EDTA 0.2 ml 0.5 M EDTA (pH 8.0)
APPENDIX E. CAMPY-CEFEX AGAR DIRECTIONS

Base (per 750 mL)
43 g Brucella Agar – BD (Fischer # B11086)

Supplement: Add to 750 mL after autoclaving and tempering to 55°C.
1. 25 mL citrated blood
2. 3 mL of sterile-filtered FS/SP/SM solution

To make solution, to 20 mL water add:
2.5g Sodium Pyruvate
2.5g Ferrous Sulfate
1.0g Sodium Metabisulfite
Note: Sterile filter solution and protect from light! Make fresh each time! Should have a red-brown appearance

3. 2mL Cefoperazone stock: Dissolve 125 mg in 10 mL of 50% Ethanol
4. 2mL Cyclohexamide stock: Dissolve 750 mg in 10 mL of 50% Ethanol

*Note: Add stir bar and leave when autoclaving. Add each ingredient to the autoclaved media separately. Mixing any of the 4 supplement solutions together will cause precipitation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration (per 750 mL)</th>
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</thead>
<tbody>
<tr>
<td>BD Brucella Agar</td>
<td>43 g</td>
</tr>
<tr>
<td>Bovine Citrated Blood</td>
<td>25 mL</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>375 mg</td>
</tr>
<tr>
<td>Ferrous Sulfate</td>
<td>375 mg</td>
</tr>
<tr>
<td>Sodium Metabisulfite</td>
<td>15 mg</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>25 mg</td>
</tr>
<tr>
<td>Cyclohexamide</td>
<td>150 mg</td>
</tr>
</tbody>
</table>
APPENDIX F. TOTAL FECAL DNA EXTRACTION

Total DNA Extraction from Fecal Samples and 3rd Stage *in vitro* Samples (Repeated Bead Beating Plus Column (RBB+C) Method)

Last modified: 6/25/07 by Ingmar

Estimated time from start to finish for two batches of 8 extractions: ~7 hours (depending on experience)

Reference:

Reagents (Not Included in Kit)

**Tris-HCl (500 mM, pH 8.0):** Place 30.28g of Tris in a 500 mL volumetric flask; add ~300 mL milliQ water. Then bring to pH 8.0 by adding concentrated (1M or higher) HCl, then add milliQ water to volume.

**EDTA (500 mM, pH 8.0):** Place 93.05g of EDTA Disodium Salt Dihydrate in a 500 mL volumetric flask; add ~300 mL milliQ water – heating helps EDTA dissolve; adjust pH close to 8.0 (EDTA only completely dissolves at ~pH 8.0 – when all is dissolved, adjust pH to 8.0, then bring to volume). Autoclave for 15 minutes (if immediately making a solution with this stock, you can use the fresh EDTA solution)

**Lysis Solution:** Place 7.3 g of NaCl and 10 g SDS in an autoclavable bottle; add 25 mL of 500mM Tris-HCl; add 25 mL of 500mM EDTA; add 200mL milliQ water. Stir on a hotplate/stirrer (medium temperature) to dissolve the SDS (this takes a while) – do NOT shake aggressively, this solution foams excessively. Take out the stir bar, then autoclave.

**NOTE:** This solution separates easily – after autoclaving, a STERILE stir bar can be added back. Stir on a medium temperature hotplate before use (solution should be clear)

**10M NH₄-Acetate:** place 38.54 g Ammonium acetate in a 50 mL volumetric flask; bring up to volume. Mild heating will ease dissolving.
APPENDIX F. CONTINUED

**TE Buffer:** pipet 10mL Tris-HCl (500 mM) and 1mL of 500mM EDTA into a 500mL volumetric flask. Bring to volume, autoclave for 15 minutes.

**70% Ethanol:** bring 70mL of 100% ethanol to 100 mL volume

**100% Ethanol**

**Isopropanol**

**DNase-free RNase:** get at the life sciences store-room (Roche catalog #11119915001) in 1 mL tube – good for ~25 extractions. If you know you need large quantities (many extractions) you may want to order directly from Roche – the store room only has a few vials in stock.

**Procedure**

Sample collection for *in vitro* samples only: Place in the -80°C as soon as possible.

**Day 1:**

**Part I:**

**Cell Lysis:**

*Suggestion for fecal samples:* do a 105 dry matter on the sample you actually extract.

*Turn on the water baths to 37 & 70°C, and let the centrifuge cool to 4°C*

*To ensure all fluid is incubated properly, you need to spin your tubes in the micro- centrifuge for 2-3 seconds – this is referred to as “quick-spin”*

- Weigh up 0.4 g of sterile zirconia/glass beads in 2-mL screw-cap tubes using the porcelain scoop
  - 0.4g *Disruption Beads* *(RPI 9830)*

- Transfer 0.25 g (or 400ul for *in vitro* samples) of sample into a fresh 2-mL screw-cap tube (acceptable weight range: 0.20 – 0.32 g) – try to keep the rim clean and to not have the sample stick to side of the tube.
APPENDIX F. CONTINUED

- Add 1 mL of lysis solution to the tube and vortex at full speed until beads and sample are reasonably mixed (make sure no sample is sticking to the wall of the tube)
- Homogenize for 3 min at maximum speed with a vortex (tube adapter)
- Incubate at 70°C for 15 min with gentle shaking (inverting) by hand every 5 min
- Centrifuge at 4°C for 5 min at 16,000 X g
- Pipet the supernatant into a fresh 2-mL Eppendorf tube
- Add 300 μL of fresh lysis buffer to the screw-cap tube
- Vortex until mixed – make SURE to break up the pellet at the bottom of the tube
- Homogenize for 3 min at maximum speed with a vortex (tube adapter)
- Incubate at 70°C for 15 min with gentle shaking (inverting) by hand every 5 min
- Centrifuge at 4°C for 5 min at 16,000 X g.
- Combine the supernatant with the supernatant collected previously

Precipitation of Nucleic Acids
- Add 260 μL of 10 M ammonium acetate to each tube, vortex to mix.
- Incubate on ice for 5 min
- Centrifuge at 4°C for 10 min at 16,000 X g
- Transfer (split) supernatant into two 1.5-mL Eppendorf tubes (use fixed volume,
600 μL will get you most, if not all supernatant)
- Add an equal volume of isopropanol to each eppendorf and mix well
- Incubate on ice for 30 min
- Centrifuge at 4°C for 15 min at 16,000 X g
- Carefully pipet off the supernatant, make sure to leave pellet intact!
APPENDIX F. CONTINUED

- Wash the nucleic acids pellet by adding 0.5 mL with 70% ethanol, centrifuge at 4°C for 1 minute at 16,000 X g (to make sure the pellet is intact), then carefully pipet off ethanol – do not damage the pellet!

NOTE: after this step, set the centrifuge to warm up to room temp for the next centrifugation steps

- Dry the pellet in a BSC for 30 mins

- Dissolve the nucleic acid pellet in 100 μL of TE (Tris-EDTA) buffer, this takes some effort – pellet is re-hydrated overnight at 4C.

Day 2:

- Pool the two aliquots of dissolved pellet into one tube

**Removal of RNA**

- Add 40 μL of DNase-free RNase (final [0.1 μg/μL]). Vortex and quick-spin.

- Incubate at 37°C for 15 min

**Part II:**

**Removal of Protein and Purification (Partial Qiagen Stool Mini Kit – Centrifugation at Room Temp)**

- Add 15 μL of proteinase K, vortex and quick-spin

- Add 200 μL of Buffer AL, vortex and quick-spin

- Incubate at 70°C for 10 min

- Add 200 μL of ethanol, vortex and quick-spin, and pipet all liquid to a QIAMP column (don’t wet the rim) and centrifuge at 16,000 X g for 1 min at room temp

- Insert columns into a new 2 mL collection tube

- Add 500 μL of Buffer AW1 and centrifuge at 16,000 X g for 1 min, insert columns into new 2 mL collection tube
APPENDIX F. CONTINUED

- Add 500 μL of Buffer AW2 and centrifuge at 16,000 X g for 1 min, insert columns into new 2 mL collection tube
- Dry the column by centrifugation at RT for 1 min (16,000 X g), insert columns into an appropriately labeled 1.5 mL eppendorf tube
- Add 200 μL of Buffer AE and incubate at RT for 4 min
- Centrifuge at RT for 1 min to elute DNA and read a 2 μL sample on the Nano-drop spec
- Freeze samples at -20°C for storage or until further use
APPENDIX G. RECOMBINANT LACTOBACILLI DOSAGE OF BROILER CHICKENS

G.1 Materials and Methods

The primary objective of this study was to evaluate the immune response of administering recombinant lactobacilli strains to Ross 308 broiler chickens. The recombinant lactobacilli strains expressed *C. jejuni* FlpA either on the surface of the bacterial cell or into the supernatant. The study consisted of seven randomly assigned groups, consisting of one control group and six recombinant *Lactobacillus* groups (Table G.1). At 6 and 16 days of age, broiler chickens were orally administered with the treatment dosage (Table G.2). Each treatment group was assigned three cages of birds, which each held five birds, for a total of fifteen birds within each treatment group. Necropsy was spread across three days, to evaluate the host immune response. Necropsy occurred on day 16, day 18, and day 20, with one cage selected from each treatment group per necropsy day.

**Broiler Chickens**

Day-old chickens were randomly divided into study groups and placed into cohabitated stainless steel batteries (Table G.1). Broiler chickens inoculated with *C. jejuni* were housed separately from control broilers, with an established workflow to reduce risk of cross-contamination. Water and standard diet without bacitracin were provided ad libitum. Fecal material was collected in a removable metal tray below the wire mesh floor of the cage. The following studies were conducted at the University of Illinois with experiments and procedures approved by the Institutional Animal Care and Use Committee (IACUC; protocol no. 13059).
APPENDIX G. CONTINUED

*Lactobacillus* Dosage

Methods in Chapter 3 detail the construction of the recombinant *Lactobacillus* strains used in this study. As previously described in Chapter 3, broiler chickens were orally gavaged with 0.5 mL of suspended *Lactobacillus* strains (~10^7 CFU/mL) on days 6 and 16 of age. A frozen stock was passed four times in MRS + ERM (2.5 µg/mL) at 37°C under anaerobic conditions. The samples were serially diluted in PBS to the proper optical density at 600 nm wavelength (OD_{600 nm}, Spec 21, Bausch and Lomb) and serial dilutions were performed to verify the concentration. The inoculated samples were plated on MRS + ERM (2.5 µg/mL) and enumerated after 48 h incubation. Control birds received PBS without *Lactobacillus*, on the same dosage timeline. Birds necropsied on d 16 did not receive the *Lactobacillus* dosage on d 16.

**Enumeration of Cecal *Lactobacillus* Populations**

As previously described under “Enumeration of Cecal *Campylobacter* Populations,” the cecal dilutions were spread plated on MRS + ERM (2.5 µg/mL) agar plates (Chapter 3). Plates were incubated anaerobically at 37°C for 48 h and plates were enumerated (round, with white- or cream-colored colonies) and recorded. All bacterial enumerations were performed in duplicate.

**Serum Collection**

Shortly after euthanasia and incision of the chest cavity, blood samples were drawn from the heart of broiler chickens to ensure sufficient sample collection. Attached to a BD Vacutainer® Needle Holder, 21-gauge BD Vacutainer® blood collection needles were placed
directly into the heart and blood collected in a 4 mL BD Vacutainer® Rapid Serum Tube (VWR, Pennsylvania, USA). Blood samples were allowed to remain at room temperature and to clot in the upright position for at least 30 min but no longer than 1 h, before being placed onto ice. Samples were then centrifuged at 4°C for 10 min at 1,000-2,000 × g. Serum aliquots of 0.5 mL were transferred into plastic screw-cap vials and stored at -20°C.

Protein Verification

*Lactobacillus* cultures were grown in MRS broth for sample preparation divided into broth culture supernatant, sonicated supernatant, and resuspended pellet. For the sonicated supernatant, the initial bacterial pellets were resuspended in PBS and sonicated for 10 min. The resuspended pellet in PBS was recovered from the post-sonicated sample, due to difficulty in centrifuging the sample. All three samples were mixed with 1× laemelli buffer and boiled 10 min (>95°C) before running the samples on gels for blotting. The protein sequence of *C. jejuni* flpA Domain II is predicted to have a size of 11 kD.

G.2 Results and Discussion

Contaminating growth was evident on all cecal content dilutions, with *Lactobacillus* colonies difficult to visualize on higher dilutions. *Lactobacillus* isolates were not further analyzed to verify the identity of the strains. Future studies must verify the ability of the direct-plating method to select for the organism of interest in the presence of background growth.

Upon further analysis of the inoculated recombinant *Lactobacillus* strains using Western Blot analysis, a protein band of the predicted 11 kD was not present on the gel. In addition, a
APPENDIX G. CONTINUED

plasmid producing C. jejuni flpA could not be verified. Based on the lack of protein expression by the Lactobacillus strains, it was determined that the supposed recombinant strains did not actually produce the C. jejuni FlpA DII as desired. As the strains did not express the protein of interest, further work did not evaluate the immune response of the broiler chicken. Future analysis of the recombinant Lactobacillus strains must take into account the stability of the plasmid under laboratory conditions as well as the gastrointestinal tract. The vector structure must be evaluated to verify the functionality of the translational components, including the promoter and ribosome binding site.
APPENDIX G. CONTINUED

Table G.1 Orally gavaged *Lactobacillus* strain groups. Treatment groups orally gavaged to broiler chickens on d 6 and d 16.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Strain</th>
<th><em>Lactobacillus</em> strain</th>
<th>Protein Expression</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n/a</td>
<td>n/a</td>
<td>Negative Control</td>
<td>n/a</td>
</tr>
<tr>
<td>2</td>
<td>MJM274</td>
<td><em>L. crispatus</em> CC1-1</td>
<td>Control</td>
<td>pMJM8</td>
</tr>
<tr>
<td>3</td>
<td>MJM270</td>
<td><em>L. crispatus</em> CC1-1</td>
<td>Secreted + Anchored</td>
<td>pMJM13</td>
</tr>
<tr>
<td>4</td>
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<td><em>L. crispatus</em> CC1-1</td>
<td>Secreted</td>
<td>pMJM14</td>
</tr>
<tr>
<td>5</td>
<td>MJM275</td>
<td><em>L. gallinarum</em> ATCC 33199</td>
<td>Control</td>
<td>pMJM8</td>
</tr>
<tr>
<td>6</td>
<td>MJM272</td>
<td><em>L. gallinarum</em> ATCC 33199</td>
<td>Secreted + Anchored</td>
<td>pMJM13</td>
</tr>
<tr>
<td>7</td>
<td>MJM273</td>
<td><em>L. gallinarum</em> ATCC 33199</td>
<td>Secreted</td>
<td>pMJM14</td>
</tr>
</tbody>
</table>
APPENDIX G. CONTINUED

**Table G.2** Assess the probiotic and vaccine effect of recombinant *Lactobacillus* dosage on *C. jejuni* colonization of broiler chickens.

<table>
<thead>
<tr>
<th>DAY 6 &amp; 16</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td><em>Lactobacillus</em> dosage</td>
<td>PBS only</td>
<td>MJM 274</td>
<td>MJM 270</td>
<td>MJM 271</td>
<td>MJM 275</td>
<td>MJM 272</td>
<td>MJM 273</td>
</tr>
<tr>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
</tr>
</tbody>
</table>

| DAY 16, 18 & 20 |  |  |  |  |  |  |  |
| Necropsy | SAMPLES= 1) Cecum | 3) Serum | 2) Freeze cecum sample |