THE COAGGREGATIVE ABILITIES OF LACTOBACILLI AND *CAMPYLOBACTER JEJUNI*

BY

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THESIS

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ABSTRACT

Campylobacter jejuni is a pathogen commonly found in poultry that is one of the main bacterial causes of foodborne diarrheal disease in humans. The aim of this study was to analyze the coaggregative abilities of lactic acid bacteria and *C. jejuni* as a means to reduce the colonization of *C. jejuni* in poultry. In this study, various lactic acid bacteria were screened to analyze their coaggregative abilities with *C. jejuni*. The coaggregative abilities of LAB strains within the same species were compared. *L. crispatus*' ability to coaggregate with *C. jejuni* mutant strains was tested. The generation of non-coaggregating *L. crispatus* variants was performed. DNA sequencing was performed on the wildtype strains of *L. crispatus* (MJM 207 and 207) along with three non-coaggregating variants. All of these measures were used to help pinpoint a genetic determinant for the coaggregative ability of the LAB strains tested. *L. plantarum*, *L. acidophilus*, and *L. fermentum* did not display significant variation of coaggregative abilities with *C. jejuni*. *L. crispatus* was not able to coaggregate with *C. jejuni* mutant strains (MJM 376, 378, 379, 381 and 382). Findings from this study can provide knowledge to help better understanding the coaggregative relationship between lactobacilli and *C. jejuni*.

Keywords: *Lactobacillus*, *Campylobacter jejuni*, coaggregation

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CHAPTER 1: INTRODUCTION

Campylobacter jejuni is a gram-negative, spiral shaped bacterium. It is the most common bacterial cause of foodborne illness and diarrheal disease in the United States (Konkel et al. 2007). Consumption of contaminated chicken is one of the most common ways humans contract diseases caused by *C. jejuni.* This bacterium is a commensal organism of chickens and other avian species (Young, Davis, & DiRita, 2007) but can be harmful to humans that ingest products contaminated with *C.jejuni*. *C. jejuni* colonizes the gastrointestinal tract of chickens primarily in the mucosal layer. The ability of *C. jejuni* to adhere to epithelial cells lining the gastrointestinal tract is shown to be an important virulence attribute. Also, the ability of *C. jejuni* to bind to receptors on cells lining the intestinal tracts of birds appears to be required for the colonization process (Konkel et al., 2007). With the growing prevalence of *C. jejuni* associated infection, an effective intervention method needs to be developed to reduce or prevent *C .jejuni* colonization in chickens. The intervention strategy being analyzed in this study is the use of lactic acid bacteria as a way to help reduce *C. jejuni* colonization in chickens.

Lactic acid bacteria (LAB) have been found to be beneficial to both humans and animals. The relationship between *C. jejuni* and LAB, especially probiotics, is of importance because of the ability of probiotics to possibly competitively exclude *C. jejuni* in the epithelial cells lining the gastrointestinal tract of chickens. A mechanism of interest that relates to this competitive relationship is coaggregation (Janković, Frece, Abram, & Gobin, 2012). Coaggregation, which is the clumping of genetically distinct bacteria to each other, has been identified as a possible mechanism that facilitates the competitive relationship between probiotics and *C. jejuni* (Tareb, Bernardeau, Gueguen & Vernoux, (2013).

Further understanding the properties behind the coaggregation of probiotics and *C. jejuni in vitro* could possibly lead to an intervention method to help prevent or reduce *C. jejuni* colonization in poultry. The overall goal for the future would be to conduct an *in vivo* chicken study to evaluate the effect of coaggregation between LAB and *C. jejuni* on the colonization ability of *C. jejuni* in chickens. In order to conduct such a study, LAB bacterial candidates that coaggregate and do not coaggregate strongly with *C. jejuni* need to be identified. The first objective of this study is to analyze if the coaggregative abilities of LAB with *C. jejuni* are strain dependent. The second objective is to pinpoint the possible mechanism that is facilitating coaggregation between lactobacilli and *C .jejuni*. It is hypothesized that identifying a specific mechanism or gene as the facilitator of the coaggregative relationship could help lead to better understanding the interaction. This would lead to the ultimate objective which is to conduct an *in vivo* chicken study comparing the effects of the coaggregative abilities of these strains on reduction of *C. jejuni* colonization in chickens.

CHAPTER 2: LITERATURE REVIEW

2.1. *Campylobacter jejuni*

Campylobacter jejuni is one of the most common causes of foodborne diarrheal disease in the United States (Centers for Disease Control and Prevention [CDC], 2014). *C. jejuni* commonly colonizes the intestinal tract of humans and other animals. Humans that ingest products contaminated with *C. jejuni* are at risk of contracting a disease called campylobacteriosis. Symptoms of campylobacteriosis include diarrhea, cramping, abdominal pain, and fever. Bloody diarrhea is another possible symptom and can be accompanied by nausea and vomiting (CDC, 2014). Consumption of contaminated poultry products has been correlated with many cases of campylobacteriosis (Williams et al., 2015). In rare instances, *C. jejuni* infection can lead to a more detrimental disease called Guillain-Barré syndrome which causes acute neuromuscular paralysis in humans (Konkel et al., 2007). Interestingly, *C. jejuni* is a commensal of chickens and other avian species (Young, Davis, & DiRita, 2007). Consequently, the bacterium imposes no harm to poultry but can cause detrimental effects to other animals that consume or come into contact with products contaminated with *C. jejuni*. Studies have shown that there is a linear relationship between *C. jejuni* flock prevalence and the probability of human campylobacteriosis (Newell & Fearnley, 2003). Therefore, finding an intervention method to reduce the colonization of *C. jejuni* in flocks could reduce the probability of humans contracting campylobacteriosis. The ability of *C. jejuni* to bind to receptors on cells lining the intestinal tracts of poultry appears to be required for the colonization process to occur (Konkel et al., 2007). *C. jejuni*'s ability to adhere to epithelial cells lining the gastrointestinal tract is shown to be an important virulence attribute. *C. jejuni* is often able to survive routine poultry slaughtering and food processing. Identifying a solution to help reduce *C. jejuni* colonization in chickens

could potentially reduce the amount of *C .jejuni* that survive through these steps in food production.

2.2. Strategies to reduce *Campylobacter jejuni*

C .jejuni can spread through a poultry farm very rapidly, so an intervention strategy needs to be implemented that reduces *C. jejuni* introduction and transmission in poultry flocks (Hermans et al., 2011). There have been many studies attempting to identify an effective intervention method to reduce *C. jejuni* colonization in chickens. Reducing or ultimately eliminating the presence of *C. jejuni* in chicken does not have a finite solution. Studies have been conducted to reduce the presence of *C. jejuni* during all stages of food production including measures to: prevent chicken exposure to *C. jejuni*, reduce the load of *C. jejuni* within birds, reduce contamination during slaughter and remove/kill *C. jejuni* from the surface of meat products (Hermans et al., 2011a). The primary measure of interest for this study is reducing the load of *C. jejuni* within birds. Methods that have been analyzed include reducing environmental exposure through increasing poultry's host resistance to reduce *Campylobacter* carriage in the gut (e.g., competitive exclusion, vaccination, and host genetics selection), and using antimicrobial alternatives to reduce and even eliminate *Campylobacter* from colonized chickens (Lin, 2009). Hygienic and biosecurity measures, water treatment, bacteriophage application and passive immunization are additional intervention strategies tested to reduce or eliminate *C. jejuni* colonization in poultry (Hermans et al., 2011a). Further research and understanding of these methods could lead to an efficient and applicable intervention strategy.

Bacteriocins and bacteriophages are possible strategies studied to reduce *C. jejuni* colonization in poultry. Bacteriocins are antimicrobial peptides produced by bacteria with varied host ranges (Lin, 2009). Lactic acid bacteria are among the bacteria that can produce

bacteriocins. It has been shown that bacteriocins may function as a barrier against pathogens in the gut (Zacharof & Lovitt, 2012). An advantage of bacteriocins is that they are natural, nontoxic and can commonly be found in food products. The food industry uses bacteriocins such as nicin and pediocin PA1/AcH to aid in preservation (Lin, 2009). Anti-*Campylobacter* bacteriocins have been developed and tested in poultry studies. Stern et al., 2008 concluded in an *in vivo* study that bacteriocins may not be the most important mechanism involved in competitive exclusion against pathogens (Stern et al., 2008). They came to this conclusion because the anti-*Campylobacter* bacteriocins failed to exclude *C. jejuni* but it is possible that other bacteriocins may be found to be effective.

Bacteriophages are another method tested to reduce *C. jejuni* in the chicken gut. The first trial was conducted with *Campylobacter* phages (campylophages) in commercial broiler flocks. Results showed that phages lead to a reduction of up to $\log_{10}$ 3.2 CFU in *Campylobacter* loads (Kittler et al., 2013). Though in order for implementation of this method, timing and suitable campylophage cocktails needed to be optimized (Kittler et al., 2013). However, a major drawback of using bacteriophages to reduce *C. jejuni* colonization in chickens is phage resistance (Hammerl et al., 2014).

Competitive exclusion, vaccination, and host genetics selection are all methods being tested to help increase poultry's host resistance to *C. jejuni*. Studies have shown that colonization of chickens by *Campylobacter* can be inhibited by competitive exclusion bacteria if effective microbial strains are used (Zhang, Ma & Doyle, 2007). In a simulated chicken digestive model, it was shown that selected lactobacilli had an antagonistic effect on *C. jejuni*. (Chang & Chen, 2000). A conclusion drawn from this study was that reducing the incidence of *C. jejuni* in poultry could be done by direct feeding lactic acid bacteria but the intestinal tract is very

complex so more *in vivo* studies need to be conducted to analyze this further (Chang & Chen, 2000). Neal-McKinney et al., 2014 analyzed the strategy of reducing *C. jejuni* colonization in poultry by vaccination. This study analyzed whether the vaccination of chickens with *C. jejuni* surface-exposed colonization proteins could reduce the ability of *C. jejuni* to colonize chickens. A limitation of this study was that the antigens were delivered through intramuscular injection; however *C. jejuni* is normally found in the digestive tract of chickens (Neal-McKinney et al., 2014). Delivering the vaccine through intramuscular injection did result in a reduction in *C. jejuni* colonization but it would have been more practical if the researchers had developed a method that delivered the vaccine orally to the digestive tract via mucosal delivery since *C. jejuni*'s pathogenic activity occurs in the intestinal mucosa of chickens. Also, it would be challenging to deliver a vaccine through intramuscular injection in a large chicken flock. Currently, no commercial vaccine is yet available to control *Campylobacter* infection in poultry, but there is the possibility of manipulating the intestinal microflora of chicks to increase resistance by competitive exclusion (Chang & Chen, 2000). Better understanding the mechanisms that facilitate lactobacilli's ability to competitively exclude *C. jejuni* within the intestinal tract could potentially lead to improved intervention methods to reduce *C. jejuni* colonization in chickens.

2.3. Probiotics

Research has shown that probiotics can competitively exclude pathogens. Probiotics are bacteria that confer health benefits to the host when administered in adequate amounts (FAO/WHO, 2002). These health benefits include but are not limited to antimicrobial properties and resistance to enteric pathogens (Nagpal et al., 2012). Probiotics can be found in many products including yogurt and cheese. To be characterized as a probiotic, the bacteria must be able to survive

transit through the gastrointestinal environment, as well as withstand exposure to bile and pancreatic juice in the upper small intestine. The bacterium can then exert beneficial effects in the lower small intestine and the colon (Ljungh & Wadström, 2006). Several studies have also suggested that adhesive probiotic bacteria could prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract (Lee et al., 2000). Probiotics' anti-pathogenic mechanisms are divided into three classes which are direct antagonism, immunomodulation, and exclusion. Probiotic exclusion mechanisms include improving epithelial barrier function in the GI tract and interfering with pathogen binding (Preidis et al., 2011). As stated before, the mechanism of interest in the current study is to analyze probiotics' ability to competitively exclude pathogens.

2.4. Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are bacteria which produce lactic acid as their major fermentation product (Masood et al., 2011). Benefits of LAB in humans and other animals include prevention of diarrhea, stimulation of the immune system and playing a role in infectious disease prevention (Masood et al., 2011). Lactobacilli are the largest genus of lactic acid bacteria and are of interest because many possess probiotic characteristics (Claesson, Sinderen, & O'Toole, 2007). The interaction between probiotics and pathogens shows promise for helping counteract the harmful effects of pathogens such as *C. jejuni*. Lactic acid bacteria are of main interest when it relates to the competitive exclusion of pathogens because they are already found naturally in the chicken gut and are introduced to suppress the growth of harmful bacteria (Chang & Chen, 2000). *Lactobacillus crispatus* is a lactic acid bacterium that has gained increased interest because of its probiotic properties *in vitro* and *in vivo*. In a study conducted by Neal-McKinney et al., 2012, experiments were conducted to evaluate the ability of four

Lactobacillus strains to reduce colonization of *C. jejuni* in commercial broiler chickens. Of the four strains, *L. crispatus* was the most effective in reducing the number of chickens colonized with *C. jejuni.* It was concluded that the production of lactic acid was most likely the mechanism behind *L. crispatus*' reduction of *C. jejuni* (Neal-McKinney et al., 2012). *L. crispatus* is a potential bacterial candidate for use as an intervention method to help reduce *C. jejuni* colonization in chickens.

2.5. Relationship between *Campylobacter jejuni* and Probiotics

C. jejuni colonizes the intestinal mucosal lining of animals and probiotics also have this capability. Adhesion to the intestinal mucosa is considered one of the main criteria for the selection of potential probiotics, as it may increase their persistence in the intestine. (Kolida, Saulnier, & Gibson, 2006). An important mechanism associated with probiotics is their ability to compete with pathogens for binding sites on epithelial cells (Jankovic et al., 2012). This competition prevents pathogens such as *C. jejuni* from adhering to intestinal epithelial cells and even reduces pathogen colonization and infection (Jankovic et al., 2012). Several studies have suggested that adhesive probiotic bacteria could prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract (Lee et al., 2000). Probiotics' ability to adhere to the surface of epithelial cells or mucus enables them to form a protective layer and block contact between pathogens and host cells (Popova et al., 2012). In a study by Ghareeb et al., 2012 the efficacy of an avian-specific probiotic against *C. jejuni* growth and colonization was analyzed *in vitro* and *in vivo*. For *in vitro* tests, the research group used a co-cultivation agar plate assay to observe the inhibition of *C. jejuni* growth by probiotic bacteria. For *in vivo* tests two different dosing regimens of a probiotic product were administered. Either a single dose (2 mg/bird per day) was administered at each stage of the experiment or two different doses at

different stages in the experiment (2 mg/bird per day or 20 mg/bird per day). These treatments were used to compare the effects of the different dosing regimens on reducing the cecal colonization of *C. jejuni* in broiler chickens. The probiotic product consisted of *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* microorganisms (Ghareeb et al., 2012). Results from the *in vitro* study demonstrated that *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* all demonstrated high inhibition indexes against *C. jejuni* growth. The research group suggested that these are promising microorganisms for probiotic application to reduce *C. jejuni* colonization. This result is also promising in regards to the current study because our study specifically focuses on lactobacilli and two of the probiotic strains were from this genus. For the *in vivo* chicken study, results showed that there was no significant difference between the different probiotic dose treatment groups. In both *in vivo* experiments, chickens that received the probiotic treatment were significantly less colonized with *C. jejuni* than the chickens in the control group (Ghareeb et al., 2012). Findings from this study reinforce the idea that feeding probiotics to chickens could be a possible way to reduce the colonization of *C. jejuni* in chickens. A drawback of this study and many other related studies is that the actual mechanism behind why probiotic bacteria have inhibitory effects on *C. jejuni* was not identified. Better understanding the mechanisms behind the competitive relationship between probiotic bacteria and pathogens could help aid in developing more effective intervention methods.

2.6. Coaggregation of Bacteria

One mechanism of action that plays a role in the relationship between probiotics and pathogens is aggregation. Aggregation is defined as the reversible accumulation of cells, causing them to spontaneously precipitate in the medium in which they are suspended (Janković, Frece,

Abram, & Gobin, 2012). Two sub-categories of aggregation are coaggregation and autoaggregation. Coaggregation occurs when genetically distinct bacteria become attached to one another via specific molecules (Rickard, Gilbert, High, Kolenbrander, Handley, 2003). Alternatively, autoaggregation is when cells from the same species clump together and precipitate in the medium in which they are suspended. The ability for a probiotic to aggregate within the GI tract is a desirable characteristic. Strains that are able to autoaggregate can adhere to surface mucosa which increases probiotic persistence in the intestine (García-Cayuela et al., 2014). When probiotics coaggregate with pathogens, it allows pathogens to be more easily removed from the intestinal environment (García-Cayuela et al., 2014). The coaggregation of probiotic bacteria and pathogenic bacteria is of primary importance for the current study. Through coaggregation, probiotic bacteria may form a barrier that prevents pathogenic bacteria from colonizing the gastrointestinal tract (Kos et al., 2003) of chickens. A study by Tareb et al., 2013 analyzed the coaggregative abilities of various lactobacilli with pathogens including *C. jejuni*. Their results showed that all of the lactobacilli effectively prevented the adhesion of *C. jejuni* to mucin (Tareb et al., 2013). This was significant because mucin supports *C. jejuni* reproduction and enhances its adhesion to epithelial cells (Alemka et al., 2010). There have been various *in vitro* and *in vivo* studies analyzing the coaggregative interactions of lactobacilli with pathogens. In a study by Nishiyama et al., 2014, the effect of the coaggregative abilities of *Lactobacillus gasseri* SBT205 with *C. jejuni* were analyzed in an *in vivo* chicken model. They were specifically looking at the effect of coaggregation between the two bacteria on the reduction of *C. jejuni* colonization. In this study, the coaggregative abilities of *L. gasseri* SBT2055 (LG2055) with *C. jejuni* 81-176 were analyzed. Methanol (MeOH) fixation and proteinase K (ProK) treatments of LG2055 were performed. The purpose of the ProK and MeOH

treated LG2055 tests were to analyze the effects of inhibiting surface components on *C. jejuni*'s invasion and adhesion. Results showed that ProK treatment eliminated LG2055-mediated inhibition of *C. jejuni* 81-176 invasion and adhesion. In the *in vivo* study, the inhibitory effect of LG2055 on *C. jejuni* colonization in chicks was analyzed. Results from the chicken study showed that LG2055 was able to significantly reduce colonization in chicks at 14 days post-inoculation. The research group concluded that LG2055 could be useful in suppressing pathogen colonization of the chicks at early growth stages and could help prevent pathogen infection (Nishiyama et al., 2014). A shortcoming of this study was that although Nishiyama et al. conducted inhibitory tests to identify the surface components contributing to coaggregation, they did not pinpoint the specific mechanisms that facilitate the coaggregation between LG2055 and *C. jejuni*. One of their conclusions was that the coaggregation phenotype and/or adhesion mediated by proteinaceous surface components of LG2055 might be responsible for the reduction in *C. jejuni* infection and colonization (Nishiyama et al., 2014). This was an inference that was not confirmed further other than the results of the ProK treatment test. It would have been beneficial if the research study had further confirmed the mechanism that caused the reduction in *C. jejuni* infection and colonization. For LG2055 to be useful in suppressing pathogen colonization, the specific mechanisms behind coaggregation need to be specifically identified. Identifying a specific mechanism or gene that facilitates coaggregation would help to better characterize a point to target in a chicken study and later in an intervention strategy. An approach that is lacking in this study and the literature as a whole is comparing isogenic strains of lactobacilli that do and do not coaggregate. Finding *Lactobacillus* strains with similar genotypes but different coaggregative abilities would be helpful in possibly identifying the mechanism or gene facilitating coaggregation with *C. jejuni*. Knowledge of this specific gene

could lead to further *in vitro* studies analyzing the coaggregative abilities of wildtype *Lactobacillus* strains versus gene knockout strains. Findings from these studies could lead to an *in vivo* study comparing the effects of the coaggregative abilities of these strains on reduction of *C. jejuni* colonization in chickens. This type of study is lacking in the literature but results from a study like this could be a step towards finding an effective strategy to reduce *C. jejuni* colonization in poultry.

2.7. Bacterial Mutant Selection

Selection of bacterial mutant strains is a procedure that has been developed over the years. Selection can be performed where stringent conditions prevent the growth of the parent bacterial strain and only allow the pre-existing mutants to grow (Roth et al., 2006). Furukawa et al., 2012 developed mutants of *L. plantarum* ML11-11 that were deficient in coaggregation with yeast. In this study, the authors selected for the mutant strain through spontaneous mutant selection. Results showed that after repeating the non-coaggregative cell enrichment process twenty times, fifteen colonies were selected as possible non-coaggregative mutants (Furukawa et al., 2012). Selecting for a mutant bacterial strain could help identify specific mechanisms facilitating coaggregation by comparing sequences of the mutant and wild type *Lactobacillus strain*. Identifying a particular LAB strain that can prevent the adhesion of *C. jejuni* to mucin through coaggregation could be the first step towards developing a strategy to reduce infection caused by *C. jejuni*. More studies need to be conducted to identify such a strain and coaggregation assays should be tested under different conditions including in an i*n vivo* chicken model. The focus of the current study is analyzing the coaggregation between lactobacilli and *C. jejuni*. Better understanding the relationship between coaggregation and *C. jejuni* colonization in poultry could help lead to an intervention method using LAB in the future.

CHAPTER 3: THE COAGGREGATIVE ABILITIES OF LACTOBACILLI AND *CAMPYLOBACTER JEJUNI*

3.1. ABSTRACT

Campylobacter jejuni is a pathogen commonly found in poultry that is one of the main bacterial causes of diarrheal disease. The aim of this study was to analyze the coaggregative abilities of lactic acid bacteria and *C. jejuni* as a means to possibly reduce the colonization of *C. jejuni* in poultry. Initially, eighteen strains of lactobacilli were screened for their ability to coaggregate with *C. jejuni* NCTC 11168. Six strains (*Lactobacillus johnsonii* ATCC 11506, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 9595, *Lactobacillus salivarius subsp. salivarius* ATCC 11741, *Lactobacillus fermentum* CECT5716 and *Lactobacillus crispatus* JCM5810) were identified that strongly coaggregated with *C. jejuni* yet did not autoaggregate. Subsequently, we tested several different strains of *L. acidophilus*, *L. fermentum, L. plantarum*, and *L. crispatus* and confirmed that coaggregation is a stable trait within lactobacilli species except for *L. crispatus*. Since *L. crispatus* JCM5810 has been used as a probiotic to reduce *C. jejuni* colonization previously, we investigated the mechanism further. Non-aggregating variants of *L. crispatus* JCM5810 were identified and sequenced. Potential genetic determinants in lactobacilli for coaggregation were identified. Also, various mutants of *C. jejuni* were tested with *L. crispatus* JCM5810 and it was found that several genes required for *C. jejuni* colonization and/or pathogenicity were also required for coaggregation with *L. crispatus*. Findings from this study can provide knowledge to help better understanding the coaggregative relationship between lactobacilli and *C. jejuni* and identify a genetic determinant for this relationship.

Keywords: *Lactobacillus***,** *Campylobacter jejuni,* coaggregation

3.2. INTRODUCTION

The bacterium *Campylobacter jejuni* is the leading cause of human gastroenteritis in many developed countries (Hermans et al., 2011b). *C. jejuni* favors the intestinal environment of many avian species (Newell & Fearnley, 2003) and is a commensal organism of chickens (Young, Davis, & DiRita, 2007). *C. jejuni* spreads rapidly through chicken flocks and is able to survive through food processing (Hermans et al., 2011b). As a result, human consumption of products contaminated with *C. jejuni* can lead to campylobacteriosis which includes symptoms of diarrhea, cramping, abdominal pain, and fever (CDC, 2014). *C. jejuni* colonizes the gastrointestinal tract of chickens primarily in the mucosal layer. The ability of *C. jejuni* to adhere to epithelial cells lining the gastrointestinal tract is shown to be an important virulence attribute. Also, the ability of *C. jejuni* to bind to receptors on cells lining the intestinal tracts of birds appears to be required for the colonization process (Konkel et al., 2007). With the growing prevalence of *C. jejuni* associated infection, an effective intervention method needs to be developed to reduce or prevent *C .jejuni* colonization in chickens. The intervention strategy being analyzed in this study is the use of lactic acid bacteria as a way to help reduce *C. jejuni* colonization in chickens.

Lactic acid bacteria (LAB) have been found to be beneficial to both humans and animals. The relationship between *C. jejuni* and LAB, especially probiotics, is of importance because of the ability of probiotics to possibly competitively exclude *C. jejuni* in the epithelial cells lining the gastrointestinal tract of chickens. One mechanism of action that plays a role in the relationship between probiotics and pathogens is aggregation. Aggregation is defined as the reversible accumulation of cells, causing them to spontaneously precipitate in the medium in which they are suspended (Janković, Frece, Abram, & Gobin, 2012). Coaggregation, the

clumping of genetically distinct bacteria to each other, has been identified as a possible mechanism that facilitates the competitive relationship between probiotics and *C. jejuni*. The ability for a probiotic to aggregate within the GI tract is a desirable characteristic. When probiotics coaggregate with pathogens, it allows pathogens to be more easily removed from the intestinal environment (García-Cayuela et al., 2014). The coaggregation of probiotic bacteria and pathogenic bacteria is of primary importance for the current study. Through coaggregation, probiotic bacteria may form a barrier that prevents pathogenic bacteria from colonizing the gastrointestinal tract (Kos et al., 2003) of chickens. There have been various *in vitro* and *in vivo* studies analyzing the coaggregative interactions of lactobacilli with pathogens. In this study, coaggregative abilities of lactic acid bacteria with *C. jejuni* were analyzed. Further understanding the properties behind the coaggregation of probiotics and *C. jejuni in vitro* could lead to an intervention method to help prevent or reduce *C. jejuni* colonization in poultry.

The eventual goal of this study is to conduct an *in vivo* chicken study to evaluate the effect of coaggregation between LAB and *C. jejuni* on the colonization ability of *C. jejuni* in chickens. In order to conduct such a study, LAB bacterial candidates that coaggregate and do not coaggregate strongly with *C. jejuni* needed to be identified. The first objective of this study was to analyze if the coaggregative abilities of LAB with *C. jejuni* are strain dependent. The second objective was to identify the possible mechanism that facilitates coaggregation between lactobacilli and *C .jejuni*. The overall objective was to conduct an *in vivo* chicken study evaluating the coaggregative abilities of the bacteria previously mentioned.

3.3. MATERIALS AND METHODS

3.3.1. Analysis of the Coaggregative Ability of *Campylobacter jejuni* with various lactobacilli

Bacterial Strains and Growth Conditions

Lactobacilli were grown anaerobically in de Man, Rogosa, and Sharpe (MRS) broth at 37 °C for 24 hours while *C. jejuni* F38011 (MJM 211) and *C. jejuni* 11168 (MJM 213) was grown on Mueller Hinton broth supplemented with 1.5% agar and 5% bovine citrate blood agar plates. Plates were incubated at 37 °C for 24 hours in a CO_2 incubator with a gas composition of 85% nitrogen, 10% carbon dioxide and 5% oxygen.

Autoaggregation and Coaggregation Assays

Eighteen different *Lactobacillus* strains (Tables 1 and 2) were tested to analyze if the ability to coaggregate with *C. jejuni* was strain dependent. The autoaggregation of the *Lactobacillus* strains along with their coaggregation with *C. jejuni* F38011 (MJM 211) and *C. jejuni* 11168 (MJM 213) were tested. The *Lactobacillus* strains were centrifuged at 3,000 × *g* for 5 minutes and resuspended in acetate buffer (pH 4). *C. jejuni* was centrifuged at 3,000 × *g* for 10 minutes, washed twice in PBS, and then resuspended in acetate buffer. To analyze coaggregative abilities, equal volumes of each LAB strain were mixed with a *C. jejuni* strain (MJM 211 or MJM 213). Autoaggregation tubes were also prepared with only LAB strains and *C. jejuni* strains suspended in acetate buffer. Tubes were allowed to sit with no agitation for one hour and were observed every thirty minutes for a total of three hours of observation. The tubes were analyzed visually and ranked based on degree of aggregation and pellet formation (Table 1 and Figure 1). Further comparison of autoaggregation/coaggregation tests were conducted with additional bacterial strains. *L. fermentum*, *L. plantarum* and *L. acidophilus* strains were used because there were many strains from different sources accessible in our culture collection. Also,

these species previously showed promise for successful genetic manipulation. The analysis of coaggregative variation among *Lactobacillus* strains within the same species were conducted with *C. jejuni* (MJM 213) (Table 4). The ability of *L. crispatus* (MJM 207) to coaggregate with various *C. jejuni* variant and knockout strains was also analyzed (Table 4). Autoaggregation and coaggregation tests were performed as described above.

3.3.2. Selection for Non-Coaggregative and Coaggregative Variant *Lactobacillus* Strain

Bacterial strains and Growth Conditions

Strong coaggregative strains from the initial screening process were selected for the variant selection process. Growth conditions for the *Lactobacillus* strains and *C .jejuni* were performed the same as previously mentioned. The procedure for selecting for a non-coaggregative *Lactobacillus* spontaneous mutant was patterned after Furukawa et al., 2012.The process started by mixing equal volumes of each *Lactobacillus* strain with *C. jejuni* (MJM 213) cells and coaggregation was visually observed. 30 µl was then taken from the supernatant (non-coaggregative cells) and inoculated into 3 mL of fresh MRS. 30 µl of *C. jejuni* cells suspended in acetate buffer were also added to the MRS and vortexed. Tubes were grown anaerobically at 37˚C. The selection process was repeated by conducting coaggregation tests again with the variant cells grown in MRS (pipetted from the top of the MRS tubes). The non-coaggregative cell enrichment process was repeated until a *Lactobacillus* variant was found that no longer coaggregated with *C. jejuni*. Glycerol stocks were prepared for these variants and streaked for a single colony. Tests were repeated to confirm the aggregative phenotype for the variants.

In addition, selection of a *Lactobacillus crispatus* (MJM 206) coaggregative variant strain was conducted. The procedure consisted of carrying out coaggregation tests with *C. jejuni* as previously mentioned. 30 µl was pipepeted from the bottom of the coaggregation tube and then

added to fresh MRS with 30 µl of *C. jejuni* cells and vortexed. Tubes were grown anaerobically at 37˚C. The selection process was repeated by conducting coaggregation tests again with the variant cells grown in MRS (taking from the bottom of the MRS tubes). The coaggregative cell enrichment process was repeated until a *Lactobacillus crispatus* variant was found that eventually coaggregated with *C. jejuni*.

3.3.3. Preparation of *L. crispatus* wild type and variant samples for DNA sequencing

L. crispatus (MJM 207) wild type DNA was extracted for mate-pair library preparation. DNA from *L. crispatus* variants (1, 3, and original) *L. crispatus* wild type (MJM 206) was extracted for paired-ended library preparation. DNA extraction methods were adapted from lab established protocol (S:\Miller Lab\General Lab Information\Protocols).

3.3.4. DNA sequencing

DNA sequencing was performed at the Roy J. Carver Biotechnology Center (University of Illinois). Illumina MiSeq v3 300nt paired-end reads were generated.

3.3.5. DNA sequencing analysis

Analysis of results was performed in CLC Genomics Workbench 8.5.1.

3.4. RESULTS

3.4.1. Analysis of the Coaggregative Ability of *Campylobacter jejuni* with various lactobacilli

The autoaggregative and coaggregative abilities of the eighteen strains of lactobacilli with *C. jejuni* (MJM 211 and 213) were tested (Table 2 and 3). *Lactobacillus* strains of interest were ones that within a 3 hour span showed no indication of autoaggregation but had strong indication of coaggregation with *C. jejuni*. Strains picked for further testing showed consistent coaggregative abilities with both *C. jejuni* strains (Table 2 and 3 indicated by asterisk) which included *Lactobacillus johnsonii* ATCC 11506, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 9595, *Lactobacillus salivarius subsp. salivarius* ATCC 11741, *Lactobacillus fermentum* CECT5716 and *Lactobacillus crispatus* JCM5810.

3.4.2. Analysis of coaggregative variation among *Lactobacillus* strains within the same species

While *L. crispatus*, *L. plantarum*, *L. fermentum* and *L acidophilus* were positive for coaggregation, we hypothesized that coaggregation was a variable trait within the species. To test our hypothesis, we tested several strains for these four species. (Table 4). Coaggregative abilities did not vary tremendously within *L. acidophilus*, *L. fermentum* and *L. acidophilus*. *L. crispatus* strains MJM 206 and 207 showed different coaggregative abilities with *C. jejuni*. MJM 207 was found to be a strong coaggregator but MJM 206 appeared to be a non-coaggregator. (Table 4)

3.4.3. Coaggregation tests with variant and knockout *Campylobacter jejuni* strains

The necessary properties of *C. jejuni* that mediate coaggregation were tested using several different strains including strains with deleted genes that are involved in motility and adhesion. After conducting tests with the various knockout strains (Table 5), MJM 371 was determined to be a very strong coaggregator with *L. crispatus* (MJM 207). This is a highly motile variant of MJM 213. It was determined to be a fast coaggregator and was used in further coaggregation tests. After five hours there was still no indication of coaggregation with the mutant *C. jejuni* strains (376, 378, 379, 381 and 382). Tests were replicated three separate times and showed the same results. These variant strains also did not autoaggregate.

3.4.4. Selection for Non-Coaggregative *Lactobacillus* Strain

After 10 selections, coaggregation with certain LAB ceased. Tubes were checked after 3 hours to observe any coaggregative activity. After 16 hours selection tubes were checked again and *Lactobacillus* wildtype strains MJM 39, 43, 54 and 207 (Figure 2) strongly coaggregated whereas their non-coaggregating variants did not.

L. crispatus (MJM 207) wild type strain and its non-coaggregative variant were the focus for further testing. Four additional non-coaggregative variants were created. The selection process was repeated as previously mentioned but with less bacterial passes between tests. After four selections, coaggregation with *C. jejuni* ceased. Figure 4 shows that after 3 hours the 4 additional variant strains did not coaggregate with *C. jejuni*. After 4 passes the MJM 206 variant still showed no sign of coaggregation with *C. jejuni*.

3.4.5. Mate-pair and Paired-end Library Results

The original MJM 207 variant, variant 1 and variant 3 along with the MJM 206 wild type were chosen for construction of a paired end library. A mate-pair and paired-end library were

constructed for the MJM 207 wild type. Results from the MJM 207 wild type mate-pair library were compared to the paired-end libraries of MJM 207 variants (original, variant 1, and 3) and the MJM 206 wild type. A draft genome of *L. crispatus* (MJM 207) consisting of 12 contigs was submitted to GenBank [Submission ID: 1877072].

3.5. DISCUSSION AND CONCLUSIONS

Coaggregation is a promising phenomenon to help better understand the relationship between lactic acid bacteria and pathogens. The first objective of this study was to analyze if the coaggregative abilities of LAB with *C. jejuni* are strain dependent. The second objective was to identify the possible mechanism that facilitates coaggregation between lactobacilli and *C. jejuni*.

This current study has reinforced that the ability to coaggregate is strain dependent. The different abilities of various *Lactobacillus* strains were identified and it was shown that some strains are strong coaggregators and others are not. *Lactobacillus crispatus* (MJM 207) was a particular strain that gained interest during the current study. Results showed that *L. crispatus* does not show any indication of autoaggregation but does show coaggregative abilities with *C. jejuni* (Table 2 and 3). Neal-McKinney et al., 2012 evaluated the ability of this particular *L. crispatus* strain and three other strains' ability to reduce colonization of *C. jejuni* in commercial broiler chickens. Of the four strains, *L. crispatus* was the most effective tested in reducing the number of chickens colonized with *C. jejuni.* All four *Lactobacillus* strains (*L. acidophilus* NCFM, *L. crispatus* JCM 5810, *L. gallinarum* ATCC 33199 and *L. helveticus* CNRZ32) tested in the study conducted by Neal-McKinney et al., 2012 were also tested in the initial aggregation tests of the current study (Table 2 and 3). Some of this study's findings correlated with the autoaggregation and coaggregation results found from these strains. Of the four strains, *L. crispatus* showed the best coaggregative abilities with both strains of *C. jejuni*. *L. acidophilus* NCFM and *L. gallinarum* ATCC 33199 did not show any strong indication of coaggregation with either strain of *C. jejuni*. *L. helveticus* CNRZ32's coaggregative abilities were inconsistent among both strains of *C. jejuni. L .crispatus* was the most effective in reducing the number of chickens colonized with *C .jejuni* (Neal-McKinney et al., 2012) and it was the strongest

coaggregator out of the four strains in our initial coaggregation tests. These findings make *L. crispatus* a promising bacterial candidate for further testing. Tests that could correlate the strong coaggregative abilities and the capability to reduce *C. jejuni* colonization in chickens would need to be further analyzed.

The coaggregation of *L. crispatus* was observed with various variant and knockout *C. jejuni* strains (Table 5). There were various gene knockouts in the strains including *cadF, flpA, cadF-flpA* double knockout, *flgL, flaA, flab, flaA-flabB* double knockout and *fbpA*. *L. crispatus* did not show any indication of coaggregation with any of the knockout strains. Some of the genes knocked out have significance as it relates to *C. jejuni*'s colonization abilities. For example, *cadF* and *flaA* have importance related to *C. jejuni*'s adherence, colonization and invasion capabilities (Zheng, Meng, Zhao, Singh & Song, 2006). Studies have shown that *cadF* and *flaA* are required for *Campylobacter*'s colonization and adherence to a host cell surface (Neal-McKinney & Konkel, 2012). Studies have shown that the motility of the flagella of *C. jejuni* is required for colonization of the mucus lining of the gastrointestinal tract (Guerry, 2007). The flagellum of *C. jejuni* is composed of a basal body, hook, and filament. The flagellar filament, a component of the flagellum, is made up of the proteins, *flaA* and *flaB* (Konkel et al., 2004). *FlgL* is one of the components that make up the hook of the flagellum (Neal-McKinney & Konkel, 2012) and *fbpA* is part of a class of proteins that play a role in membrane protein complexes for transport or signal transduction (Berntsson et al., 2010). Since *L. crispatus* did not show the ability to coaggregate with any of the knockout strains it can be hypothesized that these proteins play a role in the coaggregative interactions between lactobacilli and *C. jejuni*. In some cases, lack of motility may be the reason for no interaction with lactobacilli. Understanding why

the absence of certain *C. jejuni* flagellar proteins prevents coaggregation could be important for future *in vivo* studies as it relates to what role these proteins play in *C. jejuni* colonization.

Coaggregation is a complex mechanism that needs further research. In our current study, measures were taken to further understand the mechanism or protein that may be facilitating coaggregation. Analysis of the coaggregative ability of *C. jejuni* with various lactobacilli was conducted. Two different *L. crispatus* strains were found to have differing coaggregative abilities (Table 4). These strains were sent for DNA sequencing and paired-end libraries were constructed for both MJM 206 and 207 wild type strains. A mate pair library was generated for MJM 207 and these libraries were compared to identify possible differences in their genomes. Paired-end libraries were also constructed for the original variant, variant 1 and 3 (Figure 3). These results were also compared to MJM 207's wild type strain. More coaggregation studies need to be conducted to confirm the precise mechanism facilitating these interactions between lactobacilli and *C. jejuni*. In a study by Nishiyama et al., 2014, the effect of the coaggregative abilities of *Lactobacillus gasseri* SBT205 with *C. jejuni* were analyzed in an *in vivo* chicken model. They were specifically looking at the effect of coaggregation between the two bacteria on the reduction of *C. jejuni* colonization. In this study, the coaggregative abilities of *L. gasseri* SBT2055 (LG2055) with *C. jejuni* 81-176 were analyzed. One of their conclusions was that the coaggregation phenotype and/or adhesion mediated by proteinaceous surface components of LG2055 might be responsible for the reduction in *C. jejuni* infection and colonization (Nishiyama et al., 2014). This was an inference that was not confirmed further other than the results of their ProK treatment test. It would have been beneficial if the research study had further confirmed the mechanism or gene that caused the reduction in *C. jejuni* infection and colonization. For LG2055 to be useful in suppressing pathogen colonization, the specific

mechanisms behind coaggregation need to be specifically identified. Identifying a specific mechanism or gene that facilitates coaggregation would help to better characterize a point to target in a chicken study and later in an intervention strategy.

This study provided information on the coaggregative abilities of various *Lactobacillus* strains with *C. jejuni*. It was found that coaggregation with *C. jejuni* is strain dependent and *L. crispatus* was identified as a strain of interest. *L. crispatus*' coaggregative abilities in the current study and probiotic capabilities found in other studies make it a bacterial candidate for future research.

3.6. TABLES

Table 1. Ranking System for Measuring Aggregation

Degree of Aggregation
0+ For no visible aggregates in the cell suspension; an even homogeneous suspension of cells, may remain for days until settling out un-aggregated (small powdery/dense pellet may still form) **1+** For small uniform aggregates in the suspension; small clusters or sand-like grains of cells can be seen with careful observation, but remain in suspension, generally with minimal pellet formation **2+** For aggregates that are easily seen but may not settle immediately; clusters form and are distinct from supernatant or remaining suspension, but do not settle and/or do so very slowly **3+** For larger aggregates which settle and leave some turbidity in the supernatant fluid; aggregates form pellets on the bottom of the tubes, but some remain in suspension and/or some do not aggregate **4+:** For larger aggregates which settle immediately and leave clear supernatant fluid; often strong aggregation leaves clear supernatant easily visible between very large clusters in suspension
Degree of Pellet Formation
A: A small pellet or powdery collection of cells that the bottom of the tube has formed, not specifically indicative of flocculated cells so much as debris and dead cells falling out of suspension; generally slow to form **B:** An appreciable pellet forms, relatively tightly packed, but in a large enough proportion, and rapidly enough, that it is obviously from aggregates **C:** A fluffy, loose pellet or layer has formed on the bottom, indicative of aggregation in a loose network, but some flocs remain in suspension **D:** Full aggregation, no turbidity in supernatant above pellet/floc; often indicative of even separation from the surface, slowly migrating down, from uniform aggregation of all cells

Table 2. *Lactobacillus* strains screened for coaggregative abilities with *C. jejuni* (MJM 211)

MJM #	Genus	Species	Strain	AAG	COAG	
4	*Lactobacillus*	*gasseri*	ATCC 33323	++++	++++	
7	*Lactobacillus*	*acidophilus*	NCFM	++++	++++	
9	*Lactobacillus*	*rhamnosus*	ATCC 53103	++++	++++	
13	*Lactobacillus*	*johnsonii*	ATCC 11506	-	++++	*
39	*Lactobacillus*	*acidophilus*	ATCC 4356	++++	++++	
53	*Lactobacillus*	*rhamnosus*	ATCC 9595	-	++++	*
73	*Lactobacillus*	*salivarius subsp. salivarius*	ATCC 11741	-	++++	*
89	*Lactobacillus*	*johnsonii*	La-1	++++	+++	
90	*Lactobacillus*	*plantarum*	LP-66	-	+++	
96	*Lactobacillus*	*acidophilus*	La-5	++++	++++	
108	*Lactobacillus*	*fermentum*	CECT 5716	-	+++	*
110	*Lactobacillus*	*reuteri*	DSM 17938	-	++++	
149	*Lactobacillus*	*casei*	LB6	++++	++++	
155	*Lactobacillus*	*plantarum*	LB12	++	+++	
206	*Lactobacillus*	*crispatus*	CC1-1	-	-	
207	*Lactobacillus*	*crispatus*	JCM 5810	-	++++	*
208	*Lactobacillus*	*gallinarum*	ATCC 33199	++	+++	
209	*Lactobacillus*	*helveticus*	CNRZ32	+	-	

MJM: Refers to the strain number from the culture collection of Dr. Michael J. Miller
Asterisks indicate strains with strong coaggregative ability
Number of + and – symbols illustrate degree of aggregation

Table 3. *Lactobacillus* strains screened for coaggregative abilities with *C. jejuni* (MJM 213)

MJM #	Genus	Species	Strain	AAG	COAG	
4	*Lactobacillus*	*gasseri*	ATCC 33323	++++	++++	
7	*Lactobacillus*	*acidophilus*	NCFM	+++	+++	
9	*Lactobacillus*	*rhamnosus*	ATCC 53103	+++	++++	
13	*Lactobacillus*	*johnsonii*	ATCC 11506	-	++++	*
39	*Lactobacillus*	*acidophilus*	ATCC 4356	+	++++	*
53	*Lactobacillus*	*rhamnosus*	ATCC 9595	-	++++	*
73	*Lactobacillus*	*salivarius subsp. salivarius*	ATCC 11741	-	++++	*
89	*Lactobacillus*	*johnsonii*	La-1	+++	+++	
90	*Lactobacillus*	*plantarum*	LP-66	-	+++	
96	*Lactobacillus*	*acidophilus*	La-5	+++	+++	
108	*Lactobacillus*	*fermentum*	CECT 5716	-	+++	*
110	*Lactobacillus*	*reuteri*	DSM 17938	-	-	
149	*Lactobacillus*	*casei*	LB6	+++	+++	
155	*Lactobacillus*	*plantarum*	LB12	++	+++	
206	*Lactobacillus*	*crispatus*	CC1-1	++	+++	
207	*Lactobacillus*	*crispatus*	JCM 5810	-	++++	*
208	*Lactobacillus*	*gallinarum*	ATCC 33199	++	+++	
209	*Lactobacillus*	*helveticus*	CNRZ32	-	+++	

MJM: Refers to the strain number from the culture collection of Dr. Michael J. Miller
Asterisks indicate strains with strong coaggregative ability
Number of + and – symbols illustrate degree of aggregation

Table 4. *Lactobacillus* strains tested to analyze coaggregative variation among strains within the same species

MJM #	Genus	Species	Other Designation	Source	Strain	AAG	COAG
43	*Lactobacillus*	*plantarum*	NRRL B-4496	NCAUR	ATCC 14917	-	+++
54	*Lactobacillus*	*plantarum*		MicroBioLogics	ATCC 8014	-	+++
90	*Lactobacillus*	*plantarum*		Cargill	LP-66	-	+++
144	*Lactobacillus*	*plantarum*	LB1	Cerri (Italy)		-	+
170	*Lactobacillus*	*plantarum*	LB28	Cerri (Italy)		-	+
227	*Lactobacillus*	*plantarum*	GRu212	Goat Rumen (Thailand)		-	+
238	*Lactobacillus*	*plantarum*	GSI104	Goat Small Intestine (Thailand)		-	+
245	*Lactobacillus*	*plantarum*	GLI406	Goat Large Intestine (Thailand)		-	+++
38	*Lactobacillus*	*fermentum*	NRRL B-585	NCAUR	ATCC 9338	-	+
45	*Lactobacillus*	*fermentum*	NRRL B-1840	NCAUR	ATCC 14931	-	+
64	*Lactobacillus*	*fermentum*	NRRL B-1932		ATCC 11581	-	+
108	*Lactobacillus*	*fermentum*		Puleva Biotech	CECT5716	-	+++
7	*Lactobacillus*	*acidophilus*		NCK 56	NCFM	+++	+++
39	*Lactobacillus*	*acidophilus*	NRRL B-4495	NCAUR	ATCC 4356	+	++++
56	*Lactobacillus*	*acidophilus*		MicroBioLogics	ATCC 314	++++	++++
96	*Lactobacillus*	*acidophilus*		Chr.Hansen	La-5	+++	+++
198	*Lactobacillus*	*acidophilus*		ATCC	ATCC 4796	+++	+++
206	*Lactobacillus*	*crispatus*			CC1-1	++	+++
207	*Lactobacillus*	*crispatus*			JCM5810	-	++++

MJM: Refers to the strain number from the culture collection of Dr. Michael J. Miller
Asterisks indicate strains with strong coaggregative ability
Number of + and – symbols illustrate degree of aggregation

Table 5. *C. jejuni* mutant strains tested for COAG abilities with *L. crispatus* (MJM 207)

MJM #		Strain	Genotype	Phenotype	AAG	COAG
371	*C. jejuni*	NCTC 11168		Highly motile, autoaggregative	+++	++++
372	*C .jejuni*	NCTC 11168		Non-motile, non-autoaggregative	-	+
373	*C .jejuni*	F38011		Autoaggregative in PBS	++++	+++
374	*C. jejuni*	F38011		Non-autoaggregative in PBS	-	+
375	*C .jejuni*	F38011	cadF knockout		-	-
376	*C. jejuni*	F38011	flpA knockout		-	-
377	*C .jejuni*	F38011	cadF-flpA double knockout		-	-
378	*C .jejuni*	F38011	flgL knockout		-	-
379	*C .jejuni*	F38011	flaA knockout		-	-
380	*C .jejuni*	F38011	flaB knockout		-	-
381	*C .jejuni*	F38011	flaA-flaB double knockout		-	-
382	*C .jejuni*	F38011	fbpA		-	-

MJM: Refers to the strain number from the culture collection of Dr. Michael J. Miller
Asterisks indicate strains with strong coaggregative ability
Number of + and – symbols illustrate degree of aggregation

3.7. FIGURES

Figure 1. Ranking System for Measuring Aggregation

Figure 1. Examples of Degree of Aggregation. Tubes provide examples of how the degree of aggregation was ranked visually. **a.)** 0+ **b.)** 1+ **c.)** 2+ **d.)** 3+ **e.)** 3+ **f.)** 4+

Figure 2. Variant and wild type coaggregation tubes

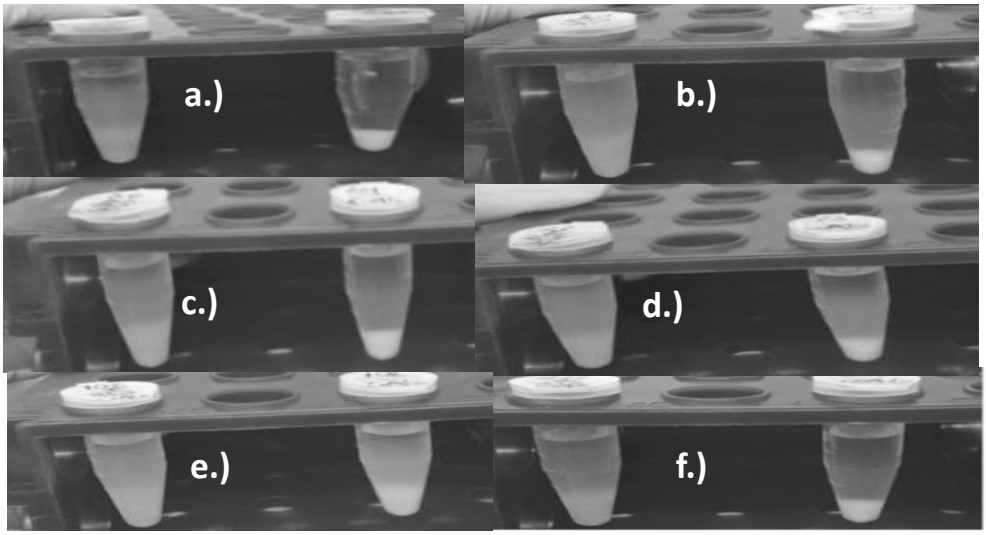

Figure 2. Coaggregative abilities of wild types and variants. Tubes show the coaggregative abilities of various *Lactobacillus* non-coaggregating variants(left) and their wild type strain (right). **a.)** MJM 39 (*L acidophilus* ATCC 4356) **b.)** MJM 43 (*L. plantarum* ATCC 14917) **c.)** MJM 54(L. *plantarum* ATCC 8014) **d.)** MJM 73(*L salivarius* subsp. *salivarius* ATCC 11741) **e.)** MJM 108 (*L. fermentum* CECT5716) **f.)** MJM 207 (*L. crispatus* JCM5810)

Figure 3. *L. crispatus* additional variants and wild type AAG and COAG tubes

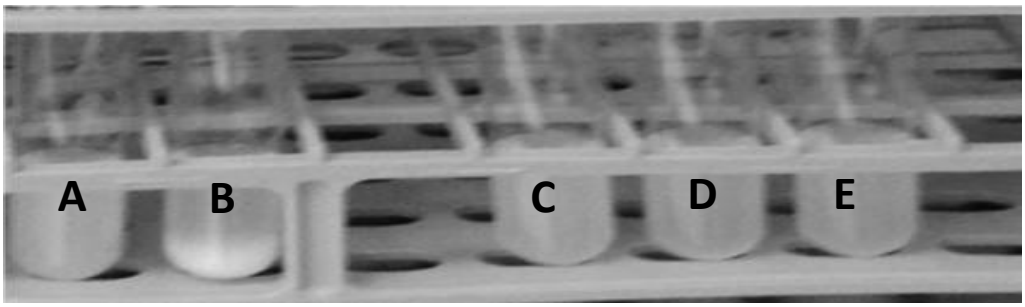

Figure 3. Coaggregative abilities of *L. crispatus* wild type s and variants. Tubes show the coaggregative abilities of the two *L. crispatus* wildtypes (MJM 206 and 207) and the MJM 207 non-coaggregating variants after 3 hours. **a.)** MJM 206 wildtype **b.)** MJM 207 wildtype **c.)** MJM 207 Variant 1 **d.)** MJM 207 Variant 3 **e.)** Original MJM 207 variant

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CHAPTER 4: FUTURE DIRECTIONS

Currently, there is not extensive research in the literature focusing on the coaggregation relationship between lactic acid bacteria and *Campylobacter jejuni*. There have been research studies studying the coaggregation of various bacterial strains but the mechanism facilitating the ability to coaggregate has not always been concretely identified. The current study provided insight for better understanding the coaggregative relationship between lactic acid bacteria and *Campylobacter jejuni.* A comprehensive list of coaggregative and non-coaggregative lactic acid bacteria was identified. More tests should be conducted with the *Lactobacillus* strains characterized as strong coaggregators. Measures were taken to identify the genetic determinant behind the coaggregation of these bacteria. Findings from this study and further research could help lead to *in vitro* studies analyzing the coaggregative abilities of wildtype *Lactobacillus* strains versus gene knockout strains. This could eventually lead to an *in vivo* study comparing the effects of the coaggregative abilities of these strains on reduction of *C. jejuni* colonization in chickens.

Lactobacillus crispatus JCM5810 was identified as a strain of interest not only because of its coaggregative abilities but also because of its probiotic properties identified in previous studies. Further testing should be conducted with *L. crispatus* to better understand whether its coaggregative abilities contribute to its ability to reduce *C. jejuni*'s colonization in chickens (as evidenced in a previous research study). The DNA sequencing results from the current study could possibly confirm what is possibly facilitating coaggregation between *L. crispatus* and *C. jejuni*. These results could help structure the approach for conducting further *in vitro* and *in vivo* experiments.

Coaggregation is a complex occurrence that needs further research conducted to be fully understood. Through more *in vitro* and *in vivo* work the correlation between coaggregation and the competitive exclusion of *C. jejuni* by lactic acid bacteria could possibly be explained. This research could significantly help lead to the development of an intervention strategy to prevent the colonization and infection of *C. jejuni* within poultry and humans.

APPENDIX A. DETAILED PROTOCOLS

Procedure

Testing *C. jejuni's* (213) ability to coaggregate with 18 different strains of Lactobacilli

Equipment used in project:

1. Centrifuge
2. Anaerobic chamber
3. Microfuge
4. Incubator
5. Vortex
6. Biosafety cabinet

Preparing LAB Strains

1. Label tubes corresponding to lactobacilli strains being tested
2. Pipet 1.5 mL of each strain into their tube (make sure to vortex out clumps)
3. Spin down LAB for 5 min at @ 3000 g in microfuge (may have to slam microfuge door to completely close)
4. After, pipet off liquid into waste beaker
5. Pipet .75 PBS into each tube; pipet up and down until homogenized then add the other .75 mL PBS later (Minimize bubbles as much as possible by pipetting directly towards pellet)

Preparing *C. jejuni* strains

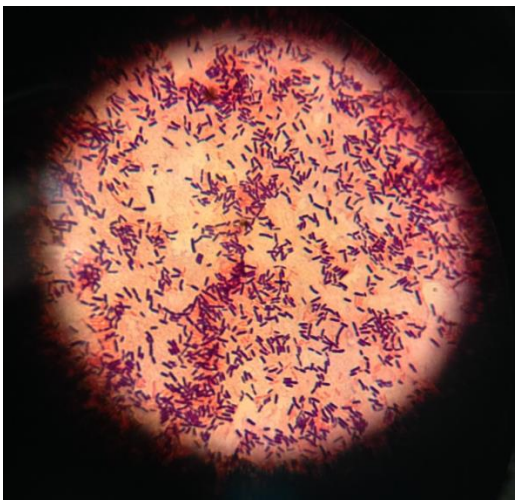
6. Pipet 10 mL PBS onto a plate with an overnight lawn of *C. jejuni* from incubator
7. Swirl plate around to suspend bacteria
8. Tilt plate to one side and pipet suspension into a labeled screw-cap 15 mL falcon tube

9. Centrifuge tube for 10 min @ 3,000 g; use bucket caps (use one hand to handle tubes and the other to open and control centrifuge)
10. When re-suspending: Pour off supernatant and add 2 mL PBS at a time then pipet up and down to homogenize.
11. Add PBS up to original volume
12. Repeat steps 9-10, then suspend in PBS up to OD_{600} of approx. 0.5
13. Pipet .75 mL into label sample tubes

Preparing Co-aggregation tubes

14. Pipet .75 mL of each LAB strain into the corresponding *C. jejuni* tubes
15. Vortex campy/LAB tubes and let sit
16. Add .75 PBS to LAB tubes (remaining .75 mL); vortex and let sit
17. Have control *C. jejuni* tube
18. When observing be careful not to agitate tube
19. Don't let too much time pass between AAG and CoAG recordings so you can compare; After 3 hours can terminate observations **C. jejuni* usually takes 1-2 hours to AAG*

APPENDIX B. GRAM STAIN PICTURE



MJM 207 and 213 (signs of coaggregation)