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Coaggregation of Lactobacilli with *Campylobacter jejuni*

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ABSTRACT

According to research, a probiotic that can coaggregate with a pathogen effectively can prevent its adhesion to intestinal epithelial cells. Therefore, better comprehension of how probiotic bacteria coaggregate with *C. jejuni* could prevent or reduce the colonization and infection of livestock and humans. Although there are studies that explain how *C. jejuni* colonizes the epithelial cells of livestock and humans, there is still no clear method to reduce colonization. In this study, the coaggregative abilities between eighteen different potential probiotic strains of *Lactobacillus* with *C. jejuni* F38011 and *C. jejuni* 11168 were tested. The results from this study showed that the coaggregative abilities of the *Lactobacillus* strains were strain dependent and that *Lactobacillus rhamnosus* ATCC 9595 had the strongest coaggregative ability with *C. jejuni*. The findings of this research could lead to further research regarding prevention of *C. jejuni* colonization within livestock and humans with the use of probiotics.

INTRODUCTION

Campylobacter jejuni is a bacterium that is now the leading cause of bacterial diarrheal disease (Hu et al., 2008). *C. jejuni* typically infects the epithelial cells within the intestinal tract of both poultry and humans. This bacterium is not harmful to chickens, yet harms humans when this bacterium colonizes the intestines (Fairchild et al., 2005; Lan, Verstegen, Tamminga, & Williams, 2005). *C. jejuni* can cause harm to humans due to its ability to adhere to intestinal epithelial cells (Backert & Hofreuter, 2013). It has been identified by researchers that *C. jejuni* is most often spread by contact with contaminated raw or undercooked poultry (Kurinčič, Berce, Zorman, & Smole Možina, 2005).

Infection with *C. jejuni* in acute stages leads to diarrhea, bloody stool, abdominal pain, nausea, muscle pain, headache, fever, and in extreme cases, Guillain-Barre syndrome (Sivadon-Tardy et al. 2013; Wagenaar, French, & Havelaar, 2013). *C. jejuni* is estimated to affect over 1.3 million individuals per year. Approximately, 76 of those infected die (Centers for Disease Control and Prevention [CDC], 2014). *C. jejuni* is a pathogen that threatens the safety of the food chain across the globe (Tareb, Bernardeau Gueguen, & Vernoux 2013). Although *C.jejuni* causes harmful effects in humans, there is a lack of a clear method to reduce its effects. A procedure needs to be developed to help reduce the harm caused by *C. jejuni*.

Not all bacteria are harmful to humans. Probiotics are defined as “live microorganisms which, when administered in adequate quantity confer health benefits to the host” (Tuo et al. 2013). Probiotics are used to normalize bowel health, prevent infections in intestines and issues caused by antibiotics, and to help maintain a natural balance of microflora in the intestines and also to maintain a healthy immune system response (Yu, Peng, Chen, Deng, & Guo 2014). An intriguing mechanism related to colonization is aggregation. Two sub-categories of aggregation are coaggregation and autoaggregation. Coaggregation is defined as “a process by which genetically distinct bacteria become attached to one another via specific molecules” (Rickard, Gilbert, High, Kolenbrander, & Handley 2003). Autoaggregation is the characteristic clumping of cells of the same species

(Schembri, Christiansen, & Klemm, 2008). In order for probiotics to confer benefits, these microorganisms should be able to aggregate effectively on the intestinal mucosa (Cayuela et al. 2014). Certain Lactic Acid Bacteria (LAB) have even been shown to inhibit the adhesion of pathogens to mucin by aggregation (Tareb et al. 2013). LAB are anaerobes that ferment glucose into lactic acid (Rattanachaikunsopon & Phumkhachorn 2010). Many of the LAB strains used in this study are probiotics such as *Lactobacillus acidophilus, casei,* and *rhamnosus.* Many other potential probiotic LAB strains will be tested as well.

Probiotics' colonization of the gastrointestinal tract can have beneficial effects in humans, but pathogens that can colonize the GI tract effectively can cause harm. When a probiotic can autoaggregate and colonize the GI tract effectively, its protective abilities can be expressed (Collado, Meriluoto, & Salminen, 2007). Probiotics are able to compete with pathogens for bonding 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).

Based on findings from previous research, this study will analyze how potential probiotic strains of *Lactobacillus* bacteria coaggregate with the foodborne pathogen, *Campylobacter jejuni.* We will evaluate whether the coaggregative abilities of different strains of *Lactobacillus* with *C. jejuni* are strain dependent. By conducting this research, we can obtain a better understanding of how beneficial bacteria interact with *C. jejuni*. This knowledge could help improve methods to prevent *C.jejuni*'s colonization and infection in livestock and humans.

LITERATURE REVIEW

There is no finite method to prevent the colonization of *C. jejuni* because the interaction between *C.jejuni* and its avian host are not completely understood. *C. jejuni* chicken colonization begins after ingestion, where it reaches the cecum and multiplies. The bacteria then establish a colony. Soon after, the majority (>95%) of birds in the flock are colonized and remain so through slaughter (Hermans et al. 2011). Studies conducted by Hermans et al. (2011) have been able to identify several colonization mechanisms, one being aggregation, that *C. jejuni* uses to colonize poultry. It was concluded that surface properties such as aggregation may lead to adhesion and colonization. It is seen that LAB can prevent the adhesion of pathogenic bacteria due to competing bonding sites on intestinal epithelial cells (Halasz, 2009). Rosenfeldt et al. (2002) conducted a study in which sixty-nine children, who were diagnosed with acute-diarrhea, received a mixture of *Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 12246 or a placebo to be established as a control group. The diarrheal phase was reduced by 20% in the patients receiving the probiotic and it was concluded that both probiotics were able to improve the acute diarrheal phase. Jankovic et al. (2012) conducted an experiment to identify how three probiotic strains of *Lactobacillus plantarum* autoaggregated and also how they coaggregated with different foodborne pathogens such as *Listeria monocytogenes* and *Escherichia coli.* This study expressed that all probiotics tested were able to coaggregate with the foodborne pathogens. In a similar study, two *Lactobacillus* strains along with their heat killed forms had their autoaggregation abilities tested along with their coaggregative abilities with several pathogenic strains such as *Campylobacter, Salmonella,* and *Escherichia coli* to identify if they could reduce their adhesion to mucin. It was shown that both strains, whether heat killed or viable, inhibited the attachment of *C. jejuni* to mucin. Several probiotic strains of *Lactobacillus* were seen to inhibit the adhesion of *S.* Typhimurium significantly by its coaggregation properties (Tareb et al. 2013). By further tests based on these studies, we hope to develop a better understanding between probiotics and pathogens.

METHODOLOGY

Bacterial Strains and Growth Conditions

Eighteen different strains of *Lactobacillus* (Table 1) were used in this study. The autoaggregation of the *Lactobacillus* strains along with their coaggregation with *C. jejuni F38011* and *C. jejuni* 11168 were tested. All tested bacteria were stored at -80 °C in glycerol. *Lactobacillus* was grown in de Man, Rogosa, and Sharpe (MRS) broth in an anaerobic chamber at 37 °C for 24 hours while *C. jejuni* was grown on bovine blood agar plates and incubated in a CO_2 incubator at 37 °C for 24 hours.

Aggregation Assays

The *Lactobacillus* strains were centrifuged for

The *Lactobacillus* strains were centrifuged for five minutes at 3,000 rcf and resuspended in Acetate Buffer. *C. jejuni* was centrifuged at 3,000 rcf for ten minutes and washed twice in PBS. After the washing process was completed, 0.5 mL of the solution was pipetted into labeled sample tubes.

Autoaggregation and coaggregation assays were conducted for both the *Lactobacillus* and *C. jejuni strains*. 0.5 mL of the *Lactobacillus* strains was pipetted into their corresponding *C. jejuni* tubes. 0.5 mL of ABS was then pipetted into the *Lactobacillus* tubes to bring to concentration back to 1 mL. A control tube containing *C. jejuni* was prepared as well to monitor its autoaggregation. The autoaggregation, coaggregation, and control tubes were then vortexed and monitored intermittently for thirty minutes within a span of two hours. The tubes were analyzed visually and microscopically and ranked.

Table 1
Lactobacillus Strains.

MJM	Genus	Species	Strai
4	*Lactobacillus*	*gasseri*	ATC
7	*Lactobacillus*	*acidophilus*	NCF
9	*Lactobacillus*	*rhamnosus*	ATC
13	*Lactobacillus*	*johnsonii*	ATC
39	*Lactobacillus*	*acidophilus*	ATCC 4356
53	*Lactobacillus*	*rhamnosus*	ATCC
73	*Lactobacillus*	*salivarius subsp. salivarius*	ATC 11741
89	*Lactobacillus*	*johnsonii*	La-1
90	*Lactobacillus*	*plantarum*	LP-6
96	*Lactobacillus*	*acidophilus*	La-5
108	*Lactobacillus*	*fermentum*	CECT 5
110	*Lactobacillus*	*reuteri*	DSM 17
149	*Lactobacillus*	*casei*	LB
155	*Lactobacillus*	*plantarum*	LB1
206	*Lactobacillus*	*crispatus*	CC1
207	*Lactobacillus*	*crispatus*	JCM 5
208	*Lactobacillus*	*gallinarum*	ATC

*MJM: Refers to the strain number from the culture collection of Prof. Michael J. Miller

FINDINGS

Autoaggregation and coaggregation of *Lactobacillus* and *C. jejuni* was tested. Many *Lactobacillus* strains that did not autoaggregate after two hours were able to coaggregate with *C. jejuni*. The autoaggregation and coaggregation tests done with *C. jejuni* F38011 were tested in triplicate and the results were averaged (Table 2). The most consistent coaggregators were tested with another strain, C. jejuni 11168 (Table 3).

The autoaggregative abilities of *C. jejuni* F38011 (MJM 211) and the *Lactobacillus* strains were tested (Table 2). *Lactobacillus* strains MJM 13, 73, 90, 108, 110, 206, and 207 averaged a score of 0+ a. MJM 53 averaged a 0+A. MJM 209 averaged a 1+. MJM 208 averaged a 2+B. MJM 155 averaged a 3+B.MJM strains 4, 7, 9, 39, 89, 96, and 149 averaged a score of 4+B. MJM 90, 108, and 208 scored a 3+C.

The autoaggregative abilities of *C. jejuni* 11168 (MJM 213) and the eighteen *Lactobacillus* strains were tested (Table 3). *Lactobacillus* strains MJM 13, 73, 90, 108, and 207 scored a 0+. MJM 53 and 96 scored a 0+A. MJM 39 scored a 1+A while MJM 213 received a 3+C.

The coaggregative abilities of *C. jejuni* F38011 with the *Lactobacillus* strains were also tested (Table 2). MJM 209 and 206 scored a 0+A. MJM 90, 108, and 208 scored a 3+C MJM 4, 7, 9, 13, 39, 89, 96, 110, 149, 155, and 207 scored a 4+C. MJM 53 and 73 both scored a 4+D.

Coaggregation of *C.jejuni* 11168 and the *Lactobacillus* strains were tested as well (Table 3). *Lactobacillus* strains MJM 90, 96, and 108 scored a 0+ while MJM 13, 39, 53, 73, and 207 scored a 4+D.

The strains that were visually observed to coaggregate without a strong autoaggregating factor were analyzed via microscopy to verify their coaggregation. Under the microscope, it was seen that the *Lactobacillus* strains that were believed to coaggregate with *C. jejuni* had varied results regarding their actual coaggregation. The *Lactobacillus* strains MJM 39, 73, 90, 108, and 207 that seemed to coaggregate with *C. jejuni* F38011 were observed. MJM 39 had impressive coaggregation with seemingly no *C. jejuni* unbound to *Lactobacillus*. MJM 73's coaggregation was difficult to assess. MJM 90 was observed to have no coaggregation. MJM 108 was a possible candidate for coaggregation but had many unbound bacteria. MJM 207 appeared to have no coaggregation.

The *Lactobacillus* strains, MJM 13, 39, 53, 73, and 207 that seemed to coaggregate with *C. jejuni* 11168 the strongest, were also observed microscopically. MJM strains 13, 39, and 207 showed no coaggregation. MJM 73 showed some coaggregation while MJM 53 showed strong coaggregation. It was concluded that MJM 53 had the strongest coaggregation out of all the strains tested. It was observed by the visual coaggregative tests conducted on all the strains of *Lactobacillus* that tubes that had a tightly packed pellet expressed stronger coaggegative ability under the microscope.

Table 2

Lactobacillus and MJM 211 autoaggregation and coaggregation.

***Lactobacillus* strains' and MJM 211's Autoaggregation ability (Averages)**

	Time points (Hours)			
MJ	**0:30**	**1:0**	**1:3**	**2:0**
4	3+B	3+B	3+B	4+B
7	3+	3+B	3+B	4+B
9	4+	3+C	3+B	4+B
13	0+	0+	0+	0+
39	2+B	3+B	3+B	4+B
53	0+A	0+	0+	0+
73	0+	0+	0+	0+
89	3+C	3+B	3+B	4+B
90	0+	0+	0+	0+
96	2+A	3+B	3+B	4+B
108	0+	0+	0+	0+
110	0+	0+	0+	0+
149	2+	3+B	3+B	4+B
155	3+B	3+B	3+B	3+B
206	0+	0+	0+	0+
207	0+	0+	0+	0+
208	0+	1+	1+	2+B
209	0+	0+	0+	1+
211	0+	0+	0+	0+

***Lactobacillus* strains' Coaggregation ability with MJM 211 (Averages)**

	Time points (Hours)			
MJ	**0:3**	**1:0**	**1:3**	**2:0**
4	0+	3+	3+	4+
7	0+	3+	3+	4+
9	0+	2+	3+	4+
13	0+	3+	3+	4+
39	0+	3+	3+	4+
53	3+	3+	3+	4+
73	3+	3+	3+	4+
89	0+	3+	3+	4+
90	0+	0+	2+	3+
96	0+	3+	3+	4+
108	0+	1+	2+	3+
110	0+	3+	3+	4+
149	0+	0+	3+	4+
155	0+	3+	4+	4+
206	0+	0+	0+	0+
207	0+	3+	3+	4+
208	2+	2+	2+	3+
209	0+	0+	0+	0+

*MJM: Refers to the strain number from the culture collection of Prof. Michael J. Miller

*0+: No visible aggregates in the cell suspension; an even homogeneous suspension of cells, may remain for days until settling out unaggregated. A 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 the supernatant or remaining suspension. These clusters do not settle 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 do not aggregate

*4+: For larger aggregates, which settle immediately and leave clear supernatant fluid; strong aggregation leaves clear supernatant easily visible between very large clusters in suspension.

*A: A small pellet or powdery collection of cells at 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 obviously forms 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 3

Lactobacillus and MJM 213 autoaggregation and coaggregation.

***Lactobacillus* strains' and MJM 213's Autoaggregation ability**

	Time Points (Hours)			
MJM	**0:30**	**1:00**	**1:30**	**2:00**
13	0+	0+	0+	0+
39	0+	0+	0+	1+A
53	0+	0+	0+	0+A
73	0+	0+	0+	0+
90	0+	0+	0+	0+
96	0+	0+	0+A	0+A
108	0+	0+	0+	0+
207	0+	0+	0+	0+
213	0+	0+	1+	3+C

***Lactobacillus* strains' and MJM 213's Coaggregation ability**

	Time Points (Hours)			
MJ	**0:3**	**1:0**	**1:3**	**2:0**
13	0+	4+D	4+D	4+D
39	0+	0+	3+C	4+D
53	3+C	4+D	4+D	4+D
73	1+A	4+D	4+D	4+D
90	0+	0+	0+	0+
96	0+	0+	0+	0+
108	0+	0+	0+	0+
207	0+	1+	4+D	4+D

*MJM: Refers to the strain number from the culture collection of Prof. Michael J. Miller

*0+: No visible aggregates in the cell suspension; an even homogeneous suspension of cells, may remain for days until settling out un-aggregated. A 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 the supernatant or remaining suspension. These clusters do not settle 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 do not aggregate

*4+: For larger aggregates, which settle immediately and leave clear supernatant fluid; strong aggregation leaves clear supernatant easily visible between very large clusters in suspension.

*A: A small pellet or powdery collection of cells at 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 obviously forms 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.

A study conducted by Tareb et al. (2013) expressed that *C. jejuni* was able to coaggregate better than any pathogen with *Lactobacillus* regardless of the strain. This study also expressed how *Lactobacillus rhamnosus* 3698 was one of the two strains able to coaggregate with all pathogens after twenty-four hours. MJM *Lactobacillus* strain 53, the most consistent coaggregator, is a part of the *rhamnosus* species. MJM 53 showed promise for future testing due to its strong coaggregative ability. Related studies have used other methods that were not utilized in this experiment. Optical density (OD) was an example of a method used in a study to monitor the aggregation properties of certain strains of *Lactobacillus* and pathogens including *C. jejuni* (Tareb et al. 2013). OD was not used in this study due to its inability to accurately measure the degree of autoaggregation or coaggregation. One study was closely related to this study due to their choice to visually observe and rate their bacteria while using a similar grading scale to the scale used in this study (Khemaleelakul, Baumgartner, & Pruksakom 2006).

CONCLUSIONS

It can be concluded that the coaggregation of *Lactobacillus* strains with *C. jejuni* was strain dependent due to Lactobacilli of the same species, but different strains, coaggregating differently. This is expressed with *Lactobacillus rhamnosus* ATCC 9595 (MJM 53) being the strongest coaggregator while another *Lactobacillus* strain of the same species (MJM 9) was not able to strongly coaggregate with *C. jejuni*. Colonization of chickens by *C. jejuni* is an issue that is not fully

understood. Although coaggregation may not have been observed under the microscope, there still might have been coaggregation between *Lactobacillus* and *C. jejuni* that was possibly disturbed by the microscope slide. Another limitation to this study was that the acetate buffer used was not parallel with the actual conditions of the intestinal tract of a chicken. Further studies need to be conducted in conditions similar to this environment. Ultimately, the findings of this study could lead to further research such as an *in vivo* chicken study to test the reduction of *C. jejuni* colonization in chickens by the use of probiotics. This research could greatly aid the development of a method to prevent the colonization and infection of *C. jejuni* within livestock and humans.

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