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Reduction of Campylobacter jejuni on Chicken Wingettes by Treatment with Caprylic Acid, Chitosan or Protective Cultures of Lactobacillus spp.

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Reduction of *Campylobacter jejuni* on Chicken Wingettes by Treatment with Caprylic Acid, Chitosan or Protective Cultures of *Lactobacillus* spp.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Poultry Science

by

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Abstract

Poultry is one of the main sources of protein in the United States and in 2014 Americans ate approximately 100 lbs per person. However, consumption of poultry products is strongly associated with foodborne illness from *Campylobacter*. In the first study, chitosan, caprylic acid and their combination were evaluated as a coating treatment for the reduction of *Campylobacter jejuni* on poultry products. For the initial screening trials, chitosan of three different molecular weights (15-50 kDa, 190-310 kDa and 400-600 kDa) was evaluated at three concentrations (0.5%, 1.0%, 2.0%) and separately, caprylic acid was tested at 0.5%, 1.0% and 2.0% using chicken skin pieces. From these initial screenings a solution of 2% medium molecular weight (190-310 kDa) chitosan was chosen for continued evaluation, as was 1% and 2% caprylic acid. To determine short term and long term efficacy of the 2% chitosan, 1% caprylic acid, 2% caprylic acid individually and in combination wingettes were inoculated with *Campylobacter*, coated with a given treatment and sampled at 0, 1, 3, 5 and 7 days. The 2% chitosan and both 1% and 2% caprylic acid continually reduced *Campylobacter* counts starting at day 3 through day 7. The combination of either 1% or 2% caprylic acid plus 2% medium molecular weight chitosan continuously reduced *Campylobacter* starting at day 0 through day 5.

In the second study *Lactobacillus* spp. isolates with in-vitro anti-*Campylobacter* activity were evaluated for their efficacy as a protective culture when applied to chicken wingettes. An original 13 isolates of *Lactobacillus* were screened, resulting in the selection of 4 isolates for further evaluation. Wingettes were inoculated with *Campylobacter jejuni* and treated with either a *Lactobacillus* broth culture or a BPD control, followed by sampling at days 0, 1, 3, 5 and 7. All isolates were able to reduce *Campylobacter* counts by day 3, however two isolates produced more consistent reductions. These two isolates were combined with a 2% chitosan solution (190-310 kDa).
kDa) and applied as a coating treatment for evaluation of increased efficacy. The combination of 2% chitosan plus either *Lactobacillus* isolate failed to increase the efficacy.
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Dedication

This work is dedicated to my late mother
Charlene Theresa Delaney Lorenz

“Grief has no distance. Grief comes in waves, paroxysms, sudden apprehensions
that weaken the knees and blind the eyes and obliterate the dailiness of life.”
Stephanie Wittels Wachs
Table of Contents

Abstract
Acknowledgements
Dedication
Table of Contents
List of Tables
List of Figures

Chapter 1: Introduction

1 Justification

References

Chapter 2: Literature Review

2.1 Brief History of Campylobacter
2.2 Morphology and In-Vitro Culture Conditions
2.3 Human Infection Associated with Campylobacter
  2.3.1 Sequelae Following Infection with Campylobacter
  2.3.2 Guillain-Barre´ Syndrome (GBS)
  2.3.3 Reactive Arthritis
2.4 Campylobacter in the Environment
2.5 Rates of Human Illness from Campylobacter
  2.5.1 Culture Independent Diagnostic Tests (CIDTs) for Campylobacter
  2.5.2 The Viable but Non-Culturable (VNBC) State for Campylobacter
  2.5.3 Campylobacter and Biofilm Formation
  2.5.4 Human Sources of Infection with Campylobacter
2.6 Poultry as a Reservoir of Campylobacter
  2.6.1 Rates of Contamination on Processed Poultry
  2.6.2 Contamination in the Processing Plant
2.6.3  First Processing: Slaughter Through Chilling  29
2.6.4  Cross-Contamination During Processing  29
2.6.5  The Potential Role of Chicken Skin  30
2.6.6  *Campylobacter* Populations Within Hosts and Environments  31

2.7  Interventions During First Processing  31
   2.7.1  Physical Treatments  32
   2.7.2  Washers  32
   2.7.3  Chilling  33
   2.7.4  Irradiation  33
   2.7.5  Processing Aids  34

2.8  Chlorine Based Compounds  35
   2.8.1  Sodium hypochlorite (Chlorine)  35
   2.8.2  Chlorine dioxide  35
   2.8.3  Acidified sodium chlorite (ASC)  36
   2.8.4  Organic Acids Used as Interventions  36

2.9  Phosphate Based Compounds  36
   2.9.1  Trisodium phosphate (TSP)  36
   2.9.2  Peracetic acid (PAA)  37
   2.9.3  Cetylpyridinium chloride (CPC)  38

2.10  Post-Harvest Treatment of Raw Ready to Eat Poultry Products  38
   2.10.1  Antimicrobial Edible Coatings  38
   2.10.2  Generally Recognized as Safe (GRAS) Designation  39
   2.11.1  Chemical and Physical Characteristics of Chitosan  40
   2.11.2  Antimicrobial Activity of Chitosan  41
   2.11.3  Chitosan Coating of Poultry Products  42

2.12  Caprylic Acid in the Food Industry  43
   2.12.1  Guidelines for Use of Caprylic Acid in Food Processing  44
   2.12.2  Proposed Mechanisms of Action for Fatty Acids  44
2.12.3 Fatty Acid Treatment of Poultry Skin

2.13 Introduction to Lactic Acid Bacteria

2.13.1 Lactic Acid Bacteria as Probiotics

2.13.2 Antimicrobial Activity of Lactic Acid Bacteria

2.13.3 Use of Probiotics in Poultry Production

2.13.4 Probiotics to Prevent Campylobacter Infection in Pre-harvest Poultry

2.13.5 Application of Lactic Acid Bacterial Cultures to Raw Meat Product

References

Chapter 3: The application of caprylic acid, chitosan or their combination as a coating treatment reduces Campylobacter jejuni on inoculated chicken wingettes

3.1 Abstract

3.2 Introduction

3.3 Material and Methods

3.4 Results

3.5 Discussion

3.6 References

3.7 Research Compliance Protocol Letter

3.8 IBC Protocol Approval

Chapter 4: The efficacy of application of protective cultures of Lactobacillus spp. isolates with or without a chitosan coating to reduce Campylobacter jejuni on chicken wingettes

4.1 Abstract

4.2 Introduction

4.3 Material and Methods

4.4 Results

4.5 Discussion

4.6 References

4.7 Research Compliance Protocol Letter
4.8 IBC Protocol Approval

Conclusion
List of Tables

Chapter 3

Table 3.1. The efficacy of caprylic acid, chitosan or their combination to reduce *Campylobacter* counts on chicken wings trial 1 105

Table 3.2. The effects of caprylic acid, chitosan or their combination to reduce *Campylobacter* counts on chicken wings trial 2 106

Chapter 4

Table 4.1. The efficacy of selected *Lactobacillus* spp. isolates to reduce *C. jejuni* counts on chicken wingettes trial 1 131

Table 4.2. The efficacy of selected *Lactobacillus* spp. isolates to reduce *C. jejuni* counts on chicken wingettes trial 2 132

Table 4.3. Reductions in *C. jejuni* counts after treatment with *Lactobacillus* spp. isolates (4 or 8), chitosan or their combination on chicken wingettes trial 1 and 2 133
List of Figures

Chapter 3

Figure 3.1. Evaluation of differing concentrations of caprylic acid treatment to reduce *Campylobacter jejuni* on chicken skin 104

Chapter 4

Figure 4.1. Evaluation of *Lactobacillus* spp. isolates to reduce *Campylobacter jejuni* counts on chicken skin pieces Trial 1 134

Figure 4.2. Evaluation of *Lactobacillus* spp. isolates to reduce *Campylobacter jejuni* counts on chicken skin pieces Trial 2 135
Chapter 1
Introduction
Campylobacter is a common contaminant of poultry carcasses and raw retail poultry products (Conner et al., 2001; Suzuki and Yamamoto, 2009; USDA FSIS, 2009). This bacterium is a consistent inhabitant of the gut microflora of poultry, with the capability to colonize at high levels without negatively impacting the health of the bird (Blaser et al., 1983; Evans, 1991; Jacobs-Reitsma et al., 1995; Hermans et al., 2012; Wagenaar et al., 2015). Foodborne illness resulting from infection with Campylobacter is highly associated with consumption of poultry products and the mishandling of raw and cooked poultry (Wilson et al., 2008; Painter et al., 2013; Bondi et al., 2014). Campylobacter contamination of poultry meat is a significant problem because it is consumed as one of the major sources of protein in the United States and indeed globally (AVEC, 2013, 2014; National Chicken Council, 2015; USDA and Foreign Agricultural Service, 2015). The implications of this being that increased consumption will result in a higher frequency of illness even if the contamination rate is low (Painter et al., 2013; Wagenaar et al., 2015). This stresses the importance of the need for effective intervention strategies to reduce Campylobacter on raw poultry. The decontamination of poultry during processing and packaging is a complex problem from many aspects. Unlike other food animals, the skin of poultry is not removed during first processing which may lead to pathogenic bacteria becoming attached to or trapped in the folds of skin or feather follicles which can contribute to cross-contamination along the production line (Lillard, 1989; Corry and Atabay, 2001; Wagenaar et al., 2013). Additionally, poultry products pose challenges when applying surface antimicrobial compounds due to their heterogeneous characteristics, high pH and high protein and fat content (Davidson et al., 2014).

In the U.S. today there is a growing trend amongst consumers for knowledge about how food commodities are grown, processed and packaged—with increased focus on minimized processing and chemical treatment (Gyawali and Ibrahim, 2014; Sánchez-Ortega, 2014). As a
response to these desires food production companies have an increased interest in methods of making foods microbiologically safe by more natural interventions or treatments. A potential option for naturally safer product is by utilizing antimicrobial packaging or coating treatments (Cagri et al., 2004; Salleh et al., 2007; Pavlath and Orts, 2009; Sánchez-Ortega, 2014). The use of coating treatments on raw poultry products can be considered another intervention to add to the multiple hurdle approach for enhancing the food safety of poultry products (Ricke and Hanning, 2013). An important aspect of any food coating or material in contact with food is the understanding that the material must be food grade and frequently require designation by the FDA as Generally Recognized as Safe (GRAS)—additionally and edible coating is considered a food additive and GRAS status is necessary to demonstrate that the coating material is safe within the parameters of how it is used in the food (Pavlath and Orts, 2009; FDA, 2015).

The use of chitosan as an antimicrobial food coating and preservative is receiving increased attention owing to the many biological characteristics which are beneficial for its application in the food industry (Dutta et al., 2009). According to the literature chitosan based coatings are: (1) non-toxic and non-polluting; (2) possess antimicrobial/antifungal activity; (3) are low cost and abundant; (4) edible; (5) biodegradable; and (6) have positive chemical properties for ease of industrial use (Kong et al., 2010; Aider, 2010; Sánchez-González et al., 2011; Elsabee and Abdou, 2013). The approval of the use of chitosan as a coating is already in place in Canada and while not GRAS in the U.S. it is expected to be approved by the FDA in the near future (Baldwin, 2007; Vasilatos and Savvaidis, 2013; Zivanovic et al., 2014). Menconi and colleagues (2013) were able to reduce Salmonella Typhimurium and extend the shelf-life of chicken skin treated with a 0.5% chitosan solution. The use of chitosan and its incorporation with natural compounds for the treatment of poultry products to extend shelf-life and reduce pathogens has also generated
increased interest. Petrou and colleagues (2012) successfully extended the shelf-life of MAP packaged chicken breast fillets by 6–12 days with the application of 1.5% chitosan or 1.5% chitosan plus oregano oil. The combination of chitosan and thyme essential oil significantly reduced spoilage organisms, Enterobacteriaceae and lactic acid bacteria on packaged chicken kebabs after 12 days of storage as compared to the controls (Giatrakou et al., 2010).

A potential surface treatment for poultry meat is the naturally occurring fatty acid caprylic acid, a medium chain fatty acid found in mammalian milk, palm kernel oil and coconut oil. It is designated by the FDA (FDA, 2014) as a multipurpose food ingredient and the EPA has approved it as a sanitizer for use on inanimate objects (EPA, 2014). Caprylic acid has broad spectrum antibacterial activity, and specific activity against the foodborne illness causing organisms Listeria monocytogenes, E.coli O157:H7, Staphylococcus aureus, Salmonella spp. and Campylobacter spp. (Kabara et al., 1972; Wang and Johnson, 1992; McLay et al., 2002; Nair et al., 2004; Skrivanova et al., 2007). Riedel and colleagues (2009) treated chicken skin and meat with a 5.0% concentration of caprylic acid sodium salt and produced reductions in Campylobacter counts by up to 2.84 log_{10} on skin and 4 log_{10} on meat after treatment and storage at 4^\circ C for 24 hours.

An additional strategy under evaluation to reduce foodborne pathogens on poultry products is the use of protective cultures. The concept of protective cultures involves the addition of viable bacteria to food products that will inhibit pathogenic microorganisms by the production of metabolites which are antimicrobial (Holzapfel et al., 1995; Schillinger et al., 1996; Gálvez, Antonio et al., 2010; Garcia et al., 2010; Gaggia et al., 2011). It has been suggested by Maragkoudakis and colleagues (2009) that the use of lactic acid bacteria as a protective culture on poultry meat is a feasible and safe option due to their ubiquitous nature in the foods we already consume they have been identified as a constituent of normal flora in the human gut microbiome.
and these organisms have essentially been a part of our diet for thousands of years without significant negative implications. Protective cultures that can be consumed by humans are most frequently from the genus *Lactobacillus* (Gibson, 2008; Fliss et al., 2011; Davidson et al., 2014). There is very little information in the literature in regards to the application of protective cultures to specifically inhibit *Campylobacter*. Work by Melero and colleagues (2012, 2013) has evaluated the impact of protective cultures to reduce *Campylobacter jejuni* and *Listeria monocytogenes* in chicken products. A protective culture of *Bifidobacterium longum* was used to treat chicken legs artificially inoculated with *C. jejuni* and packaged under modified atmosphere, and they observed a reduction in *C. jejuni* counts (1.09 log CFU/g) between days 6 – 9 of the study (Melero et al., 2013).

**Justification:**

Foodborne infection with *Campylobacter* is a public health concern and the rates of illness have remained stagnant over the last two years. Currently there are no treatments to eliminate *Campylobacter* from colonizing the gastrointestinal tract of poultry. This stresses the importance for interventions during processing and at final packaging that reduce or eliminate the contamination of poultry products with *Campylobacter*. Current trends in consumer preference include a desire for a more natural product—minimally processed and no chemical treatments. There is very little literature on the use of natural antimicrobials or protective cultures to reduce *Campylobacter* counts on poultry products. More research is needed to find effective natural treatments to reduce foodborne pathogens on poultry products.
References:


CHAPTER 2

Literature Review
2.1 Brief History of *Campylobacter*

In so far as bacterial foodborne agents are concerned *Campylobacter* is a relative newcomer. Although its presence was first described by Theodore Escherich in 1886 (1886) and later confirmed to cause septic abortion in cattle and sheep (McFadyean and Stockman, 1909), it remained an organism primarily of veterinary concern up until the 1970's (Altekruse et al., 1999). The large gap in time from first observation to realization of the endemic nature of the organism can be attributed to its growth requirements and the necessity of selective culturing techniques in order to obtain pure cultures for study (Butzler, 2004). Human infection with *Campylobacter* in the 1940's and 1950's was diagnosed through direct observation of the suspected organism from blood of people suffering with gastroenteritis. The advancements in the detection of *Campylobacter* from human specimens began in the early 1970's. It was through a series of small steps that we have currently arrived at the ability to routinely culture this organism from infected patients, environmental sources and food matrices. Initially, researchers Cooper and Slee (1971) observed that an isolate of *Campylobacter* was able to grow in the presence of the antibiotic cephalothin when incubated in a microaerophilic atmosphere—leading to the first method available to laboratorians to help isolate *Campylobacter*. The main obstacle to isolation of *Campylobacter* from people with diarrhea was the overgrowth of other organisms present in the stool. The following year, Dekeyser and Butzler (1972) published a protocol utilizing a filtration technique which allowed the *Campylobacter* to be separated from other bacteria in the stool sample due to their relatively small size. Further progress was made by Martin Skirrow, when in 1977 he published a selective culture technique that was less labor intensive but with equal or greater sensitivity than the filtration technique (Skirrow, 1977). To validate this method he used the new technique over an 18 month period to culture for *Campylobacter* and other known bacterial
pathogens; he was able to determine that *Campylobacter* was responsible for diarrhea and gastroenteritis in people that also had blood cultures positive for the presence of *Campylobacter* (Skirrow, 1977). This research combined with earlier work from King in 1957 (1957) and Dekeyser in 1972 (1972) led to the suspicion that *Campylobacter* may be one of the most frequent causes of infective acute diarrhea. In the early history of *Campylobacter* infection of humans, it was isolated only infrequently from body sites believed to be sterile, or from severely ill people presenting with bacteremia or septicemia. However, work published by Blazer in 1979 (Blaser et al., 1979) redefined the role of *Campylobacter* in human disease from one of a perceived opportunistic to its significantly greater role as a frequent cause of acute gastroenteritis. From this point onward it was recognized that *Campylobacter* was pathogenic to humans.

2.2  **Morphology and In-Vitro Culture Conditions**

In the earliest history of what is now classified as *Campylobacteriaceae*, it was a recognized veterinary pathogen and assumed related to the genus *Vibrio* (McFadyean and Stockman, 1909). A few years later Smith and Taylor found a similarly described organism in tissues from aborted cattle fetuses and classified them as *Vibrio fetus* (Smith and Taylor, 1919). It was not until 1963 that researchers were able to classify the unusual *Vibrio fetus* as a distinct genus which they named *Campylobacter fetus* (Sebald and Veron, 1963). The name *Campylobacter* derives from the Greek words *kampylos* (curved) and *baktron* (rod) as this concisely describes the morphology of the bacterium. At the present time the family *Campylobacteraceae* is composed of 3 genera: *Campylobacter, Sulfurospirillum* and *Arcobacter* (Debruyne et al., 2008). Upon direct observation *Campylobacter* appear as small (0.2 to 0.8µm) curved or S-shaped, non-spore forming Gram negative rods with a single polar flagella at one or both ends of the cell (Debruyne et al., 2008). It is the polar flagella that give *Campylobacter* its most recognizable morphological feature.
of tumbling, falling or corkscrew motility. A distinguishing characteristic of the genus *Campylobacter* are its growth requirements. It requires reduced atmospheric oxygen (5% O₂, 10% CO₂ and 85% N₂) and grows best at 41.5°C, however it is capable of growth at temperatures of 30°C to 45°C (Robyn et al., 2015).

As of 2013, 24 known species of *Campylobacter* and 8 subspecies have been identified ranging in origin from environmental, human or animal sources. In addition, there are 14 completely sequenced genomes of *Campylobacter* species in the National Center for Biotechnology Information (NCBI) genome database available for more in depth molecular analysis (Pruitt et al., 2002). This has been a valuable resource for investigation into the potential mechanisms which lead to the high degree of genetic variability both within and among *Campylobacter* strains.

While there are many species of *Campylobacter*, clinical data indicates that humans are most frequently sickened by *C. jejuni* and less frequently with *C. coli*, *C. lari* and *C. upsaliensis* (Friedman, 2000; Gillespie et al., 2002; Taboada et al., 2013). Infection with *Campylobacter* is by ingestion through the oral route, as most illness is caused by eating or drinking contaminated foods. The number of bacteria that survive the acidic environment of the stomach depends upon the buffering capacity of the food that was ingested along with the bacteria (Janssen et al., 2008). Several studies have been undertaken to determine the infective dose of *Campylobacter* which will lead to disease. And from the reported results it was determined that the material with which the bacterium is ingested plays a role in the rate of infectivity, as demonstrated by low levels of *Campylobacter* contamination in milk correlating with illness (Robinson, 1981). Data from Black (1988) and Medema (1996) indicate that as low as 500-800 cells can result in a high probability of infection, which makes it more infective than other bacterial pathogens including *Salmonella* spp.,
but the dose to initiate disease could not be determined. General symptoms of illness include fever, malaise, abdominal pain, diarrhea and fever within 24 to 48 hours of exposure to the bacterium (Blaser et al., 1987; Wood et al., 1992). From epidemiological studies of *Campylobacter* outbreaks, in which a source was identified, the duration from exposure to illness is variable and can occur between 1 - 7 days, but 24 to 48 hours is the most common (Blaser et al., 1987; Wood et al., 1992). For most people, development of illness occurs within 3 days of an exposure and leads to a self-limiting illness with a duration of less than a week—however continued fecal excretion of the bacteria can last for an average of 16 days and up to 69 days has been documented (Blaser, 1997; Wassenaar and Blaser, 1999; Kapperud et al., 2009).

### 2.3 Human Infection Associated with *Campylobacter*

Upon passage through the stomach *Campylobacter* adheres to the epithelial cells and the mucus layer of the intestines and proliferates (Janssen et al., 2008). Then, once infection and colonization has taken place, a person may become an asymptomatic carrier (Christenson et al., 1983; Cawthraw et al., 2000) or diarrheal illness may proceed (Blaser et al., 1979). The disease manifestation of *Campylobacter* enteritis can be generally grouped into two categories (Hu and Kopecko, 2000) i) a secretory non-inflammatory diarrhea resulting from alterations in fluid resorption in the intestines from toxins (Wassenaar, 1997) or ii) bloody diarrhea as a result of the inflammatory response to *Campylobacter* invasion and proliferation within the intestinal mucosa (Janssen et al., 2008). In rare cases, intestinal infection with *Campylobacter* can produce cholecystitis, pancreatitis, or significant hemorrhage of the gastrointestinal tract (Acheson and Allos, 2001). *Campylobacter* is also capable of extraintestinal infection, though it is very rare. When *Campylobacter* disseminates beyond the intestinal tract, meningitis, endocarditis, septic arthritis osteomyelitis, sepsis or bacteremia can occur (Acheson and Allos, 2001). People that are
immunocompromised, very young or advanced in age are most susceptible to these more severe disease outcomes (Acheson and Allos, 2001; Samuel et al., 2004; Louwen et al., 2012). While no exact number of immunocompromised people in the United States has been calculated, the number is estimated at approximately 10 million or 3.6% of the population (Kemper et al., 2002). Ultimately, the severity of the disease in humans is determined by the specific strain of bacterium and factors of the host including immune system status (Newell, 2002).

2.3.1 Sequelae Following Infection with *Campylobacter*

Very soon after the development of selective stool culture techniques to isolate *Campylobacter*, researchers found connections between development of acute paralysis and prior infection with *Campylobacter*. Doctors were already aware of the link between gastrointestinal illness and development of what was then termed acute infective polyneuritis (Campbell, 1958). However, clinical proof of was not published until 1982 by Rhodes and and Tattersfield (1982) which documented a confirmed infection with *Campylobacter* and subsequent development of loss of motor development function, known as Guillain-Barre´ Syndrome (GBS). It is now accepted that post-infection diseases including Guillain-Barre´ syndrome / Miller Fischer Syndrome (Hughes and Cornblath, 2005; Fujimura, 2013), Reactive Arthritis (ReA) (Hannu, 2002), and immunoproliferative small intestinal disease (alpha chain disease) (Lecuit et al., 2004) are serious medical conditions that can develop after infection with *Campylobacter*.

2.3.2 Guillain-Barre´ Syndrome (GBS)

Guillain-Barre´ Syndrome, and its variant Miller Fischer Syndrome, are severe neurological diseases characterized by a rapid onset of weakness and tingling in the extremities progressing to ascending symmetrical loss of muscular function caused by biological mimicry of *Campylobacter* antigens and human gangliosides resulting in cross-reactive antibodies that attack
the peripheral nerves. The pathogenesis of GBS is rooted in the molecular mimicry between the lipooligosaccharides (LOS) on the surface of *Campylobacter* and the resultant production of antibodies that mimic areas on gangliosides within the peripheral nerves. Investigation has demonstrated that some *Campylobacter jejuni* serotypes have LOS in its outer core that coincidentally share similar antigenic epitopes to a large number of human peripheral nerve gangliosides (Yuki, 1997; Sheikh et al., 1998; Ang et al., 2000). According to Godschalk and colleagues (2004) it is estimated that between 50-60% of *Campylobacter jejuni* isolates are capable of producing and expressing the ganglioside mimicking sugars on their outer surface.

Guillain-Barre` Syndrome is now classified as a clinical syndrome caused by an acute inflammatory condition of the nervous system (Winer, 2014). The current classification scheme divides GBS into subtypes based upon neurological symptoms as related to potential different immunological mechanisms (Winer, 2014; Wakerley et al., 2014). The 3 subtypes are listed as: acute inflammatory demyelinating polyradiculoneuropathy (AIDP); acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN) (van Doorn et al., 2008; Winer, 2014). Miller Fisher syndrome is considered a variant of GBS and has distinct clinical presentations which include paralysis of eye muscles (ophthalmoplegia), loss of muscle coordination (ataxia), or loss of reflexes (areflexia)—and can then be further subdivided into incomplete and central nervous system subtypes (Mori and Kuwabara, 2011; Wakerley et al., 2014)

The likelihood of development of GBS after *Campylobacter* infection is quite low, and in the U.S. estimates are 1 in every 1058 infections may lead to GBS (Buzby et al., 1997). It is estimated that worldwide incidence of GBS is 1/100,000 (Yuki et al., 1993; Oomes and Jacobs, 1995; McGrogan et al., 2009; Sejvar et al., 2011). The first symptoms of the onset of GBS occur
between 10 days to 3 weeks after infection with *Campylobacter* (Blaser et al., 2008; Winer, 2014). Development of GBS is a very serious condition, reports indicate that approximately 25% of GBS patients require ventilation (Lawn et al., 2001) and between 3 -5% of patients die (Souayah et al., 2008). Disease progression reaches its apex at approximately 4 weeks followed by gradual improvements with 60% of patients regaining the ability to walk unassisted by 1 year (Winer, 2014). Continual fatigue, pain and neurological disorders are a complication from GBS (Winer, 2014). The outcome from GBS is varied and partially dependent upon host factors and *Campylobacter* strain.

### 2.3.3 Reactive Arthritis

Infection with *Campylobacter* can also trigger the development of a type of spondyloarthropathic disorder known as reactive arthritis (ReA) (Carter, 2006). Reactive arthritis was first described in 1969 (Ahvonen et al., 1969) and has since been utilized to describe a condition of acute joint inflammation with sterile synovial fluid that develops after a gastrointestinal or genitourinary infection (Berden et al., 1979; Carter, 2006). The connection between development of ReA and the confirmed antecedent of *Campylobacter* infection was first described in the late 1970's (Urman et al., 1977; Berden et al., 1979; Weir et al., 1979). Prior to the discovery of *Campylobacter* as a possible bacterium associated with ReA, it was already recognized that *Salmonella, Shigella* and *Yersinia* infection could lead to the development of ReA (Carter, 2006). The general symptoms of ReA include joint, tendon, skin, mucosal, or ocular pain and inflammation with possible malaise and fever (Carter, 2006). The onset of ReA is in general within 1 to 6 weeks of the initial bacterial infection (Carter, 2006; Singh and Karrar, 2014). As techniques in biology have continued to improve, analysis of synovial fluid has shown evidence of lipopolysaccharide and bacterial products of gram negative bacteria locating to these areas
(Carter, 2006). It has been suggested that these gram negative organisms pass through the mucus membranes in the gastrointestinal or genitourinary tract and travel to synovial tissues or fluid (Singh and Karrar, 2014). Current research on ReA has focused on the host-bacterium interaction then progressing to an autoimmune response causing the body to target the synovial space (Singh and Karrar, 2014). In 2000, researchers proposed a working classification scheme to try and assist with diagnosis of ReA (Braun et al., 1999). However, the diagnosis of ReA is not straightforward, as of 2013 there were no established diagnostic criteria (Ajene et al., 2013). Data on the rate of incidence of ReA following Campylobacter infection vary widely, Hannu and colleagues (2002) analyzed data suggesting the rate is 4.3 per 10,000 infections, while Ajene and colleagues (Ajene et al., 2013) determined it to be 9 per 1000 infections. The duration and resolution of ReA symptoms are also quite varied. It is reported that approximately half of all ReA conditions spontaneously resolve while the remaining half develop recurring long lasting arthritis (Carter, 2006). Medical care for people with ReA may last for over 1 year and include frequent interactions with medical personnel (Porter et al., 2013).

2.4 Campylobacter in the Environment

All the newly acquired information regarding Campylobacter that culminated from the work in the 1970's through 80's led to a great interest from public health officials to determine the true rates of illness and to try to determine its etiology. Research regarding sources of Campylobacter has demonstrated that it can be found in domestic animals, livestock and in the environment. Isolation from environmental sources has included abattoir effluent, surface water, sand, animal feces, shellfish and protozoa (Jones, 2001; Skelly and Weinstein, 2003; Axelsson-Olsson et al., 2005). While it is assumed that Campylobacter cannot multiply outside of a warm blooded host, studies have demonstrated that it is capable of persisting for extended periods of
time if given a favorable environment, which would include protection from direct sunlight and a cool moist atmosphere (Jones, 2001). *Campylobacter* has been isolated from many animals sources, and we now know it is a common inhabitant of the gastrointestinal system of domestic and wild animals (Blaser et al., 1983). Reservoirs for infection can include the animals we are most frequently surrounded by including domestic pets, and animals we utilize for food (Blaser et al., 1983). In addition to the wide variety of hosts from which *Campylobacter jejuni* has been isolated, further research has demonstrated that there is a large proportion of strain diversity within *C. jejuni* strains found in these different hosts and some strains exhibit host specificity (Griekspoor et al., 2013). Of particular importance to human health is the research which indicates that certain genetic populations of *Campylobacter jejuni* strains are widely distributed between hosts including food animals and humans—which may increase its potential as a zoonotic pathogen (Griekspoor et al., 2013).

### 2.5 Rates of Human Illness from *Campylobacter*

The CDC has been monitoring *Campylobacter* infections since 1982 and started with an initial 11 states (Finch and Riley, 1984). In July of 1995 the Foodborne Diseases Active Surveillance Network (FoodNet) was initiated (CDC, 2012). This network of 10 state health departments along with USDA, FDA and CDC provides active surveillance of laboratory diagnosed infections from 8 bacterial pathogens, including *Campylobacter*, that are most frequently transmitted through food. The goal of this program is to provide reports on trends in foodborne illness and analyze the impact of national food safety policy. The most recent data published by FoodNet provides preliminary estimates of the number of *Campylobacter* infections for 2013 at 6,621 including 1,010 hospitalizations and 12 deaths (Crim et al., 2014). It is important for our understanding of these numbers to realize that the true number of infections is
underreported, as diagnosed infections only account for an estimated 1 in 20 to 1 in 30 illnesses (Scallan et al., 2011; CDC, 2012). When adjusted for multiple factors, it is estimated that the true numbers of illnesses caused by food contaminated with *Campylobacter* is 1.3 million per year (Scallan et al., 2011).

### 2.5.1 Culture Independent Diagnostic Tests (CIDTs) for *Campylobacter*

Interestingly, quite recently some clinical labs have adopted the usage of culture independent diagnostic tests (CIDTs) in the diagnosis of enteric infections. In a report by Crim and colleagues (Crim et al., 2014) they note that of the 1,017 cases of positive CIDT for *Campylobacter* 42% came from samples that were not cultured for the bacterium and 58% were negative upon laboratory culture. While this technology is a great improvement for the ability to determine the causative agent of an infection—it does not integrate into our current model of foodborne illness tracing which involves characterization of bacterial isolates to detect clusters of similar pulsotypes (Cronquist et al., 2012; Braden and Tauxe, 2013). Wide spread adoption of these testing methods may increase the numbers of diagnosed infections without increasing the ability to detect trends and clusters of infection (Cronquist et al., 2012; Braden and Tauxe, 2013). This is especially important in regards to *Campylobacter*, due in great part to the relative difficulty of culturing the organism, the lack of a common standardized protocol used in clinical laboratories which could potentially lead to more reliance on CIDTs (Cronquist et al., 2012; Crim et al., 2014).

### 2.5.2 The Viable but Non-Culturable (VNBC) State for *Campylobacter*

*Campylobacter* is also one of the growing list of pathogenic bacteria that are capable of entering into a stasis like existence that has been termed viable but non-culturable (VNBC) when encountering periods of stress (Oliver, 2010). The importance of the VNBC state in regards to
poultry processing and raw retail product is due to the multiples stressors during processing (including reduced pH and starvation conditions) that may induce the VNBC state and the resultant potential for bacterial survival for long periods of time (Chaveerach et al., 2003; Mihaljevic et al., 2007). During the VNBC state the bacterium is unable to be resuscitated with normal culturing techniques (Oliver, 2000a; Li et al., 2014). The change from viable to non–culturable in *Campylobacter* also results in a morphological change from spiral or curved shaped to a coccoid shape (Rollins and Colwell, 1986). Bacterial cells are induced into the non–culturable state by environmental stressors including lack of nutrients, temperature shifts and osmotic stress (Oliver, 2000b). This is important in the context of food safety and production because these stressors are among many of the methods employed to eliminate pathogens during food processing (Oliver, 2005). It has also been reported that *Campylobacter* in the VNBC state has an increased tolerance to low pH, salinity, ethanol and chlorine (Rowe et al., 1998). *Campylobacter* cells that have entered the VNBC state have been brought back to culturability with the use of embryonic chicken eggs as the host (Cappelier et al., 1999; Talibart et al., 2000). Currently the mechanisms which enable a bacterium to be brought out of the VNBC state are unknown, but it is suspected that it is a result of bacterial-host interaction (Li et al., 2014). There is still dispute about the virulence potential VNBC *Campylobacter* cells and their level, if any, of infectivity (Oliver, 2005). But given the data from the embryonic egg model, it is most prudent to assume that VNBC cells of *Campylobacter* have the potential to be a risk factor for humans and animal infection alike (Talibart et al., 2000). In addition, the current gold standard to detection and enumeration of *Campylobacter* in animals and environmental samples is culture on selective media, and with the loss of culturability there can be an underestimate of the number of viable cells which may lead to negative health impacts (Li et al., 2014).
2.5.3 *Campylobacter* and Biofilm Formation

In the laboratory environment *Campylobacter* has very particular growth conditions and can prove quite challenging. It has been demonstrated to be sensitive to sodium chloride concentrations of greater than 2% w/v, desiccation, atmospheric oxygen, freezing, heat and pH below 4.9 or above 9.0 (Solomon and Hoover, 1999; Martínez-Rodriguez et al., 2004; Murphy et al., 2006; Wagenaar et al., 2015). However, *Campylobacter* is commonly found in environmental sources which would seem inhospitable. Much research on *Campylobacter* in the environment has shown that it is capable of survival in water, agricultural runoff, manure compost, and in frozen poultry products (Blaser et al., 1983; Friedman et al., 2004; Martínez-Rodriguez et al., 2004; Nicholson et al., 2005; Havelaar et al., 2007; Inglis et al., 2010). Another potential mechanism which *Campylobacter* may utilize for survival in times of stress is the ability to become part of or produce biofilms. (Reeser et al., 2007; Reuter and Mallett, 2010; Siringan et al., 2011; Ica et al., 2012). Biofilms are composed to sessile bacterial colonies from either single of multiple species of bacteria, which become covered in an extracellular matrix (Siringan et al., 2011). *Campylobacter* has been found in biofilms located within the gastrointestinal tract of animals and in industrial settings including water pipes, which could serve as a potential reservoir for infecting food animals (Brown et al., 2014). Biofilms are advantageous for bacteria because they serve as a mechanism for survival during periods of stress, and in addition provide increased resistance to disinfectants, antimicrobials and antibiotics (Reuter and Mallett, 2010; Sofos and Geornaras, 2010). In the poultry processing environment biofilms have been detected on many food contact surfaces including conveyor belts and stainless steel tables, which may become sources of contamination for foodstuffs with pathogenic organisms including *Campylobacter* (Lindsay et al., 1996; Peyrat et al., 2008). Interestingly Reuter and colleagues (2010) documented that biofilm
formation by *Campylobacter* was increased in the presence of atmospheric oxygen and Brown and colleagues (2014) determined that juice from poultry meat can act as a conditioning agent on abiotic surfaces and enhance the ability of the bacterium to attach to biofilms. It is most recently proposed that *Campylobacter* in a food production environment is unlikely to create de novo biofilms and instead attaches and survives within already established multi-species biofilms (Teh et al., 2014).

### 2.5.4 Human Sources of Infection with *Campylobacter*

While it is recognized that there are at least 24 species of *Campylobacter*, data from confirmed cases of campylobacteriosis indicates that up to 90% of infections are with *C. jejuni* and the remaining 10% is made up of *C. coli, C. lari* and *C. upsaliensis* (Friedman, 2000; Gillespie et al., 2002; Taboada et al., 2013). Epidemiological analysis has determined that the major vehicles for human exposure to *Campylobacter* include untreated water, consumption of raw milk, produce and consumption of contaminated poultry products (Friedman et al., 2004; Taylor et al., 2013). Outbreaks of campylobacteriosis are rare and most frequently are caused by the consumption of unpasteurized dairy products (Taylor et al., 2013). Produce is also on the rise as a source for *Campylobacter* and the FDA is in the development stages of updating the food safety regulations for fresh produce (FDA, 2009; Taylor et al., 2013). The majority of human illness in the United States caused by *Campylobacter* are classified as sporadic and therefore are not epidemiologically linked to an outbreak (CDC, 2009). However, Wilson and colleagues (Wilson et al., 2008) claim that up to 97% of sporadic cases of *Campylobacter* are related to consumption of poultry and beef by analysis of population genetics.
The area of source attribution is a rapidly growing methodology which seeks to provide an estimate of relative contribution of all reservoirs towards the total amount of human illness (Wagenaar et al., 2015). The full potential of this model is focused upon risk assessment, providing information in order to direct intervention efforts on the most important sources of infection (Wagenaar et al., 2015). The current proposed framework utilizes two main approaches to Campylobacter attribution 1) microbiological and 2) epidemiological (WHO, 2012; Wagenaar et al., 2013).

Analysis of data from Campylobacter infections that were utilized in the source attribution model for both the microbiology and epidemiology pathways concluded that poultry, specifically chicken is responsible for the majority of infections (Wagenaar et al., 2015). However, it should be noted that other pathways to exposure with Campylobacter including infected domestic animals and agricultural exposure are also responsible for human illness (Wagenaar et al., 2013). Most commonly, contact with poultry and poultry products are the leading cause of Campylobacter infection in humans (Harris et al., 1986; Wilson et al., 2008). Painter and colleagues (Painter et al., 2013) analyzed outbreak data as a result of foodborne illness between 1998-2008 in order to determine food commodities linked to illness. They determined that poultry was a significant commodity that was linked to bacterial foodborne illness, poultry was also the source of the most deaths (Painter et al., 2013). More specifically the Campylobacter-poultry combination accounted for an estimated $1257 million in health care related costs (Painter et al., 2013). In 2013 the United States processed over 8,500,000 broiler chickens and in 2012 the average per capita consumption of broiler meat was 83.1 lbs, by comparison the per person consumption of beef for the same year was 57.4 lbs (North American Meat Institute, 2015; U.S. Poultry and Egg, 2015). Chicken has become a popular choice as a food source due to its relative low price and high protein content. So it should be recognized that the high production rate and consumption rate make it a significant
source for human exposure even if the potential pathogen load was very small (Painter et al., 2013; Wagenaar et al., 2015).

### 2.6 Poultry as a Reservoir of *Campylobacter*

Poultry and wild birds are a natural host for *Campylobacter*. The body temperature of the chicken is 40-42°C—the optimum temperature for *C. jejuni* replication (Horrocks et al., 2009). The primary site of *Campylobacter* colonization in birds is the ceca, which are a pair of blind ended pouches that lay between the ileum and colon in the intestinal tract (Duke, 1986). Within the ceca *Campylobacter* are most frequently found in the mucus layer deep within the cecal crypts (Beery et al., 1988; Meade et al., 2009). *Campylobacter* may preferentially colonize the mucus layer due to their chemoattraction to mucin which can act as an energy source (Beery et al., 1988; Hugdahl et al., 1988). *Campylobacter* resides within the bird as a commensal organism—as a part of the cecal microbiota (Hermans et al., 2012). Functional genomic analysis of the chicken cecal microbiome has suggested that *Campylobacter* may function as a hydrogen sink to facilitate acetate production within its niche environment (Sergeant et al., 2014). Upon infection, the immune system of the chicken is poorly activated and studies indicate that *Campylobacter* down regulates the production of antimicrobial peptides of the chicken, both of which enable *Campylobacter* to persist and colonize in high levels within the ceca (Meade et al., 2009; Hermans et al., 2011). The density of *Campylobacter* with the ceca can reach from between $10^4$ to upwards of $10^8$ CFU/g of cecal contents (Evans, 1991; Jones et al., 1991; Berndtson et al., 1992; Musgrove et al., 2001). As of now there are no available treatments to eliminate *Campylobacter* from within the bird prior to processing—this makes post-harvest interventions essential (Wagenaar et al., 2015).
2.6.1 Rates of Contamination on Processed Poultry

During July 2007 through June of 2008 the USDA FSIS sampled rinsate from young chicken carcasses at commercial poultry processing plants. One of the objectives of this survey included data analysis to determine a baseline level of *Salmonella* and *Campylobacter* on carcasses at re-hang and post-chill points within the evisceration process (USDA FSIS, 2008). According to their statistics the estimated national prevalence of *Campylobacter* on post-chill carcasses is 46.7% (USDA FSIS, 2008). In addition to the determination of a baseline level of *Campylobacter*, the study also evaluated the effects of interventions used by comparing bacterial levels between re-hang and post-chill. For *Campylobacter*, the difference in rates of carcass contamination were significantly reduced (from 71.36% to 10.66%) between the two processing points indicating that currently implemented interventions had a large effect in reducing *Campylobacter* contamination (USDA FSIS, 2008).

In the United States the majority of raw retail poultry products are boneless skinless broiler meat pieces sold in tray packs (Williams and Oyarzabal, 2012). From January to August of 2012 the FSIS USDA initiated a new study to compare the levels of *Salmonella* and *Campylobacter* on whole carcasses at post-chill and then again as raw chicken parts (USDA FSIS, 2012). For this analysis the baseline *Campylobacter* level was adjusted statistically to take into account multiple variables including size of the processing establishment (USDA FSIS, 2012). When the young chicken baseline study *Campylobacter* data were adjusted, the adjusted percent positive rate was 10.6% and that was the number used for calculation. The results of this survey concluded that the estimated national prevalence of *Campylobacter* on raw chicken parts is 21.7% (USDA FSIS, 2012). When comparing the incidence of carcass contamination to cut up parts, the percentage of positive parts is nearly double that of the whole carcass (USDA FSIS, 2012). The FSIS has
suggested that the almost doubling of the *Campylobacter* rate on chicken parts could be due to positive carcasses being broken down into multiple parts which can spread the bacterium to multiple other pieces of chicken (USDA FSIS, 2012).

In a study by Scherer and colleagues (2006), investigation into the incidence of *Campylobacter* on chicken skin samples versus internal muscle tissue and found that skin samples were 66% positive and internal samples were 27% positive. This is in agreement with an earlier studies by Luber and colleagues (2004; 2007) in which they found the greatest levels of *Campylobacter* were on the surface of the meat as compared to internal levels, and skin-on chicken products had much higher concentrations of *Campylobacter* than skin-off parts. When the USDA FSIS tested skin-on versus skin-off chicken parts to detect if there was any significant differences in *Campylobacter* levels, they found that skin-on was significantly greater for *Campylobacter* than skin-off (USDA FSIS, 2012). Analysis was also performed on various skin-on chicken parts and table 2 lists the percent positive by type of part. Currently there are no USDA FSIS performance standards that encompass foodborne pathogens in retail broiler meat.

### 2.6.2 Contamination in the Processing Plant

Contamination of chicken carcasses during processing is a function of broilers entering the facility with high levels of the bacterium within their intestinal tract as well as on their feathers—*Campylobacter* can then spread throughout the processing facility (Keener et al., 2004). As much as 90% of broiler chickens in the United States are colonized with *Campylobacter*, resulting in an almost constant influx of the bacterium into the processing plant (Stern et al., 2001). Multiple steps during the slaughter and evisceration process can expose the carcass to external contamination.

### 2.6.3 First Processing: Slaughter Through Chilling
The steps in first processing of poultry include: unloading of the birds and placing them in shackles, from there they are stunned and exsanguinated followed by scalding and picking, evisceration and finally chilling (Bashor, 2003).

2.6.4 Cross-Contamination During Processing

Stern and colleagues (2001) found that during scalding and picking cross-contamination can occur possibly from leakage of cloacal contents and the dilation and subsequent contraction of the feather follicles which may trap the bacteria. *Campylobacter* has been isolated from the scald water and rubber picker fingers used in the picking process leading to potential sources of contamination for subsequent carcasses (Stern et al., 2001). However, an optimized scald tank is capable of a 2-3 log_{10} reduction of the concentration of *Campylobacter* on carcasses (Oosterom and Notermans, 1983; Izat et al., 1988; Berrang et al., 2000). When assessing the potential points at which *Campylobacter* can be introduced into the processing facility and the possibility for dissemination the major factors are:

1) the high concentration of *Campylobacter* within the alimentary tract (Keener et al., 2004)

2) scalding and picking processes, which take place before evisceration, and increase the potential for fecal contents being expelled (Stern et al., 2001; Byrd et al., 2005).

3) the evisceration process itself that can lead to carcass contamination if viscera being removed ruptures and spills its contents (Allen et al., 2007; Wagenaar et al., 2015)

4) the high throughput (upwards of 12,000 to 13,000 birds per hour) and automation which creates a system that makes cross-contamination among and between flocks a significant challenge to prevent (Lillard, 1989; Elvers and Morris, 2011; Wagenaar et al., 2015)
Certain processes that are unique to poultry processing, as opposed to red meat and pork, enhance the potential for contamination of the carcass. Factors that promote the cross-contamination of poultry carcasses include mechanical evisceration, intact skin, and the liberal use of water which keeps the carcass and processing environment wet (Butzler and Oosterom, 1991; Corry and Atabay, 2001).

### 2.6.5 The Potential Role of Chicken Skin

Chicken skin is recognized as an important source for the survival of *Campylobacter* during the multiple stages of processing—including scalding, picking and chilling—leading to cross-contamination (Wempe and Genigeorgis, 1983; Corry and Atabay, 2001; Stern et al., 2001; Tan et al., 2014). Skin and its associated fat is an innate barrier to any environmental animate or inanimate object making contact with the underlying tissue—and as such the skin is an excellent site for attachment and entrapment of bacteria. Lillard and colleagues (1989) propose that during exposure to water (chilling, scalding, rinses) the skin absorbs water—which may already contain fecal bacteria—and they loosely attach to the skin by physiochemical bonds. The loosely attached bacteria are able to form a biofilm in the thin layer of water covering the surface of the carcass, strengthening their attachment and making them much more resistant to being physically removed. In addition, microbes can persist in the folds, crevices and feather follicles of the skin as it swells with water and become protected from contact with applications of various disinfectants (Corry and Atabay, 2001). However, in research trials where direct observation of chicken skin inoculated with *Campylobacter jejuni* and subsequently exposed to sanitizing treatments, the viable *Campylobacter* remained at a higher depth than the feather follicles and crevasses indicating that these sites may not play a role in the protection of *Campylobacter* (Chantarapanont et al., 2004).

### 2.6.6 *Campylobacter* Populations Within Hosts and Environments
How is *Campylobacter* able to survive on the poultry carcass and in the environment of the poultry plant—besides protection from the skin? The answer to this is believed to be related to the heterogeneous mixture of *Campylobacter jejuni* strains that exist within their niche environment. Researchers have studied the composition of bacterial populations from ‘Farm to Fork’ in an effort to trace the points at which specific bacteria are able to pass from live bird to raw retail product (Melero et al., 2012). Diverse strains of *Campylobacter jejuni* colonize the gastrointestinal tract of broilers—when following these individual strains through processing the data showed that only certain strains can survive (Petersen et al., 2001; Newell et al., 2001; Melero et al., 2012). Strain diversity within *Campylobacter jejuni* potentially enables specific stable or more robust subtypes to remain in the processing facility and on the carcass (Newell et al., 2001; Peyrat et al., 2008; El-Shibiny et al., 2009; Elvers and Morris, 2011; Melero et al., 2012; Duffy et al., 2015).

### 2.7 Interventions During First Processing

Currently in poultry production there are no consistently effective treatments to reduce or eliminate *Campylobacter* from colonizing the birds (Lin, 2009). Due to our inability to prevent colonization during grow out, the need for post-harvest methods of pathogen elimination is of the utmost importance (Wagenaar et al., 2015). The decontamination process uses the multiple hurdle approach—a hurdle being a process in which the goal is to reduce or eliminate unwanted microbes—and multiple hurdles used together or sequentially can drastically reduce the bacterial load on a food product (Leistner, 2000; Hugas and Tsigarida, 2008; Loretz et al., 2010). Important to hurdle methods is the succession of treatments applied to the target product (Geornaras and Sofos, 2005; Hugas and Tsigarida, 2008). The importance of reducing the levels of *Campylobacter* on carcasses during processing should not be underestimated—predictive modelling has determined that if *Campylobacter* counts can be reduced by $2 \log_{10}$ on the carcass the incidence of
Campylobacteriosis would be reduced 30 fold (Rosenquist and Nielsen, 2003). Methods of decontamination for the carcass during processing can be grouped into 3 classifications physical, chemical or biological (Hugas and Tsigarida, 2008). Importantly these methods are required to be economically feasible, safe and easily implemented into the production scheme, not hazardous to wastewater treatment and finally – not result in negative attributes to the final meat product (Loretz et al., 2010).

2.7.1 Physical Treatments

The goal of physical treatments is removal of fecal contamination and microbial flora of the skin on the whole carcass. Physical approaches include but are not limited to water, temperature, and radiation treatments.

2.7.2 Washers

Water is used copiously during poultry processing. Washing and rinsing of carcasses can be accomplished by immersion, spraying and chilling. Since 1978 carcass washing has been utilized for removal of gross contaminants on the carcass—and multiple types of washing devices have been developed. Common wash systems include brush washers, cabinet washers and inside/outside wash cabinets (Conner et al., 2001; Bashor et al., 2004). Depending on the needs of the plant, multiple washer systems can be used throughout the processing work flow. To the water in these washer devices chemical treatments may be added (most commonly chlorine) and the pressure of the water as it contacts the carcass can be adjusted. The pressure is important as a balance must be made between too high, which can force bacteria into the skin, and low enough to allow for removal of organic material on the carcass (Conner et al., 2001). Overall the effect of carcass washing is shown to reduce the bacteria on the carcass by 1 -3 log_{10} (Hugas and Tsigarida, 2008; Wagenaar et al., 2015).
2.7.3 Chilling

Chilling systems are used to reduce the temperature of the broiler carcass after first processing from body temperature to $4^\circ C$ or lower within 4 hours (Sams, 2001). Most commonly in the United States immersion style chillers are used, however air-dry systems and evaporative air chillers are also available (El-Shibiny et al., 2009). The goal of carcass chilling is to reduce the growth of microbes on the surface of the carcass for both food safety and shelf-life considerations (Sams, 2001). Interestingly, research has demonstrated that *Campylobacter* populations on the skin of the carcasses can be resistant to inactivation by chilling depending upon the conditions in which the carcass was frozen—and some research demonstrated increased survivability of *Campylobacter* from super-chilling treatments and storage at $4^\circ C$ (Zhao and Ezeike, 2003; El-Shibiny et al., 2009; Ivić-Kolevska et al., 2012).

2.7.4 Irradiation

Irradiation is a poorly accepted yet highly effective means by which pathogenic bacteria, including *Salmonella* spp. and *Campylobacter* spp. can be inactivated on the surface of the carcass or meat products (MacRitchie et al., 2014; Wagenaar et al., 2015). Gamma and X-ray irradiation treatments expose the surface matrix to ionizing radiant energy that damages the DNA of the microbe rendering it unable to survive and multiply (CDC, 2005). The investigation into the utilization of irradiation—more specifically gamma irradiation—of poultry products contaminated with *Campylobacter* has determined the dose at which 90% of the bacterium are killed is between 0.12 and 0.31 kGy—dependent upon the packaging of the product and strain of *Campylobacter* (Lambert and Maxcy, 1984; Patterson, 1995; Kudra et al., 2012; Raut et al., 2012). In the early 1990’s the FDA and USDA approved the use of 1.5 to 3 kGy on fresh or frozen raw packaged poultry products (USDA, 1999; CDC, 2005).
2.7.5 Processing Aids

During first processing most if not all large poultry processors supplement their physical interventions with processing aids. As defined in the FDA CFR 21 CFR 101.100 processing aids are:

1. Substances that are added to a food during the processing of such food but are removed in some manner from the food before it is packaged in its finished form.
2. Substances that are added to a food during processing, are converted into constituents normally present in the food, and do not significantly increase the amount of the constituents naturally found in the food.
3. Substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food (FDA, 2014).

Antimicrobial compounds utilized during poultry processing to reduce foodborne pathogens on the surface of meat are classified as processing aids (National Chicken Council,). These compounds are approved by the FDA and USDA and many are listed as Generally Recognized as Safe (GRAS). The three primary classes of chemical processing aids are chlorine compounds, phosphate based compounds and organic acids (Hugas and Tsigarida, 2008; Loretz et al., 2010; Wagenaar et al., 2015). In a survey of U.S. commercial poultry operations, peracetic acid (PAA) was the leading chemical intervention and used with less frequency was cetlypyridium chloride (CPC), chlorine, acidified sodium chloride (ASC) and acids with a pH of 2.0 (McKee, 2011). USDA FSIS provides specific information on acceptable levels of permitted compounds in the FSIS directive 7120.1—listing approved substance levels within the mode that they are used in the processing plant (USDA FSIS, 2015).

2.8 Chlorine Based Compounds

2.8.1 Sodium Hypochlorite (Chlorine)

Historically chlorine has been the most frequently used chemical in poultry operations, with the benefits of being readily available and low cost. It can be added to processing water in
either a gas form or as the liquid sodium hypochlorite (Keener et al., 2004). The USDA has set the allowable levels; 20 ppm in bird washers, 50 ppm in potable water to be used in the primary chillers and 5 ppm in the recycled water used in the pre-chiller (USDA FSIS, 2015). In practice the antimicrobial effectiveness of chlorine—as used in poultry processing—has limited efficacy due to its dependence on pH of the water, long contact time and inactivation by organic material present in the water (Keener et al., 2004; Oyarzabal, 2005). When specifically investigating Campylobacter levels on poultry in relation to the age of the chiller water Yang and colleagues (Yang et al., 2001) found that chiller water with 10 ppm chlorine had a 3.3 log CFU/mL reduction when fresh but at 8 hours the reduction was less than 0.5 CFU/mL.

### 2.8.2 Chlorine Dioxide

Chlorine dioxide is also acceptable to be added to water used in poultry processing—it has advantages of being a more stable compound and a more effective bactericidal agent in the presence of organic matter—with less impact on organoleptic qualities of the meat (Conner et al., 2001). It is allowed to be used at up to 3 ppm in water that has direct contact with whole fresh poultry carcasses (USDA FSIS, 2015). Comparisons of chlorine to chlorine dioxide indicate that chlorine dioxide is effective at lower concentrations and has up to 7 times more activity than chlorine (Lillard, 1979). Campylobacter cells are susceptible to injury from chlorine dioxide and it is reported that up to 97% of C. jejuni cells became injured when exposed to 20 ppm for 2 minutes (Smigic and Rajkovic, 2011).

### 2.8.3 Acidified Sodium Chlorite (ASC)

Acidified sodium chlorite as used in the poultry processing plant is a mixture of sodium chlorite and citric acid and works as a broad spectrum oxidative antimicrobial. It can be applied by either spraying or dipping during pre-chill, chill and/or post-chill processes. Work by Orzybal
and colleagues (2004) demonstrated that a post-chill application of ASC significantly reduced *Campylobacter* to less than 0.2 log CFU/mL.

### 2.8.4 Organic Acids Used as Interventions

Organic acids can be added as part of an intervention plan to reduce microbial counts on poultry carcasses. They have the benefit of being more stable in the presence of organic material. The FSIS directive states that the organic acids can be applied as a small droplet rinse, fog or a mist at aqueous concentrations up to 2.5% (USDA FSIS, 2015). Organic acids including acetic acid, lactic acid, citric acid and succinic acid have demonstrated antimicrobial activity when used in poultry processing—however care must be used when applying these acids due to their negative effects on the sensory characteristics of the poultry meat (Blankenship et al., 1990; Bilgili and Conner, 1998; Keener et al., 2004). Overall, the general efficacy of organic acids is variable and results depend upon contact time, concentration and the temperature at the time of application (Dickson and Anderson, 1992). The addition of 0.1% acetic acid to scald water was able to reduce *Campylobacter* levels by 1.5 log$_{10}$ CFU/mL—when added directly to chicken wings a 1% lactic acid treatment was found to be less effective than 0.5, 1.0, 1.5 and 2.0% acid concentrations (Okrend et al., 1986; Zhao and Doyle, 2006). When acetic acid (2%) and lactic acid (3%) were used in combination to treat the surface of leg and breast samples the concentration of *Campylobacter* was reduced by 0.36 – 1.98 log$_{10}$ CFU/cm$^2$ when compared to treatment with a water control (Coşansu and Ayhan, 2010).

### 2.9 Phosphate Based Compounds

#### 2.9.1 Trisodium Phosphate (TSP)

The USDA has declared trisodium phosphate to be a GRAS compound approved for use during pre-chill and post-chill poultry processing at 8–12% (USDA FSIS, 2015). Trisodium
phosphate has several characteristics that make it a beneficial compound in regards to food safety. It is reported to be more effective at reducing Gram negative bacteria including the pathogens *Salmonella, E.coli* and *Campylobacter* (Bashor et al., 2004; Keener et al., 2004). It is theorized that the mechanisms involved in bacterial reductions on the surface of carcasses treated with TSP are caused by: the high pH (9–11 at 12%) which disrupts the bacterial cell membranes in addition to the removal of a negligible amount of surface fat that helps remove loosely attached bacteria during washing (Keener et al., 2004). Evaluation of a post-chill application of 10% TSP on poultry carcasses and subsequent analysis of *Campylobacter* levels at 0, 1 or 6 days revealed a significant decrease in levels at both 1 and 6 days (Slavik et al., 1994). Pre-chill applications of TSP, while effective, can have a negative impact on the treatment effect of the compound used in the chill tank. TSP is a highly basic compound with a pH of 11.8 at a 12% solution—introduction of TSP into chiller water from the surface of treated carcasses can increase the pH to levels which prevent the antimicrobial activity of chlorine compounds (Keener et al., 2004).

### 2.9.2 Peracetic Acid (PAA)

Peracetic acid is produced as a mixture of acetic acid and hydrogen peroxide in water and is a strong oxidizer and acid—in contact with cell membranes it disrupts permeability and interferes with protein synthesis (Baldry and Fraser, 1988; Wideman, 2013). It has been widely used in the food industry starting in the 1950’s when it was applied as a fungicide and antimicrobial on fruits and vegetables, however it was not until 2001 that PAA was legally permitted to treat poultry carcasses and parts (FDA, 2013a). The antimicrobial activity of peracetic acid is dependent upon the pH and temperature at which it is used. In accordance with the FSIS Directive 7120.1 the allowable upper limit of peracetic acid and hydrogen peroxide in the chiller is 220 ppm and 110 ppm respectively—when used as a post-chill dip solution the allowable concentration is 2000 ppm.
(USDA FSIS, 2015). The three stages of first processing at which PAA is most frequently utilized is (1) on pre-chill carcasses (2) during chilling and (3) as a post-chill dip or spray (EFSA BIOHAZ Panel, 2014). When PAA was evaluated against chlorine in a commercial setting as applied during chilling the overall percentage of Campylobacter positive carcasses decreased by 43% as compared to the chlorine control (Bauermeister et al., 2008).

2.9.3 Cetylpyridinium Chloride (CPC)

Cetylpyridinium chloride is a cationic quaternary ammonium compound and is most commonly found in mouthwash and throat lozenges. Its application in poultry processing can be at pre-chill, post-chill or as a pre-package intervention. It is allowed by USDA to be applied at concentrations of up to 0.8% (USDA FSIS, 2015). CPC is able to interact with negatively charged molecules on the surface of bacteria which leads to cellular leakage, interference with cellular metabolism and ultimately cell death (Scheie, 1989; Smith et al., 1991). Trials with CPC have demonstrated that at 0.5% it is highly effective against Campylobacter. Waldroup and colleagues (2000) reduced Campylobacter to non-detectable levels with 10 second dip in 0.5% CPC and Cargi and co-workers (2004) found a 99.7% reduction also with a 0.5% solution.

2.10 Post-Harvest Treatment of Raw Ready to Eat Poultry Products

2.10.1 Antimicrobial Edible Coatings

The use of coating treatments on raw poultry products can be considered another intervention to add to the multiple hurdle approach for enhancing the food safety of poultry products (Ricke and Hanning, 2013). The concept of coating a food item in order to keep it fresh longer (inhibition of spoilage microorganisms) dates back hundreds of years to practices in Asia—in 12th century China wax coatings were applied to fruits to slow water loss, and in Japan during
the 15th century the first edible food coating was developed from soymilk (Hardenburg, 1967; Gennadios et al., 1993).

In use today are edible coatings prepared from various substances including protein, polysaccharides and lipids. The food grade substances are made into a solution which may then be sprayed, dipped or spread onto the surface of the food matrix and allowed to dry into a transparent layer; this coating is defined as part of the final product (McHugh, 2000; Han and Gennadios, 2005; Falguera et al., 2011). To enhance the functionality of the coating treatment antimicrobial substances can be integrated into it to deliver another layer of food safety (Sánchez-Ortega, 2014). Edible coatings with antimicrobial activity can add value to food commodities while improving food safety—in addition these coatings and antimicrobials are being developed from natural materials including chitin, fatty acids and essential oils in place of chemical preservatives—which is favorable to consumers (Gennadios et al., 1997; Salleh et al., 2007; Sánchez-Ortega, 2014).

2.10.2 Generally Recognized as Safe (GRAS) Designation

Due to the fact that edible coatings are physically added to the food product and there is no expectation of the coating to be removed before consumption it is considered a food additive; it is important that the coating and any antimicrobials added to it are made from food-safe materials, most frequently with FDA approved Generally Recognized As Safe (GRAS) designated constituents (FDA, 2015). The use of GRAS labelled compounds as food additives does not require a premarket review and FDA approval. Food additives can be grouped into 1 of 3 different GRAS categories:

(1) Self-affirmed, where the manufacturer has carried out necessary work and is ready to defend GRAS status if challenged,
(2) FDA pending, where results of research have been submitted to FDA for approval, and
(3) No comment- which is the response of FDA if after review, it has no challenges (Pavlath and Orts, 2009).
However, it is essential to understand that if the material is designated as GRAS by FDA it does not provide a guarantee of absolute food safety (Pavlath and Orts, 2009).

2.11.1 Chemical and Physical Characteristics of Chitosan

In the area of edible and antimicrobial food coatings, chitosan (derived from chitin) is a polymer that has received a great amount of interest due to its numerous favorable attributes (Dutta et al., 2012). Published reports list the positive aspects of chitosan based coatings include (1) non-toxic and non-polluting, (2) possessing antimicrobial/antifungal activity, (3) low cost and abundance of source material (4) edibility (5) biodegradable, (6) positive chemical properties for ease of industrial use (Kong et al., 2010; Aider, 2010; Sánchez-González et al., 2011; Elsabee and Abdou, 2013). Chitosan is currently used in food industries outside of the U.S. as a coating, it has been approved since 1983 in Japan and is also approved for use in Canada—while it currently does not have FDA GRAS status it is expected that it will receive approval in the near future (Baldwin, 2007; Vasilatos and Savvidis, 2013; Zivanovic et al., 2014). Chitin was first discovered in the early 1800’s due to its unusual stability when exposed to common acids and subsequently received a name derived from the Greek ‘chiton’ meaning a coat of mail (Lower, 1984; Jeuniaux, 1996). Chitin soon came to be recognized as the major component of the exoskeletons of arthropods and is also found in fungi, mushrooms, and worms (Arcidiacono and Kaplan, 1992; Khoushab and Yamabhai, 2010). Chitosan is produced by the removal of an acetyl group from the original chitin molecule in the presence of sodium hydroxide. In the marketplace chitosan is characterized by its degree of deacetylation and average molecular weight; both of which have direct impacts on its functional qualities (No and Meyers, 1995; Cho et al., 1998; Elsabee and Abdou, 2013).

Chitosan is a highly versatile biopolymer and is one of the most abundant in nature (second only to cellulose)—estimates are that $10^{11}$ tons of chitin is produced globally each year by natural
processes and industrial use of chitin is approximately 10,000 tons per year (Muzzarelli et al., 1986; Arcidiacono and Kaplan, 1992; Kim, 2010). The crab and shrimp industry generates the greatest amount of chitosan as a byproduct of processing and due to the extended amount of time required for it to degrade the accumulation of shellfish waste is a significant concern of the shellfish industry and so finding a use for this byproduct is very helpful (Shahidi and Synowiecki, 1991).

2.11.2 Antimicrobial Activity of Chitosan

The antimicrobial mode of action for chitosan is still under investigation, but there is consensus that its activity is directly related to the degree of deacetylation, the molecular weight, concentration in solution, pH of the chitosan solution, temperature and food matrix in which the chitosan is applied (Dutta et al., 2009; Aider, 2010; Elsabee and Abdou, 2013). The theorized mechanisms of action include: interaction of the positively charged amino group of the chitosan disrupting the negatively charged cell membrane which will lead to leaking of cellular contents (Young et al., 1982; Shahidi et al., 1999; Kim and Thomas, 2003), chitosan may stimulate the production of chitinases and other defense proteins in host cells (Ghaouth et al., 1992), chitosan is able to chelate trace metals leading to inhibition of microbial growth (Cuero et al., 1991) and lastly, if chitosan penetrates the cell wall it disrupts protein synthesis by binding to host DNA (Sudarshan et al., 1992). It is reported in the literature that the more highly deacetylated chitosan has a greater antimicrobial effect and, in addition, the lower the pH of the chitosan the more effective it is (Sekiguchi et al., 1993; Dutta et al., 2009). More specifically, Zheng and colleagues (Zheng and Zhu, 2003) investigated the differing antimicrobial mechanisms of chitosan against Gram-positive and Gram-negative bacteria and concluded that as the molecular weight of chitosan increased it was more bactericidal towards Gram-positives and the direct opposite was true for Gram-negative
organisms. It is their theory that for Gram-positive bacterial the chitosan forms a barrier that block nutrients from entering the cell and for the Gram-negative organism chitosan enters the cell and disrupts normal cellular functions. The utilization of chitosan to inhibit the growth of foodborne disease causing bacteria has received a great amount of attention and chitosan has been shown to be very effective against *S. aureus*, *E. coli*, non-typhoidal *Salmonella*, *Listeria* spp., *Vibrio* spp., *B. cereus* and *Campylobacter* spp. (Chhabra et al., 2006; Beverlya et al., 2008; Ganan, 2009; Friedman and Juneja, 2010). Research into the application of chitosan onto raw chicken skin has produced positive results—Menconi and colleagues (2013) were able to reduce *Salmonella Typhimurium* and extend the shelf-life of skin treated with a 0.5% chitosan solution, thus demonstrating the role for chitosan in enhancing food safety and improving quality. In addition to studies on planktonic cells, chitosan and its derivatives were tested on mature biofilms of *Listeria monocytogenes* and *Salmonella enterica* and produced significant reductions in the attached populations of the pathogens (Orgaz et al., 2011).

### 2.11.3 Chitosan Coating of Poultry Products

Research and development into the utilization of chitosan in the food industry is directly related to the increase in consumer interest for fresh, microbiologically safer, minimally processed food products—and chitosan films and coating are especially useful because of their antimicrobial and antifungal properties plus their functional capabilities as carriers of other inhibitory compounds, including spices, organic acids, essential oils, and nutraceuticals (Ouattara et al., 1997; Appendini and Hotchkiss, 2002; Tapia and Rojas Graü, 2007; Petrou et al., 2012; Vasilatos and Savvaidis, 2013; Fernández-Pan et al., 2014). Many natural compounds including plant derived antimicrobials and essential oils are themselves inhibitory to foodborne pathogens, however to obtain maximum effect high concentrations are sometimes needed, potentially
affecting organoleptic properties of the food (Ntzimani et al., 2011; Sánchez-González et al., 2011; Petrou et al., 2012). There is much interest in the combination of chitosan and these natural compounds—to reduce the concentrations of natural compounds necessary for efficacy and to evaluate potential synergism (Elsabee and Abdou, 2013). The use of chitosan and its incorporation with natural compounds for the treatment of poultry products to extend shelf-life and reduce pathogens has generated much positive data. Petrou and colleagues (2012) successfully extended the shelf-life of MAP packaged chicken breast fillets by 6–12 days with the application of 1.5% chitosan or 1.5% chitosan plus oregano oil. The combination of chitosan and thyme essential oil was able to significantly reduce spoilage organisms, Enterobacteriaceae and lactic acid bacteria on packaged chicken kebabs after 12 days of storage as compared to the controls (Giatrakou et al., 2010). Ready-to-eat products are an especially important product for the control of foodborne pathogens. When chitosan and mixtures of chitosan plus lauric arginate or nisin were applied to turkey deli meat Listeria innocua was reduced by up to 4 log CFU/cm² (Guo et al., 2014). Similarly, Zheng and co-workers (2011) significantly reduced Listeria monocytogenes on roasted turkey meat by treatment with chitosan coatings incorporated with sodium lactate or sodium diacetate. The liquid purge that accumulates in the packaging of raw poultry products is another potential source for microbial growth of spoilage organisms and pathogens. The treatment of chicken purge, artificially inoculated with E. coli, by a chitosan-arginine solution was able to reduce both the actual microbial counts and the metabolic activity of E.coli (Lahmer et al., 2012).

2.12 Caprylic Acid in the Food Industry

The increased consumer preference for less chemically treated foods has turned more attention to plant derived antimicrobials as an alternative. It has been reported that the possible benefits for the use of antimicrobials derived from plants instead of organic chemicals include lack
of inciting bacterial resistance and less potential for negative environmental effects (Wyk and Gericke, 2000; Ohno et al., 2003; Ali et al., 2005; EFSA BIOHAZ Panel, 2014). Caprylic acid—a medium chain fatty acid found in breast milk, cow milk, palm kernel oil and coconut oil—has many positive characteristics for its use as a decontaminant on edible products (Jensen and Ferris, 1990; Sprong et al., 2001; Al Shahib and Marshall, 2003). Caprylic acid has reported broad spectrum antibacterial activity, and specific activity against the foodborne illness causing organisms Listeria monocytogenes, E.coli O157:H7, Staphylococcus aureus, Salmonella spp. and Campylobacter spp. (Kabara et al., 1972; Wang and Johnson, 1992; McLay et al., 2002; Nair and Vasudevan, 2004; Skrivanova, 2007).

2.12.1 Guidelines for Use of Caprylic Acid in Food Processing

The FDA (21CFR184.1025) has designated caprylic acid as a GRAS multipurpose food ingredient—the maximum acceptable levels for its inclusion in foods is up to 10 mg/kg and in snack foods the tolerance is up to 160 mg/kg (FDA, 2014). It is also approved by EPA as an antimicrobial pesticide—it is approved for use as a sanitizer to kill or inhibit microbial growth on inanimate objects in commercial kitchens, health care facilities, dairy and food processing plants (EPA, 2014). The toxicity of caprylic acid is very low, the EPA lists the oral LD₅₀ (in rats) as a range of 1283 mg/kg to 10,080mg/kg of bodyweight and the dermal LD₅₀ (in rabbits) is greater than 5000 mg/kg; in addition when supplemented at levels up to 50% of dietary fat in a balanced diet no toxic effects were observed (Traul et al., 2000; EPA, 2014).

2.12.2 Proposed Mechanisms of Action for Fatty Acids

Fatty acids from animal and vegetable sources have been used for thousands of years in soap making and it is the type and amount of fatty acids that determine the physical properties of the soap (Oghome et al., 2012). Today bar soap is generally 80% mixed carrier oils and
approximately 20% coconut oil; which is a natural source for saturated medium chain fatty acids (including caprylic acid) and are valued for their efficient removal of dirt and the production of foamy lather (Oghome et al., 2012). Several mechanisms have been proposed for the antimicrobial properties of fatty acids most of which are related to the aliphatic nature of saturated fatty acids and their ability to interact with cell membranes (Freese et al., 1973). Included in the potential mechanisms of action are (1) the creation of pores in the cell membrane (Petschow et al., 1996) (2) the diffusion of fatty acids into the cell resulting in a lowered intracellular pH that interferes with energy transport (Gauthier, 2002; Sun et al., 2002) (3) the inhibition of cellular glycolysis (Brul and Coote, 1999). As a consequence of fatty acids having many potential mechanisms for killing or inhibiting microbial growth, the selection for resistance within the microbial community is not likely to occur (Lacey and Lord, 1981; Petschow et al., 1996; Sun, 2003).

2.12.3 Fatty Acid Treatment of Poultry Skin

The application of fatty acids or their salts to reduce foodborne pathogens on chicken carcasses and skin has demonstrated the potential for these natural molecules to serve as part of the scheme of reducing foodborne illness from poultry. During in-vitro testing, Campylobacter was found to be particularly susceptible to capric acid (C10) and its derivative monocaprin when exposed in a slightly acidic medium, a pH 4-5 (Thormar, 2006). In a follow up study, Thormar and colleagues (2006) created a stable emulsion of the monocaprin and applied it to chicken legs and neck skins; there were significant log reductions in the recovery of Campylobacter from the treated legs (1.6-2.58 log) and neck skin (1.89-3.04 log) as compared to a treatment with water or samples left untreated. Oleic acid, when applied to poultry skin, was able to reduce Campylobacter significantly, and in addition, it reduced the total amount of aerobic bacteria attached to the skin surface (Hinton and Ingram, 2000).
Limited research on the use of caprylic acid or its derivatives as a treatment for the decontamination of chicken skin to reduce foodborne illness has shown promising results. Treatment of chicken skin and meat with a 5.0% concentration of caprylic acid sodium salt produced significant reductions in *Campylobacter* counts by up to $2.84 \log_{10}$ on skin and $4 \log_{10}$ on meat after treatment and storage at $4^\circ$C for 24 hours (Riedel and Brøndsted, 2009). On chicken skin artificially contaminated with *Salmonella* spp., the application of 0.75 mg/mL of caprylic acid significantly reduced the amount of recoverable *Salmonella* at days 1, 2, and 3 as compared to a saline control treatment (Skřivanová et al., 2012).

### 2.13 Introduction to Lactic Acid Bacteria

The utilization of bacteria by humans for the processing of foods is thousands of years old—bacterial fermentation to produce sourdough bread and dairy products including butter, yogurt and cheese has been discovered in cultures that date back as far as 5000 years ago (Währen, 1990). Interest in bacteria that produce lactic acid (and provide us fermented foods) has been ongoing since the mid 1850’s when Pasteur studied the process of lactic acid fermentation and Lister became the first person to document the isolation of a pure bacterial culture (*Bacterium lactis*) in 1873 (Lister, 1873; Stiles and Holzapfel, 1997). The use of lactic acid bacteria for the industrialized production of fermented food was started in 1890 in Copenhagen and is still in use today (Stiles and Holzapfel, 1997). It was in the early 1900’s that the first pioneers in microbiology conceived of the idea to classify these lactic acid producing bacteria in a group they called lactic acid bacteria (LAB) (Orla-Jensen, 1919). This group was composed of gram-positive, non-motile, non-sporeforming, rod or coccus shaped bacterium which are able to grow in microaerophilic to strict anaerobic conditions and which produce mainly lactic acid as their end product during fermentation of carbohydrates (Orla-Jensen, 1919; Stiles and Holzapfel, 1997). The interest in
these organisms for their possible healthful properties to humans was pioneered by Elie Metchnikoff while at the Pasteur Institute, he believed that this group of bacteria could help prevent and treat illness from harmful bacteria and prolong the duration of a person's life (Metchnikoff, 1907; Bibel, 1988).

2.13.1 Lactic Acid Bacteria as Probiotics

Today these lactic acid bacteria are most frequently associated with probiotics. The definition of probiotic—from the Greek, meaning for life—has gone through many iterations since it was first introduced in 1965 (Lilly and Stillwell, 1965). According to the International Scientific Association for Probiotics and Prebiotics the agreed upon consensus for the definition of probiotic is:

‘Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.’ (Hill et al., 2014)

Lactic acid bacteria most commonly used as commercial probiotics include *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* (Hamilton-Miller, 1997; Stiles and Holzapfel, 1997; Klein et al., 1998; Naidu et al., 1999). It is agreed upon that probiotic cultures must be safe within the conditions of their use and while there is no 100% certainty for the potential of adverse effects many of these strains have a long history of use without significant associations with pathogenicity within normal healthy populations (Saxelin and Chuang, 1996; Hamilton-Miller, 1997; Boyle et al., 2006; Ljungh and Wadstrom, 2006; Vankerckhoven and Huys, 2008; Sanders, 2009).

2.13.2 Antimicrobial Activity of Lactic Acid Bacteria

Probiotic lactic acid bacterial strains are reported to produce a diverse variety of activity to promote health, which can be broadly grouped into nutritional, physiological and antimicrobial activity (Naidu et al., 1999). Investigation into the many mechanisms by which lactic acid bacteria
are capable of inhibiting or killing other bacteria, including food-borne pathogens, has demonstrated the production of: (1) short chain fatty acids (SCFA) including acetic, lactic and propionic acid, (2) production of hydrogen peroxide in anaerobic conditions and (3) production of specific inhibitory molecules (bacteriocins) (Havenaar et al., 1992; Sanders, 1993; Gibson et al., 1997; Robyn et al., 2015). In addition to the production of inhibitory substances and molecules other mechanisms have been proposed including competition for nutrients, attachment sites and the interference with pathogenic mechanisms of Enterbacteriaceae (Havenaar et al., 1992; Sanders, 1993; Saavedra, 1995; Naidu et al., 1999). It is important to understand that the mechanisms of antibacterial activity and production of inhibitory substance are strain specific and strains should be characterized on an individual basis to determine if they have probiotic traits (Ljungh and Wadstrom, 2006).

2.13.3 Use of Probiotics in Poultry Production

The administration of probiotic bacteria to broilers and turkeys is a common practice and there are many commercially available direct fed microbials available on the market. Probiotics are provided to poultry for the main objectives of promoting gut health, prevention of disease related to environmental stress of the bird, and for the promotion of performance characteristics important to the commercial poultry industry (Davis and Anderson, 2002; Talebi et al., 2008; Wideman et al., 2012; Neal-McKinney et al., 2012; Huff et al., 2015). Interest in probiotics is continually increasing—mainly due to consumer and regulatory pressure for the discontinuation of antibiotic growth promoters and the increasing demand for antibiotic free poultry meat (FDA, 2012, 2013b; Kesmodel et al., 2014). Commercial poultry companies and allied health industries
are interested in the potential for probiotic bacterial strains to replace antibiotic growth promoters in the poultry industry.

**2.13.4 Probiotics to Prevent *Campylobacter* Infection in Pre-harvest Poultry**

It is well documented that within the chicken the greatest numbers of *Campylobacter* spp. inhabit the cecum and thrive within the cecal crypts—chemoattraction to mucin within this environment supplies the bacteria with energy (Beery et al., 1988; Hugdahl et al., 1988; Meinersmann et al., 1991; Achen et al., 1998; Van Deun et al., 2008). There is much literature on the proposed idea of isolating LAB strains which (1) produce anti-*Campylobacter* metabolites and (2) can competitively inhibit *Campylobacter* within its niche environment (Nurmi and Rantala, 1973; Schoeni and Doyle, 1992; Gaggia et al., 2011). By preventing *Campylobacter* from colonizing within the bird, the downstream effects would be the reduction of foodborne illness from contaminated poultry meat (Andreoletti et al., 2011). Many studies have demonstrated that it is possible to isolate—from chickens or other species—strains of LAB that produce substances which are inhibitory to *Campylobacter* in-vitro (Chaveerach et al., 2004; Stern and Svetoch, 2006; Zhang et al., 2007; Bhaskaran et al., 2011; Ganan et al., 2013; Cean et al., 2015). The administration of these LAB strains to broiler chickens have been evaluated for their ability to reduce *Campylobacter* within the bird's gastrointestinal tract, Aguiar and colleagues (2013) were able to produce a reduction in the colonization of broiler chickens by administration of motility enhanced probiotic isolates and Neal-McKinney and colleagues (2012) also decreased *Campylobacter* in broiler chicks with strains of *Lactobacillus* evaluated for their in-vitro production of lactic acid. Additionally, researchers have evaluated methods to increase the efficacy of specific LAB with in-vivo anti-*Campylobacter* activity by the addition of fructooligosaccharide or mannan oligosaccharide as a prebiotic treatment in feed. Arsi and
colleagues (2015a) demonstrated that the addition of 0.04% mannan oligosaccharide in the feed in combination with an isolate of *Lactobacillus salivarius* reduced *Campylobacter* counts in the ceca by 3 logs as compared to the control. Frequently, in trials which involve the testing of LAB isolates with *in-vitro* inhibitory activity in live birds by oral administration of the cultures there is a failure to see reductions in *Campylobacter* levels within the gastrointestinal tract. A recently proposed model to more efficiently assess the antimicrobial effects of specific LAB cultures within the cecal environment has demonstrated that intra-cloacal inoculation of LAB isolates was more specific at determining *in-vivo* efficacy than oral administration as a screening tool (Arsi et al., 2015b).

In addition to the antimicrobial activity of the lactic acid produced by LAB, many researchers have investigated the production of bacteriocins specific to killing *Campylobacter* and evaluated their ability to be administered to poultry as either a therapeutic or prophylactic treatment to suppress or eliminate *Campylobacter* infection. Trials involving purification, characterization and ultimately dosing of broiler chickens with anti-*Campylobacter* bacteriocins has demonstrated these molecules are highly effective at reducing *Campylobacter* *in-vivo* (Stern and Svetoch, 2006; Line and Svetoch, 2008; Svetoch and Eruslanov, 2008; Stern et al., 2009; Svetoch and Stern, 2010). However, when performing a survey of the literature regarding the use of probiotics in poultry the general trend is toward a wide degree of variability of results—factors that influence the variability include the strain of bacterium used, its proposed mechanism of action, how it interacts with the host and the environment in which it ultimately settles within the host (Otutumi et al., 2012).
2.13.5 Application of Lactic Acid Bacterial Cultures to Raw Meat Product

The application of lactic acid bacteria—to perform as a protective culture or biological preservative—on the surface of poultry carcasses or raw retail product may be another hurdle that could be added to the food safety programs currently in use (Holzapfel et al., 1995). The concept of biological preservation may not be a term readily encountered but it is a technique that has been used for thousands of years—specifically to make fermented foods and beverages (Stiles, 1996). While we may not accept the idea of fermentation in the context of our raw poultry products the application of bacterial cultures onto meat may provide several advantages: (1) live organisms may produce metabolites which are inhibitory to other spoilage bacteria, leading to increased shelf-life of the product (2) and/or the production of substance inhibitory to pathogenic microorganisms which may increase the food safety aspect of the product (3) there may be potential health benefits to the consumer by ingestion of the added probiotic culture (4) biopreservation with natural lactic acid bacterial cultures might be a more acceptable treatment for edible products, as compared chemical treatments by consumers (Holzapfel et al., 1995; Schillinger et al., 1996; Stiles, 1996; Gálvez et al., 2010; Garcia et al., 2010; Gaggia et al., 2011).

In an opinion by Maragkoudakis and colleagues (2009) the use of lactic acid bacteria as a protective culture on poultry meat is a feasible and safe option due to their ubiquitous nature in the foods we already consume, they have been identified as a constituent of normal flora in the human gut microbiome and these organisms have essentially been a part of our diet for thousands of years without significant negative implications.
Currently there is no official opinion or guideline from our government in regards to protective cultures—however the strains of bacteria that could potentially be used in this manner share many attributes with probiotic bacterial strains, and so guidelines in place for probiotics may be useful to developers of protective cultures. Due to the increased interest world-wide in the development and use of probiotic cultures in foods, the World Health Organization together with the Food and Agriculture Organization of the United Nations has proposed guidelines on the selection of probiotic cultures (FAO, WHO, 2006). It is the intent of the guidelines to help researchers and the food industry to provide to consumers probiotic cultures, as related to food items, which provide consistent results, are well characterized and safe for human consumption.

Research trials involving the application of live protective cultures onto raw poultry meat surfaces for the reduction of the foodborne pathogen *Campylobacter* are sparse. There is literature, however, on application of live protective cultures to reduce *Salmonella* spp. and *Listeria* spp. in raw poultry meat. In a study by Maragkoudakis and colleagues (2009) they surveyed 635 lactic acid bacterial cultures for their potential to act as protective cultures, displaying antagonistic activity against *Listeria monocytogenes* and *Salmonella enteritidis* specifically, in chicken meat. After performing in-vitro tests on the 635 isolates, which included screening for antibiotic resistance profiles, the presence of bacteriocin producing genes and survivability within food processing and gastrointestinal tract environments, 2 strains were selected for further testing on the chicken meat itself. The results demonstrated that the two cultures, *E. faecium* and *L. fermentum* were able to decrease the total population of both pathogens by what they speculate is not a direct killing effect but an inhibition of the growth rate of the pathogens and in addition, the application of these bacterial strains did not produce detrimental effects in regards to spoilage of the meat. In a more recent trial Sakaridis and
colleagues (2014) tested the application of lactic acid bacteria against *Salmonella* spp. and *Listeria* spp. on chicken skin and meat. The application of *L. salivarius* to chicken skin that was inoculated with either *Salmonella* spp. or *Listeria* spp. produced a significant, but modest, reduction in the amount of pathogen remaining on the skin after 7 days. The log reduction in *Salmonella* spp. was 0.54 log CFU/cm² and for *Listeria* spp. was 0.71 log CFU/cm² at the day 7 time point. Trials involving chicken meat in lieu of skin produced similar results with the exception being that significant reductions were observed a day earlier, at day 6. The log reduction of *Salmonella* spp. on meat were 0.51 log CFU/cm² and for *Listeria* spp. the reductions were 0.67 CFU/cm². The addition of a live protective culture has also been investigated against *E. coli* O157:H7 in chicken meat. Brashears and coworkers (1998) inoculated boneless-skinless chicken breast pieces with *E.coli* O157:H7 and then treated the breast samples by dipping them in a solution of *L. lactis*, followed by storage in refrigerated temperatures for 0, 3, 5 and 7 days. The samples treated with *L. lactis* significantly reduced the *E. coli* O157:H7 at day 5 as compared to the controls, and in addition it was noted that the control samples were putrid smelling and had a slimy texture at day 5 whereas the *L. lactis* coated samples did not.
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67


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Chapter 3

The application of caprylic acid, chitosan or their combination as a coating treatment reduces *Campylobacter jejuni* on inoculated chicken wingettes.
The application of caprylic acid, chitosan or their combination as a coating treatment reduces *Campylobacter jejuni* on inoculated chicken wingettes


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Abstract

In this study, chitosan, caprylic acid and their combination were evaluated as a coating treatment for the reduction of *Campylobacter jejuni* on poultry products. For the initial screenings, chitosan of three different molecular weights (15-50 kDa, 190-310 kDa and 400-600 kDa) were evaluated at three concentrations (0.5%, 1.0%, 2.0%) on 2 g chicken skin pieces in order to determine the most effective molecular weight/concentration treatment. In addition, caprylic acid at 0.5%, 1.0% and 2.0% was tested for efficacy against *Campylobacter jejuni* on chicken skin pieces. From these initial screenings a solution of 2% medium molecular weight (190-310 kDa) chitosan was chosen for follow up evaluation, as was 1% and 2% caprylic acid. To assess the short term and long term efficacy of 2% medium molecular weight chitosan, 1% caprylic acid, 2% caprylic acid individually and in combination, chicken wingettes were inoculated with *Campylobacter*, coated with a given treatment and microbial analysis was performed at 0, 1, 3, 5 and 7 days post-treatment. In replicate trials both 1% and 2% caprylic acid continuously reduced *Campylobacter* counts starting at day 3 through day 7 as compared with controls. Interestingly, there was no improved efficacy from the 2% caprylic acid as opposed to the 1% caprylic acid. The coating of the wingettes with 2% medium molecular weight chitosan also produced reductions in *Campylobacter* counts starting at day 3 lasting until day 7. The combination of either 1% or 2% caprylic acid plus 2% medium molecular weight chitosan continuously reduced *Campylobacter* counts starting at day 0 through day 5. With the exception of a single time point in trial 2 there was no observed increase in efficacy between the 1% caprylic acid with chitosan and the 2% caprylic acid plus chitosan. These results demonstrate that the coating of raw poultry products with chitosan, caprylic acid or its combination may be an effective treatment option for the reduction of *Campylobacter* contamination.
Introduction

The contamination of poultry products with *Campylobacter* spp. has been receiving increased attention in the United States from the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS). In 2011, in conjunction with the passage of the Food Safety Modernization Act (FSMA), the FSIS implemented new performance standards for the contamination of chilled carcasses with *Campylobacter* (FDA, 2011; FSIS, 2011). In addition to this, the Department of Health and Human Services (HHS) initiated the Healthy People 2020 program which, among other goals, has an objective to reduce the incidence of human *Campylobacter* infection by 33% by the year 2020 (HHS, 2015). In order to work towards this goal FSIS recently enacted additional performance standards for *Campylobacter* that include the testing of raw chicken parts (including breast, thigh, and wing) as well as ground pieces (USDA and FSIS, 2015).

The most recent estimates of foodborne illness incidence from *Campylobacter*, as determined by FoodNet, has calculated a rate of 13.4 per 100,000 people—the Healthy People 2020 goal is 8.5 (Crim et al., 2015). When compared against incidence rates from 2006-2008 *Campylobacter* infection has risen by 13%, and even with the new programs initiated to address *Campylobacter* in poultry products this rate has remained stagnant for the last 2 years (CDC, 2014, 2015).

In the United States, poultry is a main source of protein, the estimated amount of poultry consumed per person in 2014 was approximately 45 kg (National Chicken Council, 2015). As a consequence of the large volume of poultry consumed, the potential for foodborne illness is increased even if the rate of contamination is low (Painter et al., 2013). Modern poultry processing involves many sequential steps and procedures which can lead to fecal contamination or cross-
contamination of carcasses—while this potential for contamination exists, it is currently an unavoidable consequence in the practice of producing poultry meat (Corry and Atabay, 2001; Sampers et al., 2008; Elvers et al., 2011; Alonso-Hernando et al., 2013). To date there are no consistent, effective methods for the elimination of *Campylobacter* from the gastrointestinal tract of broiler chickens, the result of which is more focus on the options for post-harvest treatments to reduce contamination of poultry from bacterial pathogens (Wagenaar et al., 2015). It has been suggested that the greatest effect on reduction of human *Campylobacter* illness can be obtained not from focusing on prevention of the infection of chicken, but by reducing the level of *Campylobacter* on carcasses and raw poultry products (Nauta et al., 2009). Simulations involving multiple intervention options and their predicted effect on the human incidence rate of *Campylobacter* have proposed that a 2 log decrease in the amount of *Campylobacter* on the carcass should lead to a 30x decrease in human infection (Rosenquist et al., 2003; Lindqvist and Lindblad, 2008; Loretz et al., 2010).

The application of antimicrobial coatings onto the surface of raw poultry for the reduction of pathogens, including *Campylobacter*, may be an additional intervention to supplement post-harvest decontamination treatments on poultry carcasses (Ricke and Hanning, 2013). An edible coating is most frequently a liquid in which proteins, polysaccharides or lipids are suspended and applied to the food product by dipping, spraying or spreading—the coating is then treated as part of the final product to be consumed and classified as a food additive (McHugh, 2000; Han and Gennadios, 2005). Chitosan is a molecule with many physical and biological attributes which may be utilized by the poultry industry to increase the food safety of their products. Characteristics of chitosan which are desirable for the food industry include its relative abundance, ability to form gels and films, antimicrobial and antifungal activity, GRAS designation and ability to have other
antimicrobial compounds incorporated into it (Aider, 2010; Kong et al., 2010; Sánchez-González et al., 2011; Elsabee and Abdou, 2013). Menconi and colleagues (2013) were able to reduce *Salmonella* Typhimurium and show improvements in shelf-life of chicken skin treated with a solution of 0.5% chitosan.

The ability of chitosan to be combined with other antimicrobial compounds and applied as a coating to poultry products offers great potential—the possible synergism between the two compounds may increase the antimicrobial effect as compared to each compound individually (Elsabee and Abdou, 2013). Research into the application of chitosan coatings in combination with natural compounds has demonstrated promising results. Chitosan and its combination with thyme essential oil was able to reduce overall levels of spoilage bacteria and *Enterobacteriaceae* on raw retail chicken kebabs after 12 days of storage (Giatrakou et al., 2010). Raw chicken breasts have shown improvements in shelf-life by the coating of chitosan plus plant derived antimicrobials including cinnamon, oregano, clove, and rosemary (Fernández-Pan et al., 2014).

Fatty acids are being investigated for their potential to reduce pathogens in food products. The medium chain fatty acid caprylic acid has reported broad spectrum antibacterial activity against many foodborne illness causing bacteria including *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes* and *Campylobacter* spp. (Kabara et al., 1972; McLay et al., 2002; Nair et al., 2005; Skrivanova, 2007). Caprylic acid is naturally found in mammalian milk and coconut oil—additionally it has GRAS status as conferred by the FDA for use in foods (Jensen et al., 1990; Sprong et al., 2001; USDA FSIS, 2015). When used as an antimicrobial treatment for *Salmonella* on chicken skin, it was found that a 0.75mg/mL dose was able to reduce *Salmonella* during sampling at 1, 2 and 3 days post treatment (Skřivanová et al., 2012). In trials by Riedel and colleagues (2009) the application of a 5.0% solution of caprylic acid sodium salt reduced
Campylobacter counts on chicken skin and legs by 2.84 log and 4 log, respectively, after treatment and 24 hours storage at 4°C.

The objective of the present study was to evaluate the antimicrobial activity of chitosan, caprylic acid or their combination to reduce Campylobacter on chicken skin and wingettes for its potential use as a post-harvest intervention.

Materials and Methods

Preparation of Campylobacter jejuni Inoculum

A frozen wild type strain of Campylobacter jejuni, previously isolated by our laboratory, was used as the inoculum for this study. One loopful of frozen glycerol stock of the wild-type strain Campylobacter jejuni was inoculated into 5 mL of Campylobacter Enrichment Broth (CEB, Neogen, Lansing, MI) and incubated at 42°C in a microaerophilic atmosphere (5% O₂, 10% CO₂, and 85% N₂) for 48 hours. The Campylobacter inoculum for each trial was prepared as described by Akins and colleagues (2009). Briefly, an aliquot of Campylobacter in CEB was transferred to a biphasic culture condition consisting of a Mueller Hinton agar (Difco, Sparks, MD) coated cell culture flask with an overlay of Mueller Hinton broth (Difco, Sparks, MD) and incubated at 42°C for 12 hours in a microaerophilic atmosphere. At each inoculation time point, an aliquot of biphasic grown Campylobacter was centrifuged at 3000 rpm for 12 minutes, the supernatant discarded and the cell pellet resuspended in 2x the volume of the original aliquot in Butterfield’s Phosphate Diluent (BPD).

Chitosan
Powdered chitosan of high molecular weight (400-600 kDa) and 90% deacetylation was purchased from Spectrum Chemical (Spectrum Chemical Mfg. Corp., Gardena, CA). Medium molecular weight chitosan powder was 190-310 kDa (75%-85% deacetylated) and procured from Sigma (Sigma-Aldrich, Co., St. Louis, MO) and the low molecular weight chitosan was 15-50 kDa, at least 85% deacetylated, and purchased from Polysciences, Inc. (Polysciences, Inc., Warrington, PA). Caprylic acid was purchased from Acros Organics (Thermo Fisher Scientific Inc., Geel Belgium).

**Preparation of the High, Medium and Low Molecular Weight Chitosan Coating Treatments**

Chitosan solutions were prepared according to a previously published method (Upadhyay et al., 2014). A 50 mM solution of acetic acid was prepared with glacial acetic acid (BDH Aristar, West Chester, PA) in deionized water (vol/vol). To prepare the treatments each molecular weight of powdered chitosan was solubilized in 50 mM acetic acid to make a 0.5% (wt/vol), 1.0% (wt/vol) or 2.0% (wt/vol) solution, the pH was adjusted as necessary with HCl (Fisher Scientific) to obtain complete solubilization and stirred overnight at room temperature to ensure complete incorporation of the chitosan powder. The 50 mM acetic acid control was pH adjusted as necessary with 10N NaOH to be similar to the chitosan treatments.

**Evaluation of Antimicrobial Activity of Chitosan on Chicken Skin**

Chicken thigh skin was obtained from commercially available bone-in skin-on chicken thighs. The skin was removed from the muscle tissue and cut into 2 g pieces which were then stored at -20°C until needed. On the day of the experiment the skin pieces were allowed to reach room temperature and then attached to clips which were equidistantly mounted on a rod. Coating of the chicken skin pieces was based on the protocol by Olaimat and colleagues (2014) with minor
modifications. While attached to the clips, the skin pieces were laid flat on a piece of aluminum foil and 50 µl (~1x10^7 CFU/mL) total volume of the *Campylobacter* inoculum was added dropwise across the surface on each piece of skin. The skin samples were kept flat for 30 minutes at room temperature to allow time for the *Campylobacter* to adhere to the skin. For each treatment group the rod with the clips and skin attached was raised, allowing all skin samples to hang freely without contact to each other and then lowered over a rack holding 50 mL conical tubes with 10 mL of treatment solution in each tube. This allowed the skin samples to be dipped into each treatment tube simultaneously. Chicken skin pieces were dipped into treatment groups which included 50 mM acetic acid (chitosan control), 0.5% HMW, MMW or LMW chitosan; 1.0% HMW, MMW or LMW chitosan and 2.0% HMW, MMW or LMW chitosan. For each treatment group, the inoculated skin samples were simultaneously dipped into 10 mL of treatment in 50 mL conical tubes with a contact time of 5 minutes followed directly by air drying for 30 minutes, after which point in time each skin sample was individually placed into a sterile sample bag and held at 4°C for 18 hours until sampled for microbial analysis. There were 5 chicken skin samples per treatment.

**Antimicrobial Efficacy of Caprylic Acid on Chicken Skin**

The antimicrobial effect of caprylic acid was evaluated the same as above with the few modifications being: treatments were 0.5%, 1% or 2% caprylic acid prepared in BPD (vol/vol) and the control was BPD alone. Treatment solutions were 10 mL aliquots in 50 mL conical tubes and the exposure time was 30 seconds, skin pieces were immediately transferred to 50 mL conical tubes followed by microbial analysis. For each treatment 5 chicken skin pieces were tested.

**Preparation of Chitosan, Caprylic Acid and their Combinations as Coating Treatments**
For the chitosan coating treatment a 2% solution of medium molecular weight chitosan (wt/vol) was prepared in 50 mM acetic acid as described previously. Caprylic acid coating treatments were prepared by dilution of stock caprylic acid with BPD (vol/vol) to obtain a 1% or 2% solution. The chitosan plus caprylic acid treatments were made by adding equal volumes of 1% or 2% caprylic acid in BPD with the 2% medium molecular weight chitosan solution. For the combination control of BPD and acetic acid, an equal volume of BPD and 50 mM acetic acid was combined. The 50 mM acetic acid and the BPD plus acetic acid controls were adjusted with 10N NaOH as necessary to obtain pH values similar to the chitosan and caprylic acid treatments.

**Coating of Chicken Wingettes**

Whole chicken wings were received from the University of Arkansas Poultry Pilot Processing Plant (Fayetteville, AR) and separated into drumettes, wingettes and wing tips. The wingette portion was only utilized in these trials. Wingettes were stored at -20°C. For each of the replicate trials, frozen chicken wingettes were thawed overnight at 4°C and on the day of the trial were brought to room temperature. Wingettes were divided into groups of 5 per treatment group. To each wingette an inoculum of approximately 50 µl (~1x10^7 CFU/mL) total volume of *Campylobacter* was added drop wise to the surface and allowed to adhere for 30 minutes. To coat the wingettes, each one individually was placed in a sample bag containing 10 mL of the respective treatments. The treatments included 1% and 2% caprylic acid plus its control (BPD), 2% MMW chitosan and 50 mM acetic acid as its control, and finally 1% caprylic acid plus 2% MMW chitosan and 2% caprylic acid plus 2% MMW chitosan with a 1:1 BPD:acetic acid control. Working with one treatment group at a time, wingettes were vigorously shaken for 30 seconds in order to obtain a complete coating. After coating, the wingettes were placed on metal drying racks and allowed to dry for 15 minutes on one side and then turned over to dry for 15 minutes on the opposite side.
To process the day 0 samples microbial analysis was done immediately after a total of 30 minutes drying. For the samples tested at days 1, 3, 5 or 7, wingettes were vacuum sealed by a commercially available sealer (Ziploc V021) and stored at 4°C until the day of sampling.

**Microbial Analysis**

For all experiments, the samples were diluted with sterile BPD dilution blanks and plated using the spread plate technique onto *Campylobacter* Line agar (Line, 2001) followed by incubation at 42°C for 48 hours under microaerophilic atmosphere. For sample testing of the 2 gram chicken skin pieces, 18 mL of BPD was added to the sample bag or 50 mL conical tube and agitated/vortexed for 30 seconds. The chicken wingettes were individually removed from their vacuum packaging and aseptically transferred to a sterile stomacher bag, weighed and an equal wt/vol of BPD was added followed by blending for 30 seconds at 250 rpm (Stomacher 400 Circulator). For all samples ten-fold serial dilutions were prepared from each initial dilution. After 48 hours incubation colonies were enumerated and CFU per milliliter was calculated. The detection limit of the plating assay used in these experiments was 2.0x10¹ CFU/mL.

**Statistical Analysis**

The *C. jejuni* counts were log₁₀ transformed (log₁₀ CFU/mL) for analysis to achieve homogeneity of variance (Byrd et al., 2003). Log transformed data were analyzed using ANOVA with the PROC MIXED procedure in the SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC). Treatment and control means were partitioned by LSMEANS analysis (SAS Institute Inc., 2010) and probability of p < 0.05 was required for statistical significance.
Results

Comparison of the Efficacy to Reduce Campylobacter jejuni on Chicken Skin by Dipping in Chitosan Solutions

The 9 total chitosan treatments (3 molecular weights x 3 concentrations) plus the acetic acid control were evaluated for anti-Campylobacter activity after a 5 minute contact time followed by 18 hours of storage at 4°C. The mean ± SEM log_{10} CFU/mL counts for low molecular weight chitosan at each concentration were: 0.50% (3.72 ± 0.15), 1.0% (3.97 ± 0.14) and 2.0% (3.68 ± 0.20). The low molecular weight chitosan solution failed to reduce Campylobacter counts at 0.5%, 1.0% or 2.0% as compared to the acetic acid control (4.12 ± 0.23). The medium molecular weight chitosan at 0.50% (3.84 ± 0.11) and 1.0% (3.87 ± 0.19) similarly failed to reduce Campylobacter counts, however, the 2.0% medium molecular weight chitosan reduced Campylobacter counts by approximately 1.3 log_{10} (3.09 ± 0.35) when compared to acetic acid alone. High molecular weight chitosan at 0.50% (3.84 ± 0.11), 1.0% (3.65 ± 0.17) or 2.0% (3.68 ± 0.10) was also unable to reduce Campylobacter counts on the skin by 18 hours as compared to the control.

Efficacy of Caprylic Acid to Reduce Campylobacter jejuni on Chicken Skin

Figure 1 shows the effect of caprylic acid at 3 different concentrations to determine its immediate efficacy against Campylobacter jejuni on artificially inoculated chicken skin. The lowest concentration (0.50%) caprylic acid failed to reduce Campylobacter counts as compared to the BPD control during a 30 second exposure time in all three trials. The 1% caprylic acid solution produce variable results with reductions in Campylobacter counts of 1.44 log_{10} in trial 1 and 0.57 log_{10} in trial 3 as compared with the BPD control. However, in trial 2 the 1% caprylic acid failed to reduce Campylobacter counts in comparison with BPD. In contrast to the 0.5% and 1.0%
caprylic acid solutions, the highest concentration of caprylic acid (2.0%) reduced *Campylobacter* counts in all three trials. *Campylobacter* counts were reduced by 1.37 log$_{10}$, 0.63 log$_{10}$ and 0.38 log$_{10}$ in trials 1, 2 and 3 respectively as compared with the controls.

*Treatment of Chicken Wings with Chitosan, Caprylic Acid or their Combination to Reduce Campylobacter*

Further testing was done with the 2% MMW chitosan, 1% caprylic acid and 2% caprylic acid, to investigate their potential individually or in combination, as a coating treatment to reduce *Campylobacter* counts on chicken wings with microbial analysis at days 0, 1, 3, 5 and 7. As shown in Table 1, the 1% and 2% caprylic acid treatments alone were able to reduce *Campylobacter* counts at days 0, 3, 5 and 7 when compared to the BPD control, and by day 7 there was a 2.5 log$_{10}$ reduction in counts from the 1% and 2% treatments (Table 1). Similarly, in trial 2 the 1% and 2% caprylic acid treatments reduced *Campylobacter* counts at days 3, 5 and 7 (Table 2).

In trial 1, the 2% medium molecular weight chitosan coating reduced *Campylobacter* counts on the chicken wings by day 3, 5 and 7, as compared with the acetic acid controls (Table 1). In the second trial the chitosan coating reduced *Campylobacter* counts starting at day 1 and reductions were also determined at day 3 and day 5 (Table 2). In both trials the 2% medium molecular weight chitosan coating reduced the *Campylobacter* counts to at or just above the detection limits of the plating assay. The overall log$_{10}$ reductions were variable, in trial 1 at day 7 the reduction was 2.9 log$_{10}$ and in trial 2 it was a more modest 0.32 log$_{10}$, in comparison with the acetic acid control.

In contrast to the individual treatments, the combination treatments had efficacy against *Campylobacter* at the earliest time point—with reproducible decreases in *Campylobacter* counts
at day 0 and continually through day 7 in trial 1 (Table 1) and day 0 through 5 in the second trial (Table 2). The *Campylobacter* counts were reduced by 2.3 log$_{10}$ for either the 1% or 2% caprylic acid plus chitosan coatings in trial 1 at day 0, and in trial 2 at day 0 they were reduced by 0.91 log$_{10}$ and 0.64 log$_{10}$ respectively, as compared with the BPD:acetic acid control. Overall, for the 1% caprylic acid plus chitosan the mean log$_{10}$ reductions ranged from 1.3 log$_{10}$ to 2.5 log$_{10}$ over all the sampling time points in the first trial and 0.91 log$_{10}$ to 2.5 log$_{10}$ reduction during the second trial. The 2% caprylic acid plus chitosan produced similar results, in trial 1 the range in mean reductions was 1.3 log$_{10}$ through 2.5 log$_{10}$ from day 0 to day 7, and in trial 2 the mean log reductions were between 0.43 log$_{10}$ and 1.7 log$_{10}$.

**Discussion**

In this study chicken skin pieces or wingettes were used as a model for the treatment of a whole carcass to investigate the potential of natural compounds, specifically chitosan and the medium chain fatty acid caprylic acid, to reduce *Campylobacter* on the skin surface. The coating of chicken carcasses and raw poultry products with antimicrobial compounds is a potential treatment strategy that could be added to the food safety interventions currently in use to reduce the presence of pathogens. A wash step post-treatment was not included in the skin studies due to our interest in evaluation of chitosan and caprylic acid as coating treatments which would not be removed after application.

For the evaluation of chitosan, 9 treatment combinations were evaluated to determine a molecular weight and concentration (vol/vol) with the greatest efficacy on the skin itself. Three molecular weights of chitosan (400-600 kDa, 190-310 kDa and 15-50 kDa) were evaluated at three concentrations (0.5%, 1.0% and 2.0%). The antimicrobial activity of chitosan is directly related
to its average molecular weight and studies have shown that the efficacy of chitosan is also dependent upon the particular target microorganism (Zheng and Zhu, 2003; Elsabee and Abdou, 2013). In this study Campylobacter on chicken skin was most sensitive to the 190-310 kDa chitosan when prepared as a 2% solution. However, the low (15-50 kDa) and high (400-600 kDa) chitosan at any concentration tested (0.5%, 1.0% or 2.0%) were not effective at reducing Campylobacter counts on the skin. During in-vitro studies Ganan and colleagues (2009) were able to demonstrate that Campylobacter is highly sensitive to 0.05% solutions of chitosan at molecular weights including 120 kDa, 400 kDa and 643 kDa. It is not entirely unexpected that the antimicrobial activity of the various molecular weight chitosan solutions would be different when tested in different matrices, as Dutta and coworkers have reported this finding (Dutta et al., 2009). The 1.3 log_{10} reduction in Campylobacter counts from the 2% medium molecular weight (190-310 kDa) during a 5 minute application time and 18 hours of storage at 4°C suggest that this combination has the best properties to be used as an antimicrobial coating for chicken carcasses.

In addition to chitosan, caprylic acid was also evaluated for its potential to act as an antimicrobial coating to reduce Campylobacter counts. The medium chain fatty acid caprylic acid was chosen based on its reported broad spectrum antimicrobial activity and its potential acceptability by the food industry and consumers (Sprong et al., 2001; Ganan et al., 2009). Caprylic acid is a natural compound from cow milk, human milk and coconut oil and additionally it has GRAS status from the FDA to be used on foods (Jensen et al., 1990; Sprong et al., 2001; USDA FSIS, 2015). In this trial chicken skin, previously inoculated with Campylobacter was dipped into treatments consisting of 0.5%, 1.0% and 2.0% caprylic acid for 30 seconds followed by immediate microbial evaluation. The brief exposure time of the chicken skin pieces (30 seconds) was chosen as this would more closely reflect the amount of contact time used in
industrial in-line poultry processing (Bauermeister et al., 2008). Among the concentrations tested, the most effective treatment was the highest concentration tested (2%) which produced mean $\log_{10}$ reductions of 1.37 $\log_{10}$, 0.63 $\log_{10}$ and 0.38 $\log_{10}$ over the course of the three trials. Treatment with 1% caprylic acid produced more variable results, with mean reductions of 1.44 $\log_{10}$ and 0.57 $\log_{10}$ in two of the three trials. Chicken skin treated with the 0.5% caprylic acid did not reduce *Campylobacter* counts in any of the three trials. The 2% caprylic acid treatment had immediate efficacy on the chicken skin to reduce *Campylobacter* counts, which shows its potential to be used as a food safe natural coating on chicken carcasses or cut up pieces.

For the final set of trials 2% MMW chitosan and 1% or 2% caprylic acid were selected based upon their demonstrated efficacy in the previous tests for evaluation of their potential to reduce *Campylobacter* counts over an extended period of time. In addition, the selected compounds were also assessed for possible synergistic activity by combination of each concentration of caprylic acid (1% or 2%) with the 2% MMW chitosan. Again, the treatment protocol did not involve rinsing the wingettes after application of the coating treatments as the objective was evaluation of the coating as a final treatment meant to be left on the raw poultry product. Chicken wingettes were chosen as the matrix for these trials as they potentially more closely emulate the various surface properties of the whole carcass as compared to a smaller discrete section of skin from the thigh area. The combination of either concentration of caprylic acid (1% or 2%) with 2% MMW chitosan reduced *Campylobacter* counts on the wingettes more rapidly than either treatment alone—as evidenced by reduced counts at day 0 with the combination treatments but not until day 3 with the individual treatments. This would lead to speculation that there was a possible additive effect when caprylic acid and chitosan are combined into one treatment. In regards to the sustained effect, in trials 1 and 2 the individual caprylic acid treatments
reduced *Campylobacter* counts by day 3 and continually through day 7 to levels below or just above the detection threshold, with overall log$_{10}$ reductions of between 1.67 to 2.53 logs. Interestingly there was no noticeable increased efficacy from the 2% caprylic acid as compared to the 1% caprylic acid. Similar to the caprylic acid, the 2% MMW chitosan coating provided long acting reductions in *Campylobacter* counts from day 3 through 7 in trial 1 and day 1 through 5 in trial 2.

In conclusion, the application of a 2% MMW solution of chitosan or caprylic acid at 1% or 2% as a coating is effective as an antimicrobial coating treatment to reduce *Campylobacter* on the skin surface. For a more immediate reduction in *Campylobacter* on the skin surface a coating made from equal volumes of 2% MMW chitosan and caprylic acid provides reductions immediately upon application and lasts through at least day 5. Further evaluation is needed for these compounds in areas including shelf-life studies and sensory analysis prior to implementation in an industrial setting.
References:


Figure 3.1. Evaluation of differing concentrations of caprylic acid treatment to reduce *Campylobacter jejuni* on chicken skin$^{1,2}$

$^1$*C. jejuni* log$_{10}$ counts (mean ± SEM) on chicken skin pieces inoculated with 50 µl (~$9 \times 10^7$ CFU/mL) of a single strain of *C. jejuni* and exposed to a 30 second dipping treatment in 0.5%, 1.0%, 2.0% caprylic acid or a BPD control followed by serial dilution in BPD and direct plating.

$^2$*C. jejuni* counts within trials with no common superscript differ significantly (p > 0.05).
Table 3.1. The efficacy of caprylic acid, chitosan or their combination to reduce *Campylobacter* counts on chicken wings trial 1\(^{1,2}\)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log(_{10}) CFU/mL</td>
<td>Log(_{10}) CFU/mL</td>
<td>Log(_{10}) CFU/mL</td>
<td>Log(_{10}) CFU/mL</td>
<td>Log(_{10}) CFU/mL</td>
</tr>
<tr>
<td>BPD control</td>
<td>3.95 ± 0.10(^a)</td>
<td>2.18 ± 0.13(^a)</td>
<td>3.12 ± 0.27(^a)</td>
<td>3.48 ± 0.12(^a)</td>
<td>3.83 ± 0.20(^a)</td>
</tr>
<tr>
<td>1% caprylic acid</td>
<td>2.78 ± 0.39(^b)</td>
<td>2.55 ± 0.24(^a)</td>
<td>1.88 ± 0.17(^b)</td>
<td>2.09 ± 0.32(^b)</td>
<td>1.30 ± 0.00(^b)</td>
</tr>
<tr>
<td>2% caprylic acid</td>
<td>3.00 ± 0.33(^b)</td>
<td>2.17 ± 0.55(^a)</td>
<td>1.92 ± 0.30(^b)</td>
<td>1.52 ± 0.22(^b)</td>
<td>1.30 ± 0.00(^b)</td>
</tr>
<tr>
<td>50mM acetic acid control</td>
<td>3.85 ± 0.46(^a)</td>
<td>3.35 ± 0.29(^a)</td>
<td>3.98 ± 0.09(^a)</td>
<td>3.71 ± 0.04(^a)</td>
<td>4.25 ± 0.11(^a)</td>
</tr>
<tr>
<td>2% MMW chitosan</td>
<td>3.30 ± 0.20(^a)</td>
<td>3.46 ± 0.29(^a)</td>
<td>2.38 ± 0.11(^b)</td>
<td>3.01 ± 0.26(^b)</td>
<td>1.30 ± 0.00(^b)</td>
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<tr>
<td>BPD:acetic acid control</td>
<td>4.31 ± 0.29(^a)</td>
<td>3.30 ± 0.33(^a)</td>
<td>3.50 ± 0.18(^a)</td>
<td>4.37 ± 0.07(^a)</td>
<td>3.81 ± 0.25(^a)</td>
</tr>
<tr>
<td>1% caprylic acid + chitosan</td>
<td>1.92 ± 0.08(^b)</td>
<td>1.63 ± 0.33(^b)</td>
<td>2.15 ± 0.29(^b)</td>
<td>2.19 ± 0.16(^b)</td>
<td>1.30 ± 0.00(^b)</td>
</tr>
<tr>
<td>2% caprylic acid + chitosan</td>
<td>2.00 ± 0.13(^b)</td>
<td>1.83 ± 0.15(^b)</td>
<td>1.41 ± 0.24(^b)</td>
<td>1.38 ± 0.05(^c)</td>
<td>1.36 ± 0.06(^b)</td>
</tr>
</tbody>
</table>

\(^{1}\)Individual chicken wings (n=5 per treatment group) were inoculated with 50 µl (~1x10\(^7\) CFU/mL) of *C. jejuni* followed by 30 sec. coating treatments, 30 min. air drying and vacuum packaging. Sampling was performed immediately (day 0) and on days 1, 3, 5 or 7 during which time samples were vacuum sealed and held at 4\(^0\)C. The *Campylobacter jejuni* counts were logarithmically transformed (log\(_{10}\) CFU/mL) before analysis to achieve homogeneity of variance (Byrd et al., 2003).

\(^{2}\)Data presented as mean ± SEM of log\(_{10}\) *C. jejuni* counts.

\(^{a}, \(^{b}, \(^{c}\)}Treatment means within columns and within treatment groups were partitioned by LSMEANS analysis (SAS Institute, 2010) and probability of \(p<0.05\) was required for statistical significance.

Table 3.2. The effects of caprylic acid, chitosan or their combination to reduce *Campylobacter* counts on chicken wings trial 2\(^{1,2}\)
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log$_{10}$ CFU/mL</td>
<td>Log$_{10}$ CFU/mL</td>
<td>Log$_{10}$ CFU/mL</td>
<td>Log$_{10}$ CFU/mL</td>
<td>Log$_{10}$ CFU/mL</td>
</tr>
<tr>
<td>BPD control</td>
<td>3.86 ± 0.20$^a$</td>
<td>4.11 ± 0.19$^a$</td>
<td>4.58 ± 0.12$^a$</td>
<td>4.54 ± 0.12$^a$</td>
<td>3.45 ± 0.06$^a$</td>
</tr>
<tr>
<td>1% caprylic acid</td>
<td>3.83 ± 0.34$^a$</td>
<td>4.21 ± 0.15$^a$</td>
<td>3.96 ± 0.16$^b$</td>
<td>3.31 ± 0.15$^b$</td>
<td>1.77 ± 0.16$^b$</td>
</tr>
<tr>
<td>2% caprylic acid</td>
<td>4.05 ± 0.13$^a$</td>
<td>3.81 ± 0.09$^a$</td>
<td>3.82 ± 0.09$^b$</td>
<td>2.84 ± 0.21$^b$</td>
<td>1.75 ± 0.26$^b$</td>
</tr>
<tr>
<td>50mM acetic acid control</td>
<td>3.92 ± 0.23$^a$</td>
<td>4.56 ± 0.13$^a$</td>
<td>4.53 ± 0.09$^a$</td>
<td>4.61 ± 0.09$^a$</td>
<td>2.25 ± 0.29$^a$</td>
</tr>
<tr>
<td>2% MMW chitosan</td>
<td>4.59 ± 0.29$^a$</td>
<td>3.54 ± 0.20$^b$</td>
<td>2.72 ± 0.21$^b$</td>
<td>2.96 ± 0.19$^b$</td>
<td>1.93 ± 0.20$^a$</td>
</tr>
<tr>
<td>BPD:acetic acid control</td>
<td>4.25 ± 0.20$^a$</td>
<td>4.54 ± 0.07$^a$</td>
<td>4.54 ± 0.07$^a$</td>
<td>4.75 ± 0.09$^a$</td>
<td>2.84 ± 0.45$^a$</td>
</tr>
<tr>
<td>1% caprylic acid +chitosan</td>
<td>3.34 ± 0.22$^b$</td>
<td>3.18 ± 0.10$^b$</td>
<td>3.18 ± 0.10$^b$</td>
<td>3.28 ± 0.25$^b$</td>
<td>2.13 ± 0.32$^b$</td>
</tr>
<tr>
<td>2% caprylic acid +chitosan</td>
<td>3.61 ± 0.10$^b$</td>
<td>2.85 ± 0.36$^b$</td>
<td>2.85 ± 0.36$^b$</td>
<td>3.16 ± 0.22$^b$</td>
<td>2.41 ± 0.36$^a$</td>
</tr>
</tbody>
</table>

1 Individual chicken wings (n=5 per treatment group) were inoculated with 50 µl (~1x10$^7$ CFU/mL) of *C. jejuni* followed by 30 sec. coating treatments, 30 min. air drying and vacuum packaging. Sampling was performed immediately (day 0) and on days 1, 3, 5 or 7 during which time samples were vacuum sealed and held at 4°C. The *Campylobacter jejuni* counts were logarithmically transformed (log$_{10}$ CFU/mL) before analysis to achieve homogeneity of variance (Byrd et al., 2003).

2 Data presented as mean ± SEM of log$_{10}$ *C. jejuni* counts.

a, b, c Treatment means within columns and within treatment groups were partitioned by LSMEANS analysis (SAS Institute, 2010) and probability of p<0.05 was required for statistical significance.
Attn: University of Arkansas Graduate School

August 21, 2015

To Whom it May Concern;

I attest that Ann Woo-Ming was first author of the manuscript cited below and completed at least 51% of the work for the paper.

A. Woo-Ming, K. Arsi, B. R. Wagle, S. Shrestha, P. J. Blore, A. M. Donoghue, K. Venkitanarayanan, D. J. Donoghue. The application of caprylic acid, chitosan or their combination as a coating treatment reduces Campylobacter jejuni on inoculated chicken wingettes.

Regards,

Dan J. Donoghue,
Professor,
Department of Poultry Science
POSC O-114
University of Arkansas
Fayetteville, AR 72701
Phone: (479) 575-2913
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February 9, 2012

MEMORANDUM

TO: Dr. Dan Donoghue

FROM: W. Roy Penney

Institutional BioSaf.

RE: IBC Protocol Approval

IBC Protocol #: 06021

Protocol Title: “Reducing Food Borne Pathogens in Poultry”

Approved Project Period: Start Date: February 14, 2012
Expiration Date: February 13, 2015

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 06021, “Reducing Food Borne Pathogens in Poultry”. You may continue your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.
Chapter 4

The efficacy of application of protective cultures of *Lactobacillus* spp. isolates with or without a chitosan coating to reduce *Campylobacter jejuni* on chicken wingettes
The efficacy of application of protective cultures of Lactobacillus spp. isolates with or without a chitosan coating to reduce Campylobacter jejuni on chicken wingettes


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Key words: Campylobacter jejuni, food safety, protective culture, poultry

1Corresponding author: ddonogh@uark.edu
Abstract

The presence of *Campylobacter* on poultry products remains one of the leading causes for foodborne illness in the U.S. Increased consumer preference for more natural and less processed food products has led to an increased focus on alternative forms of improving food safety. The use of lactic acid bacteria (LAB) as a biopreservative or protective culture in food commodities is an ancient technology that is safe and natural. In this study, 13 *Lactobacillus* spp. isolates were screened by a chicken skin dipping model to evaluate for the potential to reduce *Campylobacter jejuni* counts. From the 13 original isolates four were chosen for further evaluation based upon their ability to inhibit *Campylobacter* counts, *in vitro*. Of the four isolates selected three were *Lactobacillus salivarius* and one was *Lactobacillus hamsteri*. They were evaluated for short term and long term efficacy against *Campylobacter jejuni* in a chicken wingette model. Chicken wingettes were inoculated with *Campylobacter jejuni* and treated with either a *Lactobacillus* broth culture or a BPD control, and *Campylobacter* counts were determined at days 0, 1, 3, 5 and 7. Many isolates were able to reduce *Campylobacter* counts by day 3, however two isolates (4 and 8) produced more consistent reductions when compared to the BPD control.
Introduction

*Campylobacter* is a common contaminant of poultry carcasses and raw retail poultry products (Suzuki and Yamamoto, 2009; USDA FSIS, 2009). Strong correlation exists between foodborne illnesses and consumption of contaminated or mishandled raw/cooked poultry products. (Painter et al., 2013; Bondi et al., 2014). *Campylobacter* contamination of poultry meat is a significant problem because it is consumed as one of the major sources of protein in the United States and indeed globally (AVEC, 2013, 2014; National Chicken Council, 2015; USDA and Foreign Agricultural Service, 2015). The implications of this being, increased consumption will result in a higher frequency of illness even if the contamination rate is low (Painter et al., 2013). This stresses the importance of the need for effective intervention strategies to reduce *Campylobacter* counts on raw poultry.

The challenge to poultry processors arises from increased consumer demand for minimally processed foods while maintaining a microbiologically safe product (Sofos et al., 1998; Davidson et al., 2013). This in turn has led to an increased interest in the use of natural antimicrobials or biopreservatives (Holzapfel et al., 1995; Davidson et al., 2014). An emerging strategy for the improvement of food safety without the use of chemicals is by the addition of lactic acid bacteria (LAB) as a protective culture or biopreservative. This method works by introduction of live organisms onto the food matrix and the subsequent inhibition of growth of microbial pathogens or spoilage organisms, thereby increasing the safety of the food product and extending its shelf-life (Holzapfel et al., 1995; Schillinger et al., 1996; Gaggia et al., 2011). Lactic acid bacteria have a long and safe history in regards to foods. Many beneficial microbes have been used for thousands of years to produce fermented food (Währén, 1990). Additionally they are an abundant and normal inhabitant of the human and animal gut microbiome (Maragkoudakis et al., 2009). It is well
documented that LAB exert significant antimicrobial activity by various mechanisms including secretion of bacteriocins, production of organic acids and hydrogen peroxide (Schillinger et al., 1996; Gálvez et al., 2010; Garcia et al., 2010; Gaggia et al., 2011). However, favorable characteristics of LAB are generally strain specific and therefore screening of each strain is necessary to determine its application (Ljungh and Wadstrom, 2006). Maragkoudakis and colleagues (2009) screened 635 LAB from various food sources to assess for the potential to function as protective cultures on chicken meat. Two LAB strains namely, Enterococcus faecium and Lactobacillus fermentum significantly reduced Listeria monocytogenes and Salmonella Enteritidis when applied on meat. In addition, no negative effects on the meat quality were produced by the probiotic strains (Maragkoudakis et al., 2009).

Advances in packaging technology now include the use of edible films or coatings. The use of the natural compound chitosan is an appropriate choice for use as an edible coating—it has many favorable biological characteristics including broad spectrum antimicrobial and antifungal activity, ability to form gels and films and its ability to readily combine with other substances (Kong et al., 2010; Aider, 2010; Sánchez-González et al., 2011; Elsabee and Abdou, 2013; Azevedo et al., 2014). Prior research performed in our laboratory has demonstrated that a 2% concentration of 190-310 kDa chitosan, when applied as a surface coating, reduces Campylobacter jejuni counts on chicken wingettes (first manuscript in this dissertation). Tapia and colleagues (2007) have investigated increasing the functional ability of edible coatings by incorporation of the probiotic bacterium Bifidobacterium lactis to be applied to fresh cut fruit, in conclusion they found that the culture was able to remain viable at levels greater than 10^6 CFU/g during 10 days of refrigerated storage.
This study investigated the potential of previously characterized LAB isolates in reducing *Campylobacter jejuni* on chicken wingettes either alone or in combination with chitosan.

**Materials and Methods**

*Isolation and Identification of Poultry Associated Lactobacillus spp.*

The LAB strains used in this study were previously isolated and characterized in our laboratory (Aguiar et al., 2013). In brief, ceca from healthy broiler chickens were aseptically removed and the cecal contents transferred to sterile tubes followed by serial dilution with Butterfields Peptone Diluent (BPD). Dilutions were plated on blood agar plates (BAP, Difco Becton Dickinson and Company, MD) and deMan Rogosa and Sharpe Agar (MRS, Difco Becton Dickinson and Company, MD) followed by aerobic incubation at 37°C for 24 hours. Isolated colonies were selected and repeatedly subcultured on fresh BAP to obtain pure cultures. Gram staining was utilized for the initial identification. Final identification was made using the Biolog system (“Biolog Microbial ID System,”) as previously described (Aguiar et al., 2013). Isolates identified as *Lactobacillus* spp. were selected and glycerol stock was made and stored at -80°C (Bielke et al., 2003; Bhaskaran et al., 2011; Aguiar et al., 2013).

*Screening for In-vitro Anti-Campylobacter Activity of Poultry Associated Lactobacillus spp. Isolates*

Each *Lactobacillus* spp. isolate used in this study was previously evaluated for anti-*Campylobacter* activity by use of the soft agar overlay method as described below (Gratia, 1936; Aguiar et al., 2013; Arsi et al., 2015). *Lactobacillus* spp. isolates were initially grown in 5 mL of Tryptic Soy Broth (TSB, BBL Becton Dickinson and Company, MD) and incubated aerobically for 24 hours at 37°C. Concurrently, glycerol stock of *Campylobacter jejuni* was inoculated into 5
mL of *Campylobacter* Enrichment Broth (CEB, Neogen, Lansing, MI) and revived for 48 hours at 42°C under microaerophilic (5% O₂, 10% CO₂, and 85% N₂) conditions. From the 24 hour *Lactobacillus* broth culture 100µl was spot inoculated into the middle of a Tryptic Soy Agar (TSA, BBL Becton Dickinson and Company, MD) plate followed by incubation at 37°C for 24 hours. A 100 µl aliquot of the 48 hour *Campylobacter* culture was transferred to fresh CEB and incubated microaerophilically for 24 hours at 42°C. To produce the overlay, 100 µl of 24 hour *Campylobacter* broth culture was added to 2 mL of pre-melted soft agar (0.65% agar), mixed and gently poured over the surface of the previously spot inoculated TSA plate with a *Lactobacillus* culture in the center. The overlay was briefly allowed to solidify and then the plates were incubated in a microaerophilic atmosphere for 48 hours at 42°C. Overlay plates were visually evaluated for anti-*Campylobacter* activity of *Lactobacillus* cultures by observation of a clear zone of inhibition of *Campylobacter* in the overlay surrounding the *Lactobacillus* colony—the larger the zone the greater the inhibitory activity of the *Lactobacillus* culture. Isolates producing the largest zone were selected for further evaluation.

**Preparation of Campylobacter jejuni Inoculum**

A frozen wild type strain of *Campylobacter jejuni*, previously isolated by our laboratory, was used as the inoculum for this study. One loopful of frozen glycerol stock of the wild-type strain *Campylobacter jejuni* was inoculated into 5 mL of CEB and incubated at 42°C in a microaerophilic atmosphere for 48 hours. The *Campylobacter* inoculum for each trial was prepared as described by Akins and colleagues (2009). Briefly, an aliquot of *Campylobacter* in CEB was transferred to a biphasic culture condition consisting of a Mueller Hinton agar (Difco, Sparks, MD) coated cell culture flask with an overlay of Mueller Hinton broth (Difco, Sparks, MD) and incubated at 42°C for 18 hours in a microaerophilic atmosphere. At each inoculation
time point, an aliquot of biphasic grown *Campylobacter* was centrifuged at 3000 rpm for 12 minutes, the supernatant discarded and the cell pellet resuspended in 2x the volume of the original aliquot in Butterfield’s Phosphate Diluent (BPD, Difco Becton Dickinson, MD).

**Preparation of Lactobacillus spp. Cultures as Experimental Treatments**

From the glycerol stock of the pure *Lactobacillus* isolates a loopful was transferred to 5 mL of MRS broth and incubated for 24 hours at 37°C. One day before the experiment 100 µl of the 24 hour culture was inoculated into a larger volume of fresh MRS broth and incubated at 37°C for 24 hours, after which time 20 mL was dispensed into 50 mL conical tubes (for the skin experiment) or 10 mL was transferred to sterile sample bags (for the wingettes) to be used as a dipping or coating treatment.

**Evaluation of Antimicrobial Activity of Poultry Associated Lactobacillus spp. on Chicken Skin**

Chicken thigh skin was obtained from commercially available bone-in skin-on chicken thighs. The skin was removed from the muscle tissue and cut into 2g pieces which were then stored at -20°C until needed. On the day of the experiment the skin pieces were allowed to reach room temperature and then attached to clips which were equidistantly mounted on a rod. While attached to the clips, the skin pieces were laid flat on a piece of aluminum foil and 50 µl (~1x10⁷ CFU/mL) total volume of the *Campylobacter* inoculum was added dropwise across the surface each piece of skin. The skin samples were kept flat for 30 minutes at room temperature to allow time for *Campylobacter* attachment to the skin. For each treatment group the rod with the clips and skin attached was raised, allowing all skin samples to hang freely without contact to each other and then lowered over a rack holding 50 mL conical tubes with 20 mL of *Lactobacillus* broth culture in each tube. This allowed the skin samples to be dipped into each treatment tube.
simultaneously. For each treatment group (and BPD control) the skin pieces had a contact time of 5 minutes then removal from the treatment and 2 minutes of drip drying to allow excess treatment to be removed from the skin and then directly to microbial analysis. There were 5 chicken skin samples per treatment.

**Preparation and Inoculation of Chick Wingettes with Campylobacter jejuni**

Whole chicken wings were received from the University of Arkansas Poultry Pilot Processing Plant (Fayetteville, AR) and separated into drumettes, wingettes and wing tips. The wingette portion was only utilized in these trials. Wingettes were stored at -20°C. For each of the replicate trials frozen chicken wingettes were thawed overnight at 4°C and on the day of the trial were brought to room temperature. To each wingette an inoculum of 50 µl (~approximately 1x10^7 CFU/mL) of *Campylobacter jejuni* was added drop-wise to the surface and allowed to adhere for 30 minutes at room temperature.

**Preparation of the Medium Molecular Weight Chitosan Coating Treatment**

Powdered chitosan of 190-310kDa (75%-85% deacetylated) was purchased from Sigma (Sigma-Aldrich, Co., St. Louis, MO). The chitosan solution was prepared according to a previously published method (Upadhyay et al., 2014). A 50mM solution of acetic acid was prepared with glacial acetic acid (BDH Aristar, West Chester, PA) in deionized water (vol/vol). Medium molecular weight chitosan powder was solubilized in 50mM acetic acid to make a 2.0% (wt/vol) solution, the pH was adjusted as necessary with HCl (Fisher Scientific, Pittsburgh, PA) to obtain solubilization and stirred overnight at room temperature to ensure complete incorporation of the chitosan powder. The 50mM acetic acid control was pH adjusted as necessary with 10N NaOH to be similar to the chitosan treatment.
Preparation of the Combination Treatment of 2% MMW Chitosan and Lactobacillus spp. Broth Cultures as Coating Treatments

The chitosan plus *Lactobacillus* spp. treatments were produced by adding equal volumes of each *Lactobacillus* 24 hour broth culture with the 2% medium molecular weight chitosan solution. For the combination control made up of BPD and acetic acid, an equal volume of BPD and 50mM acetic acid was combined. The 50mM acetic acid and the BPD plus acetic acid controls were adjusted with 10N NaOH as necessary to obtain pH values similar to the chitosan and *Lactobacillus* treatments.

Application of Coating Treatments to Chicken Wingettes

Wingettes were divided into treatments groups, 5 wingettes per treatment group. Coating of the chicken wingettes was based on the protocol by Olaimat and colleagues (2014) with minor adjustments. Each wingette was individually aseptically transferred to a sample bag containing 10 mL of treatment or control solution. Working with one treatment group at a time, the sample bags containing the treatment and wingette were vigorously shaken for 30 seconds in order to obtain a complete coating. After coating, the wingettes were placed on metal drying racks and allowed to dry for 15 minutes on one side and then turned over to dry for 15 minutes on the opposite side. To process the day 0 samples microbial analysis was performed immediately after a total of 30 minutes drying. For the samples tested at days 1, 3, 5 or 7, wingettes were vacuum sealed by a commercially available sealer (Ziploc V021) and stored at 4°C until the day of sampling.

For the first set of coating experiments the wingettes were coated with *Lactobacillus* spp. broth cultures or a BPD control. From these replicate trials two *Lactobacillus* spp. isolates that produced the greatest reduction in *Campylobacter* counts were chosen for further evaluation. In
the final set of experiments the *Lactobacillus* cultures alone or in combination with 2% MMW chitosan were applied as a coating treatment to wingettes. The treatments included isolate 1 and 2 individually and a BPD control, a 2% MMW chitosan treatment alone and its 50 mM acetic acid control and lastly the combination of *Lactobacillus* isolate 1 plus 2% MMW chitosan, *Lactobacillus* isolate 2 combined with 2% MMW chitosan and a control treatment of BPD and 50 mM acetic acid combined.

**Microbial Analysis**

For all experiments, the samples were diluted with sterile BPD dilution blanks and plated using the spread plate technique onto *Campylobacter* Line agar (Line, 2001) followed by incubation at 42°C for 48 hours under microaerophilic atmosphere. For sample testing of the 2 gram chicken skin pieces, 18 mL of BPD was added to the 50 mL conical tube and agitated/vortexed for 30 seconds. The chicken wingettes were individually removed from their vacuum packaging and aseptically transferred to a sterile stomacher bag, weighed and an equal wt/vol of BPD was added followed by blending for 30 seconds at 250 rpm (Stomacher 400 Circulator). For all samples ten-fold serial dilutions were prepared from each initial dilution. After 48 hours incubation colonies were enumerated and CFU per milliliter was calculated. The detection limit of the plating assay used in these experiments was 2.0x10¹ CFU/mL.

**Statistical analysis**

The *C. jejuni* counts were log₁₀ transformed (log₁₀ CFU/mL) for analysis to achieve homogeneity of variance (Byrd et al., 2003). Log transformed data was analyzed using ANOVA with the PROC MIXED procedure in the SAS statistical software, version 9.3 (SAS Institute Inc.,
Cary, NC). Treatment and control means were partitioned by LSMEANS analysis (SAS Institute Inc., 2010) and probability of p < 0.05 was required for statistical significance.

**Results**

*Evaluation of Lactobacillus spp. Isolates to Reduce Campylobacter Counts on Chicken Skin*

For this study a total of 13 *Lactobacillus* spp. strains previous isolated, identified and tested for *in-vitro* anti-*Campylobacter* activity (Arsi et al., 2015) by our laboratory and were chosen for further evaluation as a dipping treatment to reduce *Campylobacter* numbers on artificially inoculated chicken skin pieces.

A total of thirteen isolates were tested in two separate trials for immediate efficacy to reduce *Campylobacter jejuni* counts on chicken skin during a 5 minute exposure to the broth culture. In the first trial, out of the 5 *Lactobacillus* spp. isolates tested (isolates 1-5), isolate 1 failed to reduce *Campylobacter* counts by this testing method, but isolates 2-5 reduced *Campylobacter* counts by 1.31, 1.21, 1.21 and 1.22 log_{10} CFU/mL respectively when compared to the BPD control (Figure 1). In trial 2, an additional 8 *Lactobacillus* spp. isolates were evaluated and their results are shown in Figure 2. Isolates 7, 9, 11, 12 and 13 did not produce reductions when compared to dipping in BPD. *Lactobacillus* isolate 6 had a modest reduction of 0.91 log_{10} CFU/mL and isolates 8 and 10 had the greatest reductions of 1.37 and 1.24 log_{10} CFU/mL as compared to the control. From these two trials, 4 *Lactobacillus* isolates (3, 4, 8 and 10) were chosen to investigate their potential efficacy at long term reductions in *Campylobacter* counts using a chicken wingette model. Three of the isolates selected (3, 8 and 10) were identified as *Lactobacillus salivarius* and the fourth isolate (4) was *Lactobacillus hamsteri*. 

120
Determination of Potential Long Term Anti-Campylobacter Activity by Lactobacillus spp.

Isolates

Results from the microbial analysis of the treated wingettes are shown in Table 1 and 2. All Lactobacillus cultures had variable results on reducing Campylobacter counts on day 0 or 1. Isolate 3 was able to reduce Campylobacter counts in both trial 1 and 2 at sampling time points of day 3, 5 and 7 as compared to wingettes coated by BPD. Isolate 10 also had variable results on day 0 in trials 1 and 2 and additionally did not reduce Campylobacter counts in either trial at day 1. At days 3 and 5, isolate 10 reduced Campylobacter counts on the wingettes by around 1.5 log$_{10}$ CFU/mL in both trials 1 and 2. Campylobacter counts continued to be reduced at day 7 in both trials, with a 0.71 log$_{10}$ CFU/mL reduction in trial 1 and 1.21 log$_{10}$ CFU/mL in trial 2. Isolates 4 and 8 consistently reduced Campylobacter counts starting at day 1 and at each sampling point through day 7, in both trials. Isolate 4 produced the greatest reductions at days 3 and 5—at day 3 the reductions were 2.48 and 1.62 log$_{10}$ CFU/mL in trial 1 and 2 respectively and at day 5 Campylobacter counts were reduced by 1.5 and 1.86 log$_{10}$ CFU/mL as compared to the control. Similarly, isolate 8 had the greatest efficacy at days 3 and 5; the reductions at day 3 were 1.66 and 1.07 log$_{10}$ CFU/mL and at day 5 log$_{10}$ CFU/mL reductions were 1.82 and 2.22.

Treatment of Chicken Wingettes with Lactobacillus spp. Isolates, Chitosan or their Combination to Reduce Campylobacter

Of the 4 isolates tested for long term efficacy against Campylobacter, 2 were selected Lactobacillus hamsteri (4) and Lactobacillus salivarius (8), for additional testing aimed at assessing potential synergistic activity between the Lactobacillus isolates and their combination with a 2% MMW chitosan solution. Listed in Table 3 are the Campylobacter counts for each
treatment and controls from replicate trials. *Lactobacillus* isolate 4, when applied singly, decreased the counts at the initial time point (day 0) and continually through the final time point (day 7) in both trials as compared to the BPD treatment. The combination of isolate 4 plus 2% MMW chitosan similarly produced reductions at day 0 and consistently through each sampling point up to day 7 in both of the trials as compared to its BPD:acetic acid control. Isolate 8 was unable to consistently decrease the *Campylobacter* counts at day 0 but counts at days 1, 3, 5 and 7 were less than the control. Likewise, isolate 8 combined with 2% MMW chitosan did not produce consistent reductions at day 0 but counts were decrease at each subsequent sampling point through day 7. The 2% MMW chitosan applied alone was not able to reproducibly lower the *Campylobacter* counts at the first sampling time but each time point thereafter did have counts lower than the control treatment.

**Discussion**

*Campylobacter* continues to be one of the top two bacterial foodborne pathogens which causes illness in the U. S. each year (Crim et al., 2015). Despite many interventions utilized within the poultry processing facility, *Campylobacter* remains a persistent contaminant on raw poultry products (Stern et al., 2001). Poultry producers face a two-fold challenge with the desire to make product with an extended shelf-life yet the consumer is concerned about eating a product with minimal processing and chemical treatment—all while maintaining a microbiologically safe product (Sofos et al., 1998; David et al., 2013; Sakaridis et al., 2014). A potential strategy for the reduction of foodborne pathogens is by the treatment of raw poultry products with protective cultures. In this study we investigated the efficacy of poultry derived *Lactobacillus* isolates with demonstrated in-vitro anti-*Campylobacter* activity for use as a protective culture in a chicken wingette model.
In the first two trials a chicken skin dipping model was utilized to screen 13 *Lactobacillus* isolates with previously demonstrated anti-*Campylobacter* activity. The results of these trials indicated that select isolates are capable of producing up to a 1.3 log CFU/mL reduction in *Campylobacter* counts after a 5 minute exposure to the broth culture. Differences in efficacy between testing matrices are expected and there are many examples in the literature of lactic acid bacteria isolates with antimicrobial activity in a laboratory setting but upon testing in a more complex system only a small number of isolates have repeated efficacy (Aguiar et al., 2013; Arsi et al., 2015; Bratz et al., 2015). From this screening assay 4 isolates which produced greater than a 1 log reduction in *Campylobacter* counts were chosen for further evaluation in a chicken wingette model.

The chicken wingette model was used to more closely resemble the heterogeneous nature of the skin on a chicken carcass and in addition, the treatment exposure time of 30 seconds more closely approximates a realistic exposure time in terms of integrating the coating treatment with intervention strategies already in place within the processing plant (Bauermeister et al., 2008; Davidson et al., 2014). It should be noted that the protocol for treatment of the wingettes did not include a rinse step after exposure to the treatments, as coating of the wingette surface with the treatment is meant to be consumed with the product. To date there is very little literature on the use of protective cultures to enhance the shelf-life and food safety of raw poultry products, and specifically for *Campylobacter jejuni*. Sakaridis and colleagues (2014) evaluated lactic acid bacteria (LAB) isolated from poultry carcasses as a protective culture to reduce the growth of *Salmonella* spp. and *Listeria monocytogenes* on chicken skin and meat. They selected a single *Lactobacillus salivarius* culture for testing and evaluated the skin and meat from days 1 through 7. They did not find significant reductions in the growth of *Salmonella* or *Listeria* until day 6, and
the reductions at that time point were between $0.51 \log_{10} \text{CFU/cm}^2$ and $0.71 \log_{10} \text{CFU/cm}^2$. Melero and colleagues (2012, 2013) have evaluated the impact of protective cultures to reduce *C. jejuni* and *L. monocytogenes* in chicken products. When a protective culture of *Bifidobacterium longum* was used to treat chicken legs artificially inoculated with *C. jejuni* and packaged under modified atmosphere they observed a reduction in *C. jejuni* counts (1.09 log CFU/g) between days 6 – 9 of the study (Melero et al., 2013). In contrast, during the testing of *Lactobacillus* isolates 3, 4, 8 and 10 all of them produced reductions in *Campylobacter* counts by day 3 and at day 7 the range of mean $\log_{10}$ reductions was $0.63 \log_{10} \text{CFU/mL} – 1.65 \log_{10} \text{CFU/mL}$ in trial 1 and $0.74 \log_{10} \text{CFU/mL} – 1.21 \log_{10} \text{CFU/mL}$ in trial 2. The testing of these 4 *Lactobacillus* isolates has demonstrated the effectiveness of using specific lactic acid bacterial cultures as a protective culture to reduce the foodborne pathogen *C. jejuni*.

In an attempt to improve upon the efficacy of the *Lactobacillus* isolates when applied as a protective culture, *Lactobacillus hamsteri* (4) and *Lactobacillus salivarius* (8) were selected to be combined with a 2% chitosan solution and applied as a coating on the wingettes. In earlier work done by our laboratory (first manuscript in this dissertation) it was shown that a 2% solution of medium molecular chitosan (190-310 kDa) was effective at prolonged reductions in *Campylobacter* counts on chicken wingettes. Additionally, chitosan is an advantageous choice as a surface coating due to its favorable characteristics including gel and film forming capabilities, ability to be combined with other compounds and Generally Regarded as Safe (GRAS) status (Kong et al., 2010; Aider, 2010; Sánchez-González et al., 2011; Elsabee and Abdou, 2013). From these trials we can observe that both *Lactobacillus salivarius* (8) and *Lactobacillus hamsteri* (4) when applied singly as a protective culture, reproducibly lower the *Campylobacter* levels ($\log_{10}$ CFU/mL) continuously through day 7 (Tables 1, 2 and 3).
In conclusion, from this study we have identified 2 isolates, *Lactobacillus salivarius* and *Lactobacillus hamsteri*, which consistently reduced the number of surviving *Campylobacter* on wingettes and show potential to be used as a protective culture on raw poultry meat to supplement the interventions already in use. Additional testing of these isolates for assessment of their impact on sensory characteristics of the poultry products, effects on food spoilage causing bacteria and in combination with modified atmosphere packaging for shelf-life extension are the logical next steps.
References:


Biolog Microbial ID System, Biolog Hayward, CA.


Table 4.1. The efficacy of selected *Lactobacillus* spp. isolates to reduce *C. jejuni* counts on chicken wingettes trial 1.2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPD</td>
<td>4.00 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.68 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.53 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.65 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.13 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>3.75 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.51 ± 0.23&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.32 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.07 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.56 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>3.93 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.22 ± 0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.05 ± 0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.15 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.81 ± 0.24&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>3.92 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.82 ± 0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.87 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.83 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.88 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>3.82 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.10 ± 0.34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.94 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.14 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.42 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1 Individual chicken wingettes (n=5) were inoculated with 50 µl (~1x10<sup>7</sup> CFU/mL) of *C. jejuni* followed by 30 sec. coating treatments, 30 min. air drying and vacuum packaging. Sampling was performed immediately (day 0) and on days 1, 3, 5 or 7 during which time samples were vacuum sealed and held at 4°C. The *Campylobacter jejuni* counts were logarithmically transformed (log<sub>10</sub> CFU/mL) before analysis to achieve homogeneity of variance (Byrd et al., 2003).

2 Mean ± SEM log<sub>10</sub> *C. jejuni* counts.

<sup>a, b, c</sup> Treatment means within columns and within treatment groups were partitioned by LSMEANS analysis (SAS Institute, 2010) and probability of p<0.05 was required for statistical significance.
Table 4.2. The efficacy of selected *Lactobacillus* spp. isolates to reduce *C. jejuni* counts on chicken wingettes trial 2<sup>1,2</sup>

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPD</td>
<td>4.36 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.35 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.17 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.38 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.38 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>3.47 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.73 ± 0.30&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.67 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.22 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.48 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>3.19 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.77 ± 0.35&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.55 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.52 ± 0.25&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.64 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>3.38 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.47 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.10 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.16 ± 0.29&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.40 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>3.15 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.06 ± 0.37&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.62 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.79 ± 0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.17 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Individual chicken wingettes (n=5) were inoculated with 50 µl (~1x10<sup>7</sup> CFU/mL) of *C. jejuni* followed by 30 sec. coating treatments, 30 min. air drying and vacuum packaging. Sampling was performed immediately (day 0) and on days 1, 3, 5 or 7 during which time samples were vacuum sealed and held at 4<sup>0</sup>C. The *Campylobacter jejuni* counts were logarithmically transformed (log<sub>10</sub> CFU/mL) before analysis to achieve homogeneity of variance (Byrd et al., 2003).

<sup>2</sup>Mean ± SEM of log<sub>10</sub> *C. jejuni* counts.

<sup>a, b, c</sup>Treatment means within columns and within treatment groups were partitioned by LSMEANS analysis (SAS Institute, 2010) and probability of p<0.05 was required for statistical significance.
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>BPD</td>
<td>4.43 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.87 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.59 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.50 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.42 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.45 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>3.23 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.50 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.84 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.24 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.34 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.59 ± 0.24&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>3.64 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.55 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.51 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.26 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.36 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.63 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AA&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.39 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.67 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.59 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.38 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.48 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.10 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2% chitosan</td>
<td>4.26 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.02 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.71 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.78 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.95 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.74 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B:A&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.25 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.22 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.54 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.78 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.69 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.47 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4ch&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.88 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.87 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.00 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.77 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.71 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.10 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8ch&lt;sup&gt;**&lt;/sup&gt;</td>
<td>4.06 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.41 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.92 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.95 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.62 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.82 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1 Individual chicken wingettes (n=5) were inoculated with 50 µl (~1x10<sup>7</sup> CFU/mL) of <i>C. jejuni</i> followed by 30 sec. coating treatments, 30 min. air drying and vacuum packaging. Sampling was performed immediately (day 0) and on days 1, 3, 5 or 7 during which time samples were vacuum sealed and held at 4°C. The <i>Campylobacter jejuni</i> counts were logarithmically transformed (log<sub>10</sub> CFU/mL) before analysis to achieve homogeneity of variance (Byrd et al., 2003).

2 Mean ± SEM of log<sub>10</sub> <i>C. jejuni</i> counts.

<sup>a</sup>, <sup>b</sup>, <sup>c</sup> Treatment means within columns and within treatment groups were partitioned by LSMEANS analysis (SAS Institute, 2010) and probability of p<0.05 was required for statistical significance.

<sup>*</sup>AA= 50mM acetic acid control; B:A = BPD:acetic acid control,

<sup>**</sup>4ch = isolate 4 plus 2% MMW chitosan; 8ch = isolate 8 plus 2% MMW chitosan
Figure 4.1. Evaluation of *Lactobacillus* spp. isolates to reduce *Campylobacter jejuni* counts on chicken skin pieces Trial 1\(^1,2\)

\(^1\) *jejuni* log\(_{10}\) counts (mean ± SEM) on chicken skin pieces (n=5) inoculated with approximately 50 µl (~1 x 10\(^7\) CFU/mL) of a single strain of *C. jejuni* and exposed to a *Lactobacillus* spp. broth culture or BPD control for 5 minutes followed by 2 minutes of drip drying and microbial analysis by direct plating.

\(^{a,b,c}\) Treatments with no common superscript differ significantly (p > 0.05).

*Isolates chosen for further evaluation*
Figure 4.2 Evaluation of *Lactobacillus* spp. isolates to reduce *Campylobacter jejuni* counts on chicken skin pieces Trial 2 \(^1,2\)

\(^1\) *C. jejuni* log\(10\) counts (mean ± SEM) on chicken skin pieces (n=5) inoculated with approximately 50 µl (~1 x 10\(^7\) CFU/mL) of a single strain of *C. jejuni* and exposed to a *Lactobacillus* spp. broth culture or BPD control for 5 minutes followed by 2 minutes of drip drying and microbial analysis by direct plating.

\(^{a,b,c}\) Treatments with no common superscript differ significantly (p > 0.05).

*Isolates chosen for further evaluation*
Attn: University of Arkansas Graduate School

August 21, 2015

To Whom it May Concern;

I attest that Ann Woo-Ming was first author of the manuscript cited below and completed at least 51% of the work for the paper.

A. Woo-Ming, K. Arsi, B. R. Wagle, S. Shrestha, P. J. Blore, A. M. Donoghue, K. Venkatanarayanan, D. J. Donoghue. The efficacy of application of protective cultures of Lactobacillus spp. isolates with or without a chitosan coating to reduce Campylobacter jejuni on chicken wingettes.

Regards,

Dan J. Donoghue,
Professor,
Department of Poultry Science
POSC O-114
University of Arkansas
Fayetteville, AR 72701
Phone: (479) 575-2913
Email: ddonogh@uark.edu
February 9, 2012

MEMORANDUM

TO: Dr. Dan Donoghue

FROM: W. Roy Penney
       Institutional Biosafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 06021

Protocol Title: "Reducing Food Borne Pathogens in Poultry"

Approved Project Period: Start Date: February 14, 2012
                        Expiration Date: February 13, 2015

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol
06021, "Reducing Food Borne Pathogens in Poultry". You
may continue your study.

If further modifications are made to the protocol during the study, please submit a
written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and
Federal guidelines for research involving hazardous biological materials.
Conclusions

In the United States, foodborne illness from ingestion of *Campylobacter* is under increased scrutiny—it is one of the leading causes of bacterial food poisoning as well as a direct target of the Healthy People 2020 federal program which aims to reduce the incidence by 33% in the year 2020. Several factors make the reduction of *Campylobacter* illness a significant challenge; infection with *Campylobacter* is closely associated with mishandling or consumption of contaminated poultry products meanwhile poultry meat is one of the mains sources of protein in the U. S. and some estimates report that up to 90% of raw retail poultry meat is contaminated with this bacterium. *Campylobacter* is frequently a normal part of the gut microbiota of poultry and to date there are no consistent effective treatments to eliminate it from this source. This emphasizes the need to implement post-harvest strategies for ensuring the microbial safety of poultry products.

In this project we evaluated the efficacy of natural compounds and Lactic Acid Bacteria (LAB) to reduce *Campylobacter* when applied as a coating treatment and evaluated at days 0, 1, 3, 5 and 7. As an edible antimicrobial coating, a 2% medium molecular weight (190-310 kDa) chitosan solution was effective at sustained (up to day 7) reductions of *Campylobacter* on surface contaminated chicken wingettes. Also evaluated was caprylic acid, a medium chain fatty acid with demonstrated broad spectrum antimicrobial activity. We found that both 1% and 2% concentrations of caprylic acid, when applied as an edible coating, was effective at reducing *Campylobacter* from day 3 through 7. When either 1% or 2% caprylic acid was combined with 2% medium molecular weight chitosan and applied as a coating the efficacy was increased and reductions in *Campylobacter* were observed from day 0 through day 7.

Also evaluated in this study was the method of applying *Lactobacillus* spp. isolates onto chicken wingettes for reducing *Campylobacter*. After screening 13 isolates of *Lactobacillus* spp.
on chicken skin pieces, four specific isolates were chosen for further evaluation in a chicken wingette model. Each of these 4 *Lactobacillus* isolates reduced *Campylobacter* on the wingettes from day 3 through 7. Two of the four isolates evaluated (L. salivarius and L. hamsteri) produced the most consistent reductions and were therefore chosen to be evaluate when combined with 2% medium molecular weight chitosan. When each *Lactobacillus* isolate was combined individually with a 2% medium molecular weight chitosan solution and applied as a coating to wingettes reductions continued to be observed, however there was no increase in efficacy.

The natural compounds and LAB evaluated in this study show promising potential as treatments to reduce the incidence of *Campylobacter* contamination on poultry products. Follow-up testing of these compounds for their effect on the sensory qualities of the poultry products on which they are applied is a natural progression of this study.