Optimization of Enrichment Protocol for *Campylobacter* Detection

by

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Abstract

Poultry meat represents the primary source of *Campylobacter*-associated foodborne illness. The current methods set forth by USDA-FSIS for qualitative detection of *Campylobacter* from poultry involve selective enrichment to enhance recovery of cells within a sample and can take days to accomplish complete analysis. A great deal of time and expense associated with these methods is accumulated in order to detect *Campylobacter*, especially for companies testing a high volume of samples. Therefore, there is a demand for more effective and economical methods of analysis that can satisfy challenging criteria. The current research was conducted in order to optimize selective enrichment of *Campylobacter* and reduce the time and cost associated with detection.

The objectives of this study were to optimize selective enrichment of *Campylobacter* by evaluating growth of *Campylobacter jejuni* throughout 48 h in various modifications to enrichment media as well as to compare detection of *Campylobacter* from poultry rinse samples after enrichment in optimized media to the current USDA enrichment method. When growth comparisons in a novel inhibitory broth, Brucella-FBP and blood-free Bolton's enrichment broth supplemented with Bolton's selective supplement were made and incubated in a microaerophilic atmosphere, growth of *C. jejuni* in Brucella-FBP was better ($P \leq 0.05$) at 12, 24, and 48 h.

Therefore, modified Brucella broth (Brucella-FBP incorporated with cefoperazone and amphotericin B) was developed and optimized for *Campylobacter*

enrichment. Evaluation of modified Brucella broth demonstrated better growth ($P \leq 0.05$) of *C. jejuni* at both 12 h and 48 h than Bolton's enrichment broth base incorporated with the same inhibitors. Furthermore, enrichment of poultry rinse samples in modified Brucella for 12 h showed better detection ($P \leq 0.05$) of natural levels of *Campylobacter* on carcasses than enrichment in blood-free Bolton's enrichment broth.

Additionally, cost analysis of one liter of prepared medium shows that the expense associated with the media evaluated for selective enrichment of *Campylobacter* can be greatly reduced without impacting recovery of cells by enriching samples in modified Brucella rather than blood-free Bolton's broth. The results from this study will provide industry with improved methods for enrichment and detection of *Campylobacter* while also reducing the cost associated with analysis.

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CHAPTER I.

INTRODUCTION

Campylobacter is a foodborne bacterial pathogen that has been estimated to cause illness in approximately 0.8 million people each year in the United States (Scallan et al., 2011) and sometimes results in serious long-term complications from acute infection. *Campylobacter* spp. are primarily associated with ready-to-cook poultry meat and in terms of annual disease burden, *Campylobacter* in poultry is ranked first in the top 10 pathogen-food combinations. Responsible for an estimated $2.4 billion in annual disease burden, contaminated poultry meat has the most significant public health impact among food sources. *Campylobacter* is responsible for 72% of the illnesses attributed to poultry and 12% of the total cost of illness (Batz et al., 2011).

The overall annual production of chicken in the United States is nearly 9 billion, and it is the top consumed protein source in Americans' diets (NCC, 2012). Therefore, it is crucial that the poultry industry as a whole takes measures to prevent foodborne illness and produce safe and wholesome products for consumers. To aid in the prevention of *Campylobacter*-associated foodborne illness, the USDA requires poultry sampled at processing facilities to be below 10.4% positive to meet the current performance standards established for *Campylobacter* (USDA-FSIS, 2011).

Although the current *Campylobacter* sampling protocol required by USDA is a quantitative method for enumeration of *Campylobacter*, qualitative methodology which

requires enrichment of samples is used for internal analysis and to detect low levels of contamination. Isolation of *Campylobacter* from foods can be difficult since low populations of these bacteria may be present along with high levels of competing organisms (Baylis et al., 2000). Therefore, selective enrichment of target organisms is often required for detection and has been shown to increase sensitivity for recovery of *Campylobacter* spp. compared to direct plating (Park et al., 1983). Before rapid detection methods or qualitative analysis can be performed, *Campylobacter* samples must be enriched for approximately 48 h, followed by direct plating on selective growth media and incubation for 48 h. Therefore, the current time required for detection of *Campylobacter* is 96 h before additional confirmation of colonies through phase contrast microscopy and latex agglutination.

Furthermore, the conditions for culturing *Campylobacter* are exacting. *Campylobacter* does not ferment carbohydrates and requires incubation in a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) for active growth of bacterial populations. In addition to essential growth factors, most media include addition of ingredients that protect the bacteria from the toxic effects of substances that form in the presence of oxygen and light (Bolton et al., 1984). Some of these include: blood, haemin, charcoal, ferrous sulfate, sodium pyruvate, and sodium metabisulfite (Corry et al., 1995). Media used to culture *Campylobacter* must likewise contain various antibiotics to inhibit the overgrowth of background microflora in or on the media. Typically, antibiotic supplements are the most costly components of the media.

The materials required to generate a microaerophilic atmosphere for *Campylobacter* sampling are likewise very expensive. Previous research has indicated

that *Campylobacter* spp. can grow aerobically in media supplemented with a combination of organic acids. However, selective enrichment to obtain higher *Campylobacter* populations requires 72 h (Hinton, Jr., 2012). While eliminating the need for a modified atmosphere for growth and recovery of *Campylobacter* from food samples would be monumental, continued research on methods to attain detectable levels promptly and reliably is needed.

The amount of time necessary to promote growth of *Campylobacter* and therefore, the time required for selective enrichment requires days to achieve complete analysis. These qualitative methods are time-consuming and costly due to the requirement for selective supplements. Thus, there is a demand for effective and more economical methods of analysis that can satisfy challenging criteria. By optimizing physical methods such as the enrichment of samples, the detection of *Campylobacter* may be enhanced and accomplished more rapidly than with other media currently used.

Therefore, the current research was conducted in order to optimize selective enrichment of *Campylobacter* and reduce the time and cost associated with qualitative analysis. Results of this study indicate that modified Brucella broth (Brucella-FBP incorporated with cefoperazone and amphotericin B antibiotics) demonstrated better performance ($P \leq 0.05$) on the growth rate of *Campylobacter jejuni* populations throughout 48 h of incubation in a microaerophilic atmosphere when compared to other media evaluated. Enrichment of samples in modified Brucella broth rather than blood-free Bolton's enrichment broth also resulted in improved recovery ($P \leq 0.05$) of *Campylobacter* from chicken carcasses within 12 h of enrichment. Additionally, it is shown that by reducing the amount of selective agents incorporated into the optimized media, the cost

required for enrichment can be greatly reduced without impacting recovery of C*ampylobacter* from samples.

CHAPTER II.

LITERATURE REVIEW

Campylobacter is a bacterial pathogen that is a major cause of foodborne enteric illness. It is estimated that *Campylobacter* results in 845,024 illnesses, 8,463 hospitalizations, and 76 deaths each year in the U. S. (Scallan et al., 2011). Moreover, the total basic cost of illness associated with *Campylobacter* in the U.S. was reported as $1.56 million (Sharff, 2011), making infection with this foodborne pathogen a serious economic burden in addition to the impacts of illness and loss of life.

Campylobacter infection has been linked to food sources such as raw milk, pork, beef, lamb, seafood, and produce, although the primary source of *Campylobacter*-associated foodborne illness is due to poultry meat. Contaminated poultry meat represents the greatest public health impact among foods and is responsible for an estimated $2.4 billion in annual disease burden. Out of the illnesses attributed to poultry, *Campylobacter* is responsible for 72% and contributes 12% of the total cost of illness (Batz et al., 2011). Furthermore, a study conducted by Consumer Reports (2010), estimated that 62% of broiler chickens at retail were contaminated with *Campylobacter*. Due to a low infective dose and high pathogenicity of *Campylobacter*, poultry products may pose a serious health concern for consumers if mishandled or not cooked thoroughly.

Although poultry has been cited as a major source of *Campylobacter* related illness, performance standards for *Campylobacter* in poultry had not been established

prior to 2011. This was primarily due to the lack of national prevalence data and the requisite for adopted and implemented sampling and detection methods (McKee, 2012). Upon establishing validated testing methods, the USDA Food Safety and Inspection Service (FSIS) collected pathogen reduction data during the Nationwide Microbiological Baseline Data Collection Programs: The Young Chicken Baseline Survey (YCBS) of 2007-2008 and the Young Turkey Baseline Survey (YTBS) of 2008-2009. These data indicated that *Campylobacter* prevalence in post-chill samples from young chickens was 46.7% and 1.09% in turkeys.

Additionally, the U. S. Department of Health and Human Services (HHS) published Healthy People 2010 which set objectives pertaining to the overall reduction of foodborne illness. The responsibility in producing safe and wholesome products for consumers still lies with the food industry and regulatory officials to strengthen policies and improve practices which are compelled by the best available scientific evidence and knowledge. The Healthy People 2010 goal for *Campylobacter* was 12.3 illnesses per 100,000 people. However, FoodNet case rate data from 2010 estimated the number of *Campylobacter* illnesses to be 13.6 per 100,000 people. The association of contaminated poultry as a major source for infection, baseline data as well as the failure to achieve Healthy People 2010 targets, became the driving force for implementing performance standards for *Campylobacter* for the first time in the history of the FSIS. The new performance standards for all poultry processing facilities became effective for sample sets that began on or after July 1, 2011 and require that the percentage of *Campylobacter*-positive samples be below 10.4%. Establishments will pass the *Campylobacter* standards if FSIS finds no more than eight positive samples in a 51-sample set for young chickens

and no greater than three positive samples in a 56-sample set for turkeys (USDA-FSIS, 2011).

The objective for *Campylobacter* in Healthy People 2020 is a 33% reduction in illnesses with 8.5 cases per 100,000 people. However, 2012 FoodNet data showed that there has been a lack of recent progress in decreasing foodborne infections. *Campylobacter* is the 2nd most common infection reported in FoodNet and case rate data indicated that the incidence of lab-confirmed *Campylobacter* infection has increased 14% since 2006-2008, reaching 14.3 cases per 100,000 people in 2012 which emphasizes the importance to continue to identify and address areas where improvement in food safety may be targeted. Furthermore, the Raw Chicken Parts Baseline Survey (RCBS) was conducted by FSIS from January 2012 to August 2012 with the intent to establish microbiological criteria for anticipated industry performance standards. The estimated national prevalence of *Campylobacter* in chicken parts is 21.7%. The survey also indicated that while there was no difference in the percent of *Salmonella* positive samples in parts regardless of whether they were skin-on or skin-off, higher *Campylobacter* counts and positive samples were found in parts with skin-on. This could be due to the nature of the organism with its small size and darting motility which further enable cells to intercalate into feather tracks and pores in the skin of chicken. Currently, baseline approaches are under consideration for ground product.

The public health significance of *Campylobacter*, especially in association with poultry products, merits the need for a comprehensive understanding of its persistence in the environment and food supply. Thus, this review will focus on characteristics and pathogenesis, stress response, rising antimicrobial resistance, intervention strategies

throughout the poultry production continuum, and challenges associated with detection of this fastidious organism.

Characteristics of *Campylobacter*

Campylobacter is the type genus of bacteria belonging to the family *Campylobacteraceae*. The bacteria are spiral rods that are 0.2 to 0.8 μm wide and 0.5 to 5 μm long. They are Gram negative and do not form spores. In old cultures, cells have been known to form spherical or coccoid bodies which are considered degenerative forms rather than a state of dormancy. *Campylobacter* spp. exhibit characteristic corkscrew-like motility by means of a single polar unsheathed flagellum at one or both ends.

Campylobacter spp. differ from other pathogens associated with foodborne illness because they are microaerophilic and capnophilic. They grow best in an atmosphere containing 10% CO_2 and 5% O_2. Carbohydrates are neither fermented nor oxidized. Amino acids or tricarboxylic acid cycle intermediates serve as the source of energy rather than carbohydrates. For the majority of species, typical biochemical characteristics include reduction of fumarate to succinate; negative methyl red reaction and acetoin and indole production; nitrate reduction, absence of hippurate hydrolysis, and presence of oxidase activity (Debruyne et al., 2008).

Currently, there are 17 conclusively named *Campylobacter* species although most foodborne illnesses are caused by *C. jejuni* and *C. coli* (Corry and Atabay, 2001; Friis et al., 2005), which are clinically indistinguishable. The species pathogenic to humans are considered modest thermophiles and have an optimal growth temperature of 42°C. Optimal pH for *Campylobacter* growth and survival is 6.5-7.5, or near neutral, and cells are readily killed at pH 2.3 (Blaser et al., 1980). The genomes of *C. jejuni* and *C. coli* are

approximately 1.7 Mb in size which is about one-third the size of the *E. coli* genome (Taylor, 1992). From a biochemical perspective, *C. jejuni* and *C. coli* differ only in their ability to hydrolyze hippurate, for which *C. coli* is negative. *C. jejuni* is fragile and sensitive to environmental stress. The organism does not grow at temperatures below 30°C and dry, high-oxygen conditions, and low pH are detrimental to growth and survival (Doyle and Jones, 1992). Additionally, *Campylobacter* cells have been shown to enter a viable but nonculturable (VBNC) form in which the bacteria change from a spiral to coccoid morphology causing loss of culturability although the cells are still viable. This evidence suggests that in a protective environment such as chicken skin, *Campylobacter* can be difficult to eliminate (Rollins and Colwell, 1986).

Campylobacteriosis causes mild to severe gastroenteritis and is typically associated with lower gastrointestinal tract infection. Most cases of *Campylobacter*-associated gastroenteritis worldwide result from infection with *C. jejuni* (Friedman et al., 2000). The majority of cases reported have been due to isolated, sporadic occurrences and not associated with large outbreaks (Stern et al., 2001; Jacobs-Reitsma et al., 2008). Symptoms of infection include diarrhea, fever, abdominal pain and cramps within two to five days following ingestion. Illness typically lasts one week. Antibiotic treatment for uncomplicated *Campylobacter* infection is rarely necessary. However, antimicrobial resistance to drugs used for treatment, especially floroquinolones and macrolides, has been increasingly reported (CDC, 2013). In addition, there has been evidence that persons who become infected with antibiotic-resistant strains suffer more complications than those infected with sensitive strains (Helms et al., 2005). Seldomly, infection with *Campylobacter* may result in long-term consequences and association with an

autoimmune component. It is estimated that approximately one in every 1,000 reported *Campylobacter* illnesses leads to Guillain-Barré syndrome (GBS), which can result in acute neuromuscular paralysis (CDC, 2013). Reactive arthritis has also been reported as a potential sequella to infection.

Virulence Factors and Pathogenesis

While specific virulence mechanisms have not yet been clearly elucidated for *Campylobacter*, motility, adherence, invasion, protein secretion, intracellular survival, and toxin production have collectively been cited as mechanisms by which *C. jejuni* cause disease in the human host (Larson et al., 2008). Although influenced by the pathogenicity of the *C. jejuni* strain and host immune response, the infective dose of *C. jejuni* is often low. Based on experimental human infection, the infective dose was shown to be between 500-800 cells (Robinson, 1981; Black et al., 1988). Infection with *Campylobacter* causes acute inflammatory changes in both the small and large intestine (Ketley, 1997). Bacterial adhesion and invasion of the intestinal epithelium are critical in the pathogenesis of *Campylobacter* and must take place before initiation of inflammatory processes and diarrheal development. The various mechanisms of the intestinal epithelium in addition to other innate components active in the intestine (such as bile) encompass the primary barrier against gastrointestinal infections (Iovine, 2008).

The first step in the infectious process is the ingestion of viable *C. jejuni* cells. Once ingested by the host, flagellar locomotion enables penetration of the mucosal boundary lining the host intestine. Flagella-mediated motility in *C. jejuni* is essential in establishing human disease and for successful colonization in animals (Wassenaar et al., 1993). The *flaA* gene involved in expression of the flagellar filament is responsible for

expression of adherence, colonization of the GI tract and invasion of host epithelial cells consequently hindering immune response (Jain et al., 2008). It is also thought that flagella may possess the ability to secrete non-flagellar proteins that may be related to virulence (Poly and Guerry, 2008). Furthermore, motility allows *C. jejuni* to colonize particular niches within the host. Within these niches, the bacteria are able to escape peristalsis and flushing of the intestine (Larson et al., 2008).

Campylobacter adheres to colonic epithelial cells and triggers signal transduction events that induce host cytoskeletal rearrangements, bacterial internalization, and translocation across the mucosa (Hu and Kopeco, 2008). Certain adhesins or binding proteins of *C. jejuni* have been identified and include fibronectin-binding outer membrane protein CadF (Konkel et al., 1999), autotransporter CapA, periplasmic binding protein PEB1 (Pei and Blaser, 1993), and the surface-exposed lipoprotein JlpA (Jin et al., 2001). In addition, lipooligosaccharides and polysaccharide capsules are the key surface antigens involved in interaction with the environment and/or host. These molecules in *C. jejuni* likewise aid in host mucosal adherence. Upon binding with the cell surface, *C. jejuni* synthesizes new proteins which correspond to a prompt increase in internalization and rearrangement of host cytoskeletal structures. Some of the newly synthesized proteins are secreted and translocated in the cytoplasm of target cells which enhances host cell signaling events required for *C. jejuni* uptake. After *C. jejuni* binds and invades host cells, the *Campylobacter*-infected cells release inflammatory cytokines, including interleukin-8, that stimulate recruitment of lymphocytes and phagocytic cells to the site of infection (Nachamkin, 2001). The secreted proteins, toxins, and other molecules produced by *Campylobacter* upon adhering to/and or invading host cells can have

adverse impacts in the host. Disease symptoms (e.g., abdominal cramps, diarrhea) are due to these host cell alterations (Hu and Kopeco, 2008).

C. jejuni releases several toxins which vary by strain and correlate to the severity of illness. These include mainly enterotoxin and cytotoxins (Wallis, 1994). Cytolethal distending toxin (CDT) is highly conserved among many Gram negative bacteria and is the best defined of the toxins produced by *Campylobacter* spp. Furthermore, it has been regarded as an important virulence factor of the pathogen (Asakura et al., 2008). CDT is an A/B toxin that causes DNA damage through nuclease activity of its subunit (Lara-Tejero and Galan, 2000; Whitehouse et al., 1998). Specifically, CDT arrests the cell cycle at the G2/M phase, accelerates cellular distention, and causes chromatin fragmentation and cell death (Frisan et al., 2002; Lara-Tejero and Galan, 2001; Whitehouse et al., 1998). Moreover, CDT production by *C. jejuni* has been reported to induce secretion of the pro-inflammatory cytokine interleukin-8 from intestinal epithelial cells (Hickey et al., 2000). The stress responses resulting in cytokine production may be a result of the DNA damage itself.

After infection with *Campylobacter*, protective immunity, which is seemingly antibody-mediated, develops. In addition to the various immune responses, cytokine production during intestinal infection may have a role in immunity. Baqar et al. (1993) demonstrated that oral administration of interleukin-5 and interleukin-6 reduced the level of *C. jejuni* in mice. Futhermore, flagellin is a major immunogen during *Campylobacter*-related illness, and antibodies produced against this protein correspond with protective immunity (Guerry et al., 2000).

Survival Mechanisms of *Campylobacter jejuni*

Food microbiota typically experience stresses from pre-harvest environmental factors and the post-harvest preservation applications. Stress response, at the molecular level, includes transcription leading to the synthesis of regulatory proteins. This regulation may then lead to synthesis of additional proteins that aid in survival despite the imposed stress. Microbial stress response may result in (1) production of proteins that repair damage, maintain cellular activity, or eliminate the stress agent (2) temporary increase in resistance to injurious factors (3) cell transformation to a dormant state such as spore formation or VBNC state (4) evasion of host defense mechanisms, and (5) adaptive mutations (Yousef and Courtney, 2003).

Although it is a significant and extremely prevalent enteric pathogen, *Campylobacter jejuni* has fastidious growth and survival requirements, and is very fragile to environmental stress when compared to other enterics like *Escherichia coli* and *Salmonella* spp. The genome of *C. jejuni* lacks numerous well-established stress response genes including stationary phase sigma factor RpoS, oxidative stress response regulatory proteins SoxRS and OxyR, and osmotic shock protectants like BetAB. Consequently, stationary phase *C. jejuni* has been shown to exhibit increased sensitivities to heat, oxidative, and acid stress (Kelly et al., 2001; Murphy et al., 2003; Park, 2005). However, *C. jejuni* must be capable of survival and even thriving in the various environments encountered in poultry and other zoonotic hosts, within susceptible human hosts, and conditions in the natural environment and food production chain. Therefore, the organism must have mechanisms to protect itself from unfavorable conditions.

Considered a modest thermophile, *C. jejuni* has ideal growth temperatures ranging from 37 to 42°C. Although it lacks apparent cold shock genes such as *csp*A, *C. jejuni* is capable of surviving and maintaining metabolic activity for an extraordinarily long time at refrigeration temperatures (Chan et al., 2001). Contrary to the fact that heat stress accelerates the spiral-to-coccoid transition with temperatures greater than 55°C resulting in rapid cell death (Klancnik et al., 2006; Nguyen et al., 2006; Tangwatcharin et al., 2006), several notable heat shock responses that aid in maintenance of viability have been described. Heat shock triggers a physiological response leading to the synthesis of highly conserved heat shock proteins (HSPs) (Schlesinger, 1990; Lindquist, 1986). These HSPs increase the potential of bacteria to endure subsequent stresses and may enhance their survival in foods during exposure to high temperatures.

Konkel et al. (1998) described 24 proteins that were synthesized in response to 46°C heat shock. Of these, DnaJ was shown to be required for heat shock survival and chick colonization. Furthermore, other studies have indicated that the *Ion* protease gene, groESL, clpB, and dnaK are upregulated in response to heat stress (Thies et al., 1998, 1999a, 1999b, 1999c). Heat shock is severely injurious to *C. jejuni* and has been shown to impair its ability to adhere to and invade host cells (Mihaljevic et al., 2007). Potentially novel mechanisms such as starvation, however, have been shown to improve the ability of *C. jejuni* to resist heat shock (Cappelier et al., 2000; Klancnik et al., 2006).

In addition, atmospheric oxygen levels and reactive oxygen species (ROS) generated under aerobic conditions present challenges to the survival of *C. jejuni*. *C. jejuni* shows more susceptibility to reactive oxygen species such as superoxide, hydrogen peroxide (H_2O_2), and halogenated O_2 molecules, than aero-tolerant bacteria (Hoffman et

al., 1979; Juven and Rosenthal, 1985) which results in its inability to grow actively unless in a microaerobic environment (Krieg and Hoffman, 1986). As noted earlier, this is due in large part to the lack of oxidative stress-induced genes that are part of the OxyR and SoxRS regulons. These oxidants cause damage to cellular proteins, lipids, and nucleic acids (Yousef and Courtney, 2003). Superoxide dismutase (SOD) catalyzes the breakdown of superoxide into H_2O_2 and dioxygen. Unlike other bacteria such as *E. coli*, *C. jejuni* possesses only one SOD response gene—*sodB* (Pesci et al., 1994; Purdy and Park, 1994). Likewise, *C. jejuni* and *C. coli* possess only one gene encoding catalase (*katA*) which is responsible for the conversion of H_2O_2 to H_2O and O_2. Mutant studies have indicated that catalase plays a role in *C. jejuni* H_2O_2 resistance and intramacrophage cell survival (Day et al., 2000; Grant and Park, 1995). Moreover, Stead and Park (2000) demonstrated that SOD and catalase are both required for resistance to freeze-thaw stress under aerobic conditions.

While it is well-established that O_2 is stressful to *C. jejuni*, some reports differ in their conclusions of exactly how O_2 affects the bacterium. It has been found that the transition to the VBNC and/or coccoid form occurred much faster with 24 h exposure to O_2 (Klancnik et al., 2006). However, Mihaljevic et al. (2007) found that upon making comparisons to heat shock and starvation, 15 h exposure to O_2 had very little deleterious effect on the culturability and viability of *C. jejuni*. In fact, the authors indicated an improved ability of the bacterium to adhere to and invade host epithelial cells. Similarly, it was reported that aerobiosis improved the acid adaptive tolerance response (Murphy et al., 2003). Two genes identified as important for short-term O_2 survival are heat shock protease *htrA* and regulator *hspR* which suggests a correlation between the heat shock

pathway and aerobic tolerance (Andersen et al., 2005, Brondsted et al., 2005). In addition, *htrB* was upregulated during aerobic stress (Phongsisay et al., 2007). Furthermore, Jones et al. (1993) demonstrated that *C. jejuni* was capable of adaptation to an aerobic environment. It was shown to grow better aerobically versus microaerobically when exposed to an aerobic environment for two days. However, this acquired adaptation may result in decreased ability of *C. jejuni* to colonize the microaerobic or anaerobic environment in vivo. In addition, due to lack of a stationary phase stress response comparable to most other Gram negative foodborne pathogens, resistance of *Campylobacter jejuni* to thermal stress (50°C) or aerobic atmospheres was found to be greatest in the exponential phase of growth and declined in early stationary phase (Kelly et al., 2001).

C. jejuni has also been shown to have increased sensitivity to salt when compared to other foodborne bacterial pathogens and is usually unable to grow at concentrations above 2% NaCl. Additionally, *C. jejuni* is relatively sensitive to acid and therefore, the low pH encountered in the stomach is considered a major host defense mechanism preventing *C. jejuni* colonization (Doyle and Roman, 1981). Some data suggest that the type of infected food ingested may have a significant impact as to whether the *C. jejuni* cells may subsequently establish intestinal colonization (Svensson et al., 2008).

Under adverse growth conditions and environmental stress, *C. jejuni* has been shown to enter a VBNC state. During this state of dormancy, the bacteria remain viable, but do not grow on media normally used for culture of the organism (Moore, 2001; Oliver, 2005). Certain characteristics are exhibited by bacteria entering a VBNC state. These include reduction in size (such as transformation from a rod to coccoid form),

decrease in metabolic activity, maintenance of ATP levels, sustained gene expression, and a modification of cell wall structures (Oliver, 2005). The viability assay can be useful in determining whether an organism is in a VBNC state and not dead. Viability assays measure an aspect of metabolic activity or cellular integrity (Oliver, 2005).

In fact, *Campylobacter* has various morphological forms (Vandamme, 2000). In fresh, actively growing cultures, the characteristic spiral morphology is the predominate form (Butzler and Skirrow, 1979). Under stressful conditions, the organism transitions through several intermediate forms to a coccoid morphology (Griffiths, 1993; Ng et al., 1985). In cultures of *C. jejuni* and *C. coli* grown on solid media and liquid cultures at 37°C for 24 h, spiral forms predominate. However, within 48 h, the culture largely contains bacteria with coccoid morphology (Alonso et al., 2002; Buck et al., 1983; Griffiths, 1993).

It has been debated throughout literature as to whether *Campylobacter* really enters a VBNC state or if the coccoid form is simply a degenerate state. As an explanation, Hazeleger et al. (1995) suggested that various types of coccoid cells with different characteristics that depend on conditions of formation exist. For example, it has been shown that coccoid cells formed at higher temperatures and in nutrient dense environments exhibit more degradation (Moran and Upton, 1986, 1987) as well as more rapid decline in culturability possibly due to a higher metabolic rate (Boucher et al., 1994; Hazeleger et al., 1995; Rollins and Colwell, 1986). However, when formed at lower temperatures and at low nutrient levels, the coccoid cells have characteristics that resemble the spiral bacteria. Furthermore, Boucher et al. (1994) demonstrated that

aeration increases the rate of coccoid formation but is not required for transformation to occur.

Moreover, biofilm formation has been suggested to play a role in survival of *C. jejuni* and allow the organism to survive up to twice as long under atmospheric conditions (Asakura et al., 2007; Joshua et al., 2006) and in aquatic environments (Lehtola et al., 2006). Upon formation of a biofilm, the microorganisms become sessile, and the polysaccharide matrix forms a protective barrier against the effects of antimicrobial agents. For the organisms forming biofilms, two purposes are served including protection from harsh environmental conditions as well as entrapment of nutrients (Mattila-Sandholm and Wirtanen, 1992). Bacteria in a biofilm are exposed to oxidative stresses, starvation, and dehydration, and as a result, they adapt to these stresses. Additionally, this exposure may lead to cross-protection against other stresses (Ravishankar and Juneja, 2003).

Conditions that mimic stresses that *C. jejuni* may encounter during pathogenesis appear to influence biofilm formation, and data suggest that biofilms may be a mechanism for survival within the gastrointestinal tract of commensal or susceptible hosts (Svensson et al., 2008). Biofilm formation in *C. jejuni* is supported by growth in conditions void of readily available nutrients such as those encountered in water systems (Reeser et al., 2007). Furthermore, Reuter et al. (2010) demonstrated that biofilm formation is more rapid under aerobic conditions than microaerobic conditions and that loss of motility negatively impacts biofilm formation in *C. jejuni*.

Biofilms play a significant role in the persistence of *C. jejuni* within commercial poultry flocks. It has been shown that drinking water within poultry grow-out facilities is

a common source of *Campylobacter* infection for newly hatched birds, and *C. jejuni* within biofilms on drinking nipples is strongly associated with colonization of a particular flock (Zimmer et al., 2003). Furthermore, bacteria growing within biofilms are more resistant to antimicrobial agents. Resistance to sanitizers is a significant concern for the prevention of *C. jejuni* persistence and dissemination within poultry grow-out and processing facilities as well as treatment of potable water contaminated by agricultural run-off. It has been indicated that when compared to planktonic cells, *C. jejuni* biofilms are less sensitive to commonly used sanitizers such as quaternary ammonium compounds, peracetic acid, and chlorine-based antimicrobials (Trachoo and Frank, 2002).

Antibiotic Resistance in *Campylobacter*

The increasing resistance of *Campylobacter* to antimicrobials has become a significant public health concern. Since food of animal origin is most commonly implicated in *Campylobacter* related illness, the development of antimicrobial resistance in *Campylobacter* due to use of antimicrobial agents in food animals is also basis for concern. Although patients usually recover without antibiotic treatment, some patients with severe and persistent illness may require therapy. As a method to track trends in antimicrobial susceptibility of enteric bacteria found in those suffering from illness, retail meats, and food animals in the U. S., the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) program was established in 1996. Macrolides are typically considered the drug of choice for *Campylobacter* infections, but fluoroquinolones (FQ) are also administered and preferred for treatment of undiagnosed diarrheal illness (Goodman et al., 1990; Petruccelli et al., 1992; Salazar-Lindo et al., 1986; Skirrow and Blaser, 2002). However, numerous reports have indicated increases in

the occurrence of *Campylobacter* infections that are resistant to macrolides and FQs. *Campylobacter* also shows resistance to tetracycline and β-lactam agents, but these compounds are not typically used for treatment of *Campylobacter*-associated illness.

Fluoroquinolone (FQ) antimicrobials, such as ciprofloxacin, enrofloxacin, and levofloxacin are among the most important class of antibiotics because of their broad spectrum activity and safety, and ease of administration (Appelbaum and Hunter, 2000; Chu, 1999b). Resistance to FQ antimicrobials is conferred by point mutations in the *gyrA* gene along with the function of the multidrug efflux pump CmeABC. A distinctive characteristic of FQ resistance in *Campylobacter* is that a single point mutation in the *gyrA* gene can result in clinically significant levels of resistance to FQ antibiotics (Ge et al., 2005; Luo et al., 2003; Zhang et al., 2003). In addition, GyrA mutations have been shown to enhance the fitness of *C. jejuni* in chickens in the absence of selection pressure (Luo et al., 2005).

Emergence of resistance among *Campylobacter* spp. and other enteric pathogens to nalidixic acid and fluoroquinolones became prominent during the 1990s. Before then, quinolone resistance was seldom reported. In 1995, FQ antibiotics were approved for use in poultry flocks. Evidence suggests that FQ resistance in *Campylobacter* spp. is associated with the use of these compounds in poultry. A review by Smith et al. (2000) presented the following arguments in support of this theory. First, poultry is a major source of *Campylobacter* infection and is rarely transmitted between individuals. The introduction of experimental treatment of chickens with enrofloxacin did not eradicate the bacteria, but rather selected for FQ resistance. FQ compounds were used in food animals extensively throughout the world. Furthermore, a direct relationship existed

between the approval of FQ use in food animals and the successive increase in FQ-resistant *Campylobacter* in human infections. FQ-resistant *Campylobacter* was isolated from retail poultry meat with resistance rates that paralleled the prevalence in human infections. Smith et al. (2000) also demonstrated FQ-resistant *Campylobacter* from human infections and poultry had the same molecular types. Lastly, FQ antimicrobials were used in human therapy long before the emergence of resistant strains and could not account for such a rise in resistance observed in human infections.

Macrolide antibiotics such as erythromycin, clarithromycin, azithromycin, and telithromycin have been widely used in the treatment of humans for infections caused by *Helicobacter pylori* and *Campylobacter* (Chu, 1999a). In addition, this class of compounds including erythromycin, tylosin, spiramycin, tilmicosin, and roxithromycin are approved for therapeutic use and growth promotion in animal agriculture (McEwen and Fedorka-Cray, 2002; Prescott, 2000). Macrolides such as erythromycin have commonly been used in treatment of campylobacteriosis, and studies reveal a trend of rising resistance in *Campylobacter* to some of these compounds (Engberg et al., 2001; Gibreel and Taylor, 2006). Target site modification and drug efflux are mechanisms responsible for macrolide resistance in *Campylobacter*. A notable difference from FQ resistance acquired by *gyrA* mutations exists in that spontaneous mutation rates for macrolide resistance seem to be much lower in *C. jejuni* and *C. coli*. A study conducted by Lin et al. (2007) indicated that when chickens inoculated with *Campylobacter* were given three therapeutic courses of tylosin treatment, this did not select for erythromycin-resistant *Campylobacter*. Conversely, FQ-resistant *Campylobacter* rapidly developed in chickens treated with enrofloxacin (Luo et al., 2003). However, when the infected

chickens were fed a growth-promoting does of tylosin, macrolide-resistant *Campylobacter* developed after several weeks of treatment. This indicates that macrolide-resistant *Campylobacter* requires an extended selection process (Lin et al., 2007). Thus, it stands to reason that the prevalence of macrolide-resistant *Campylobacter* isolates are less than FQ-resistant *Campylobacter* because of the low spontaneous mutation rate and slow process of resistance development.

Therefore, the U. S. Food and Drug Administration (FDA) proposed the withdrawal of fluoroquinolone approval for use in poultry in 2000. The FDA implemented the FQ ban in poultry in September 2005 when ciprofloxacin resistance in *C. coli* from chicken breast meat increased from 10% in 2002 to its peak of 29.1% in 2005. Since the FQ ban in poultry, ciprofloxacin resistance in *C. coli* decreased to 18.1 % in 2011 while the resistance in *C. jejuni* increased from 15.2% in 2002 to 22.4% in 2011. Moreover, it is well-established that *C. coli* tend to be more resistant than *C. jejuni* regardless of source although 2011 NARMS retail data showed quinolones and tetracycline to be an exception. Multi-drug resistance in *Campylobacter* is rare and only 9 of 634 isolates in poultry were found to be resistant to ≥ 3 antimicrobial classes (FDA, 2011).

The CDC (2013) estimates that there are 310,000 drug-resistant *Campylobacter* infections per year. In recent years, physicians are relying on drugs like ciprofloxacin and azithromycin to treat severe cases of infection. Resistance to ciprofloxacin is seen in almost 25% of *Campylobacter* tested and resistance to azithromycin in approximately 2%. Moreover, the percentage of human *Campylobacter* isolates tested through NARMS that were resistant to ciprofloxacin increased from 13% in 1997 to almost 25% in 2011.

Higher costs are anticipated with resistant infections because antibiotic-resistant *Campylobacter* infections tend to persist in patients for a longer time period.

Poultry Colonization with *Campylobacter*

Chickens are known to be natural reservoirs for pathogenic bacteria such as *Salmonella* and *Campylobacter*. Within broilers, *Campylobacter* is a commensal organism. Colonization takes place primarily in the ceca and is confined to the intestinal mucous layer over the crypts of villi (Beery et al., 1988). Although there is no detectable adhesion or invasion into intestinal epithelial cells, *Campylobacter* can be recovered from the liver and spleen which implies translocation across the intestinal epithelium (Knudsen et al., 2006). Following colonization, mucosal and systemic antibody responses are stimulated and increase over time (Cawthraw et al., 1994; Rice et al., 1997). Most of the broiler parent flocks are colonized with *Campylobacter* and therefore, chicks have high levels of anti-*Campylobacter*-specific maternal immunoglobulin (Ig) G at day of hatch which decrease over 2 to 3 weeks. Furthermore, studies have shown a delay in colonization in chicks from *C. jejuni*-colonized hens (Sahin et al., 2003). This signifies that maternal antibodies play a protective role as revealed by the 2 to 3 week lag phase of infection.

The most important source of *Campylobacter* is thought to be the external environment such as water, insects, feces of animals and other birds (Stern et al., 2002). While horizontal transmission into the flock unmistakably occurs, the role of vertical transmission in the epidemiology of *Campylobacter* in chicken production continues to be debated. *Campylobacter* has been recovered, though at a low incidence and level, from ovarian follicles (Cox et al., 2005) and from semen (Buhr et al., 2005). Byrd et al. (2007)

likewise reported that it was recovered from tray liners in hatcheries. However, it has yet to be shown explicitly that *Campylobacter* derived from parents are a significant source of colonization for descending flocks. Vertical transmission into parent flocks was not detectable when molecular epidemiological studies were conducted on grandparent flocks (Callicott et al., 2006). Since evidence suggests that vertical transmission may occur on a rare basis, environmental sources are significantly more important to target for infection control.

Once colonization of the first birds occurs, rapid dissemination of infection throughout the flock follows due to high levels of fecal shedding (at $>10^6$ per g of feces) and coprophagy (Wagenaar et al., 2008). When related to breeder flocks, *Campylobacter* prevalence is typically lower in the spring of the year (Berndtson et al., 1996). It was also found that *Campylobacter* infection rate increases as the size of the flock increases. In fact, the majority of flocks become positive for *Campylobacter* after 2-3 weeks of age. By 7 weeks of age, 90% of the flocks are found positive (Evans and Sayers, 2000). There are likewise seasonal trends concerning the detection of *Campylobacter* during grow-out. *Campylobacter* is typically detected more frequently in the spring and summer, with small increases during the fourth week of grow-out (Stern et al., 2001). Moreover, FoodNet case rate data (2011) on the seasonality of infection showed *Campylobacter* infection rates to be highest in the summer months of July and August, which correlates with seasonal prevalence rates in broilers.

Strategies to Control *Campylobacter* during Poultry Production and Processing

Campylobacter control in poultry can be targeted and implemented on the poultry farm during rearing, between the farm and slaughter during catching and transport, and

during poultry processing. The greatest impact might be seen if the colonization of live animals with *Campylobacter* could be prevented or reduced, thus avoiding the introduction of high levels of *Campylobacter* into the subsequent steps of the production continuum. However, controlling *Campylobacter* during primary production is a challenging proposition. Currently, commercial vaccine strategies for controlling *Campylobacter* in breeders or the grow-out flock have yet to be developed. These approaches must provide protection from day one until the time of slaughter, eliminate colonization by the point of slaughter, or reduce *Campylobacter* levels in the ceca. However, there is only a brief time in which to induce effective antibody responses since the average lifespan of broiler chickens is only six weeks and they are not immunologically developed. The main challenges associated with development of a vaccine in poultry include identifying cross-protective antigens, generating a prompt and effective immune response, and developing unique adjuvants to enhance immunity against *Campylobacter* antigens (reviewed by De Zoete et al., 2007). Studies have indicated passive immunization as an alternative strategy. Orally administered anti-*Campylobacter* antibodies have been shown to have therapeutic and protective properties in chickens (Stern et al., 1990; Tsubokura et al., 1997). While immunizing parent flocks would be feasible for breeder flocks, however, it would be too costly for broilers.

Since vaccination of poultry against *Campylobacter* is not currently available, biosecurity and overall farm management are critical in its control. A survey conducted by McKee (2012) which represented 10,317 grow out facilities for broilers indicated that strict biosecurity, litter management, and water acidifying treatments were among the best practices for controlling *Campylobacter* at the farm level. Evidence suggests that

Campylobacter is introduced into a poultry house from the external environment. Potential vectors include farm personnel, other authorized visitors, rodents, insects, and wild birds. Biosecurity measures alone are not likely to consistently protect poultry flocks from colonization with *Campylobacter*. While a correlation has been identified between stringent biosecurity practices and the absence of *Campylobacter* under commercial conditions (Van de Giessen et al., 1998), even a very high degree of biosecurity such as in grandparent flocks does not assure a *Campylobacter*-negative flock.

Considering that the organism is sensitive to drying or desiccation, litter management is another important factor in controlling dissemination of *Campylobacter*. Litter amendments such as alum have likewise been shown to result in significant reductions in *Campylobacter jejuni* prevalence in poultry litter (Rothrock et al., 2008). Additionally, research on competitive exclusion (CE) and the use of probiotics is ongoing. While such approaches have been successful for *Salmonella*, the efficacy for *Campylobacter* is unpredictable. Some commercially available CE agents have been reported to yield significant reductions in the level and prevalence of *Campylobacter* colonization in some experiments (Hakinen and Schneitz, 1999; Wieliczko, 1995) whereas other similar agents showed no effect regardless of efficacy against *Salmonella* (Aho et al., 1992). The variable nature of the CE agent and of the susceptibility of the *Campylobacter* strain is a possible conclusion for the inconsistencies in experiments. It has now been well-established that effective CE agents may produce anti-*Campylobacter* metabolites (Mead et al., 1996; Schoeni and Wong, 1994) which have been defined from several antimicrobial agents, including *Bacillus circulans*, *Paenibacillus polymyxa* and *Lactobacillus salivarius* (Stern et al., 2005, 2006). The mechanisms of action of these

bacteriocins may point toward the introduction of changes in gut morphology (Cole et al., 2006). However, more underlying research on the colonization of *Campylobacter* and its capacity to occupy a unique ecological niche in the avian gut is needed.

Campylobacter, often in high levels, enter the processing plant on the birds and may be spread to equipment during processing steps. Berrang and Dickens (2000) demonstrated that 4-5 log cfu/mL could be recovered in the early stages of processing. Furthermore, it was found that the cecal contents of 100 birds from six different *Campylobacter*-positive flocks harbored between 5.8 to over 9 log cfu/g of *Campylobacter* (Berndtson et al., 1992). Within the poultry processing plant, there are numerous opportunities for cross-contamination. Picking and evisceration are two major points during processing of poultry where microbial contamination occurs since *Salmonella* and *Campylobacter* are present in the feces of carrier birds and can be transferred from the intestines to the skin surface (Byrd and McKee, 2005). These steps may require particular attention due to the high rate of cross-contamination (Saleha et al., 1998).

During the picking or defeathering stage of processing, cross-contamination of and among carcasses may be increased due to considerable dispersion of microorganisms (Hafez, 1999). In addition, when rubber picking fingers become contaminated with bacteria, the warm, humid environment of the defeathering apparatus provides an atmosphere that may aid in the survival and growth of the pathogens (Mead et al., 1980). While most processors may prefer that picking remain a neutral step with regards to cross-contamination, this is rarely the case. *Camplylobacter* populations have been shown to increase 1-2 logs following picking (Berrang and Dickens, 2000). In fact, researchers

have reported that when a single carcass becomes contaminated during defeathering it can contaminate more than 200 other carcasses (Van Schothorst et al., 1972; Mead et al., 1975). However, some research has indicated that *Campylobacter* levels are reduced throughout processing, but positive samples are still detected following carcass chilling (Hinton et al., 2004).

It may be possible to eliminate most of the naturally occurring levels of *Campylobacter* that might remain on carcasses post-chill by targeting a 2-log reduction in bacterial counts. When post-chill locations in a processing plant were sampled for *Campylobacter*, a mean log cfu/mL of 1.5 was recovered (Berrang and Dickens, 2000), signifying that the normal chilling step is not effective enough in reducing all pathogens. Utilizing antimicrobials with intervention strategies that can yield a 2-log reduction should therefore effectively reduce pathogens remaining on the carcass after chilling. Furthermore, while a 2-log reduction may not seem remarkable, it has been predicted that a 2-log reduction in *Campylobacter* populations on poultry would lead to a 30-fold decrease in human campylobacteriosis (Rosenquist et al., 2003).

Significant improvements to food safety have been made by using the multi-hurdle approach to pathogen reduction. Online reprocessing (OLR) or spray cabinets following evisceration represent the first stage where antimicrobials may be added to reduce pathogens. Some bacterial reduction may be achieved with these methods, although results may be inconsistent because of contact time, bird coverage, and spray pressure. Therefore, these interventions are typically used as part of a multi-hurdle strategy. The immersion chiller is one of the primary sites in poultry processing facilities where antimicrobials are applied for reduction of *Salmonella* and *Campylobacter* on

carcasses. Chilling in the poultry industry is a necessary process to reduce carcass temperatures to 4°C or less within 4 hours of slaughter (USDA, 1995) as well as inhibit microbial growth to meet regulatory requirements and improve shelf-life of the product.

In recent years, methods for reducing pathogens during poultry processing have advanced with innovations in technology. Intervention strategies such as post-chill decontamination tanks have become part of the multi-hurdle approach for pathogen reduction during poultry processing. These decontamination tanks are placed directly after the primary immersion chiller and have different operating parameters such as a short dwell time (generally 30s), higher antimicrobial concentrations, and much lower organic load as compared to the primary chiller. In addition, applying antimicrobials in a post-chill dip tank in comparison to primary chillers which hold 20,000 to 50,000 gallons (dwell time of 1.5- 2.0 h) is much more efficient and cost-effective (McKee, 2011) with less likelihood of antimicrobial causing negative impacts on carcass quality. By adopting a multi-hurdle approach and combining these interventions with effective antimicrobials throughout processing, best practices in pathogen control can be achieved to meet regulatory requirements for *Salmonella* and *Campylobacter*.

Currently approved antimicrobials for use in poultry applications are described in FSIS Directive 7120.1 Revision 9 (USDA-FSIS, 2011). In the U. S., chlorine has historically been a common antimicrobial utilized for prevention of bacterial carcass cross-contamination in immersion chilling systems and throughout the poultry processing plant (McKee, 2011). However, the efficacy of chlorine as an antimicrobial is affected by pH, the presence of organic material, and contact time. In a survey conducted by McKee (2011), peracetic acid (PAA) was indicated by processors as the predominant

antimicrobial used in the poultry chiller and post-chill dip applications and has replaced chlorine as the industry standard for antimicrobial application in poultry processing. Furthermore, Bauermeister et al. (2008) reported a 43.4% reduction in *Campylobacter* when 85 ppm PAA was used in a chiller compared to a 12.8% reduction when 30 ppm chlorine was applied, indicating that PAA is more effective in reducing *Campylobacter* on broiler carcasses than chlorine when applied in the primary chiller. Nagel et al. (2013) likewise demonstrated that utilizing 400 ppm PAA in a Finishing Chiller® application with a 20 s contact time was more effective than chlorine and provided a 2-log reduction in *Campylobacter* levels to comply with industry performance standards. Cetylpyridinium chloride (CPC) is another approved antimicrobial that has been found to be effective in reducing *Campylobacter* on poultry. In unpublished data (Zhang et al., 2013), it was demonstrated that utilizing 0.6 % CPC on inoculated poultry parts in a post-chill parts decontamination unit was more effective than PAA and resulted in approximately a 4-log reduction in *Campylobacter* counts. With the anticipation of industry performance standards for poultry parts, this particular research will provide industry with new technologies and chemical interventions to effectively reduce *Salmonella* and *Campylobacter* on poultry parts.

Campylobacter Isolation and Culture Techniques

There are many challenges associated with the microbiological analysis of food due to several inherent factors. The composition and complexity of food matrices presents obstacles to the development of effective sampling, sample preparation, and rapid testing methodologies. Successful separation of target pathogens is difficult in meat, poultry, and egg products since high levels of normal flora associated with raw

foods may interfere with selective identification and isolation of specific pathogens (Feng, 2007). Pathogens in food are typically prevalent in low numbers and may be injured during food processing, thereby requiring better detection sensitivity and pre-enrichment to revive injured cells. Therefore, eliminating enrichment of samples from the process of pathogen detection would be problematic, since enrichment serves as an approach to increase the concentration of target pathogens in foods as well as reduce the effects of inhibitors and normal flora on testing methodologies (Feng, 2007). In addition, diverse distribution of pathogens in foods makes it difficult to conduct effective sampling procedures (Meng et al., 2007). This is especially true for foodborne pathogens such as *Campylobacter* which is capable of causing illness in humans with a very low infectious dose.

Campylobacter is typically isolated from food samples through a combination of enrichment broth with selective plating or direct plating on selective agars (Speegle et al., 2009). However, the isolation of *Campylobacter* from foods is particularly challenging because these bacteria may be present in low numbers and often in conjunction with high numbers of competing organisms (Baylis et al., 2000). Due to the slow and complicated growth requirements of *Campylobacter* spp., there is a need for more rapid and/or better optimized methods for *Campylobacter* detection. Filtration techniques have been used to isolate *Campylobacter* spp. from food samples (Baggerman and Koster, 1992). Because of its small size and high motility, *Campylobacter* cells can pass through the pores of membrane filters very easily, whereas the extent to which other microorganisms and particulates can penetrate small pore sizes is much less. *Campylobacter* can pass through membrane filters with pore sizes of 0.45 to 0.65 μm (Wells et al., 1989). Membrane

filters are used to concentrate target organisms to improve detection limit, to remove growth inhibitors, and to transfer organisms between media without causing injury to cells through re-suspension (Mandal et al., 2011). Another application used in food analysis to selectively concentrate bacterial cells is immunomagnetic separation (IMS). Using this technology, specific antibodies are coupled to magnetic beads to capture target pathogens from pre-enrichment media. This method may be a better alternative to cultural enrichment since it alleviates the necessity for selective enrichment, reagents, or antibiotics and also shortens the time required for sample analysis (Feng, 2001).

Campylobacter spp. are susceptible to stresses such as drying, low pH, heat, freezing and prolonged storage, however, they persist well in poultry. The growth requirements for *Campylobacter* are complicated since *Campylobacter* does not ferment carbohydrates and requires a microaerophilic (5% O_2, 10% CO_2, and 85% N_2) environment for growth. Media must be supplemented with essential growth factors such as enzymatic digest of animal tissue, lysed horse blood, hemin, and ingredients to provide nitrogen, carbon, amino acid and vitamin sources. Likewise, ferrous sulfate, sodium pyruvate, and sodium metabisulfite must be incorporated to increase the aerotolerance of *Campylobacter* oxygen scavengers (Corry et al., 1995). In addition to basic growth ingredients, media used to culture *Campylobacter* must contain various selective agents such as vancomycin, rifampicin, polymyxin, trimethoprim, amphotericin B, cephalosporins and cycloheximide to inhibit background contaminants like other bacteria and fungi that hinder its growth (Martin et al., 2002; Nachamkin et al., 2000; Corry et al., 1995; Humphrey, 1990). Typical enrichment media are Bolton's enrichment broth (BEB)

which is supplemented with lysed horse blood or blood-free Bolton's enrichment broth (BF-BEB), both of which include addition of antibiotics.

Recently, it has been indicated that supplementing media with a combination of organic acids can achieve aerobic growth of *Campylobacter* (Hinton, Jr., 2012). The same author also reported that supplementing a basal broth with various concentrations (2.5, 5 or 7.5%) of beef extract supported aerobic growth of *Campylobacter* in the media incubated for 48 or 72 h (Hinton, Jr., 2014). While these alternative culture methods would alleviate the cost and requirement for materials necessary to create the microaerophilic atmosphere for *Campylobacter* growth and have shown efficacy with pure cultures, they may not be practical for poultry rinse samples. The media evaluated in these studies were not supplemented with inhibitors which are necessary to prevent overgrowth of background microflora that would outcompete natural levels of *Campylobacter* found on poultry carcasses. Furthermore, the time required for enrichment of *Campylobacter* was not reduced or may actually be longer to achieve detectable levels. Therefore, additional research is necessary in order to apply these methods to industry and eliminate the modified atmosphere required to effectively detect *Campylobacter* from poultry.

Some plating media used for detection and enumeration of *Campylobacter* spp. have been reported to improve recovery and exclusion of background microflora (Potturi-Venkata et al., 2007; Oyarzabal et al., 2005; Line, 2001; Merino et al., 1986). In addition, particular media can affect the growth of *Campylobacter* colonies on the plate. Among the most common selective plating media used are charcoal cefoperazone deoxycholate (CCDA) agar or modified forms as well as Campy-cefex (Potturi-Venkata et al., 2007).

On charcoal based media such as modified CCDA, characteristic *Campylobacter* colonies are grayish, flat and moistened, with a tendency to spread and may have a metallic sheen. It has been indicated in previous research that the best recovery rate from poultry carcass rinses was obtained with Campy-cefex incubated at 42°C for 48 h (Oyarzabal, et al., 2005). However, in our lab, colonies have been observed by 24 h of incubation at 42°C on mCCDA.

There are rapid method assays commercially available for *Campylobacter*, but the ideal detection system would require high specificity and sensitivity, quick response time, and be capable of mass production. It should also include simplification of sample preparation and with minimal stress on the sample. The caveat is the absence of actual "real-time" procedures from sampling to results (Ge and Meng, 2009). Thus, most rapid methods, in themselves, can be accomplished in a few minutes to a few hours and are therefore more rapid than traditional culturing techniques. However, due to constraints inherent in food analysis as discussed earlier, samples must still be enriched in growth media before detection since selective enrichment broths improve sensitivity for recovery of *Campylobacter* cells within a sample. Furthermore, the detection sensitivity of many rapid assays is usually about 10^4 to 10^5 cfu/mL for bacterial cells, and with multiple pathogen intervention strategies implemented throughout poultry processing facilities, *Campylobacter* would not be found in such high levels from post-chill samples.

In addition to *Campylobacter* detection throughout the numerous steps of poultry processing, a lot of consideration is now given to quantification of the presence of the bacteria since these data are necessary for risk assessment studies which are primarily conducted in the poultry production chain (Nauta et al., 2005; Rosenquist et al., 2003;

Uyttendaele et al., 2006). As such, USDA requires a quantitative culture method for *Campylobacter* analysis in poultry. For chicken rinses, testing includes the whole carcass rinse method with buffered peptone water (BPW) and then directly plating 1 mL of the 400 mL post-chill rinsate onto Campy-cefex agar or other AOAC approved plating media. Plates are incubated under microaerophilic conditions for 48 h at 42°C, and positive colonies are confirmed via microscopy and latex agglutination. Turkey sampling is conducted by swabbing the carcass using sponges which are added to 25 mL BPW. Direct plating for turkeys involves plating 1 mL from the sponge sample in 100 µL aliquots distributed among 2 plates which are incubated and confirmed as mentioned previously. These methods are currently being employed to determine pass/fail for the sets collected by FSIS and can be found in the USDA Microbiological Laboratory Guidebook.

A qualitative analysis of *Campylobacter* in poultry was initially proposed. Testing methodology would require enrichment in double-strength blood-free Bolton's enrichment broth (BF-BEB). However, the poultry industry argued against using this method for determining pass/fail since enrichment would ultimately detect low numbers. The likelihood of many plants' failure to meet the *Campylobacter* performance standards would have been considerable due to high incoming loads and high prevalence rates. Alternatively, USDA uses enriched samples for internal analysis (Federal Register Notice vol. 76, no 54, 2011).The poultry industry is in need of more rapid methods with high sensitivity for *Campylobacter* detection in order to provide prompt information on the possible presence of the pathogen in raw product before reaching consumers, to monitor

pathogen control practices throughout the processing plant, and to reduce human error as well as save time and cost.

Traditional sampling procedures for detection of *Campylobacter* in foods involve selective enrichment for approximately 48 h followed by direct plating on selective media and incubation for an additional 48 h. Therefore, there is a need for more fundamental research to optimize detection time and culture methods as well as reduce the significant cost associated with culturing *Campylobacter*. The objectives of the current study were to optimize selective enrichment for *Campylobacter* and validate its efficacy for recovery of *Campylobacter* from poultry rinse samples. Growth of *Campylobacter jejuni* populations in various modifications to enrichment media in a microaerophilic atmosphere were evaluated in vitro. Once an optimized enrichment broth was identified, the efficacy for *Campylobacter* detection was determined through microbial analyses of chicken carcass rinse samples and compared to current USDA enrichment methodology. This study provides the poultry industry with an improved and more cost-effective approach for qualitative analysis of *Campylobacter* in poultry.

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CHAPTER III.

MATERIALS AND METHODS

Bacterial Culture Preparation

Tubes containing 9 mL of Brucella-FBP (Acumedia, Lansing, MI) broth were inoculated with 1 mL of *Campylobacter jejuni* and incubated for 24 h at 42°C in an Anaero-Jar (Oxoid, Ogdensburg, NY) containing a CampyGen™ sachet (Oxoid) to generate a microaerophilic mixture of 5% O_2, 10% CO_2, and 85% N_2. The culture was streaked for isolation onto modified charcoal cefoperazone deoxycholate agar (mCCDA; Acumedia) and incubated at 42°C for 48 h in microaerophilic conditions as previously described. *C. jejuni* was removed from the agar and inoculated into Brucella-FBP broth and further incubated for 24 h at 42°C to generate approximately 6 log CFU/mL. *Campylobacter* cultures were confirmed via phase contrast microscopy.

Lower concentrations of *C. jejuni* were obtained by serially diluting individual bacterial suspensions. High and low concentrations of *C. jejuni* on chicken carcasses were obtained by inoculating birds with 5 log CFU/mL and 2 log CFU/mL suspensions, respectively.

Selection of Media for Evaluation of *Campylobacter jejuni* Growth

Campylobacter enrichment media evaluated throughout this study were selected based upon current USDA enrichment methodology, previous studies on culturing *Campylobacter*, and novel broth formulation in conjunction with the Neogen

Corporation. Blood-free Bolton's enrichment broth (BF-BEB) is used for enrichment of poultry rinse samples by USDA-FSIS for *Campylobacter* recovery (USDA-FSIS, 2013). Brucella broth with the FBP supplement (ferrous sulfate, sodium bisulfite, and sodium pyruvate) is often used to enrich *Campylobacter jejuni* and has demonstrated good growth in previous work with *Campylobacter* culture methods (Bauermeister et al., 2008; Nagel et al., 2013; Chen et al., 2014). Furthermore, the FBP supplement is used to neutralize toxic effects of oxygen derivatives (Bolton et al., 1984) to improve *Campylobacter* recovery. Novel broth formulations were based upon identifying key nutrients and oxygen scavengers that satisfy the unique growth requirements of *Campylobacter*.

Modifications to these media were made to include inhibitors and organic salts necessary to inhibit background contaminants from poultry rinse samples and increase the aerotolerance of *Campylobacter* oxygen scavengers, respectively. Cefoperazone combined with amphotericin B comprise the CCDA supplement antibiotics. These particular inhibitors were selected to incorporate into the media because of their inhibition of the background microflora that are typically found on post-chill poultry. Cefoperazone is used to suppress Gram (-) enteric flora, has demonstrated increased selectivity than previous cephalosporins used (Bolton et al., 1984) and is included in the majority of media for isolation of *Campylobacter*. Amphotericin B is used to suppress growth of yeast and fungal contaminants without impacting recovery of *Campylobacter* (Martin et al., 2002). The particular organic salts (100 mM pyruvate and 50 mM fumarate) used in media formulation were selected based on their ability to scavenge

oxygen in research demonstrating aerobic growth of *Campylobacter* spp. (Hinton, Jr., 2012).

Evaluation of *Campylobacter jejuni* Growth in Enrichment Media

From stock cultures of approximately 6 log CFU/mL *Campylobacter jejuni*, serial dilutions were made and 1 mL was inoculated into 99 mL of various enrichment media (Trial 1: 2 inoculum samples X 3 treatments X 2 replications; Trial 2: 2 inoculum samples X 5 treatments X 2 replications) in sterile tissue culture flasks to achieve a starting concentration of approximately 2.5 log CFU/mL. Media evaluated throughout the experiment consisted of Blood-Free Bolton's enrichment broth (BF-BEB; Acumedia, Lansing, MI) incorporated with Bolton broth selective supplement (Acumedia), Brucella-FBP, and several novel broth formulations included to optimize *Campylobacter* detection and reduce cost. These formulations included an inhibitory broth formulation, Brucella-FBP with inhibitors (modified Brucella broth), Brucella-FBP with inhibitors and organic salts, Bolton's broth base with inhibitors, and Bolton's broth supplemented with inhibitors and organic salts as described in detail in Table 1.

Table 1. Composition of enrichment media used in the experiments

Medium	Base	Supplement
Blood-Free Bolton's[a,c]	Enzymatic digest of animal tissue (10 g/L), lactalbumin (5 g/L), yeast extract (5 g/L), sodium chloride (5 g/L), Hemin (0.01 g/L), sodium pyruvate (0.5 g/L), α-ketoglutaric acid (1 g/L), sodium metabisulfite (0.5 g/L), sodium carbonate (0.6 g/L), deionized water (1 L)	Cefoperazone (20 mg), cyclohexamide (50 mg), trimethoprim (20 mg), vancomycin (20 mg)

Brucella[a]-FBP	Enzymatic digest of animal tissue (10 g/L), enzymatic tissue of casein (10 g/L), yeast extract (5 g/L), sodium chloride (5 g/L), dextrose (1 g/L), sodium bisulfite (0.1 mg/L), deionized water (1 L)	Ferrous sulfate (0.5 g/L)[b], sodium bisulfite (0.2 g/L)[b], sodium pyruvate (0.5 g/L)[b]
Inhibitory broth	Nutrient broth no. 2 (20 g/L), α-ketoglutaric acid (1 g/L)[b], ferrous sulfate (0.5 g/L), sodium bisulfite (0.2 g/L), deionized water (1 L)	Pyruvate (100 mM)[b], fumarate (50 mM)[b], cefoperazone (33 mg)[b], amphotericin B (10 mg)[b]
Brucella-FBP with inhibitors (modified Brucella)	Brucella broth (28 g/L), deionized water (1 L)	Ferrous sulfate (0.5 g/L), sodium bisulfite (0.2 g/L), sodium pyruvate (0.5 g/L), cefoperazone (33 mg), amphotericin B (10 mg)
Brucella-FBP with inhibitors and organic salts	Brucella broth (28 g/L), deionized water (1 L)	Ferrous sulfate (0.5 g/L), sodium bisulfite (0.2 g/L), sodium pyruvate (0.5 g/L), cefoperazone (33 mg), amphotericin B (10 mg), pyruvate (100 mM), fumarate (50 mM)
Bolton's with inhibitors	Bolton's broth (27.6 g/L), deionized water (1 L)	cefoperazone (33 mg), amphotericin B (10 mg)
Bolton's with inhibitors and organic salts	Bolton's broth (27.6 g/L), deionized water (1 L)	cefoperazone (33 mg), amphotericin B (10 mg), pyruvate (100 mM), fumarate (50 mM)

[a]Acumedia, Lansing, MI

[b]Sigma-Aldrich, St. Louis, MO

[c]*Campylobacter* selective supplement (7998, Acumedia).

Enrichment media were incubated at 42°C under microaerophilic (5% O_2, 10% CO_2, and 85% N_2) conditions to evaluate *Campylobacter jejuni* growth. Serial dilutions

were performed on each sample using buffered peptone water (BPW; Acumedia) and spread plated in duplicate onto mCCDA at 0, 12, 24, and 48 h for enumeration of *C. jejuni*. Plates were incubated at 42°C for 48 h in a microaerophilic environment. Bacterial populations were converted to log values per mL. Results are therefore reported as log colony forming units per mL.

Qualitative Detection of *Campylobacter* from Poultry Rinse Samples

A total of 90 broiler carcasses were sampled (15 carcasses X 3 treatments X 2 replications). During each replication, 45 carcasses were commercially obtained the day of sampling. Thirty carcasses per replication were inoculated on the skin of the breast portion of the carcass with 1 mL of *Campylobacter jejuni* (2 populations: high (5 log CFU/mL) or low (2 log CFU/mL) in each of two trials). For both the high and low inoculum treatments, the birds were allowed to set for 30 minutes after inoculation to ensure adequate contact time for bacterial attachment prior to rinsing. Additionally, 15 carcasses per replication were sampled for background *Campylobacter* that may be found on post-chill broiler carcasses in a commercial processing plant.

Individual birds were placed into a sterile rinse bag and rinsed according to the USDA whole-carcass rinse method with 200 mL of BPW for 1 min. The USDA methods described in the *Microbiological Laboratory Guidebook* for qualitative analysis of *Campylobacter* in poultry rinse samples were used for bacterial sampling and detection (USDA-FSIS, 2013). For each treatment, 30 mL of the rinsate was transferred into 30 mL of double-strength (2X) BF-BEB or 2X modified Brucella broth in sterile whirl pack bags and incubated at 42°C in a microaerophilic atmosphere.

At 12 and 24 h of enrichment, samples from both enrichment media from each bacterial treatment level were serially diluted and 0.1 mL was spread plated onto mCCDA and incubated for 48 h at 42°C under microaerophilic conditions. Samples were analyzed for the presence or absence of *Campylobacter*. Isolated colonies representing each colony type typical for *Campylobacter* were confirmed by examining for corkscrew morphology and darting motility using phase contrast microscopy.

Statistical Analysis

Two replicates were conducted for the experiments. For enumeration of *Campylobacter* in enrichment media, bacterial counts were converted to log colony-forming units per mL. *Campylobacter* prevalence in carcass rinses was analyzed in a 3 x 2 factorial arrangement of bacterial level and media type by indicating positive samples with a 1, and negative samples with a 0. All data were reported as least square means and analyzed using the general linear model of SAS (SAS Institute, 2003). Pdiff was used to separate the means and significance was indicated by a $P \leq 0.05$.

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CHAPTER IV.

RESULTS AND DISCUSSION

In order to culture *Campylobacter* spp., specialized growth temperatures, gaseous environments, and a nutrient rich basal medium are necessary to achieve the complicated growth requirements of these fastidious organisms. Selective enrichment media are used to enhance the recovery of *Campylobacter* cells within a sample (Humphrey, 1989). As a result of low populations of bacteria and constraints by food components, *Campylobacter* samples must be enriched for approximately 48 h, followed by direct plating on selective media and incubation for an additional 48 h to complete qualitative analysis. Therefore, due to the extensive amount of time required for enrichment and detection of *Campylobacter* as well as the expense associated with culturing methods, there is a need for improved enrichment methods that offer a cost-effective approach to analysis.

To decrease the time and cost associated with *Campylobacter* detection, optimization of poultry rinse and sample preparation using a filter method was initially proposed. Filtration of samples would reduce the amount of time necessary to promote growth of *Campylobacter* and therefore, the time required for selective enrichment. Additionally, by filtering organic matter and other background microorganisms from samples, the requirement for antibiotics and other supplements for increased selectivity in media are reduced. *Campylobacter jejuni,* is approximately 0.5 μm in diameter and 2 μm in length, whereas the size of other bacteria averages 0.7-1.5 μm in diameter and 2-5 μm

in length. Likewise, blood cells present in organic matter in poultry carcass rinse samples are much larger—approximately 7 µm in diameter and 8 µm in length.

Campylobacter can pass through membrane filters with pore sizes of 0.45 to 0.65 µm (Fitzgerald et al., 2008). Therefore, it was thought that by using filtration methods to reduce the presence of other microorganisms and matter that may out-compete *Campylobacter*, better isolation might be achieved. Evaluation of the filtration method in this study consisted of coarse filtering the carcass rinsate using a 0.33 mm filter in order to entrap organic matter and other particulates. This was directly followed by using a membrane filter with a pore size of 0.65 µm placed on top of the 0.2 µm filter contained inside a sterile filter flask to capture the *Campylobacter* cells. However, after coarse filtering the carcass rinsate, the rinsate would not permeate through the membrane filters in the flask, even upon incorporating a vacuum to pull the rinsate through. As a result, this methodology was not feasible to target isolation of *Campylobacter* cells.

Therefore, the primary focus of this research became evaluation of various modifications to enrichment media in order to improve recovery of *Campylobacter* following enrichment and reduce cost. The first objective of the study was to develop an optimized enrichment media by determining the ideal concentration of key nutrients and oxygen scavengers which satisfy the unique growth requirements of *Campylobacter*. Recently, it has been demonstrated that aerobic growth of *Campylobacter* spp. can be achieved by supplementing media with a combination of organic acids, thereby alleviating the cost and requirement for materials to create the microaerophilic atmosphere. However, 72 h of enrichment in these media was required to achieve levels

of 6-7 log CFU/mL (Hinton, Jr., 2012) which is an additional 24 h of enrichment than what is necessary in a microaerophilic incubation atmosphere.

Based on the research by Hinton, Jr. (2012), growth evaluation of *C. jejuni* began with the aim to achieve aerobic growth in a shorter time span by optimizing current nutrients and oxygen scavengers in the enrichment media. To determine the potential for aerobic growth of *C. jejuni* in enrichment media, an inhibitory enrichment broth formulation was developed and evaluated in an aerobic environment. The inhibitory broth was comprised of an unsophisticated basal media and supplemented with organic salts (50 mM fumarate and 100 mM pyruvate) which increase the aerotolerance of *Campylobacter* oxygen scavengers in addition to inhibitors (cefoperazone and amphotericin B) necessary for subsequent evaluation of *Campylobacter* recovery from chicken carcasses. With a starting concentration of approximately 4.5 log CFU/mL, *C. jejuni* populations demonstrated approximately a 1 log decrease within 12 h of incubation before achieving a final concentration of 6 log CFU/mL by 48 h of incubation (data not shown). Promoting survival by sacrificing vegetative growth is a common strategy used by bacteria in response to less than optimal conditions (Ryall et al., 2012) as evidenced by the decrease in counts during the lag phase.

In addition, because Brucella-FBP broth has consistently demonstrated good growth of *C. jejuni* in a microaerophilic atmosphere in previous studies, this broth was likewise modified to incorporate the organic salts (50 mM fumarate and 100 mM pyruvate) and inhibitors (cefoperazone and amphotericin B) in order to determine whether essential growth factors in the basal media may impact growth of *C. jejuni* populations when incubated under aerobic conditions. The starting concentration of *C.*

jejuni for the growth trial was reduced to 2.5 log CFU/mL in order to better synchronize cellular division and also because higher populations of *Campylobacter* spp. would not typically be found on post-chill poultry meat. Growth of *C. jejuni* was inconsistent and bacterial populations were no longer detectable by 12 h of incubation with lower populations (data not shown). Aerobic growth of *C. jejuni* could not be replicated with the enrichment media evaluated. While eliminating the requirement for a modified atmosphere for growth of *Campylobacter* spp. from food samples would be a tremendous benefit, more research on methods to achieve detectable levels more rapidly and consistently is necessary.

Although the association between *C. jejuni* and oxygen is a key defining aspect of the biology of this foodborne pathogen, it remains one of the least investigated and understood. The oxygen content within an environment as well as transition from an aerobic to anaerobic environment (and vice versa) can impact adaptation to the new environment. However, many of the global regulatory systems that are present in "model" aerobic bacteria are absent in *C. jejuni*. There are few transcriptional regulators, no RpoS-type stationary-phase sigma factor, and the SoxRS and OxyR oxidative stress regulators are absent (Park-hill et al., 2000). Therefore, *C. jejuni* is more susceptible to reactive oxygen species and halogenated oxygen molecules than aero-tolerant bacteria (Hoffman et al., 1979; Juven and Rosenthal, 1985).

It was indicated by Ryall et al. (2012) that many of the factors in adaptation to a new environment are dependent on population size. Thus, it makes a difference whether a population that experiences an environmental transition is small (e.g., 3 log to 4 log CFU/mL) or large (8 log to 10 log CFU/mL). Furthermore, the authors reported that if

environmental transition involves a loss of viability, the survivors may be a small fraction of the ancestral population. Consequently, the population size resulting from the survivors can impact the degree of adaptation to a new environment, and it stands to reason that currently aerobic growth at a constant rate may not be feasible for smaller bacterial populations. But realistically, *Campylobacter* levels on post-chill poultry meat would not be found in higher levels. Therefore, additional research is necessary to determine the potential for low levels of *Campylobacter* to actively grow without the modified atmospheric requirement.

Accordingly, *C. jejuni* growth in various enrichment media incubated in a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) for 48 h was examined in the current study. Growth of *C. jejuni* cultured in enrichment media consisting of blood-free Bolton's enrichment broth (BF-BEB), Brucella-FBP, and the inhibitory enrichment broth formulated with essential growth factors and supplemented with 50 mM fumarate and 100 mM pyruvate as well as cefoperazone and amphotericin B inhibitory agents were evaluated for 48 h in a microaerophilic environment. Since USDA requires enrichment in BF-BEB, this media was used for comparison in this growth trial. The starting concentration of *C. jejuni* was approximately 2.5 log CFU/mL for the growth trials.

C. jejuni populations in Brucella-FBP immediately entered exponential growth and demonstrated higher counts ($P \leq 0.05$) at 12, 24, and 48 h when compared to BF-BEB, achieving a final concentration of 8.38 CFU/mL by 48 h of incubation. The inhibitory broth formulation resulted in a much slower growth rate of *C. jejuni* throughout 48 h with lower counts ($P \leq 0.05$) at 12 and 24 h (Figure 1). The difference in performance of these

enrichment broths could be due to the type of peptones or other ingredients used in the media, but this would require further investigation.

Because Brucella-FBP broth demonstrated improved growth of *C. jejuni* throughout 48 h (Figure 1), modifications to this media as well as to Bolton's broth base were evaluated (Figure 2). Brucella-FBP was modified to contain cefoperazone and amphotericin B inhibitory agents in order to suppress competitive organisms, which is necessary for isolation of *Campylobacter* spp. from chicken rinses. These particular antibiotics inhibit enteric flora (*Pseudomonas aeruginosa*, *Enterobacter* spp., *Proteus* spp.) and yeast and fungal contaminants, respectively. Cefoperazone was selected as an inhibitory agent used throughout the study since it is incorporated into the majority of *Campylobacter* isolation media and was reported to increase the probability of recovering *C. coli* when substituted for Cephalothin (Corry et al., 1995). Amphotericin B was likewise selected since it has often been used to replace cycloheximide in the preparation of *Campylobacter* enrichment broth and has been reported to sufficiently suppress fungal growth without inhibiting *Campylobacter* isolation (Martin et al., 2002).

Bolton's broth was likewise evaluated with addition of cefoperazone and amphotericin B rather than the Bolton's selective supplement (vancomycin, selective against Gram positives; cefoperazone, predominantly selective against Gram negatives; trimethoprim, selective against *Proteus* spp. and Gram positives; and cycloheximide, selective against yeast and molds). Some antibiotics have been shown to be inhibitory to injured cells (Humphrey, 1990) and therefore, it was thought that recovery of *Campylobacter* may be improved by reducing the combination of antibiotics used in the media while simultaneously reducing cost. Additionally, growth of *C. jejuni* populations

in Brucella-FBP and Bolton's base incorporated with inhibitors (cefoperazone and amphotericin B) as well as organic salts (100 mM pyruvate and 50 mM fumarate) was evaluated.

When compared to Brucella-FBP enrichment broth, Brucella-FBP incorporated with inhibitors showed no differences ($P>0.05$) in *C. jejuni* populations within 48 h of incubation and achieved a final concentration of 8.81 log CFU/mL. Brucella-FBP likewise resulted in higher *C. jejuni* counts ($P\leq0.05$) than Bolton's base with inhibitors at 12, 24, and 48 h. However, when both the Brucella-FBP and Bolton's plus inhibitors were supplemented with organic salts, the lag phase of growth was extended and resulted in lower populations ($P\leq0.05$) of *C. jejuni* at each sampling hour. While addition of organic salts at greater concentrations may be necessary to accomplish aerobic growth of *Campylobacter* spp. in enrichment media, they did not perform as well under microaerophilic conditions and resulted in a lower growth rate ($P\leq0.05$) at each sampling time compared to the same media without organic salts. Brucella-FBP and its modification provided the best results ($P\leq0.05$) in *C. jejuni* growth throughout 48 h (Figure 2). The Brucella-FBP broth incorporated with the inhibitors (cefoperazone and amphotericin B) was subsequently renamed modified Brucella.

After identifying an optimized *Campylobacter* enrichment media, the next objective of this study was to evaluate its efficacy for detection of *Campylobacter* from post-chill chicken carcasses. Evaluation of rinsates from post-chill carcasses permitted the methods in the current study to be analyzed for *Campylobacter* recovery from samples which contain populations of competing organisms or injured cells may be present. Because blood-free Bolton's enrichment broth (BF-BEB) is used by USDA for

qualitative analysis of *Campylobacter* from poultry rinse samples, this media served as the standard broth for comparison to the modified Brucella broth. Additionally, USDA methodology requires a 1:1 dilution of enrichment media to carcass rinse solution and therefore enrichment broths were prepared as double-strength (USDA-FSIS, 2013).

In the present study, *Campylobacter* recovery after enrichment in both media was evaluated at 12 and 24 h of enrichment with incubation in a microaerophilic atmosphere. For both media, enrichment of inoculated samples with high (5 log CFU/mL) and low (2 log CFU/mL) levels showed no differences ($P>0.05$) in *Campylobacter* prevalence on carcasses at 12 h as well as 24 h. Following enrichment in modified Brucella broth and BF-BEB, all carcasses sampled were positive for *Campylobacter* (Table 2). When the prevalence of background *Campylobacter* on post-chill carcasses after enrichment for 12 h was evaluated, however, enrichment in modified Brucella broth showed a higher recovery ($P\leq0.05$) of *Campylobacter* with 96.7% prevalence compared to 86.7% after enrichment in BF-BEB (Table 2). Furthermore, no differences ($P>0.05$) in the recovery of background *Campylobacter* after 24 h of enrichment in modified Brucella (100%) and BF-BEB (96.7%) were found (Table 2).

Media have been demonstrated to impact the recovery and exclusion of competing organisms (Potti-Venkata et al., 2007; Oyarzabal et al., 2005, Line, 2001; Merino et al., 1986) although both of the enrichment media evaluated for detection of *Campylobacter* from chicken in this study showed similar inhibition against the background flora present in the chicken rinses tested. However, the BF-BEB contained the Bolton's selective supplement which consisted of four antimicrobial compounds (vancomycin, cefoperazone, trimethoprim, cycloheximide) whereas the modified

Brucella was only supplemented with two antimicrobial compounds (cefoperazone and amphotericin B). Although background microflora are present on post-chill carcasses, microbial contamination is relatively low so a more highly selective media may not be required (Oyarzabal et al., 2005) for enrichment of samples. Furthermore, use of fewer antibiotics in the media may also result in less inhibition to rapid methods.

In addition to assessment of *Campylobacter* growth performance and recovery in various media, a cost analysis of the modified Brucella and BF-BEB enrichment media was conducted (Table 3). The total cost per liter of prepared BF-BEB was approximately 2.5 times more expensive than the total cost of the modified Brucella enrichment broth. For chicken rinse samples, this translates to approximately $0.75 more per sample. Furthermore, by selecting modified Brucella broth as an alternative to BF-BEB for enrichment of *Campylobacter* from rinse samples, there is a cost savings of 63%. For companies testing a high volume of samples, a lot of expense could be saved by using modified Brucella. While the cost of the base for each broth was comparable, addition of the Bolton's selective supplement, containing four antimicrobial compounds, resulted in the increased cost associated with this media. For enrichment of chicken carcass rinses using USDA qualitative testing methodology, basal media and supplement addition should be prepared as double-strength although this was not reflected in the cost analysis in Table 3.

From this research it can be concluded that currently, aerobic growth of *Campylobacter* in enrichment media may not be practical for levels that are recovered from post-chill poultry meat, especially with regards to reducing detection time. Due to

the variable nature of the organism and the many factors that impact bacterial adaptation, more fundamental research on the association of *Campylobacter* and oxygen as well as environmental transition is essential.

It is shown that ingredients (essential growth factors, organic salts, i.e. oxygen scavengers, and inhibitors) used to formulate media for selective enrichment of *Campylobacter* can impact the growth of *C. jejuni* populations. High concentrations of organic salts (fumarate and pyruvate) incorporated into enrichment media in this study did not perform well under microaerophilic conditions and resulted in much slower growth of *C. jejuni* throughout 48 h. In the current study, modified Brucella broth demonstrated better performance ($P \leq 0.05$) on the growth rate of *C. jejuni* throughout 48 h as well as improved recovery ($P \leq 0.05$) of background *Campylobacter* from chicken carcasses within 12 h of enrichment.

Moreover, by reducing the number of antibiotics in the media, the cost associated with enrichment media evaluated can be reduced without negatively impacting recovery of *Campylobacter*. Hence, the use of modified Brucella for selective enrichment provides the poultry industry with a cost effective alternative to current USDA methodology using BF-BEB for qualitative detection of *Campylobacter* from post-chill chicken by decreasing the time necessary to promote growth of *Campylobacter* and the expense associated with media formulation. This research indicates that qualitative analysis of post-chill chicken carcasses sampled from processing facilities could be revised to enrichment in modified Brucella broth for 12 h followed by direct plating onto a selective agar (modified CCDA for faster colony growth) and incubated in a microaerophilic environment to reduce the total analysis time required for *Campylobacter* detection.

Additionally, this work was done in conjunction with the Neogen Corporation and further application could include combining enrichment of *Campylobacter* from poultry rinse samples in modified Brucella broth for 12 to 24 h (depending on the detection limit) with a real-time RNA detection platform that the company is currently working to develop. Use of this method has the potential to decrease the total analysis time for detection of *Campylobacter* from chicken to 24 h or less.

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Table 2. Detection of *Campylobacter* on post-chill chicken carcasses following enrichment for 12 and 24 h in 2X blood-free Bolton's enrichment broth (BF-BEB) and 2X modified Brucella broth.

Treatment	Sampling Hour	% Positive after Enrichment in 2X Blood-Free Bolton's Broth	% Positive after Enrichment in 2X Modified Brucella Broth
Background	12	86.7[b]	96.7[a]
	24	96.7[a]	100[a]
Low Inoculum	12	100[a]	100[a]
	24	100[a]	100[a]
High Inoculum	12	100[a]	100[a]
	24	100[a]	100[a]

[a-b]Means with no common superscript differ significantly ($P \leq 0.05$).

Table 3. Cost of formulation of 1 liter of medium.

Medium	Cost of Supplies ($)[a]		Total Cost per liter ($)
	Base	Supplement	
Modified Brucella	7.01	7.47	14.48
Blood-free Bolton's	6.85	32.00	38.85

[a]Based on prices during spring 2014.

*Basal media and supplement amounts must be doubled for enrichment of chicken carcass rinses (not reflected in cost).

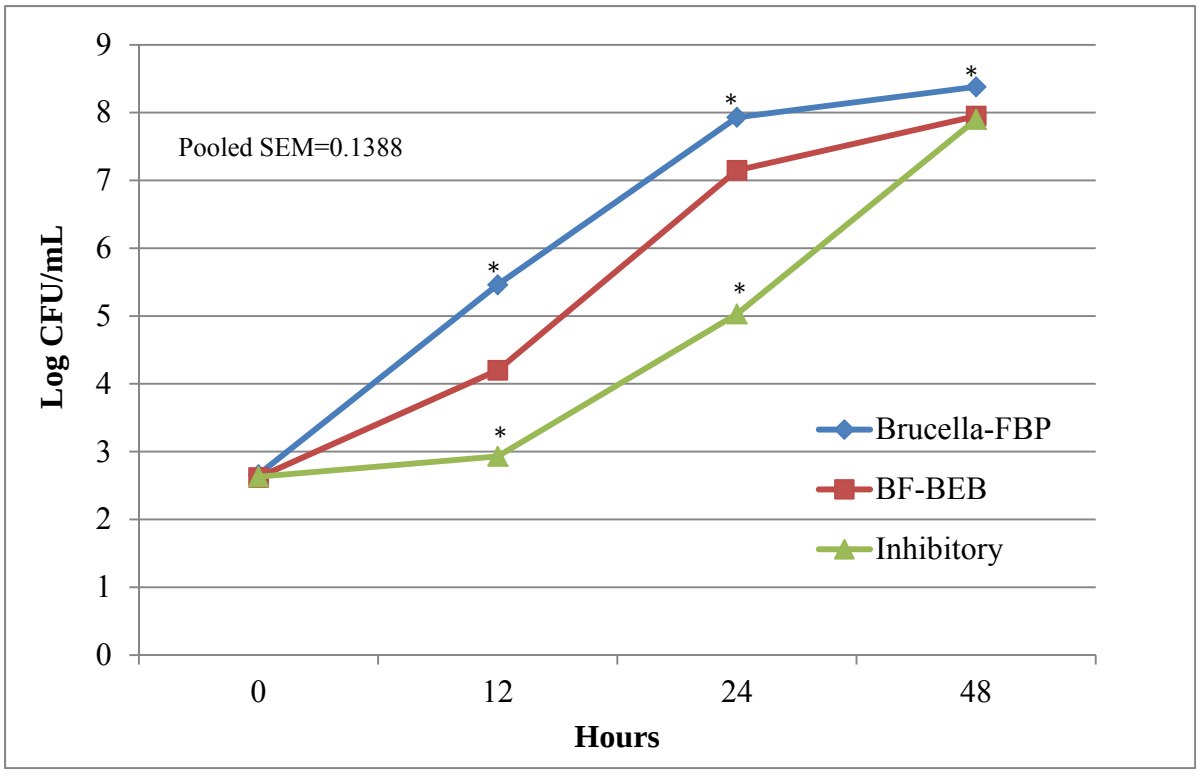

Figure 1. Growth of *Campylobacter jejuni* cultured in Brucella-FBP, blood-free Bolton's enrichment broth (BF-BEB), and inhibitory broth formulation under microaerophilic conditions for 48 h.

*indicates difference (P≤0.05) in *C. jejuni* counts compared to BF-BEB within 48 h.

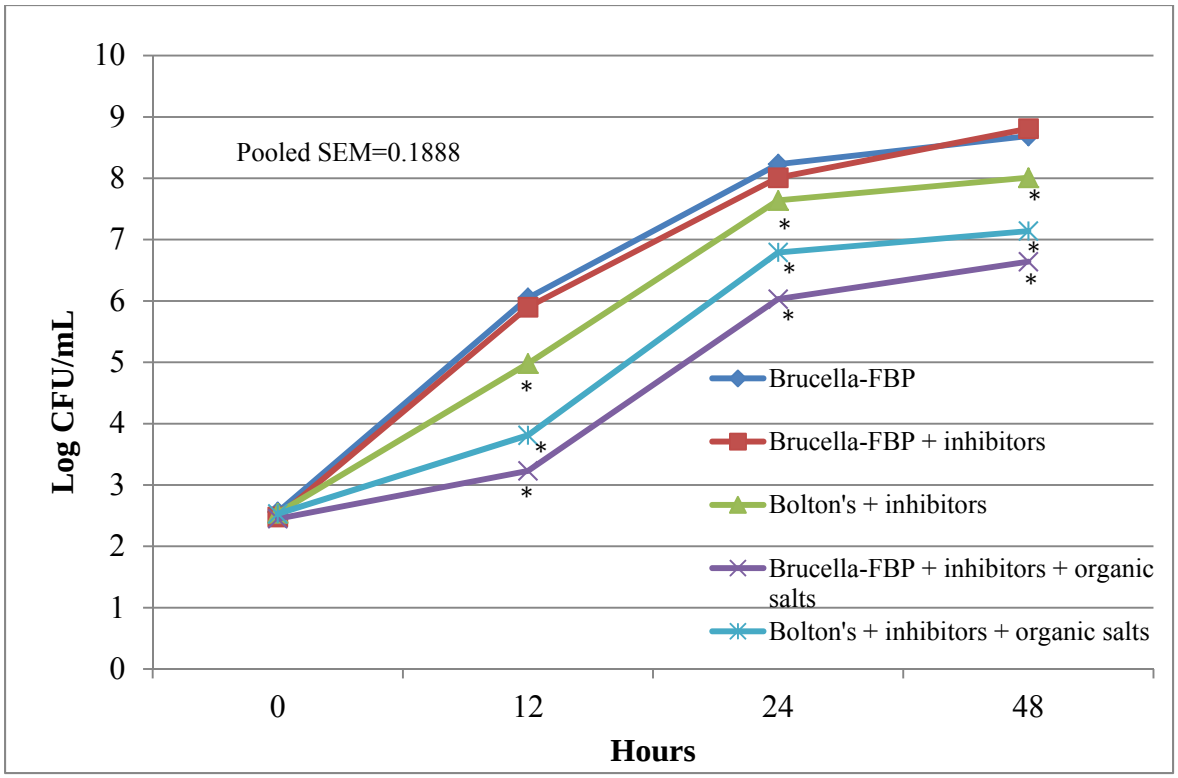

Figure 2. Growth of *Campylobacter jejuni* cultured in Brucella-FBP and Bolton's broth incorporated with inhibitors and organic salts under microaerophilic conditions for 48 h.

*indicates difference (P≤0.05) in *C. jejuni* counts compared to Brucella-FBP within 48 h.

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