The Development and Optimization of a RT-PCR assay for Rapid *Campylobacter* spp. Detection and Quantification in Poultry Rinsates

by
Aaron Rashad Bodie

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The dissertation is approved by the following members of the Final Oral Committee:
Steven C. Ricke, Professor, Animal and Dairy Sciences
Jeffrey Sindelar, Professor, Animal and Dairy Sciences
Vanessa Leone, Associate Professor, Animal and Dairy Sciences
Kathleen Glass, Faculty, Food Research Institute

ABSTRACT

Campylobacter is a foodborne pathogen mainly associated with poultry products. Currently, prevalence-based data is used to determine process control during poultry processing. However, this method is not sensitive enough, labor intensive and does not provide information on the level and subsequent risk of *Campylobacter* contamination. As a result, reliable alternative methods are imperative to detect and quantify *Campylobacter* contamination throughout the harvest process. Therefore, the goal of this dissertation was to develop and optimize a real-time polymerase chain reaction (RT-PCR) assay for rapid detection and quantification of *Campylobacter* spp. in poultry rinsates using the United States Department of Agriculture – Food Safety Inspection Services protocol and sampling techniques. The first project was focused on identifying the compositional diversity of pooled colonies enumerated from post-chill poultry carcass samples inoculated with *Campylobacter jejuni, coli,* and *lari* on various selective and non-selective media, Campy-Cefex, mCCDA, and TSA; (Chapter 2). The objective of the second study was to generate growth curves for *Campylobacter jejuni, coli,* and *lari* under the same growth conditions recommended by the International standard organization (Chapter 3). The last chapter aimed to develop and verify a rapid quantification method for *Campylobacter species* (CampyQuant™) in post-chill poultry rinsates using the BAX® System Real-Time PCR Assay for *Campylobacter* (Chapter 4). The results of this dissertation were that BAX® System CampyQuant™ assay was able to detect *Campylobacter jejuni, coli,* and *lari* after 20 h enrichment and quantify the pre-enrichment concentration in a range from 1.00 to 4.00 Log_{10} CFU/mL. The study suggests that the CampyQuant™ BAX® RT-PCR assay can provide the food industry with a rapid, accurate, and efficient alternative method for *Campylobacter* spp.

enumeration to ensure that process controls are working adequately to provide safe products to consumers.

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DEDICATION

This dissertation is dedicated to my family. To my parents, George and Lolita Bodie, my brothers George jr. and Joshua Bodie, my grandmother Evangeline Rahming, my nephews Harper and George IV, Breanna, A. Wil, A.J, and all my other family members, we did it.

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INTRODUCTION

Campylobacter is a foodborne pathogen that is the number one cause of gastroenteritis in the U.S. (Center for Control Disease (CDC), 2019). *Campylobacter* spp. are Gram-negative, microaerophilic microorganisms that are ubiquitous in the environment (Silva et al., 2011). Currently, there are approximately 25 known species of Campylobacter, with most human diseases associated with *C. jejuni*, *C. coli*, and *C. lari* (Kaakoush et al., 2015). *Campylobacter* causes the disease campylobacteriosis, which can occur by ingesting contaminated food with as few as 800 CFU/mL of *Campylobacter* present. The CDC estimates the incidence of campylobacteriosis to be about 20 cases per 100,000 people, with an estimate of 1.5 million persons diagnosed each year (CDC, 2019). Symptoms of campylobacteriosis include vomiting, headaches, and diarrhea. However, severe cases can develop into irritable bowel syndrome (IBS), reactive arthritis, and Guillain–Barré syndrome (GBS; Janssen et al., 2008).

The main vehicle of *Campylobacter* transmission to humans are poultry products. The gastrointestinal tract (GIT) of poultry is a reservoir for *Campylobacter* spp. to grow and proliferate while the bird host does not exhibit external symptoms (Heem and Lu, 2021). The GIT serves as a reservoir primarily since *Campylobacter* spp. grow at temperatures between 37 and 42 °C, with the upper range being close to the core body temperature of chickens (Tang et al., 2017; Heem and Lu, 2021). Furthermore, *Campylobacter* spp. are microaerophilic, and some regions of the chicken GIT exhibit microaerophilic conditions, allowing them to become well adapted and survive (Gabriel et al., 2006). Moreover, *Campylobacter* is a commensal with chickens and go unnoticed without causing harm. These characteristics demonstrate just some examples of why *Campylobacter* is a major concern for poultry products.

Currently, the United States Department of Agriculture – Food Safety Inspection Services (USDA-FSIS) uses poultry whole carcass rinse sampling to estimate the prevalence of foodborne pathogens such as *Campylobacter* and *Salmonella* in chickens (USDA-FSIS, 2021). The method consists of rinsing the carcass in buffered peptone water (BPW), which serves as a means to recover microorganisms from the carcass' surfaces and subsequently test for the prevalence of foodborne pathogens. In a sampling bag with 400 mL of BPW, a carcass is added to the bag by removing it from the sample line, followed by agitation for one minute, and subsequent rehanging on the sample line (USDA-FSIS, 2021). For *Campylobacter* exclusively, once rinsate samples are collected, they are subsampled and enriched in Bolton Broth for 24 h, followed by plating on Campy-Cefex media, then incubating for an additional 24 to 48 h (USDA-FSIS, 2021). Although this method is universally accepted in the poultry industry, problems still exist. Culture-based plating is not always reliable for growth and detection due to the fastidious nature of *Campylobacte*r. When *Campylobacter* spp. become stressed, they can occasionally enter a viable, but non-cultivable (VBNC) state, and as a result, will not be recovered by CFU plating for detection and enumeration (Silva et al., 2011). In addition, colonies can only be identified at a genus level because plating does not allow for visual differentiation of species based solely on morphology (Frano-Duarte et al., 2019). Species identification is critical because it provides information as to whether the microorganism possesses pathogenic traits that can cause human disease. Furthermore, the current method is time consuming, taking approximately three days before results are available (USDA-FSIS, 2022). For an efficient food production system, faster results are needed to make food safety decisions, such as alterations in intervention methods.

The rinsate medium BPW is non-selective, intended to recover multiple pathogens such as *Salmonella* and *E. coli*, from the environments in which they originate (Edel and

Kampelmacher, 1973; Ogden et al., 2001). Previous studies indicated that these organisms have different nutrient requirements and degradation pathways, therefore, the use of BPW for recovering all foodborne pathogens for detection can cause sensitivity issues for some microorganisms such as *Campylobacter* spp. (Wages et al., 2022). In addition, the use of different media has had a critical effect on research because there are no standards to compare results for *Campylobacter* studies. The most effective media and culture techniques to grow and detect *Campylobacter* from poultry and other food sources remain unclear. Interpreting results from non-selective media can be confounded by uncertainty of recovering multiple microorganisms, not just *Campylobacter* spp. Numerous research studies have compared different *Campylobacter* selective and/or enrichment media, however, all lead to varied conclusions (Bolton et al., 1983; Endtz et al., 199; Oakley et al., 2012).

With *Campylobacter* being a fastidious organism, a universal medium remains somewhat unresolved. Furthermore, the culture method for *Campylobacter* growth and detection in commercial operations is still preferred. Further insight into *Campylobacter* growth requirements can potentially drive the development of improved detection techniques.

While these studies reveal limitations in our ability to detect and enumerate *Campylobacter*, research has shown that polymerase chain reaction (PCR) assays have proven to be an effective alternative for *Campylobacter* detection (Sails et al., 2003; Lund et al., 2004). To reliably detect *Campylobacte*r spp., PCR uses specific DNA target sequences, which make them reliable for genomic-based specific taxonomic identification (Wassenaar and Newell, 2000). However, limitations do exist for PCR use. First, the assay's sensitivity needs to be defined to determine the lowest concentration of *Campylobacte*r that can be detected by the assay (threshold detection limit). In addition, much of the research has focused exclusively on *C.*

jejuni and less on the other disease-causing *Campylobacter*, namely *coli* and *lari*. Therefore, additional research is needed to elucidate a broader view of *Campylobacter* responses in media and further optimize PCR for growth and detection.

As such, this dissertation research aims to develop and optimize a RT-PCR assay (CampyQuant™) for rapid detection and quantification of *Campylobacter* spp. in poultry rinsates using the USDA-FSIS protocol and sampling techniques. The central hypothesis of the dissertation is that the use of CampyQuant™ RT-PCR assay will detect and quantify *Campylobacter* spp. in poultry rinsates more rapidly compared to current methods. To do this, I developed four objectives:

1. Review of *Campylobacter* spp., and the culture-based and molecular methods available for their detection and quantitation in poultry processing samples. (**Chapter 1**). This information will provide a comprehensive background of *Campylobacter* and the historical evolution of selective and enrichment culture-based, as well as molecular methods, available for detection and enumeration of *Campylobacter*. This will provide the background for the individual goals of the current research being presented in this thesis.
2. A next step is identifying the compositional diversity of colonies enumerated from post-chill poultry carcass samples inoculated with *Campylobacter jejuni*, *coli*, and *lari* on various selective and non-selective media (Campy-Cefex, mCCDA, and TSA; **Chapter 2**). I hypothesized that the respective *Campylobacter* spp. will dominate the community of the selective and non-selective plates, however, it will not be a completely specific monoculture as other microorganisms that typically inhabit the poultry rinsate matrix may be present. Understanding the microbial community of colonies on selective and

non-selective plates will give us a better understanding of the specificity of selective plates, as well as other communities that such media can support along with *Campylobacte*r from poultry rinsates. This information will allow us to designate the best-fit media based on selectivity for *Campylobacter* spp., to use for future development.

3. Develop growth curves and calculate growth kinetic parameters for C. jejuni, *C. coli* and *C. lari* in pure culture using blood free Bolton broth (**Chapter 3**). To optimize a PCR for rapid detection, growth kinetics are needed for enumeration methods to provide quantitative information on the growth response of *Campylobacter* spp. to the environment provided. Understanding the different growth parameters, such as doubling time and exponential phase time, should help to make the timing for generating PCR results more predictable. Therefore, this chapter aims to understand the relationship between *Campylobacter* spp. and the USDA-FSIS' recommended media for growth and detection, in order to optimize time needed to reach level of detection for PCR assays. I hypothesized that the *Campylobacter* spp. will grow at different rates in enrichment media. The kinetic parameters generated from this study will provide a standardized enrichment time prior to conducting the quantitative PCR assay, taking into account any growth physiology differences that may occur among the three *Campylobacter* spp.
4. Develop and validate a rapid detection and quantification method for *Campylobacter* spp. in post-chill poultry rinsates using the CampyQuant™ BAX® System Real-Time PCR Assay. (**Chapter 4**). The method for the development of the BAX® System Real-Time PCR Assay for *Campylobacte*r was performed following USDA-FSIS protocols for sampling techniques such as rinsate medium (Whole-Carcass rinsate in BPW), enrichment medium (blood free Bolton broth), and quantification medium (Campy-

Cefex). The development was done by identifying the length of time enrichment is needed, using growth kinetic parameters to define enrichment timepoints and then validating this information by quantifying the enumerable range of *Campylobacter* spp. detected using CampyQuant™. This allowed for the development of standard curves for quantifying *Campylobacter* spp. from poultry rinsates. For this study, I hypothesized that enrichment is needed for low cell population concentration samples, and the longest enrichment defined from the kinetic calculations were used to allow *Campylobacter* to adapt to the poultry environment before growth is initiated. Additionally, spread plating allowed for quantifying enumerable ranges, therefore, standard curves were developed for quantification. The goal was to determine whether the BAX® System Real-Time PCR Assay for *Campylobacter* can directly apply to the matrices encountered in commercial poultry processing.

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CHAPTER 1

LITERATURE REVIEW

Culture-Based and Molecular Methods for Detection and Quantitation of *Campylobacter* spp. in Poultry Processing Samples

Aaron Bodie

Abstract

Campylobacter is considered a major foodborne pathogen of public health interest because it is one of the leading causes of gastroenteritis in the United States. *Campylobacter* is a fastidious organism, needing specialized requirements for growth such as ambient temperatures, microaerophilic environment, and nutrient-rich media for growth. Consequently, growth and detection remain a challenge for inexperienced laboratory personnel. Over time, several selective media have been developed to recover *Campylobacter* from various matrices. However, variable media conditions and subsequent ongoing media development for optimizing *Campylobacter* growth and detection remains a major research effort. The use of molecular methods has been pursued as an alternative for *Campylobacter* detection of which polymerase chain reaction (PCR) assays are among the more developed molecular methods for foodborne pathogen detection.

Application of PCR for *Campylobacter* detection is considered a promising option for routine use. Research has shown that this molecular method is more sensitive and rapid than using culture media. Nevertheless, to use current PCR methods, samples must be enriched or diluted in media and the type of media can affect PCR results. A better understanding of *Campylobacter* kinetics using selective media will help optimize the development of a future PCR assay. Therefore, this review will focus on the historical and current developments in media used for the growth and detection of *Campylobacter.* In addition, requirements for evaluation and optimization of using PCR as an alternative for *Campylobacte*r spp. detection will be discussed. Since there is no preferred method for *Campylobacter* growth and detection, the aim of this review is to gain a deeper understanding of *Campylobacter* to optimize growth and detection through media choice and PCR methods.

1. Introduction

Campylobacter is a prominent foodborne pathogen that is of global public health concern. This genus is non-spore-forming, motile, S-curved or spiral shaped, and approximately 0.2 to 0.8 µm by 0.5 to 5 µm long (Silva et al., 2011; Kaakoush et al., 2015). Of the 22 species of *Campylobacter*, only *C. jejuni, C. coli, and C. lari* have been established as causes of gastroenteritis in humans (Huang et al., 2015; Liu et al., 2018). *Campylobacter* spp. are known to undergo substantial morphological changes in broth and on agar media. Under different incubation conditions, the organism may shift from a Gram-negative, spiral rod possessing a flagellum to a round, semi-round, donut, or coccoid morphology. Additionally, there are a variety of intermediate forms *Campylobacter* can generate as it becomes established in different environmental niches (Bhaduri and Cottrell, 2004; Ghaffar et al., 2015). The morphological shift of *Campylobacter* spp. may result from various stress factors placed on the organism under sub-optimal growth conditions.

Campylobacter can be viable but non-culturable (VBNC), therefore, initial detection from some sample matrices can be difficult. Specific cultivation conditions are required for *Campylobacter* growth, such as thermophilic temperature (50 to 80°C), microaerophilic gaseous environment, and nutrient rich medium, which can be challenging for incubation conditions in a routine laboratory setting (Goossens et al., 1992; Stern et al., 1992).
As *Campylobacter* has become more understood, further developments in enrichment and selective media have enhanced the ability to grow *Campylobacter* spp. routinely. Further improvement has been made possible by improvements on the optimal recovery conditions for *Campylobacter* detection from different food matrices (Kiess et al., 2010). However, when growing *Campylobacter* spp. from other foods matrices, background non-

Campylobacter organisms can be problematic. Cross contamination can also occur as *Campylobacter* cultures are typically overgrown by coliform bacteria, Proteus, and yeasts (Jefferey et al., 2000). Ideally, media should allow *Campylobacter* to grow into well separated and distinct colonies without the growth and/or interference of other microorganisms.

Over time several culture media have been developed to recover *Campylobacter* from food matrices (Eberle and Kiess, 2012 Ricke et al., 2019). With various media available for *Campylobacter* growth, there remains a lack of consistency for adopting a standard culture media in the laboratory (Benkova and Marek, 2020). Problems exist because the quality of culture media directly affects observations and inferences drawn, leading to confusing interpretations due to the cultural characteristics of competing microorganisms potentially present on the selective media (Basu et al., 2005). Each medium has different parameters for metabolism and growth, which creates the possibility of false positive cell counts (Buss et al., 2019). Standardization of *Campylobacter* media can also impact the accuracy of antimicrobial resistance breakpoints. For thermophilic *Campylobacter*, several antimicrobial susceptibility tests and resistance breakpoints have been used in various studies (Guévremont et al., 2006; Luangtongkum et al., 2007). In addition, each selective media has a unique compositional battery of antimicrobials employed for *Campylobacter* selection. As a result, depending on the medium of choice, the efficacy of the antimicrobials will differ depending on the microbial community contained in the complex samples along *Campylobacter* spp. present (Luangtongkum et al., 2007).

When isolated from samples, media can influence the selectivity and detection of *Campylobacter*; therefore, a better understanding of media can potentially improve future studies (Basu et al., 2015). This review will focus on *Campylobacter* growth and detection using

past and current culture media and explore polymerase chain reaction (PCR) assays as an alternative to culture-based methods. PCR is a molecular method that uses specific DNA sequences to amplify DNA (Garibyan and Avashia, 2013). This review aims to achieve a comprehensive understanding of C*ampylobacter*, including its metabolism for growth and detection in culture media. Understanding *Campylobacter* metabolism in growth media will help optimize detection using PCR. It is likely that the development of *Campylobacter* PCR assay will be based on alterations and modifications to maximize the detection of species other than *C. jejuni* (Post, 1995).

2. *Campylobacter* History

Campylobacter spp. were first characterized in 1886 when scientist Theodor Escherich observed the microorganism in stool samples from children with diarrhea (Kist, 1985). He also identified "spiral-shaped" microorganisms in stool specimens from neonates and kittens experiencing diarrhea. However, the organisms of interest were unable to be cultured when spread plated on agar media. This was primarily because selective media for this fastidious organism was not available at that time. In the United States (U.S), the first documented case of *Campylobacter* was observed in a study by Smith and Taylor (1919) where a similar microorganism described by Escherich in1886 was isolated from aborting sheep. *Vibrio fetus* was the name proposed by Smith and Taylor (1919) for the newly observed microorganism because at the time, studies that examined related abortion specimens of cattle had already associated the *Vibrio* genus with abortions and presumed it was a similar variant (Engberg, 2006, Skirrow, 2006).

The first documented human outbreak of *Campylobacter*-linked disease did not occur in the U.S. until May 1938. The source of the outbreak was an unknown *Vibrio* spp. isolated from

contaminated milk products (Levy, 1946). This same *Vibrio* spp. was observed in 11 patients with gastroenteritis (King, 1957). Among the 11 isolates grown, seven strains were *Vibrio fetus*. In 1957, Elizabeth King isolated a *Vibrio*-like microorganism from blood samples of children with diarrhea (King, 1957; Kist, 1985; Altekruse et al., 1999). King et al. (1962), demonstrated that *V. fetus* was thermotolerant, differentiating it from *V. jejuni* and *V. coli*. The following year, a new genus, *Campylobacter* was proposed in 1963 based on a study conducted by Sebald and Veron (1963). They were able to show differences in the *Vibrio* like organism based on its unique morphology, low DNA content, microaerobic growth requirements, and non-fermentative metabolism (Sebald and Veron, 1963). In 1972, *Campylobacter* was grown from stool samples of human patients with diarrhea which initiated the development of selective media for *Campylobacter* (Kist, 1985; Altekruse et al., 1999). *Campylobacter* has since become well known as a major foodborne pathogen (Kaakoush et al., 2015).

3. Campylobacteriosis

Campylobacteriosis transmission to humans is considered a major foodborne disease, which is commonly associated with poultry products. According to the U.S. Center for Disease Control (CDC), there are approximately 1.5 million cases of *Campylobacter* infection that occur each year in the U.S. alone (CDC, 2019). Campylobacteriosis outbreaks lead to an economical cost between $1.3 to 6.8 billion dollars annually in the U.S (CDC, 2019). The disease occurs by ingesting *Campylobacter* cells, which can pass through the stomach and colonize the distal ileum and colon (Ketley 1997; Skirrow and Blaser 2000). The infectious dose is dependent on the individual host and can be caused by a dose as low as 800 colony forming units (CFU), triggering campylobacteriosis (Janssen et al., 2008; Hansson, 2018). Colony forming units are a measure of the number of bacterial cells in a sample with enumeration based on the premise that

each colony originates from a single bacterial cell (Stachura and Traver, 2011). *Campylobacter* induces illness in humans, by colonizing and attacking the intestinal lining of the human GIT tract (CDC, 2019). The average incubation period for the disease is approximately three days, with symptoms experienced over a period of two to five days (Kirby and Coudron, 1989; Skirrow and Blaser 2000). In many cases, symptoms include abdominal discomfort, diarrhea, fever, headache, and dizziness (CDC, 2019). This disease has the capability of infecting any age group causing *Campylobacter* gastroenteritis, with the highest rate of infection in the U.S. being in the over 65 age group (CDC, 2019). Older adults may be more susceptible because of anatomic changes, functional changes and reduces immune functionality caused by increased age (Yoshikawa, 2000). However, in developing countries, the peak incidence is in children under five years old (Coker et al., 2002).

Despite intense GIT symptoms, the progression of the infection to the blood stream is rare (Louwen et al., 2012). Therefore, campylobacteriosis exhibits a low mortality rate, with an estimated 100 deaths annually. Some individuals remain asymptomatic during infection, which may allow an opportunity for several secondary conditions associated with *C. jejuni* to occur. Over time, this disease can progress into various forms, such as Guillain–Barré syndrome (GBS), Irritable bowel syndrome (IBS), relapses of inflammatory bowel diseases (IBD), and sometimes reactive arthritis (Nachamkin et al., 1998; Facciola et al., 2017). Guillain–Barré syndrome is an autoimmune disorder of the peripheral nervous system in which the myelin of nerve fibers is lost, resulting in acute flaccid neuromuscular paralysis (Nachamkin et al., 1998). In the U.S., one to two persons per 100,000 are afflicted with GBS each year (CDC, 2020). Research has shown that one-third of GBS cases are associated with *Campylobacter* infections, while another third of the *Campylobacter* afflicted patients will develop GBS within two years (WHO, 2012). Persons who

develop GBS experience weakness of the limbs and respiratory muscles and areflexia (van den Berg et al., 2014). Over time, the weakness usually develops symmetrically and evolves over several days. Damage to myelin eventually leads to loss of axial, cranial, respiratory, and peripheral muscle activity, resulting in substantial immobility (van den Berg et al., 2014). Of those affected, 15% of the patients recover entirely, while 3 to 7% die due to complications (van den Berg et al., 2014). As time progresses, IBD, which includes Crohn's Disease and ulcerative colitis, is a chronic, episodic medical condition associated with abdominal pain and altered bowel habits (Reti et al., 2015). IBD occur in approximately 4% of campylobacteriosis infections (CDC, 2019).

Furthermore, *Campylobacter* has been implicated in the development of IBS but little is known about its pathogenic role (Spiller, 2017). Studies have shown that the severity of campylobacteriosis, such as bloody diarrhea, duration of illness, and hospitalization, are linked to developing IBS (Peters et al., 2021). Research has also demonstrated that one in 10 campylobacteriosis infections develop IBS, indicating that IBS can be species specific (Spiller, 2017). Moreover, it remains unclear whether different strains of *Campylobacter* can relate to an equivalent risk (Gripp et al., 2011). Currently, the lineages associated with IBS and underlying genetic drivers of *Campylobacter* spp. are still not well understood (Peters et al., 2021).

Lastly, some patients infected with campylobacteriosis may develop reactive arthritis. Reactive arthritis is a condition that causes joint inflammation, which can be triggered by gastrointestinal tract (GIT) infection (Gumpel et al., 1981; Hannu et al., 2002). The mechanism of pathogenesis of *Campylobacter* in arthritis is not fully understood, however, it has been shown to occur in 3 to 7% of campylobacteriosis infections. In the U.S., arthritis can be identified from

campylobacteriosis with an annual incidence of 4.3 per 10000 persons infected (Pope et al., 2007; WHO, 2012).

More than 70% of *Campylobacter* illnesses originate from poultry products, with approximately 98% of retail chicken meat containing *Campylobacter*, indicating poultry can serve as a major reservoir (Batz et al., 2012; Facciola et al., 2017). The consumption of raw and undercooked chicken may be one of the leading routes of sickness related to *Campylobacter*. In poultry processing facilities, whole poultry carcass rinses are routinely used as a sampling method for determining the presence of *Campylobacter*. Jorgensen et al. (2002) evaluated raw, whole chickens for *Campylobacter* and reported that 83% of the chicken carcasses were positive for *Campylobacter*. Furthermore, Wieczore et al. (2020) assessed the prevalence of *Campylobacter* on chicken carcasses from 2014 to 2018. Here, *Campylobacter* spp. was identified by culture plating and confirmed by PCR methods in 1,263 (53.4%) chicken carcasses. Poultry contamination is mainly associated with *C. jejuni* and *C. coli* species, which are responsible for most human infections (Kaakoush, 2015; Chon et al., 2017). According to several studies, *C. jejuni* is the leading cause of gastroenteritis worldwide (Acheson and Mishu Allos, 2001; Kaakoush et al., 2015). Furthermore, different *Campylobacter* strains may have very distinct virulence characteristics. Black et al. (1988) showed that of the 111 adult volunteers ingesting either *C. jejuni* A3249 or *C. jejuni* 81-176, only 18% became ill with *C. jejuni* A3249, while strain *C. jejuni* 81-176 caused 46% of volunteer illness. These findings suggest *Campylobacter* contamination is still a problem in poultry products and serves as an indication that infection and incidence differ depending on the type of species and strain present.

4. *Campylobacter* Metabolism

Campylobacter is a microaerophilic bacterium, which requires limited oxygen for active

growth (Hill et al., 2017). For optimal growth, preferred conditions are 5% O_2, 10% CO_2, and 85% N_2 (Hilbert et al., 2010; Facciolà et al., 2017). *Campylobacter* spp. grows best between 37° and 45 °C, (Hazeleger et al., 1995; Silva et al., 2011). It has been reported that *Campylobacter* spp. exhibit low survivability when the pH is below 4.9 and above 9.0 (Keener et al., 2005; Silva et al., 2011). Additionally, research has shown that *Campylobacter* can be masked by the food it contaminates, allowing it to survive stomach acid levels of pH < 4.9 for 30 to 60 min (Axelsson-Olson et al., 2010). Colony morphology is quite variable depending on the media, from a thick translucent white growth to diffused film-like transparent growth, which can become visible within 24 to 48 h of incubation (Davis et al., 2005). Differences in phenotypic morphology depend on the growth phase and environmental stress dealing with.

Campylobacter spp. lack common metabolic pathways necessary to process carbohydrate substrates, such as glucose (Thompson and Gaynor, 2008; Line et al., 2010; Hill et al., 2017). As an alternative, *Campylobacter* rely exclusively on amino acids or tricarboxylic acid cycle (TCA) intermediates for energy (Stahl et al., 2012; Gao et al., 2017). The inability to catabolize glucose differentiates *Campylobacter* from other GIT pathogens such as *Salmonella*, *Escherichia coli* and *Listeria monocytogenes* (Hofreuter, 2014). *Campylobacter* does not encode a glycolysis enzyme for phosphofructokinase, which accounts for the inability to catabolize glucose (Velayudhan and Kelly, 2002; Gao et al., 2017). However, *Campylobacter* possesses a highly branched electron transport chain, which enables respiration with multiple electron donor end products (Kelly, 2008; Guccione et al., 2009). *Campylobacter* can encode all the enzymes necessary for a complete oxidative citric acid cycle (Weingarten et al., 2009), making use of the byproducts from the citric acid cycle produced by other microorganisms in its environment which convert these intermediates to carbon dioxide, adenosine triphosphate (ATP), and

reducing equivalents (Hofreuter, 2014). Citric acid cycle intermediates such as pyruvate, fumarate, oxaloacetate, 2-oxoglutarate, succinate, and malate have also been reported as energy sources for *Campylobacter* growth (Vegge et al., 2009).

Key biochemical reactions for *Campylobacter* spp. include the reduction of citric acid metabolites fumarate to succinate, acetoin, and indole that allows *Campylobacter* to utilize an anaerobic electron transport chain (Ursing et al., 1994; Kelly, 2001). Fumarate and succinate are crucial intermediates of *Campylobacter* energy conservation because it possesses the cytoplasmic fumarate reductase FrdABC that can oxidize succinate to fumarate and catalyze the reverse reaction (Weingarten et al., 2009; Stahl et al., 2012). Succinate acts as a carbon and energy source for *Campylobacter* and serves as an electron donor. Research has shown that *Campylobacter* spp. thrive in the presence of amino acids. The amino acids are utilized in preferential order with aspartate and serine catabolized first, followed by asparagine and glutamate (Hofreuter, 2014; Choi and Coloff, 2019). These amino acids are ideal for *Campylobacter* growth as they are the most abundant amino acids in the avian and human lower intestines (Rowland et al., 2018). Glutamate and aspartate can be converted directly into 2-oxoglutarate and fumarate to feed *Campylobacter*'s TCA cycle. *Campylobacter* can also metabolize proline; however, it is not preferred and therefore only utilized when necessary (Leach et al., 1997). This is consistent with the poultry cecal niche for *Campylobacter* as Parsons (1984) reported that aspartate, glutamate, and proline make up most of the amino acids found in hen ceca. Also, there is a metabolic diversity that can be differentiated among *Campylobacter* strains. A study by Mohammed et al. (2004), demonstrated that the substrate oxidation profiles of 100 *Campylobacter* strains were determined by monitoring changes in dissolved oxygen tension using an oxygen electrode system. Of the *C. jejuni* strains tested, 91% were able to

oxidize α–ketoglutarate, succinate, fumarate and aspartic acid, while only 7% of *C. jejuni* strains were unable to metabolize α-ketoglutarate.

Hinton et al. (2006) studied the effects of organic acids (OA) on *Campylobacter* metabolism by comparing the growth response on a medium supplemented with selected OA to the growth response on commercially available media. *Campylobacter* was grown in a basal broth supplemented with an organic acid mixture of fumaric, lactic, malic, and succinic acids. Growth of cultures in basal broth and in basal broth supplemented with OA were compared to growth in Brucella broth, Mueller-Hinton broth, and liquid thioglycolate medium. Of the 15 *Campylobacter* samples, 10 yielded more bacterial cells in basal broth with the OA combination than in other broth media. In the other 5 *Campylobacter* isolates, there was no significant difference in growth between basal broth, basal broth with OA, Brucella broth, Mueller-Hinton broth, or liquid thioglycolate medium. Findings indicated that media supplemented with OA could be used as alternatives to some of the currently available commercial media for *Campylobacter* spp. growth (Hinton, 2006). Nonetheless, a better understanding of how *Campylobacter* spp. utilize the TCA cycle may help to identify metabolic pathways crucial for growth and survival. In addition, differentiating the amino acids based on growth and/or energy sources of *Campylobacter* spp. could potentially decipher the intricate metabolic diversity associated with specific *Campylobacter* strains.

5. Media for culture plate Detection

Campylobacter growth implies the division of a bacterial cell, resulting in multiplication of the cell number (Margolin 2005; Wang and Levin 2009). Measuring and observing growth can be challenging since growth and death rates can be identical due to other background microorganisms competing for the same nutrients (Axelsson-Olsson et al., 2006; Stahl 2012).

Bacterial growth is measured by the increases in the cell population, which are assessed in two ways; measuring the increase in cell number or the increase in cell mass (Jay, 2000; Todar, 2012).

For *Campylobacter* detection, the USDA-FSIS recommended agar plate Campy-Cefex, but this approach requires at least 10^2 bacterial cells present for visible colony detection from pure cultures (Persson and Olsen, 2005). In a study by Hazeleger et al. (2016), when measuring growth using a turbidimetric method, required 6 to 7 h incubation at 41.5 °C before there was sufficient change in medium turbidity for a detectable measurement of *Campylobacter* growth (Hazeleger et al., 2016). Under unfavorable growth conditions, *Campylobacter* also can form VBNC cells (Portner et al., 2007). Viable but non-culturable cells led to no growth, resulting in the inability to detect *Campylobacter* cells.

The USDA-FSIS recommends qualitative methods for *Campylobacter* detection of contaminated poultry products (USDA, FSIS, 2016). After samples are collected, they are enriched in Hunt broth for 48 h, plated on selective growth media, and further incubated for 48 h (USDA, FSIS, 2022). Colonies are confirmed using phase contrast microscopy and latex agglutination (USDA, FSIS, 2022). This method of growth and detection is generally preferred over latex agglutination because it is inexpensive, as well as less time consuming since plating is more rapid than microscopy and latex agglutination (Hill et al., 2017). In general, the ability to reduce time and cost while limiting the growth of background microorganisms must be considered in order to optimize detection methods.

Currently, existing culture media have not always been consistent for the growth and detection of *Campylobacte*r. For example, Buss et al. (2019), collected 1552 patient fecal specimens that were initially categorized as positive or negative by traditional culture. These

patient stool samples were then compared to non-cultural methods, including 16S rRNA gene qPCR, eight species-specific PCR assays, bidirectional sequencing, and a U.S. Food and Drug Administration (FDA)-cleared multiplex PCR panel. Compared to the five molecular methods, traditional *Campylobacter* culture failed to correctly detect the organism in 30% of positive samples (Buss et al., 2019). Therefore, alternative methods for detection need to be explored to improve the sensitivity and accuracy of *Campylobacter* from various sample matrices. More accurate results can lead to better diagnosis and treatment of suspected campylobacteriosis infections.

Any development and improvement of alternative detection methods must consider the metabolism of *Campylobacter* and its ability to survive in unfavorable conditions. Not all metabolic processes of *Campylobacter* are known, therefore, characterizing the complete metabolic profile of *Campylobacter* can lead to construction of culture media with optimized nutrient availability. Understanding *Campylobacter*'s metabolism should prevent misdiagnosis and false negatives of *Campylobacter* due to difficulties in culturing with currently available media.

Culture media play an essential role in *Campylobacter'*s detection by influencing growth. Seliwiorstow et al. (2016), demonstrated the impact of culture medium on the recovery of *Campylobacter* from fresh and frozen poultry meat samples. In their study, after sampling poultry products, *Campylobacter* was analyzed using enrichment in Preston broth, and enrichment in modified Bolton broth (supplemented with potassium clavulanate, triclosan, polymyxin B). The enrichment cultures were streaked onto both modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) and Rapid *Campylobacter* agar (RCA). Both fresh and frozen poultry meat samples, resulted in a greater number of *Campylobacter* detected in

samples enriched with modified Bolton broth than Preston broth (Seliwiorstow et al., 2016). When comparing media, using RCA resulted in a higher detection rate compared to mCCDA. The results suggested that the type of media used can affect the growth and sensitivity of *Campylobacter* spp. detected.

Water activity has also been shown to exert an inhibitory effect on *Campylobacter* growth, resulting in reduced detection. For example, Casaere et al. (2003), attempted to estimate the length of time over which one could reasonably expect pathogen survival and hence the potential for cross contamination. A five-strain pool of *C. jejuni* was suspended in either a phosphate-buffered saline solution or Trypticase soy broth and inoculated on 5 cm^2 samples of Formica laminate, glazed ceramic tiles, polished stainless steel and 100% cotton dishcloths. Samples inoculated with *C. jejuni* were not able to grow with a water activity concentration less than 0.987 (Cesare et al., 2003). The results from this study suggest a relationship between water activity level and *C. jejuni* growth. This study demonstrates that some *Campylobacter* spp. are unable to grow at low water activity levels.

There are a variety of laboratory approaches to measure growth. Traditional microbiological bacteria quantification includes methods such as plate enumeration (CFU) and measuring optical density (OD) through a spectrometer (Pan et al., 2014; Wilson et al., 2017). These conventional culture methodologies remain a mainstay for *Campylobacter* quantitation, as they have their own advantages. Using traditional culture methods allows for viable cell recovery, which can be used for phenotyping through plating and imaging. These methods are also needed for some molecular approaches because *Campylobacter* cells require multiplication via enrichment media prior to reaching high enough detection levels for molecular assays such as

PCR detection. Thus, consistency for culture approaches for enrichment to support microbiological and molecular detection methods are important.

6. Growth Media

Campylobacter requires a microaerophilic atmosphere for active growth (Hill et al., 2017). Therefore, media must be nutrient rich, containing oxygen quenching agents and, in the case of mixed populations, antibiotics to reduce background microorganisms (Solis-Soto et al., 2011). Variation among different media make it difficult to quantify *Campylobacter*, specifically to elucidate metabolic mechanisms (Nachamkin, 2000). For *Campylobacter* growth, direct plating and enrichment in liquid broth are primary methods employed depending on the sampling technique and the numbers of *Campylobacter* cells present (Gonsalves et al., 2016). For example, when isolating *C. jejuni* from chicken fecal samples for quantification, *C. jejuni* is expected to be at population levels above 2.00 Log_{10} CFU/mL, thus, these types of samples can be directly plated onto *Campylobacter* agar (Musgrove et al., 2001; Sahin et al., 2003). Yet, *Campylobacter* spp. are unlikely to be present at these population levels (0.00 to 2.00 Log_{10} CFU/mL; Tomasiewiscz et al.,1980). Consequently, *Campylobacter* sampling should be enriched in media before plating them onto agar for isolation (Rivoal et al., 2005).

Since *Campylobacter* is a fastidious organism, challenges still exist for detection. Sensitivity to environmental stress and external factors such as temperature and competing microorganisms, can prevent growth and detection, therefore it is essential for media to stimulate optimal conditions for *Campylobacter* (McMahon et al., 2007). The following sections will discuss commercial media used for *Campylobacter* growth and detection.

6.1 Habitat Simulating Media effects on *Campylobacter* growth

When culturing *Campylobacter*, there are several morphological forms that *Campylobacter* can generate and subsequently be identified visually. These morphological forms may be influenced by environmental stresses. When pathogenic bacteria such as *Campylobacter* are released from their hosts into different environments, they are often challenged by various environmental stresses, such as nutrient availability, osmotic shock, temperature variation, pH, and oxidative stress (Lin et al., 2009; Pogacar et al., 2009). Young cultures, particularly in the early log phase will be rod-shaped forms, including spiral, S-shaped cells (Reezal et al., 1998). However, coccoid forms are usually non-culturable and occur in the stationary or death phase. In addition, rods transform to coccoid forms when conditions are unfavorable for growth (Kelly et al., 2001). Decreasing temperature and nutrient availability in media result in a reduction of rod-shaped bacterial cells to viable but non-culturable coccoid cells (Hazeleger et al., 1995).

As *Campylobacter* cells age in broth or on agar, they take on different forms and many of those forms are smaller in size than those in the original culture. There are two mechanisms that can cause a bacterium to become reduced in size (Byrd, 2000). One way could be that multiple *Campylobacter* cells do not increase in size before dividing but subsequently become smaller after each division coupled with the transfer of at least one copy of the genome (Byrd, 2000; Dewachter et al., 2018). *Campylobacter* cells can also be reduced in size when insufficient nutrients are available for cell division. Cell size is determined by how much energy is required and a lack of nutrients can lead to decreased cell size until death occurs (Byrd, 2000; Bjorklund, 2019). Either of these processes will cause *Campylobacter* cells to lose some of their signature identifiable characteristics that they once possessed and in turn, become undetectable on culture media. When cells become reduced in size and unable to transfer their genetic material, the culture is considered to have lost viability.

In vitro work examining *Campylobacter* spp. cell growth has been shown to be influenced by environmental alterations, such as exposure to light and air. Hoffman et al. (1979) observed that the growth of *Campylobacter* spp. was substantially reduced when nutrient media plates were stored in the presence of light and air (Hoffman et al., 1979). These environmental changes can produce toxic factors, free radicals and hydrogen peroxide which can cause injury and negatively impact *Campylobacter* growth (Hofreueter, 2014). Media for *Campylobacter* spp. usually contain ingredients to neutralize the toxic effect of substances that are formed in the presence of oxygen and light (Corry et al., 2003). Oxygen quenching agents used in media can significantly improve the recovery of *Campylobacter* by protecting it from harmful agents such as light and oxygen (Oyarzabal et al., 2005). The employment of oxygen quenching agents such as blood, hemin, charcoal, ferrous sulfate, sodium pyruvate and sodium metabisulfite can be added to *Campylobacter* media to improve growth.

Media storage is also critical for *Campylobacter* growth as it may undergo unfavorable changes if not stored correctly. When preparing agar plates, plates must not be air dried for more than 20 minutes after solidification at room temperature to avoid dehydration (Wu, 2008). It is recommended that plates should be used immediately or stored anaerobically, in the dark at room temperature. *Campylobacter* agar plates can also be stored in sealed bags in a cold room (4 to 10˚C) for up to a month before use (Mialon et al., 2012). However, prepared media absorbs oxygen during storage therefore, fresh media should be used when possible.

As *Campylobacter* is removed from its environment and handled as a laboratory culture it becomes susceptible to osmotic stress when transferred to an enrichment medium. Osmolarity is a measure of solute concentration in a solution (Cameron et al., 2012). When grown in liquid media, *Campylobacter* may be hyperosmotic or dehydrated with low osmolarity in agar

depending on the medium. These changes in osmolarity will cause substantial stress on cells causing them to swell or shrink (Burgess et al., 2016). Further, *Campylobacter* has been shown to be more sensitive to NaCl as compared to other pathogenic GIT pathogens such as *E. coli* and *Salmonella*, which can survive for long periods when exposed to 30% NaCl (Doyle and Roman 1982; Kempf 1998; Wood 1999; Cameron et al., 2012). *Campylobacter* is incapable of survival when growth media is supplemented with NaCl above 2% due to osmotic stress; as water leaves *Campylobacte*r, dehydration of the cytoplasm occurs, and turgor is reduced because *Campylobacter* is unable to adapt to these conditions (Parkhill et al. 2000). Consequently, NaCl is kept at minimal to absent levels in selective media such as Mueller-Hinton and modified Charcoal Cefoperazone Deoxycholate (Hofreuter et al., 2012; Trigui et al., 2015). *Campylobacter* can also become stress when exposed to low osmotic environments when grown in liquid media without sufficient nutrients (Reezal et al., 1998; Brandl et al., 2004). Moran and Upton (1986) reported that low osmolarity in *C. jejuni* cells resulted in leakage of the cytoplasm, which caused degradation of cellular components. This insinuates that *Campylobacter* has a threshold of osmolarity needed for survival in media.

Since *Campylobacter* is a facultative intracellular pathogen that possesses restricted catabolic pathways, media must be supplemented with nutrients to support growth. In general, *Campylobacter* spp. are asaccharolytic and are unable to utilize carbohydrates for growth, forcing them rely on amino acids (Bronowski et al., 2014). The survival of *Campylobacte*r has been shown to be affected by the nutrient concentration available in media (Thomas et al., 1999). Limited nutrient availability to their cells can lead to reduced cell size, changes in morphology and shifts in protein synthesis (Cesar and Huang, 2017; Westfall and Levin, 2018). Lack of nutrients is one of the most common environmental stresses which *Campylobacter* spp.

encounter. Mihalievic et al., (2007) conducted a study to evaluate the effect of selected environmental stress factors: temperature shift, starvation, and atmospheric oxygen concentration on the ability to cultivate *C. jejuni* and the viability of two *C. jejuni* isolates. The results of these studies show that nutrient deprivation led to fewer culturable *Campylobacter* cells as compared to exposure to temperature shifts such as heat stress (55 °C) and oxidative stress (atmospheric oxygen exposure). Media is typically supplemented with peptones and dextrose to provide *a* source of energy for growth (Lagier et al., 2015). The peptones provide nitrogenous compounds, carbon, sulfur, and trace ingredients, while dextrose is utilized as an energy source. Currently, various media are available for *Campylobacter* growth and detection, thus the efficacy for each medium must be elucidated.

6.2 Selective Media for *Campylobacter* Growth

Since *Campylobacter* is a fastidious bacterium, it can be difficult to grow when taken from samples that contain a high diversity of microorganisms. *Campylobacter* spp. multiply much more slowly than other enteric bacteria, therefore there can be competition for nutrients among the *Campylobacter* and non-*Campylobacter* bacteria (Allos, 1998; Blaser, 2000). To combat this problem, selective media were developed to inhibit the growth of more rapidly growing, competing microorganisms (Ber et al., 2003). Selective media usually contain a combination of antibiotics which limit growth of other competing microorganisms, while supporting the growth of *Campylobacter* cells.

6.2.1 Skirrow's Medium

In 1972, Butzler medium was the first medium documented to isolate *Campylobacter* on agar plates in 1972 (Butzler, 1978). However, because of the stringent nutritional metabolism of *Campylobacte*r, this medium was not selective for *Campylobacter* growth. It was long thought

that *Campylobacte*r spp. was a rare bacterium, primarily because the metabolism was poorly understood (Acheson and Mishu Allos, 2001). As a result, after isolating *Campylobacter* from human cases in 1972, the development of specific culture media was needed for the detection of specific microorganisms from infected human samples to identify the causative agent of infections (Altekruse et al., 1999; Butzler, 2004). In 1977, Skirrow, developed a selective agar (Skirrow's medium) and successfully grew *Campylobacter* from human feces (Skirrow, 1977). Fecal samples from patients without diarrhea were also used to test this medium's sensitivity and resulted in no *Campylobacter* growth on Skirrow's agar. Skirrow's medium contains proteose peptone, liver digest, yeast extract, sodium chloride (Skirrow, 1977). The proteose peptone facilitates growth by providing essential nutrients such as carbon, nitrogen, and sulfur sources to support *Campylobacter* cell growth (de Souza et al., 2015). Yeast extract and liver digest were subsequently added to provide trace vitamins and micronutrients to support *Campylobacter* metabolism (Lewis, 1992; Bonnett et al., 2019). In addition to the nutritional components, selective agent vancomycin, polymyxin B, trimethoprim, and lysed horse blood were added to the medium. Vancomycin was added because it inhibited Gram-positive bacteria, polymyxin B inhibited most Gram-negative bacilli except *Proteus* and trimethoprim was also inhibitory to *Proteus* spp. (Rivera et al., 2011; Poirel et al., 2017). Five percent sheep blood was also supplemented because *sheep* blood contained hemin, a source of the iron necessary for *Campylobacter* cells to metabolize and grow (Yeh et al., 2009). As a result, Skirrow's medium has been used as a selective agar for *Campylobacter* growth and detection.

Initially, Skirrow's medium proved to be useful for identifying *Campylobacter* infections through human feces (Skirrow, 1977; Skirrow and Benjamin, 1980). However, when *Campylobacter* spp. were grown from animal and environmental samples,

Skirrow's medium appeared to not be selective for *Campylobacter* exclusively (Bolton and Robertson, 1982). Bolton and Robertson (1982) evaluated the use of Skirrow's medium for detecting *Campylobacter jejuni/coli* from human, animal, avian and environmental samples. Fifty strains were isolated from human feces, 10 from cattle rectal swabs, 23 from pig cecal swabs, ten from chicken cloacal swabs, 10 from seagull feces, and one reference strain of *Campylobacter jejuni* (NCTC 11385) were used throughout the study (Bolton and Robertson, 1982). All samples were enriched in Skirrow's medium for 48 h at 43 °C. Following enrichment, samples were plated on Skirrow's agar and incubated for 48 h at 43 °C in an atmosphere containing approximately 6 % (vol/vol) oxygen, 10 % (vol/vol) carbon dioxide, and 84% (vol/vol) hydrogen (Bolton and Robertson, 1982). After incubation, plates were evaluated phenotypically for the presence of *Campylobacter*. The results revealed that Skirrow's agar was not selective for *Campylobacte*r in avian, animal, and environmental samples, as the agar also supported pseudomonads, coliforms, fungi and *Bacillus* spp. colonies (Bolton and Robertson, 1982). Furthermore, in a study by Bi et al. (2012) they compared the detection efficacy of six methods used for *Campylobacter* growth from raw meat samples. Ninety- nine meat samples were enriched in Bolton broth and Preston broth, for 48 h at 42 °C. Enriched samples were subsequently subjected to a 10-fold serial dilution in the respective broth and plated on Skirrow's agar, mCCDA, and blood agar filtration media (Bi et al., 2012). The results demonstrated that Skirrow's medium was significantly less efficient than mCCDA and a membrane filtration method for the presence of *Campylobacter* colonies (Bi et al., 2012).

6.2.2 Preston Medium

Since Skirrow's medium was not selective in exclusively growing *Campylobacter* from animal and environmental samples, there remained a void for a selective media for detection

(Bolton and Robertson, 1982). Bolton and Robertson (1982) modified the ingredients of Skirrow's medium and proposed a selective medium referred to as Preston medium. The Preston medium was designed to incubate samples with the selective agents leading, in turn, to an improvement in isolation capabilities (Batt, 2014).

The Preston medium is supplemented with 5% saponin-lysed horse blood and contains polymyxin, rifampicin, trimethoprim, and amphotericin B (Bolton and Robertson, 1982). Polymyxin was added because of its ability to inhibit Gram-negative bacteria except for *Proteus* spp., which were suppressed by adding trimethoprim (Poirel et al., 2017). Since rifampicin exhibited a wide spectrum of activity against Gram-positive and Gram-negative bacteria it was chosen instead of vancomycin, which is prevalent in other media but has limited activity against Gram-negative organisms. In addition, amphotericin B was added to inhibit fungal growth (Murinda et al., 2006). Based on this selectivity, many of the contaminating organisms could be eliminated, which is critical since their existence could mask the presence of *Campylobacter*.

For *Campylobacter* detection from food samples with a high level of background microbiota (e.g., raw meats, raw milk, frozen poultry meat), Preston broth is used to support selective growth. Several studies have been performed comparing Preston broth efficacy for *Campylobacte*r growth and detection. Scates et al. (2003), studied the effect of incubation temperature on the growth of *C. jejuni* from food products using Preston broth. A total of 24 raw chicken and 30 raw lamb liver samples were used in the study. Samples were incubated in Preston broth at 37 °C and 42 °C and subsequently streaked onto Preston agar and incubated again at 37 °C and 42 °C. Results of this study demonstrated that incubation temperature had no significant effect on the number of positive samples detected or on the species grown (Scates et

al., 2003). Therefore, the incubation temperatures for *Campylobacter* growth in Preston broth were suggested to be set at either 37 or 42 °C.

Additionally, Preston broth has been recommended as a first step to improve *Campylobacter* detection in food samples. Habib et al. (2011) compared Preston broth against Bolton broth and Campyfood broth (Biomeriuex, 100 Rodolphe Street Durham, NC 27712) for *Campylobacter* spp. growth in naturally contaminated chicken meat. After incubation under microaerophilic conditions, samples were plated on mCCDA, CampyFood agar and Brilliance CampyCount agar. A total of 49 meat samples were enumerated for *Campylobacter* in the experiment. The results of this study showed that after 24 h of incubation, Preston broth followed by plating on mCCDA yielded a higher number of positive samples (20/49) compared to Bolton broth samples (15/49). These findings suggest Preston broth, in combination with agar media could provide significantly better detection and enumeration of *Campylobacter* in chicken meat (Habib et al., 2011).

In contrast, research has shown other media as being more effective than Preston broth for *Campylobacter* growth. Jasson et al. (2009), two *C. jejuni* strains were grown in Bolton broth and Preston broth, then incubated for 24 h at 37 °C under a micro-aerobic atmosphere. After incubation, all samples were plated on mCCDA plates for enumeration. The results observed both *C. jejuni* strains yielded a higher concentration when grown in Bolton broth, as opposed to in Preston broth. Additionally, Strakova et al. (2021), conducted a study on the efficacy of enrichment media Bolton broth and Preston broth for *Campylobacter* growth from water samples. A total of 36 water samples were collected from a commercial plant wastewater treatment. After collection, all samples were enriched for 44 to 48 h, then followed by spread plating on mCCDA and another 48 h incubation period. After incubation of the plates, the results

show Bolton broth recovered growth in 22% of the water samples while Preston broth recovered growth in only 14%. The result from these studies demonstrates Preston to be a sufficiently efficient medium for the growth of *Campylobacter*, however, further improvements and refinements on media to support *Campylobacter* continued to be developed.

6.2.3 Campy-Line Agar

Campy-line agar (CLA) is a selective medium developed in 2001 by J. Eric Line. CLA is comprised of casein, meat peptone, sulfur, sodium, yeast extract, and sheep blood (Line, 2001). Casein and meat peptone provide nitrogen, carbon, and sulfur, which are essential nutrients for *Campylobacter* (Abbasiliasi et al., 2017). Sodium is a source of essential electrolytes and maintains osmotic equilibrium (Shrimanker and Bhattarai, 2022). The yeast extract supplies B vitamins to the *Campylobacter* cells (Lewis, 1992). Sheep blood provides hemin and other necessary growth factors (Egwuatu et al., 2014). Selective antibiotics supplemented in this medium include trimethoprim, polymyxin B, cycloheximide, cefoperazone and Triphenyltetrazolium chloride (TTC). TTC is used to distinguish between subspecies of *Campylobacter*. *Campylobacter* spp. possess the ability to reduce the initially colorless tetrazolium salts to insoluble formazan compounds, which gives a red color to the growing colonies. The ability to produce red colonies allow them to be differentiated on the agar plate (Line, 2001). When grown, colonies typically exhibit phenotypic traits such as a shiny, moist appearance and are slightly raised, whereas non-*Campylobacter* contaminates have a dry, waxy appearance (Line, 2001). The uptake of TTC is generally uniform throughout colonies, resulting in a deep-magenta to burgundy-colored colonies on the plate (Line, 2001).

The first study documented using the CLA was by Line in 2001. The objective of this study was to compare the quantity of *Campylobacter* spp. colonies grown between CLA and

CLA with blood and Campy-Cefex agar from poultry carcass samples (Line, 2001). Freshly processed broiler carcasses (n=25) were obtained from a commercial processing plant for analysis. 400 ml of sterile buffered peptone water was added to each carcass in its respective sampling bag to obtain samples. The carcasses and buffered peptone water were agitated for 2 min, then a 200 ml portion of the rinse was carefully poured off into an individual sterile sample container. For plating, 0.1 ml from each original carcass rinse were spread plated on the respective medium. All plates were incubated in microaerophilic conditions for 36 to 48 h at 42 °C. Colonies were subsequently inspected for *Campylobacter* morphological characteristics. The results from this study demonstrated that the growth of *Campylobacter* populations from poultry carcasses was not significantly different on either CLA, CLA with blood and Campy-Cefex. Oakley et al. (2012) compared the efficacy of *Campylobacter* colonies on commonly used cultivation plating on CLA, Campy-Cefex agar, mCCDA, Karmali agar, CampyFood ID agar (CFA) and Campy-CVA agar (CVA). Fecal samples were collected from a commercial broiler chicken production house. Samples originated from 25 locations within a semi-regular grid covering the entire house to represent the entire flock. Fecal samples were pooled from the 25 locations. From pooled fecal samples, serial dilutions from 10^0 to 10^{-6} were plated in duplicate on each media. All plates were incubated, and the resulting pooled colonies were 16S rRNA gene sequenced for identification of *Campylobacter* spp. The results from this study revealed mCCDA was the most selective (only 2.4 % of sequences were not *Campylobacter*), followed by Cefex at 3.2 % and CLA at 7.7 % (Oakley et al., 2012). These studies suggest that CLA can support selective growth of *Campylobacter*, however more research is needed to identify the non-*Campylobacter* isolates that also come up on these selective media.

6.2.4 Campy-Cefex

Campy-Cefex is a selective plate media used for *Campylobacter* detection. Stern et al. (1992) developed Campy-Cefex Agar to grow *C. jejuni* and *C. coli* from chicken carcasses. Campy-Cefex includes peptone, yeast extract, dextrose, NaCl, ferrous sulfate, sodium bisulfite, sodium pyruvate, alpha-ketoglutaric acid, sodium carbonate, and is supplemented with laked horse blood, cefoperazone, and cycloheximide (Oyarzabal et al., 2004). The high concentration of cycloheximide in Campy-Cefex inhibits the growth of other microorganisms frequently associated with poultry products (Stern et al., 1992; Oyarzabal et al., 2004).

Campy-Cefex has recently been used as a selective agar for *Campylobacter* spp. detection. When grown on Campy-Cefex, *Campylobacter* colonies appear small, approximately one to two mm in diameter, compared to other foodborne pathogens such as *Clostridum botulinum*, resulting in large colonies greater than five mm (Nannapaneni et al., 2005). *Campylobacter* cells are mucoid, grayish, flat colonies with irregular edges and no hemolytic patterns (**Figure 1**). They can appear curved or spiral-shaped round, while glistening (Mushi et al., 2014). Colonies typically appear at 24 to 48 h, depending on the species as *C. jejuni* appears earlier than *C. coli* on agar plates (Chon et al., 2012).

In 2005, Campy-Cefex Agar was adopted by the National Advisory Committee on Microbiological Criteria for Foods for *Campylobacter* spp. detection from chicken carcasses (NACMCF, 2005). Currently, Campy-Cefex is a commonly used commercial media for *Campylobacter* studies (Berrang et al., 2016). However, there is contradictory evidence about the efficacy of Campy-Cefex. In a study to compare the growth efficacy of *Campylobacter* colonies, Line (2001) compared CLA, Campy-Line blood agar (CLBA) media, and Campy-Cefex to assess the most effective media for detection via direct plating. CLA and CLBA media were able to recover *C. jejuni* and *C. coli* populations with minimal background growth (Line, 2001).

However, less *Campylobacter* was recovered with Campy-Cefex medium with a greater amount of background non-*Campylobacter* contaminants (Line, 2001). In contrast, Sylte et al., (2018) used Campy-Cefex, CLA with sulfamethoxazole (CLA-S), and CHROMagar *Campylobacter* (CAC) to detect *Campylobacter* from turkey poults. *Campylobacter* colonies were observed on the Campy-Cefex, CLA-S, and CAC media with limited background colonies (Sylte et al., 2018). The results of these studies indicated that the contrasting efficiency of Campy-Cefex for the detection of *Campylobacter* in various matrices. (Sylte et al., 2018).

In a study by Kim et al. (2017), the authors investigated the selectivity of *Campylobacter* colonies from Campy-Cefex plates. The study investigated microbial communities between chicken carcass rinsates of recovered bacteria from Campy-Cefex. Chicken carcass rinsates were collected from 10 birds at the bleed out tunnel, picker, evisceration, and chiller. All birds were processed similarly and removed from their respective sampling points. Each chicken carcass was transferred to a sterile bag and 400 ml of sterile buffered peptone water and manually shaken for 2 min assuring that all surfaces in- and exterior of the carcass were rinsed (Kim et al., 2017). After which, 250 rinsate samples were spread plated on four Campy-Cefex plates and incubated at 42 °C for 48 h under microaerophilic conditions. Birds were collected from each processing step, and rinsate samples were inoculated onto the plates. Following microaerophilic incubation, colonies on the Camp-Cefex plates were predominantly *Campylobacter*. However, non-*Campylobacter* bacteria, such as *Lactobacillus and Enterococcus,* were also identified in the16S rDNA microbiome sequencing analysis at varying percentages in the Campy-Cefex selective media. Even though Camp-Cefex could still recover *Campylobacter*, it appears to not fully inhibit the growth of other microorganisms. It remains to be determined if this is a problem for other *Campylobacter* spp. or is only occurring with *C. jejuni*.

6.2.5 Charcoal Cefoperazone Deoxycholate Medium

The first blood free selective media for *Campylobacter* was developed by Bolton et al. (1984). This selective medium was referred to as Charcoal Cefoperazone Deoxycholate medium (CCD), with ingredients that include charcoal, ferrous sulfate, sodium pyruvate, casein hydrolysates, cefazolin, and sodium deoxycholate. CCD is typically used as an agar for detection, allowing direct plating for rapid and reliable recovery of *C. jejuni* (Acke et al., 2009; Ugarte-Ruiz et al., 2015). When compared to Preston medium, CCD medium exhibited similar growth responses to Preston media, however CCD agar was less selective (Bolton et al., 1984). A more recent blood free modified CCD (mCCD) was proposed by Karmali et al., (1986), by adding selective agents (Karmali et al., 1986; Oyarzabal et al., 2005). The mCCD medium contains nutrient broth, bacteriological charcoal, casein hydrolysate, sodium deoxycholate, ferrous sulfate, sodium pyruvate, agar and supplemented with selective agents cefoperazone and amphotericin B (Oyarzabal et al., 2005). These improvements to the modification in mCCD have been reported to enhance the recovery of *Campylobacter* spp. (Chon et al., 2012).

The mCCDA agar is a common *Campylobacter* medium utilized typically for selective plating (Berrang et al., 2016; Kim et al., 2019). On these plates, colonies are greyish, flat, and moist, often with a metallic sheen, smooth with film-like transparency (**Figure 2**). The International Organization for Standardization (ISO) protocol for *Campylobacter* detection and enumeration suggests that Bolton broth and mCCDA plates be used for growth and detection (ISO 10272: 2006). There are several studies that compared mCCDA plating of *Campylobacter* to other selective plating. In a study by Potturi-Venkata et al. (2007), they grew *C. jejuni* from cecal and fecal samples obtained from 60 broiler chickens. *C. jejuni* samples were plated on four different plate media (mCCDA, CLA, Campylobacter agar plates (CAP), and *Campylobacter*

base agar). According to the authors, mCCDA agar was the most efficient at detecting *Campylobacter* compared to the CLA, CAP, and *Campylobacter* agar base. Peterz (1991) conducted a trial among six laboratories that tested chicken liver that had been artificially inoculated with strains of *C. jejuni* and compared the recovery performance of mCCDA and Preston agar. From the results of this study, mCCDA supported less growth of contaminating microbiota, exhibiting more selectivity for *Campylobacter*. In a similar experiment, Oyarabal et al., (2005), evaluated 240 broiler carcass rinse samples for *Campylobacter* recovery using Campy-Cefex, mCCDA and CLA agar plates. Based on the results, this study concluded that Campy-Cefex and mCCDA agar resulted in distinct *Campylobacter* colonies that originated from carcass rinse samples.

6.2.6 Trypticase Soy with 5% Sheep Blood Agar (SBA)

Another enrichment medium, recommended by USDA-FSIS is Tryptic Soy Agar with 5% sheep blood (SBA). TSA w/5% Sheep Blood is a general purpose medium for the growth of a wide variety of organisms. Tryptic Soy Agar w/5% Sheep Blood is used for cultivating fastidious microorganisms and for the visualization of hemolytic reactions produced by many bacterial species (Burton, 2005). A 5% sheep blood aliquot is added to TSA because *sheep* blood provides hemin and supplies other necessary additional growth factors such as optimal ion and salt concentrations, and nutrient content.

Several studies have explored the efficacy of SBA as a growth medium for *Campylobacter* detection and enumeration. In an experiment by Krajden et al. (1987), they compared the efficacy of recovery of *C. pylori* with 5 % SBA and Skirrows media. The ability of both media to support the growth of *Campylobacter* was tested by examining viable counts of suspensions of three strains of *C. pylori*. All strains were plated and incubated

microaerophilically at 42 °C for 5 days on SBA and Skirrow's media. The respective growth of three strains of *C. pylori* on Skirrow's agar was 1.2×10^7, 2×10^7 and 4×10^6 compared to on SBA 1.5×10^7 CFU/ml, and 1.6×10^7 CFU/ml, and 4×10^6 CFU/ml. The results indicated no numerical difference between SBA and Skirrow agar when compared (Krajden et al., 1987). In this study, Krajden also noted the colonies on Skirrow's agar were slightly larger and easier to visualize than on SBA.

Another comparison study was done by Salim et al. (2014), examining the efficacy of *Campylobacte*r enrichment media SBA, mCCDA, and chocolate agar. The objective of this study was to detect *C. jejuni* from the stool specimens on routinely available blood containing laboratory media using candle jars for creating microaerophilic atmospheric conditions. A total of 50 stool samples were inoculated onto SBA, mCCDA and chocolate agar. Of these 50 stool samples, 10% contained *C. jejuni*, of which, 12.12% came from male subjects. Amongst all the media tested, the best colony morphology was observed on the SBA and mCCDA medium, while colonies were smaller on chocolate agar (Salim et al., 2014). However, a caution to using SBA is that there are opportunities for contamination with other facultative anaerobes, which may not be present in the selective media. The findings from these studies show variable results for *Campylobacter* detection. Nonetheless, the development of SBA represented further progress for media isolation and identification of *Campylobacter* in food safety studies (Salim et al., 2014).

6.2.7 Karmali Medium

Due to the efficiency of replacing blood with charcoal, Karmali et al. (1986), proposed a new blood-free, charcoal-based selective medium for *C. jejuni* and *C. coli* growth. The presence of charcoal in the medium helped neutralize the toxic metabolic products formed during

incubation to improve *Campylobacter's* utilization of nutrients. The Karmali *Campylobacter* agar base contains charcoal, hematin, and cornstarch to serve as sources of essential nutrients required for bacterial metabolism (Karmali et al., 1986). Antibiotics such as cefoperazone, vancomycin, sodium pyruvate, and cycloheximide, are recommended for selective growth. Sodium pyruvate was used to create a microaerophilic environment for *Campylobacter* by quenching the toxic forms of oxygen (Hoffman et al., 1979). Vancomycin was added to suppress Gram-positive organisms while cycloheximide has been shown to inhibit the contaminating fungal populations in animal feeds as an additional amendment for non-selective and selective *Salmonella* media (Ha et al., 1995a,b). Cefoperazone exhibits inhibitory action on Gram-negative organisms other than *Campylobacter* (Chon et al., 2012).

However, contradictory results were obtained for the level of selectivity achieved by Karmali agar for *Campylobacter* detection. In a study by Mubarak et al. (2019), Karmali agar, Butzler selective agar and SBA were compared for their efficacy of *Campylobacter* detection. In the study, *C. jejuni* ATCC 33291 and *C. coli* were grown at 42 °C for 48 h in Tryptic soy broth containing gas generating sachets to create an atmosphere containing 5% O_2, 10% CO_2 and 85% N_2 to achieve pure culture growth. After 48 h, serial 10-fold dilutions were prepared from each *Campylobacter* spp., using phosphate buffered saline. The dilution series were plated on each medium, Karmali, Butzler and SBA. The results from the study revealed that Karmali agar plates yielded the most colonies compared to Butzler's agar and SBA plates (Mubarak et al., 2019). Additionally, these results indicate that Karmali agar could be a potentially improved medium for *Campylobacter* detection in pure culture.

When complex matrices such as food and environmental sources are sampled, the selectivity of Karmali agar can be challenged. In a study by Chon and others (2011), the authors

compared three selective media, mCCDA, Karmali agar, and Preston agar, for *C. jejuni* growth from artificially contaminated ground beef and fresh-cut vegetables. Food samples, ground beef and a mix of fresh-cut vegetables composed of cabbage, cucumber, carrot, and lettuce were inoculated with three different inoculum levels of *C. jejuni*: 91, 25, and 42 cells per 500 g of ground beef and 750, 635, and 690 cells per 500 g of fresh-cut vegetables. After inoculation, food samples were enriched in Bolton broth at 42 °C for 44 h and then streaked onto the respective media, followed by incubation under microaerobic conditions at 42 °C for 48 h. The results showed no statistical differences in sensitivities between the three selective media for ground beef and fresh-cut vegetables. However, Preston agar numerically contained more *Campylobacter* colonies than mCCDA and Karmali agar. The results also demonstrated that Karmali agar had a higher rate of contamination of *E. coli* (Chon et al., 2011).

6.3 Enrichment Media used for *Campylobacter* Growth

Growth and detection of *Campylobacter* from foods can be problematic because high levels of competing organisms may be present (Hill et al., 2017). Non-*Campylobacter* microorganisms that are present can out compete *Campylobacter* for available nutrients. When detecting *Campylobacter*, ISO protocol require using enrichment media to accomplish this. Enrichment media are designed to supplement nutrients that favor *Campylobacte*r growth when a low abundance of cells is expected in the initial sample.

6.3.1 Bolton Broth

Bolton broth is a selective enrichment medium commonly used by food regulatory agencies such as the U.S. FDA and USDA-FSIS personnel. Selective enrichment media are designed both to aid the recovery of injured cells and to avoid the need for a microaerobic atmosphere (Baylis et al., 2000). Bolton broth contains peptone and yeast extract, alpha-

ketoglutaric acid, sodium pyruvate, sodium metabisulphite, and hemin. Hemin is included to overcome trimethoprim antagonism due to the inclusion of yeast extract. Sodium pyruvate and sodium metabisulphite are included to allow aerobic incubation, while sodium carbonate provides carbon dioxide during growth (Post, 1995). The complete medium also contains 5% (v/v) lysed horse blood and the antibiotics: cefoperazone, vancomycin, trimethoprim, and cycloheximide for samples with a diverse microbial community (Kavanagh et al., 2018). Cefoperazone inhibitors were incorporated because of their ability to inhibit the background microorganisms that are typically found on post-chill poultry such as *Salmonella* and *Clostridium perfringens* (Schneider-Poetsch et al., 2010). Trimethoprim interferes with the action of bacterial dihydrofolate reductase, inhibiting synthesis enzymes necessary for DNA replication. This causes Gram-negative bacteria to be starved of nucleotides necessary for DNA replication, leading to cell lethality (Giroux et al., 2017). Vancomycin is an antibiotic used to treat Gram-positive bacteria resistant to less-toxic agents (Rivera et al., 2011). Vancomycin is added to inhibit the cell wall synthesis of Gram-positive bacteria. Cycloheximide, produced by the bacterium *Streptomyces griseus*, is a protein synthesis inhibitor added to Bolton broth (Schneider-Poetsch et al., 2010). Cycloheximide exerts its effect by impeding the binding of two tRNA molecules and an mRNA molecule thus blocking the translational elongation necessary for synthesis (Schneider-Poetsch et al., 2010). Cycloheximide acts as an antifungal and has been shown to limit fungal growth in other selective plate media (Ha et al., 1995a,b) Bolton broth is formulated with blood to reduce the damage of potential oxidative toxins (Chon et al., 2012; Hill et al., 2017). However, Blood free Bolton broth is also used in numerous studies, partially for economic restrictions because laked horse blood can be expensive (Baylis et al., 2000).

The efficacy of Bolton broth has been compared to other media used for *Campylobacter* growth. Baylis et al. (2000), conducted a study comparing three enrichment media for the growth and detection of *Campylobacte*r spp. from foods. The media used were BB, *Campylobacter* Enrichment broth and Preston broth. A total of 100 raw meat products were obtained from local retailers and butcher shops. Approximately 25 grams of food sample and 25 mL of rinses were added to 225 mL of the respective medium, Bolton broth, *Campylobacter* Enrichment broth, and Preston broth, then homogenized for one min in a stomacher. Homogenates were transferred to 300 ml screw-capped and incubated aerobically at 37 °C for 4 h followed by 42 °C for 48 h. The ability of each broth to support the growth of *Campylobacter* spp. was determined by streaking a loopful of each enrichment culture onto mCCDA plates. Plate counts on Bolton broth generated the highest yield of *Campylobacter* colonies detected, with 83 samples out of 100 samples being positive for *Campylobacter*, with the least confirmed colonies negative (7) compared to *Campylobacter* Enrichment broth (9) and Preston broth (19). Similar results were also seen in a study by Solis-Soto et al. (2011). In this study, various enrichment broths including Preston, Bolton broth, Blood Free Enrichment Broth (BFEB) and Modified-BFEB (M-BFEB) were used to determine the recovery rate (Solis-Soto et al., 2011). *Campylobacter* was inoculated onto retail chicken skin and the recovery rates were determined after 24 to 48 h of enrichment at 42 C° under aerobic incubation for BFEB and M-BFEB and microaerobic incubations for Preston and Bolton broth. After incubation, all samples were plated on Brucella agar supplemented with 5 % lysed horse blood and microaerobic incubation for 24 h. Morphology of the plates was used to determine *Campylobacter*'s presence. A *Campylobacter* colony from each media plate was serially diluted in its respective medium and a PCR assay was performed. The results from the PCR assays revealed that *Campylobacter* could be recovered from all enriched broths however

Bolton broth resulted in the most sensitive broth by consistently allowing the detection of 10 *C. jejuni* cells; variable results were obtained for *C. coli*. *Campylobacter* cells recovered were followed by BFEB, M-BFEB and Preston with the lowest sensitivity of 10^3 cells (Solis-Soto et al., 2011). These findings suggest Bolton broth to be an effective medium for *Campylobacter* enrichment for growth and detection.

6.3.2 Mueller Hinton Broth

Mueller Hinton (MH) is a popular medium, typically used as a broth for growing Gram-negative bacteria. It was developed in 1941, by John Howard Mueller and Jane Hinton, for culturing gonococcus and meningococcus. The first use of MH for *Campylobacter* growth was by Butzler and colleagues in a study on the isolation of *Vibrio fetus*, which became known later as *C. fetus* (Butzler et al., 1974; Louwen et al., 2012*)*. Mueller Hinton, a blood free medium, is commonly used as a broth to culture *Campylobacter*. Mueller Hinton includes beef extract, acid hydrolysate of casein and starch. The beef extract and acid hydrolysate of casein are used for providing essential nutrients such as nitrogen, carbon, amino acids, and sulfur. Starch is added to absorb the toxic metabolites after undergoing hydrolysis and serves as a source of energy (Mueller and Miller, 1941).

For years, MH has been a reliable option for *Campylobacter* growth. Ng et al. (1985), compared MH to basal media blood base no. 2, *Brucella* base, *Campylobacter* base, Columbia blood base, for *C. jejuni* and *C. coli* growth. Four strains of *C. jejuni* and four strains of *C. coli* were used to compare the quantitative growth of *Campylobacter* cells. The results from this study showed that after 48 h incubation under microaerobic conditions, MH yielded the highest CFU count of 8.50 Log_{10} in *C. jejuni* and *C. coli*, when compared to other basal media plate counts (Ng et al., 1985).

A comparison of methods for detection and enumeration of *Campylobacter* from freshly processed broilers was conducted by Line et al., (2001). Enumeration of *Campylobacter* from processed broilers was accomplished by direct plating samples on MH agar and Campy-Cefex. Two samples of 0.1 mL from each original carcass rinsates were transferred to agar plates and spread thoroughly with sterile loops. All plates were incubated microaerobically for 36 to 48 h at 42°C. The plates were inspected for growth of *Campylobacter* following incubation. The results showed that Campy-Cefex exhibited a greater number of colonies 9.2 CFU/mL, versus MH agar with 5.3 CFU/mL however, there were no statistically significant differences between the two. In addition, no contaminants were observed on the MH agar facilitated with *Campylobacter* enumeration when compared to Campy-Cefex plates that contained some contaminants. The results from this study confirmed that *Campylobacter* cells were more visible and grew at a more frequent rate in MH agar when compared to Campy-Cefex and mCCDA plates (Line et al., 2001). The findings from both studies suggest MH could be an additional effective enrichment medium for *Campylobacter* growth and detection.

6.3.3 Brucella Broth

Another medium that the USDA-FSIS recommends for *Campylobacter* enrichment for growth and detection is known as the Brucella broth. Brucella was developed to cultivate *Brucella* species from the environment and other potentially contaminated material. Brucella enrichment media is well known to cultivate other fastidious organisms including *Campylobacter* spp. The medium is used because supports the growth of both aerobic and microaerophilic bacteria if incubated appropriately. Brucella medium consist of basal media consists of casein and peptones, which are well known medium components to facilitate the growth of a wide variety of microorganisms (Atilola et al., 2015). Yeast extract is added as a

source of trace vitamins and micronutrients, while dextrose is added as a carbohydrate source but at a minimal dosage to prevent acid build up during growth (Lewis, 1992; Bonnett et al., 2019). For *Campylobacter* spp. specifically, Brucella medium can be supplemented with hemin to facilitate recovery and growth.

According to the USDA-FSIS, Brucella broth is a reliable transport medium for viable *Campylobacter* cells. The first research on transport and storage of *Campylobacter* in Brucella broth was done by Wang et al., (1980). In their experiment, Brucella medium with 10% sheep blood was used for storage and transport of *Campylobacter fetus* and subsp. *intestinalis* at room temperature (22 °C to 25 °C) and winter temperatures (4 °C) in Denver, Colorado. In this study, the results demonstrated that under winter conditions, the enriched Brucella medium remained stable for travel of 10 or more days (Wang et al., 1980). Therefore, it was recommended for an enriched Brucella medium described for the storage of *C. fetus* or subsp. *intestinalis* isolates. They also concluded that Brucella medium could be used successfully to send cultures of *C. fetus* in the postal mail when the time in transit is under three weeks (Wang et al., 1980). Yao and others (2014) studied the protective effects of different ingredients in *C. jejuni* transport Brucella broth media at 4 and 25 °C under aerobic conditions. Approximately 10^8 CFU/mL of six *C. jejuni* strains fresh culture were inoculated in each media and dispensed into 2 mL sterile tubes with 1 mL each. The inoculated transport media were kept at either 4 °C or 25 °C. The viability of different *C. jejuni* strains in the respective transport media was measured by plate counting from Brucella broth. After being kept at 25 °C for 72 h, the survival rates of the five strains in media with 25 mmol/L L-fucose were 1.3 to 10 times higher than those in media without L-fucose. Four out of the five strains in Brucella media with 5% laked sheep blood and 25 mmol/L L-fucose contained detectable viable bacteria on the fifth day compared with one strain surviving

in Brucella media with only 5% laked sheep blood for up to 10 days. From this study, the results showed that the Brucella enriched medium, supplementation with 5% blood, and being kept at 4 and 25 °C could improve the viability of different *C. jejuni* strains during transport. Currently, Brucella broth is still widely used as an enrichment medium for *Campylobacter* growth (Hill et al., 2017; Phung et al., 2021).

6.3.4 Hunt Enrichment Broth

Hunt enrichment medium was developed in 1985, however, little research was done until the early 2000's (Hunt et al., 1985). Since 2001, The Bacteriological Analytical Manual (BAM) protocol, designed by the U.S. FDA recommends the Hunt enrichment broth for the enrichment of *Campylobacter* spp. from food and water. This medium is composed of beef extract and meat peptone that supplies essential amino acids, peptides, and nitrogenous substances (Sanchez-Rosario and Johnson, 2021). Sodium chloride supplies essential electrolytes and maintains osmotic equilibrium (Shrimanker and Bhattarai, 2022). The yeast extract provides vitamin B complex as a growth factor. It is supplemented with selective agents including sodium cefoperazone, amphotericin B, trimethoprim lactate, vancomycin, sodium metabisulfite, sodium pyruvate, and ferrous sulfate.

Several research studies have been performed to explore the selective nature for *Campylobacte*r spp. enrichment of the Hunt enrichment broth. Stern and Line (1992), conducted a study on the comparison of three enrichment methods, Doyle and Roman enrichment broth, Park, and Sanders enrichment broth, and Hunt enrichment broth, along with different sampling times and various dilutions of enrichment culture for the efficacy of *Campylobacter* spp. detection from 50 retail-level chicken carcasses. Fifty raw broiler carcasses were purchased from local retail grocers for use in their study. Broilers from several different companies were

obtained in groups of ten and stored at 4 °C. Chicken carcasses were manually washed in large plastic bags containing 200 mL sterile buffered peptone. Enrichment broths (100 mL) were then dispensed in their respective samples and bags subsequently refilled with a commercially available microaerobic gas mixture consisting of 5% O_2, 10% CO_2, and 85% N_2 and placed in a circulating water bath with constant agitation at 42 °C for 24 h. After enrichment incubation, all samples were plated and examined for *Campylobacter* detection. The results demonstrated that the highest yield of *Campylobacter* from all samples was seen after 24 h enrichment in Hunt enrichment broth (Stern and Line 1992). Kim et al. (2009), compared the efficiency of eight enrichment broths for the selective growth of *Campylobacter jejuni.* All media, Brucella-FBP, Preston, Doyle and Roman, mCCDA, Park and Sanders, BB, Hunt and Radle and Hunt broths were compared for their recovery from the four matrices, *C. jejuni* in suspension, *C. jejuni* from inoculated ground pork, heat-injured *C. jejuni* (55 °C for 20 min) in suspension and heat-injured *C. jejuni* from inoculated ground pork. Of the eight media, Hunt enrichment broth and Bolton broth yielded the highest and most rapid enrichment efficacy with above a 7.00 Log CFU/mL for the cell suspensions and ground pork, respectively. Preston, Park, and Sanders and mCCDA broths had relatively high enrichment efficiencies, while Brucella-FBP broth was significantly inferior to the other broths (Kim et al., 2009). The conclusion of these studies indicate Hunt enrichment broth as an effective enrichment media for *Campylobacter* spp. growth. Moreover, as of 2022, the USDA-FSIS Microbiology Lab Guide suggest Hunt enrichment broth as the recommended enrichment medium for *Campylobacter* growth from food matrices (USDA-FSIS, 2022).

7 Molecular Methods for *Campylobacter* Detection

7.1 General Concepts

Currently, culture microbiological techniques for *Campylobacter* detection are labor intensive requiring continuous enrichment and plating. In food safety routine applications, time sensitive results are needed for the high throughput microbial methods required for commercial food processing operations. However, the current detection methodology recommended by the USDA-FSIS requires approximately three days to retrieve results (Espy et al., 2006). Furthermore, detection methods for *Campylobacter* are prevalence-based and predominately focused on *C. jejuni*. Characterizing pathogenic other *Campylobacter* spp. as well as concentration levels of contaminated products need to be implemented in the detection system to understand level of risk. Using non-culture-based methods for *Campylobacter* detection offers the opportunity to shorten the assay time and potentially be more sensitive (Espy et al., 2006; Asc et al., 2020). Any consideration for alternative methods for *Campylobacter* detection recommends the technique be easier, less time consuming, and consistently reliable. Over the years, a wide range of serotyping and molecular based methods have been proposed and/or implemented for *Campylobacter* (Eberle and Kiess, 2012; Ricke et al., 2019). While serotyping has proven useful for identification, molecular approaches such as multilocus sequence typing (MLST), pulse-field gel electrophoresis (PFGE), ribotyping, and PCR assays have received the most attention for further development to provide diagnostic tools for rapid detection and identification of *Campylobacter* (Eberle and Kiess, 2012; Ricke et al., 2019).

7.2 Ribotyping

Ribotyping is a molecular method that research has been shown to be reliable and rapid for the identification of *Campylobacter* spp. (Kiehlbauch et al., 1991; Owens et al., 1993). Ribotyping uses the rRNA gene to distinguish between *Campylobacter* isolates. This method is based on their ability to enzymatic digest *Campylobacte*r genomic DNA hybridized with an

rRNA probe (Wassenaar et al., 2000). The procedure for this method is as follows first, *Campylobacter*'s DNA is extracted using a DNA isolation kit (Kiehlbauch et al., 1994; Austin and Paggato, 2003; Harvey and Minter, 2003). Next, DNA is digested using restriction enzymes, and a gel electrophoresis cycle is performed on samples to generate DNA fragment sizes (Austin and Paggato, 2003; Harvey and Minter, 2003). After the gel is done, DNA fragment pieces from the gel are transferred on a nylon membrane (Harvey and Minter, 2003). The fragmented DNA pieces hybridized with the rRNA are probes will then be highlighted, creating a visual fingerprint pattern, and allowing the rRNA sequence to be analyzed based on phylogeny.

While ribotyping can be a reliable and efficient method for *Campylobacte*r identification, however, it does not possess have high discriminatory power (Wassenaar et al., 2000). *Campylobacter* spp. only possess three ribosomal gene copies in their genome, therefore, differentiation at the sub.sp. and strain levels are limited (Denes et al., 1997). Currently, there are no standards for the restriction enzymes used in *Campylobacter* spp. ribotyping. As a result, identifying patterns on the agarose gel can be difficult to read based on the restriction enzyme used for digestion (Sade and Bjorkroth, 2014). Therefore, using different ribotyping assays will affect the discriminatory index of *Campylobacter* spp. identified (Brisse et al., 2000). Additional studies are needed to develop a standard before considering ribotyping as an alternative to the culture methods.

7.3 Multilocus Sequencing Typing (MLST)

Multilocus Sequencing Typing is a molecular characterization approach provides a less unambiguous identification approach for *Campylobacter* spp. (Dingle et al., 2001; Eberle and Keiss, 2012; Romanate et al., 2017). The MLST technology was adapted from the multi loci electrophoresis method that uses the genetic variation from multiple chromosomal locations but

differs because MLST assigns alleles individually to each of the 7-housekeeping genes using DNA sequences rather than electrophoretic mobility (Maiden et al., 1998; Dingle et al., 2001). MLST relies on housekeeping genes to compare to sequenced assigned allele numbers. Each of the housekeeping genes have an allele database with the sequences of each allele and assigned allele number (Maiden et al., 1998; Dingle et al., 2001). The method relies on the large array of *Campylobacter* genes present and the thousands of alleles that the corresponding genes possess. Each gene and recorded sequence are assigned a number, then alleles and numbers are compared to the housekeeping genes and their allele number. Combinations and patterns of alleles from the designated genes have unique sequence types to assign and track *Campylobacte*r. Alleles of *Campylobacter* isolates are subsequently compared, and the more sequenced patterns the *Campylobacter* isolates have in common, the more related they are to each other (Maiden et al., 1998; Dingle et al., 2001).

There are several advantages of using MLST for *Campylobacter* detection and characterization. First, since unique DNA sequenced data is being utilized, no phenotyping is necessary. Since *Campylobacter* is sensitive and can enter a VBNC state, this is critical for detection and identification. Additionally, since DNA sequences are being used, they are uploaded to an online database, which allows them to be compared for easily interpreted results (Larsen et al., 2012). Moreover, another advantage of using the MLST for *Campylobacter* detection is that it is relatively simple to use and does not require excess training or additional reagents (Belen et al., 2009). In addition, multiple *Campylobacter* spp. can be identified in a mixed culture (Belen et al., 2009). However, although MLST can provide an alternative method for *Campylobacter* detection, it is relatively expensive to use, and routine use in a food processing environment can be cost prohibitive (Levesque et al., 2008).

7.4 Pulse Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis is known as a discriminatory molecular subtyping method for *Campylobacter* spp. (Zhou, and Oyarzabal, 2015). Using PFGE, *Campylobacte*r strains can be identified based on their unique genetic content. First, the DNA is extracted in an agarose plug, to avoid mechanical shearing of the DNA molecule. Next, *Campylobacter* spp. unique DNA sequences are generated by cleaving the genome with a specific restriction enzyme, that uses a few cut sites within the genome, making it easily comparable (Oliver and Jones, 2015). The cleaved products are then separated by running a gel electric field that is designed to periodically change directions of the electric current. The voltage is conducted in three directions, vertically through the center of the gel, and two that run at 60-degree angles on each side. Finally, when running the PFGE program, the temperature is set at 14 to 15 °C (Sharma-Kuinkel et al., 2016). After electrophoresis, gel images are obtained, normalized and DNA fragment patterns are analyzed based on fragment sizes (Sharma-Kuinkel et al., 2016).

While PFGE is one of the more discriminatory typing methods for subtyping *Campylobacter* spp, the level of discrimination is based on the type of restriction enzyme used, as well as if either one or multiple enzymes are used (Ribot et al., 2001; Oliver and Jones, 2015). Numerous protocols for *Campylobacter* typing using PFGE have been created (Ribot et al., 2001). However, no standards exist, making it difficult to compare results from other PFGE studies with the use of different restriction enzymes. Online databases such as PulseNet tend to alleviate some of these differences by making protocols universally available online, ensuring inter-laboratory comparability of the generated results (Ribot et al., 2001). Although this is a highly discriminatory method for identifying *Campylobacter*, limitations do exist. One of the needs for an alternative method from culture based plating method is because it

takes substantial time to retrieve results. This technique takes longer than a normal gel electrophoresis because of the larger DNA fragment sizes generated, and they are unable to move in a straight line (Lee et al., 2012). Additionally, using PFGE for *Campylobacter* typing is labor intensive with results only becoming available within 3 to 4 days (Lee et al., 2012).

7.5. *Campylobacter* 16S rRNA gene sequencing

To develop standard curves for *Campylobacter* quantification, the plate count method is used to validate pre-enrichment concentrations. However, research has shown that some colonies from *Campylobacter* selective plates are not *Campylobacter* exclusive. Therefore, depending on the matrix, *Campylobacter* can be accompanied by competitors when isolating on plates. To ensure precise standard curve development, colonies from selective media need to be verified as *Campylobacter*. This can be done by sequencing the colonies on the selective media used for the standard curve development. Sequencing is the processes of determining the genetic information in the order of the DNA sequenced from the amplified genetic material (Janda and Abbot, 2007). 16S rRNA amplicon sequencing provides ecological information on the microbial community in the environment and can be used to link bacterial taxa to specific environmental conditions (Nakatsu et al., 2019). The ribosomal RNA gene is used as a marker for determining evolutionary relatedness because it is ubiquitous in all prokaryotic microorganism (Sun et al., 2013). The rRNA gene is composed of a large subunit (50S) and a smaller subunit (30S). The 16S rRNA gene codes for the RNA component of the 30S subunit of a prokaryotic ribosome and is approximately 1,550 base pairs (bp) with 15 regions, 9 hypervariable and 9 conserved regions (Weinroth et al., 2022). Conserved regions are sections of the sequence that are relatively similar, whereas hypervariable regions are areas in the sequence that show the most diversity between individual microorganisms (Chakravort et al., 2007). Sequencing the conserved region

makes universal amplification possible, however, for species specific discrimination between, the v3 and v4 regions are typically used because they are hypervariable regions that can identify slight changes in the sequences to reconstruct the taxonomic composition (Clarridge, 2004). The 16S rDNA sequence analysis includes trimming, screening, and aligning the amplified rRNA gene sequences which filters and/or truncates error prone read sequences (Clarridge, 2004). After filtering steps to improve quality, sequences can be assigned into Amplicon sequence variants (ASVs) generated by DADA2, which provides a sequence clustering method that applies sequencing run-specific error to reduce sequencing error (Callahan et a., 2016). Then, using QIIME 2 online database, the microbial community profiles are compared between 16S rRNA gene sequences, assign taxonomy, or construct phylogenetic trees. The resulting 16s rRNA gene sequences and assigned taxonomy should be similar, as *Campylobacter* should dominate the microbial community in the selective environment created to support *Campylobacter* cell growth.

7.6 Polymerase Chain Reaction assays

Of the molecular methods discussed, the most extensively developed molecular method for most foodborne pathogens, including *Campylobacter*, has been PCR assays (Ricke et al., 2019). Fundamentally, PCR is a nucleic acid amplification technology, able to detect organisms such as *Campylobacter* spp., and differentiate them from unrelated organisms because it is specific and discriminatory (Salis et al., 2003). A PCR assay is conducted by amplifying a specific segment of the target DNA, producing millions of copies of the *Campylobacter* spp. targeted DNA (Inglisand Kalischuk, 2003). To perform a PCR run, the exact sequence of targeted DNA must be known so that the complementary area of specific nucleotide sequences can be designed and constructed, known as a Primer (Shahzad et al., 2020). Primers act as an

initiator to replicate the targeted DNA (Coleman and Tsongalis, 2006; Shahzad et al., 2020). The PCR process is carried out over a series of three major phases: Initially, DNA denaturation occurs when the DNA molecule is heated above the melting point (95 °C), this causes the hydrogen bonds holding the double-strands together to break and separate. Secondly, the DNA strands are cooled (55 °C) to allow primers to anneal to their now single stranded DNA, which restores the original double helix structure. Once the double helix is restored, elongation occurs at 72 °C, the heat stable polymerase synthesizes a new complementary DNA strand using the nucleoside triphosphates. After the three major steps in each PCR cycle, the DNA target sequence doubles in concentration (Hill and Stewartm 1992; Baumforth et al., 1999). After the completion of the PCR run, amplified PCR products must be analyzed using either conventional methods such as agarose gel electrophoresis or Southern Blot, or the more recent introduction of real time assessment using fluorescence (Coleman and Tsongalis, 2006; Lo et al., 2006).

7.6.1 Endpoint PCR

The most used technique for PCR-based detection is referred to as Endpoint PCR. It involves an extra step to analyze PCR amplified products obtained from the endpoint of the reaction once the run has ended. After the PCR process is completed, amplicons are transferred onto an agarose gel and undergo gel electrophoresis to analyze results the based-on band sizes. However, although specific for *Campylobacter* detection, Endpoint PCR assays have some drawbacks. Limitations of Endpoint PCR include the risk of contamination of PCR products during transmission to an agarose gel, the low resolution of band sizes, lack of size-discrimination on gel, and lack of automation (Ricke et al., 2019). Therefore, real-time (RT) PCR was developed to address some of these problems.

7.6.2 Real-time PCR

RT-PCR is a gene amplification technology that can be used to detect the presence or absence of a specific gene sequence for microorganisms such as *Campylobacter* (Han et al. 2008; Shortt et al., 2016). More importantly, in some cases, amplified PCR products can be quantified (Kralic and Ricci, 2017). RT- PCR technology either uses SYBR® Green I or TaqMan™ probe-based PCR for the DNA to be amplified. SYBR® Green I is a cyanine dye able to bind to any amplified double stranded DNA (Gudnason et al., 2007; Zhang et al., 2020). The SYBR® Green I dye probes attach to the template sequence during elongation, and when the DNA polymerase is extended it removes the probe from the template and emits the fluorescent dye. As more double stranded amplicons are produced during PCR cycling, the emitted dye signal is increased, and once it reaches the detection limit, it will indicate presence or absence of *Campylobacter* (Ahrberg et al., 2015). SYBR® Green I RT-PCR binding is not specific to one target but is universal to all amplicons in the PCR assay (Pereira-Gómez et al., 2021). However, the use of SYBR® Green I RT- PCR requires distinct melt curves for each gene that can be tracked to differentiate between target amplicons and non-specific products (Ruiz-Villalba et al., 2017).

In contrast, RT-PCR incorporates the use of TaqMan™ probe-based PCR. The PCR method uses the enzyme Taq DNA polymerase which can bind to single stranded nucleic acid specific to a target region (Josefsen et al., 2007). The TaqMan™ probe in the assay is labeled with two dyes, one at the 5′-end and the quenching dye at the 3′-end (Ryazantsev et al., 2012). During the amplification process, the TaqMan™ probe is cleaved off the specific DNA segment and the light emitted from the dyes allows the RT-PCR to detect the fluorescent signal in real-time. Research has shown that the TaqMan probe system is more sensitive and specific than the SYBR® Green system for *Campylobacter* detection (Wiemer et al., 2011; Liu et al., 2019).

7.6.3 Benefits of PCR for *Campylobacter* detection

Boer et al. (2015), conducted a study that compared a PCR detection method (16S rDNA sequences of *Campylobacter* spp.) to mCCDA plating for *Campylobacter* detection. A total of 926 samples were tested from 62 broiler flocks (the Autumn season of 2007 in The Netherlands), consisting of 308 container samples and 618 cecal samples. Results from the PCR assays were compared to plate counts to assess the accuracy of both methods for *Campylobacter* spp. prevalence. The PCR assay detected *Campylobacter* in 696 of the original 926 samples, whereas using plating methods, 606 samples were considered positive for *Campylobacter*. Additionally, the PCR results were available in 4 h compared to mCCDA plates, which were recorded after 2 days. The results from this study suggest that the PCR assay for *Campylobacter* detection is a faster and a more sensitive tool for *Campylobacter* detection than plating on mCCDA. When PCR is implemented for *Campylobacter* detection, rapid results can be provided to commercial food operations with information being received in a timely fashion for critical food safety decisions.

Given *Campylobacter*'s fragility in varying environmental conditions and its ability to enter the VBNC state, PCR can help to resolve this issue. PCR can alleviate undetected VBNC *Campylobacter* grown on selective media through the use of genomic DNA with corresponding *Campylobacter* specific primers to amplify low levels of DNA (Lv et al., 2020). Moreover, plating methods rely on phenotypic traits to distinguish *Campylobacter* colonies. Phenotypic characterization can leave room for variation in operator interpretation, which can result in misidentified colonies from plating, as well as species that are indistinguishable on agar (Eriksson, and Aspan, 2007; Buchan and Ledeboer, 2014). The PCR assay uses unique gene sequences to produce positive results for detection, allowing for species identification and

enhanced sensitivity. An example of this, Mayr al. (2010), developed a RT-PCR assay for *C. jejuni*, *C. coli* and *C. lari* detection in food samples based on a segment of the *mapA* gene for *C. jejuni*, *ceuE* gene for *C. coli* to and *pepT* gene and the gyrase subunit *A* gene for *C. coli* to differentiate between *Campylobacter* spp. A total of 464 food samples (122 chicken samples, 135 duck samples, 45 turkey samples, 29 goose samples, 32 mussel samples, 29 fish samples, 45 pork samples, 17 beef samples, and 10 lettuce samples) were analyzed for the presence of *Campylobacter* spp. using the developed RT-PCR assay and compared to the culture plate method for detection. All food samples were enriched in 225 ml of Preston broth and incubated for 40 to 48 h at 42 °C under microaerobic conditions. After the enrichment, 1 mL was collected for PCR analysis, and 0.1 mL aliquots of each sample were inoculated on blood agar plates with a sterile loop and incubated for 48 h at 42 °C under microaerobic conditions. The results from this study showed that when the RT-PCR assay was used, 55.4% of samples were positive for *Campylobacter* spp., whereas only 40.3% were observed to be positive using the culture plate method. Additionally, the RT-PCR assay was able to identify differences among *Campylobacter* spp. in food samples. Using the RT-PCR in mixed culture, *Campylobacter* was detected in chicken (29.7%), turkey (12.5%), and duck samples (39.8%) (Mayr et al., 2010). Kumar et al. (2015), determined the occurrence of *C. jejuni* and *C. coli* in food samples using culture plate and RT-PCR methods. A total of 280 food samples comprising 100 chicken meat, 50 chicken offal, 50 leg muscle, and 80 raw milk samples were used in this study. For analysis, all samples were enriched in 100 mL Preston broth and incubated at 42 °C for 24 to 36 h under microaerophilic conditions. After enrichment, 0.1 mL of each sample was spread plated onto mCCDA and incubated under microaerophilic conditions at 42 °C for 48 h. In addition, 1 mL aliquots were taken for PCR analysis. The results demonstrated that the use of RT-PCR yielded

more *Campylobacter* positive samples (68; 24.29%) compared to 29 (10.36%) recovered by the cultural method. In addition, the RT-PCR used the *lpxA* gene to differentiate between *C. jejuni* and *C. coli* samples and it was reported that of the 68 samples positive for *Campylobacter*, 41 were *C. jejuni* and 17 were *C. coli* and 10 were mixed. Furthermore, all samples identified as positive by culture methods were found positive by PCR. The results from both studies observed more positive *Campylobacter* samples using the RT-PCR compared to culture plating on selective media. The RT-PCR was also able to identify the species of *Campylobacter* detected. These studies suggest that the RT-PCR is more accurate for *Campylobacter* spp. detection than plating on selective media and can differentiate *Campylobacter* spp. in various food matrices.

7.6.4 Limitations of PCR

Although the use of PCR for *Campylobacter* spp. detection has distinct advantages, some limitations still exist. A common concern with PCR analysis is its ability to detect genomic DNA from dead cells (Postollec et al., 2011; Papic et al., 2017). Before processing, samples will contain numerous microorganisms, whose remnants can still be detected even after cell death (Postollec et al., 2011). A PCR assay does not have the capability to differentiate live versus dead cells once the nucleic acids present have been annealed. This phenomenon can cause false positives because the PCR system can amplify all the DNA in the sample. One way this can be rectified is by using DNAase, which eliminates loose DNA strands from dead cells (Boada-Romero et al., 2020). In addition, enriching *Campylobacter* can also denature loose DNA present in a sample by diluting it out and allowing *Campylobacter's* DNAase enzymatic activity to occur (He et al., 2019).

In addition, *Campylobacter* spp. are ubiquitous and can be present in many different environments. As a result, these complex matrices can contain inhibitors that prevent the

amplification of nucleic acids, ultimately having a negative impact on the sensitivity and specificity of a PCR assay (Schrader et al., 2012). Inhibitors can affect the PCR process by interfering with surfaces of *Campylobacter*, the inhibitor may react with *Campylobacter* nucleic acids during sampling or extraction and inhibitors can interfere with probe binding and fluorescent signals (Schrarder et al., 2012). Common PCR inhibitors include feces, blood, hemoglobin, fat, and sample color (Borges et al., 2020). Since *Campylobacter* is predominately associated with poultry, PCR detection can be a problem with the presence of feces, fat, and blood present (depending on what is sampled). The most important step to remove inhibitors from samples is optimizing DNA purification during extraction (Rezadoost et al., 2016). In addition, growth enrichment of samples prior to PCR can reduce inhibitors present by diluting them during cell growth and concomitantly increasing *Campylobacter* concentration (Park et al., 2014). Further optimization of PCR use for *Campylobacter* detection in additional food and meat matrices will need to be considered the elimination of inhibitors potentially present in these matrices to create a more sensitive and specific method.

8. *Campylobacter* Quantitation by PCR

While *Campylobacter* detection is important from a food safety perspective, quantitation allows for assessing population levels and the potential to estimate the risk of campylobacteriosis for a particular food product. When used in this fashion, RT-PCR is commonly referred to as quantitative PCR (qPCR) because it can quantify the DNA concentration present in a sample. In the first few PCR cycles the amount of DNA is below the detection limit. As more cycles occur, the fluorescent signal intensifies and becomes detectable (Overbergh et al., 2003). Therefore, as more cycles occur and fluorescence is released, the level of the target gene after each thermal cycle can be tracked to quantify concentrations during the exponential stage of the qPCR

(Overbergh et al., 2003). Once the DNA is detectable during doubling, this is known as the exponential phase. As more copies are made, the reaction plateaus. The amount of DNA in different samples is measured using the cycle threshold (CT), which is the point where fluorescence can be detected (Overbergh et al., 2003). The CT reveals the relative amount of DNA in a sample. The greater the amount of starting DNA in a sample, the faster it will be detected via qPCR. Therefore, before qPCR analysis, *Campylobacter* cell growth enrichment from the sample's environment must be developed to optimize the starting bacterial cell concentration prior to PCR. The application of calculations for cell concentration based on bacterial growth kinetics can be used as a predictor for the exponential phase for qPCR detection and quantification. With the CT value and a cell concentration standard curve derived from growth kinetic calculations based on the exponential growth phase, the starting DNA concentration can be back calculated.

For qPCR, standard curves are needed to accompany amplification and quantify the concentration of the original sample. However, *Campylobacter* kinetics will differ depending on the matrix sampled, enrichment media used, and the PCR assay. Therefore, to fully optimize qPCR, standard curve protocols must be developed and validated for specific samples. For example, Vondrakova et al. (2014), evaluated a qPCR assay for *C. jejuni* quantification in various chicken samples (raw chicken wings, whole carcass, and fried chicken strips). Standard curves were constructed for *C. jejuni* in all chicken samples with a range of 10^0 to 10^6 CFU/mL. This study showed *C. jejuni* could be detected in all samples, while plates revealed no *C. jejuni* present. Additionally, *C. jejuni* could be quantified in the carcass rinse sample ($7.1\times10^2 \pm 1.6\times10^2$), as other samples were under the quantification limit of this assay. These findings suggest that extensive work must be done on the front end with curve development.

Thus, to ensure sensitive, specific detection and quantification, PCR assays should provide sample specific curves for PCR detection and quantification.

To optimize PCR-based quantitation, initially, the limit of detection and best fit enrichment media for detection of the PCR assay being used must be determined (**Figure 3**). To assess this, detection parameters (specificity, sensitivity, efficiency, accuracy, positive and negative likelihood ratios (PLR, NLR), prevalence, positive predictive value (PPV), and negative predictive value (NPV), for different enrichment media for each *Campylobacter* spp. in the PCR assay must be elucidated. Specificity of a PCR assay is the ability to identify truly negatives samples. Moreover, sensitivity is the ability to identify truly positive samples. PLR is the true positivity rate divided by the false positivity rate, while NLR is the false negative rate divided by the true negative rate (Bolin and Lam, 2013). The efficiency of a PCR assay is the amount of the targeted sequence amplified in one PCR cycle. A well-designed PCR assay should have an efficiency of at least 90 % (Svec et al., 2015). The accuracy of a PCR assay measures of how aligned amplified values and how true the values are after the cycle (Kralik and Ricchi, 2017). When looking at PCR prevalence, it is defined as the number of positive samples out of the total samples (Amman et al., 2020). Lastly, the PCR's PPV is the probability that a positive sample has the targeted DNA sequence being amplified, whereas NPV is the probability that a negative sample does not have the amplified DNA sequence (Parikh et al., 2008).

Uncovering PCR detection parameters for *Campylobacte*r spp. can be done by quantifying growth responses of *Campylobacter* spp. in different media. Each *Campylobacter* spp. should be enriched in its respective *Campylobacter* enrichment media for 24 to 48 h, followed by 10-fold serial dilutions. All samples and dilutions should be subsequently plated on selective media to define sample concentration, then ran on the PCR instrument to plot CT

values against quantification from the plates. Using the CT values, time, standard curve and plate counts, detection parameters can be defined to determine the limit of detection, enumerable range and the medium that best suits consistent *Campylobacter* spp. detection in the PCR assay.

Once the PCR detection parameters are defined for *Campylobacter* detection using the PCR assay, the bacterial growth kinetic response must be determined to optimize time required for enrichment incubation prior to conducting the RT-PCR assay. *Campylobacter* sample enrichment is recommended for 24 h, however by identifying growth phases and estimating doubling times from the growth kinetic calculations, it can be used as a predictive tool for enrichment time. Growth kinetics estimated from absorbance and time points can be used to develop growth curves for *Campylobacter* spp. as a function of the best fit medium for the PCR assay.

After growth kinetics have been defined for the PCR assay, this information can be used to develop and optimize a qPCR assay. From the calculated doubling time and specific growth rates from the kinetics, *Campylobacter* enrichment time can be estimated. Since *Campylobacter* is a fastidious organism and sensitive to different stressors, standard curves for the qPCR assays must be exclusive to those samples it is for an efficient quantification system. Therefore, estimated enrichment times from the bacterial growth kinetic results must be validated in different sampling environments and quantification of the enumerable range of the PCR in that sample environment must be defined. To do this, sampling methods recommended by the USDA-FSIS guidelines must be followed to collect the appropriate samples meet regulatory requirements. Samples are inoculated with known concentrations of *Campylobacter* that fit in the enumerable range of detection, from the limit of detection to the highest concentration. Plating inoculated samples on USDA-FSIS recommended plate media, prior to enrichment will provide

concentrations for samples prior to enrichment. PCR assays can be conducted on all samples and timepoints. The shortest time for the lowest level of *Campylobacter* concentration detected efficiently in the PCR should be determined as the enrichment time needed for the qPCR assay. Additionally, using the concentrations of pre-enriched samples from plate counts and CT values of the detected samples, a standard curve can be created using CT values and sample concentrations. The standard curve will allow for *Campylobacte*r spp. to be back calculated once samples with unknown levels of *Campylobacter* spp. are in the enumerable range that has been previously established.

9. Conclusions

Campylobacter is a prominent foodborne pathogen in the poultry processing environment. The detection of *Campylobacter* is required to identify contaminated food and determine effective treatments in reducing food contamination. As mentioned in the current review, *Campylobacter* has complex growth requirements, and several different media have been developed over the years for its growth and detection, each with its own limitations.

Culture methods are still common approaches for *Campylobacter* detection. However, issues such as media bias leading to consistency and selectivity for detection can derail accurate results. Currently, there are no standards on media used for *Campylobacter* isolation and multiple media can be used such as mCCDA and Campy-Cefex. As this review indicated, numerous research studies have compared different *Campylobacter* media, however, all lead to varied conclusions. The various results can be attributed to the absence of media standards for *Campylobacter* detection. Additionally, research has shown that selective plating for *Campylobacte*r lacks sensitivity and specificity for detection due to its inability to handle stressed bacterial cells and the presence of non-*Campylobacter* organisms (Bessede et al., 2011;

Kim et al., 2017). Moreover, in the food safety environment, time sensitive results are important to ensure a safe food processing system. Yet, the culture-based method requires approximately 3 days to generate results (Espy et al., 2006).

Molecular methodologies such as PCR can be an attractive alternative for future *Campylobacter* detection versus conventional agar plating methods. Molecular approaches tend to alleviate some of these concerns with rapid assessment for the prevalence and quantification of *Campylobacter* in different matrices (Ricke et al., 2019). Additionally, the plating method still relies on phenotypic traits to distinguish *Campylobacter* colonies from non-*Campylobacter* colonies. Molecular assays such as PCR rely on unique gene sequences, which are more specific and selective for *Campylobacter* detection. A common concern with PCR analysis is their ability to detect genomic DNA from dead cells. In samples where *Campylobacter* cells are present prior to processing, the remnants of these initial cells can still be detected even after cell death. One way this can be rectified is the use of DNase, which eliminates loose DNA strands that present from dead cells (Boada-Romero et al., 2020). More research is needed to optimize *Campylobacter* detection using PCR.

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Tables.

Table 1. Composition of ingredients and selective agents for *Campylobacter* media

Media	Ingredients	Antibiotics
Skirrow's Medium	Proteose peptone, liver digest, yeast extract, sodium chloride, and lysed horse blood	vancomycin, polymyxin B, trimethoprim,
Preston medium	proteose peptone, liver digest, yeast extract, sodium chloride	Polymyxin, Rifampicin, Trimethoprim and Amphotericin B
Campy-line agar	charcoal, ferrous sulfate, sodium pyruvate, casein hydrolysates, cefazolin, and sodium deoxycholate	trimethoprim, polymyxin B, cycloheximide, cefoperazone and triphenyltetrazolium chloride
Campy-Cefex	Peptone, yeast extract, dextrose, NaCl, sodium bisulfite and Agar ferrous sulfate, sodium bisulfite, sodium pyruvate, alpha-ketoglutaric acid and sodium carbonate	Laked horse blood, cefoperazone and cycloheximide
Modified charcoal cefoperazone deoxycholate agar (mCCDA)	nutrient broth, bacteriological charcoal, casein hydrolysate, sodium deoxycholate, ferrous sulfate and sodium pyruvate, agar	cefeoperazone and amphotericin B
Trypticase Soy with 5% Sheep Blood Agar (SBA	Casein, Peptic Soybean meal, sodium chloride, nutrient agar	5% sheep blood
Karmali medium	Charcoal, hematin and cornstarch	cefoperazone, vancomycin, sodium pyruvate and cycloheximide
Bolton Broth	Peptone extract, yeast extract, alpha-ketoglutaric acid, sodium pyruvate, sodium metabisulphite and hemin	cefoperazone, vancomycin, trimethoprim and cycloheximide
Mueller-Hinton (MH) medium	beef extract, acid hydrolysate of casein and starch	sulphonamides and trimethoprim
Brucella medium	Peptone, yeast extract, dextrose, NaCl, sodium bisulfite and Agar	Hemin
Hunt enrichment Broth	Beef extract, meat peptone, sodium chloride, and yeast extract	sodium cefoperazone, amphotericin B, trimethoprim lactate, vancomycin, sodium metabisulfite, sodium pyruvate and Ferrous sulfate

Figures

Figure 1. *Campylobacter jejuni* colonies on Campy-Cefex plates.

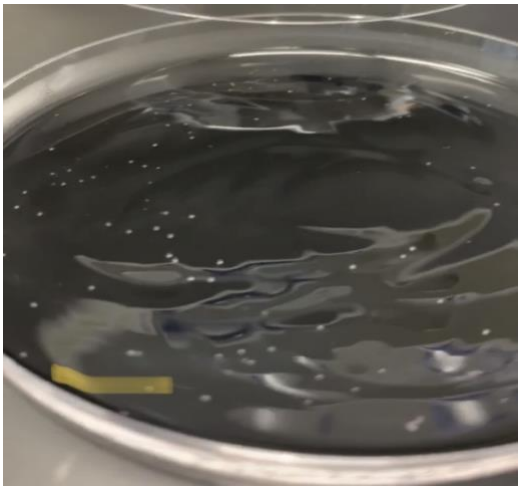

Figure 2. *Campylobacter jejuni* colonies on mCCDA plate.

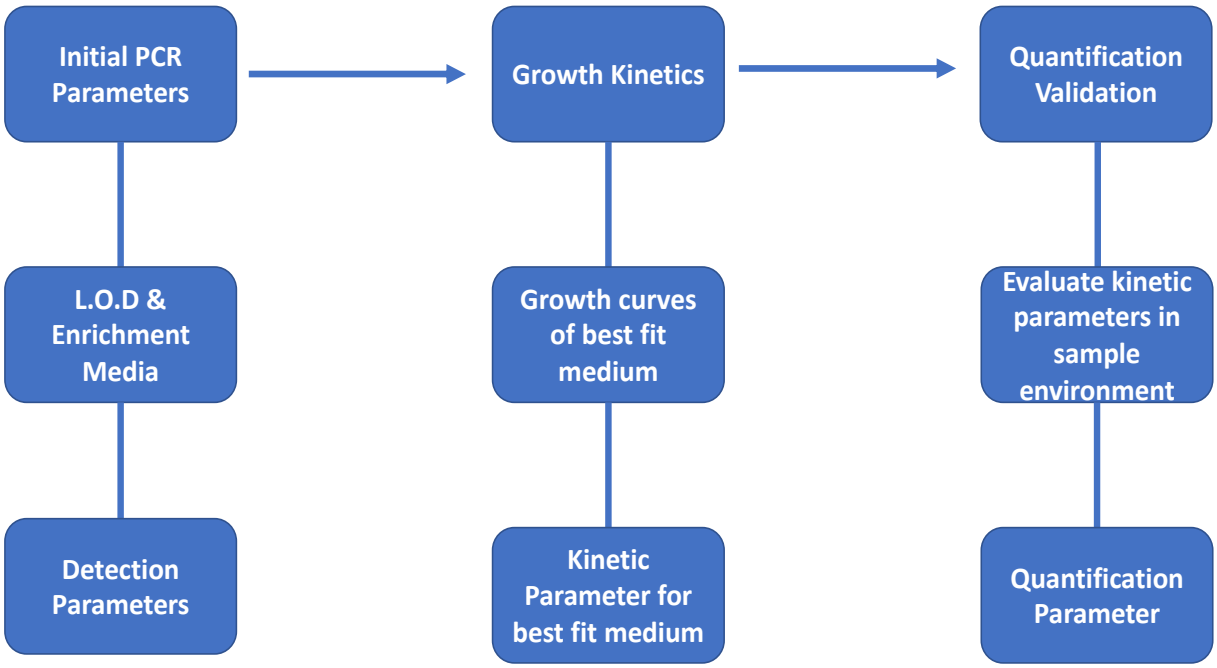

Figure 3. Flow diagram of the steps needed to develop and optimize for a qPCR assay.

Microbial diversity of selective and non-selective media for *Campylobacter jejuni, coli* and *lari* in Poultry Post-chill Whole Bird Carcass Rinsates

Aaron R. Bodie[1] Dana K. Dittoe[1], and Steven C. Ricke[1*]

[1]Meat Science and Animal Biologics Discovery Program, Dept. of Animal and Dairy Sciences, University of Wisconsin, Madison, WI, 53706, USA

[*]Corresponding Author:
Steven C. Ricke
Meat Science and Animal Biologics Discovery
Dept. of Animal and Dairy Sciences,
University of Wisconsin,
Madison, WI, 53706, USA
Email: sricke@wisc.edu

Abstract

Although *Campylobacter* is a foodborne pathogen commonly associated with poultry, it can be difficult to isolate because *Campylobacter* selective media vary in effectiveness. Therefore, using 16S rDNA gene sequencing, the relative effectiveness of selective media was determined in the current study by delineating the microbial composition of the pooled colonies on selective and non-selective media for *Campylobacter* isolation from poultry post-chill carcass rinsates. Samples were made by aliquoting 30 mL of post-chill rinsates to individual Whirl-Pak bags and inoculating them with 1.00 to 4.00 CFU/mL of either *C. jejuni, C. coli* and *C. lari*. After inoculation, 2× Blood free Bolton Broth were added to all inoculated samples and enriched for 20 h at 42 °C under microaerophilic conditions. Following incubation, samples were spread plated on Campy-Cefex, mCCDA, and TSA plates and incubated for 48 h under the corresponding conditions. The V4 region of the 16S rDNA of the subsequent pooled colonies was sequenced using an Illumina Miseq. Microbial diversity and microbiota composition (ANCOM) were analyzed in QIIME 2.2021.11 ($P \leq 0.05$; $Q \leq 0.05$). Alpha diversity revealed main effect differences for both species and media. Under Pielou's Evenness and Shannon's Entropy, *C. jejuni* had the greatest evenness and richness, followed by *C. coli* then *C. lari* ($P < 0.05$; $Q < 0.05$). For media, under Pielou's Evenness and Shannon's Entropy, TSA had the most evenly distributed taxa and most diverse taxa ($P < 0.05$; $Q < 0.05$). There was an interaction of species and media on the beta diversity ($P < 0.05$). Using Bray Curtis and Weighted Unifrac pairwise differences were seen between all species pairs ($P < 0.05$; $Q < 0.05$). Media also impacted the beta diversity with the Bray Curtis and Weighted Unifrac of TSA and mCCDA and TSA and Campy-Cefex being different ($P < 0.05$; $Q < 0.05$). Additionally, among the three *Campylobacter* spp., ANCOM revealed *Campylobacter*, Lachnospiraceae, and Oscillospiraceae

were significantly more abundant taxa (W= 218, 209, 201). The only significant more abundant taxa among the three media at the genus level was *Staphylococcus* (W=219). As a result, microbial diversity, and composition of enriched poultry rinsates appear to be affected by media used for enumeration and type of *Campylobacter* spp. present.

Keywords: *Campylobacte*r, Media, Microbiota, Microbiome, Poultry, Rinsates

1. Introduction

Campylobacter is the leading cause of gastroenteritis in the United States. Infection can be caused by a dose as low as 800 colony forming units (CFU) of *Campylobacter*, triggering campylobacteriosis (Janssen et al., 2008; Hansson, 2018). According to the World Health Organization (WHO), campylobacteriosis is responsible for 1 out of 4 causes of gastrointestinal disease worldwide (WHO, 2020). Over time, this disease can progress into various forms, such as Guillain–Barré syndrome (GBS), irritable bowel syndrome (IBS) and sometimes reactive arthritis (Facciola et al., 2017). The main reservoir for *Campylobacter* transmission to humans originates from raw poultry products (WHO, 2020). It has been reported that up to 80% of human campylobacteriosis cases can be traced to the consumption of chicken (Wagenaar et al., 2013; Melo et al., 2019). There is a commensal relationship between *Campylobacter* and the gastrointestinal tract (GIT) of chickens, making it possible to transmit to humans from chicken products (Hermans et al., 2011). The highest rates of human campylobacteriosis infections are caused by *C. jejuni*, *C. coli*, and to a lesser extent *C. lari* (Rowe and Madden, 2014; Kaakoush et al., 2015). Consequently, *Campylobacter* detection and identification research and methodology development focus primarily on these three species (Ricke et al., 2019).

Campylobacter spp. are fastidious organisms requiring microaerophilic conditions, a longer growing time, a narrow temperature range, and specialized media (Davis and DiRita, 2008). However, because of its specific niche, *Campylobacter* metabolism is poorly understood (Hilbert et al., 2010; Stahl et al., 2012). As a result, *Campylobacter* detection and enumeration remains problematic in the food industry. Poultry whole carcass rinsates are routinely used as a sampling method for determining the presence and number of quality-indicator organisms, as well as pathogenic microorganisms associated with broiler chicken carcasses in processing

facilities (United States Department of Agriculture – Food Safety Inspection Service, USDA-FSIS, 1998). Currently, the USDA-FSIS recommends Campy-Cefex agar for enumeration of *Campylobacter* spp. from poultry samples (USDA-FSIS, 2022). However, the International Organization for Standardization (ISO) protocol for *Campylobacter* isolation suggest modified charcoal-cefoperazone-deoxycholate agar (mCCDA) media for *Campylobacter* (ISO, 2017). Both methods are widely accepted in the food industry, however, the different ingredient compositional profiles between media can potentially result in microbiota composition variations between media types after *Campylobacter* enrichment (Oakley et al., 2012). Ultimately, this can impact the isolation frequency of *Campylobacter*.

Additionally, the selective enrichment of *Campylobacter* is based on providing nutrients for *Campylobacter* spp. to metabolize and the effect of antimicrobials against competing microorganisms in the environment (Kim et al., 2021). Yet, the poultry carcass matrices may consist of different microorganisms, competing with *Campylobacter* for the same nutrients (Marmion, et al., 2021; Hakeem and Lu, 2021). As a result, these competing microorganisms can potentially outcompete *Campylobacter*, causing *Campylobacter* colonies on the agar plate to be masked and/or limited by competing microorganisms. In addition, unfavorable conditions such as temperature, competition, and nutrient availability can stress *Campylobacter* spp. causing cells to be viable but non-culturable (VBNC) on selective media (Silva et al., 2011). These results ultimately hinder the effectiveness of the selective media and suggest the need for a more extensive examination of *Campylobacter* isolation and selective media to assess potential problems with competing microorganisms.

Sequencing via the 16S rDNA gene sequencing can provide a genomic means for identifying microbial communities in food matrices because the 16s rDNA gene is ubiquitous in

all microorganisms, thus offering a wide range of bacterial coverage for taxonomic bioinformatic applications (Ricke et al., 2017). Kim et al. (2017) demonstrated that 16S rDNA sequencing is an effective tool for understanding the microbial community on *Campylobacter* selective media recommended by USDA-FSIS for poultry processing microbial assessment. Kim et al. (2017) sequenced pooled colonies grown on Campy-Cefex that were plated with 100 μL of chicken carcass rinsates using an Illumina MiSeq sequencer. The results from this study concluded that Campy-Cefex plates are not entirely selective as mixed bacteria populations were present alongside *Campylobacter* spp. Therefore, the objective of the present study was to identify the compositional diversity of colonies enumerated from post-chill poultry carcass samples inoculated with *Campylobacter jejuni, coli,* and *lari* on various selective and non-selective media (Campy-Cefex, mCCDA, and TSA). It was expected that a more comprehensive identification of the microbial community of the pooled colonies enriched from the various media would allow for a delineation of *Campylobacter* ecology and the effectiveness of selective agents in isolation media. The current research was done in addition to a parallel study that inoculated post-chill poultry rinsate with the respective *Campylobacter* spp. (*jejuni, coli, lari*) at various concentrations (1.00, 2.00, 3.00, and 4.00 Log_{10} CFU/mL). The samples from that study were plated on selective and non-selective media to develop an accurate quantification method using a BAX® System Real-Time polymerase chain reaction assay (Bodie et al., submitted for publication).

2. Materials and Methods

2.1 Inoculation

The *Campylobacter* spp. used in the current study were American Type Culture Collection® (ATCC®) *C. jejuni* 700819, *C. coli* ATCC® BAA-1061 and *C. lari* ATCC® BAA-

1060. These ATCC® *Campylobacter* strains were used because they were isolated from humans and their genomes have been sequenced, allowing for a more comprehensive appraisal of responses to selective media. Prior to the onset of the experiment, each *Campylobacter* spp. (*C. jejuni*, *C. coli* and *C. lari*) were revived from stock cultures stored at -80 °C. Stock cultures were streaked for isolation on modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA; Himedia, Mumbai, India), and incubated at 42 °C for 48 h under a microaerophilic environment (5% O_2, 10% CO_2, and 85% N_2) created using the Advanced Anoxomat™ III (Advanced Instruments, Norwood, MA, USA). After incubation, a single *Campylobacter* colony was transferred to 10 mL of Blood Free Bolton Broth (BFBB; Criterion, Hardy Diagnostics, Santa Maria, CA, USA). The *Campylobacter* inoculated BFBB was incubated for 48 h at 42 °C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) using the Anoxomat™. The cell density of the inoculum used throughout the study was 9.01, 9.13, and 9.05 Log_{10} CFU/mL for *C. jejuni*, *C. coli*, and *C. lari*, respectfully.

2.2 Sample Preparation

Post-chill poultry rinsates were collected from a commercial poultry processor in the southeastern United States according to USDA-FSIS recommendations for pathogen testing (USDA-FSIS, 2021). All post-chill poultry rinsates were shipped overnight to the BSL-II food safety laboratory at the Meat Science and Animal Biologics Discovery (MSABD) program (Madison, WI, USA) in an insulated shipping cooler with frozen gel packets (ULINE, Wisconsin, USA). Samples were kept frozen until ready for use. Rinsates were subsequently thawed and combined into a bulk to alleviate variation from individual rinsate samples. Prior to enrichment, the bulk post-chill rinsate sample was screened using the BAX® System Real-Time PCR Assay. Additionally, the bulk post-chill rinsate were plated on mCCDA and TSA for

prevalence of *Campylobacter* and competing microorganisms. The mCCDA plates were incubated under microaerophilic conditions at 42 °C for 48 h, whereas TSA plates were incubated aerobically at 37 °C for 48 h.

After confirmation of *Campylobacter* free rinsates, 30 mL aliquots of the bulk post-chill rinsates were added into 24 oz sterile Whirl-Pak (Nasco, Fort Atkinson, WI, USA) bags. Post-chill poultry rinsate samples were inoculated at a targeted 1.00, 2.00, 3.00, and 4.00 Log_{10} CFU/mL of *C. jejuni* ATCC 700819, *C. coli* ATCC BAA-1061, or *C. lari* ATCC BAA-1060, respectively. For each inoculated post-chill rinsate sample, 30 mL of pre-warmed (42 ˚C) 2× BFBB with two times the antibiotic supplement was added. All inoculated post-chill rinsate samples were agitated for 10 s and 100 μL were spread plated in duplicate on Campy-Cefex, mCCDA, and TSA. Plates were incubated for 48 h at 42 ˚C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) using the Anoxomat™ system. Colonies from one respective plate of each treatment, per agar plate (Campy-Cefex, mCCDA and TSA) were pooled and saved for subsequent microbiome analysis (N = 72; n = 24 per medium). All pooled samples were stored in 200 μL of 1× Phosphate Buffered Saline (PBS) and stored at -80 ˚C until the genomic DNA could be extracted.

2.3 DNA Extraction

Prior to the experiment, samples were thawed at room temperature. Genomic DNA from each sample was extracted using the standard spin column procedure of the Qiagen DNeasy Blood & Tissue Kit and protocol (Qiagen, Valencia, CA, USA). Extracted samples were eluted in 200 μL of Buffer AE by hydrating the spin column and incubated for 5 min at ambient temperatures. Initial concentrations of samples were analyzed using a Tecan Spectrophotometer (Tecan, Raleigh, NC, USA) in a NanoQuant plate (Thermo Scientific, Waltham, MA, USA).

Samples under 10 ng/μL were precipitated to concentrate more DNA. First, 20 μL of 3M sodium acetate (Fisher Scientific, Hampton, NH, USA) was added to each sample and pulse vortexed briefly. This was followed by adding 500 μL of ice cold 100% molecular grade ethanol (Fisher Scientific, Hampton, NH, USA) to the samples and pulse vortexed briefly. The subsequent solution was subsequently incubated at -80 °C for 24 h. After 24 h, samples were centrifuged at full speed (13,000 rpm), at ambient temperature for 30 min. The pellets from the centrifuged samples were washed twice with 0.5 mL ice cold ethanol and centrifuged (13,000 rpm) at ambient temperature for 10 min. The sodium acetate and ethanol were removed and subjected to a quick spin (10-s top speed) to remove any trace amounts of both sodium acetate and ethanol. The remaining pellet was air dried and resuspended in 50 μL of Buffer AE. Afterwards, a NanoDrop One (Thermo Scientific, Waltham, MA, USA) was employed to determine the concentration purity ratios (260/280, 260/230) of genomic DNA from all extracted DNA samples. Samples above 15 ng/uL concentrations were diluted to 10 ng/uL in Buffer AE. Final concentrations of 10 ng/uL were confirmed via spot checking with a NanoDrop One. Samples were stored at -80 °C until the library was prepared.

2.4 Library Preparation

Individual DNA samples were thawed at ambient temperature prior to the study. Following Kozich et al. (2013), a sequencing library was constructed using custom primers and a high-fidelity polymerase (Accuprime Pfx DNA polymerase, Thermo Fisher Scientific, Waltham, MA, USA) that were designed to amplify the V4 region of the 16S rDNA. Sequencing library samples were amplified using a BioRad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR amplification conditions were as follows: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealed at 55 °C for 30 s, and strands extended at 68 °C for

1 min. This cycle was repeated 35 times. After the PCR cycle, DNA amplification was verified by running gel electrophoresis on the PCR products.

After verification by gel electrophoresis, the PCR products were normalized using a SequalPrep™ Normalization kit (Life Technologies, Carlsbad, CA, USA) to attain equimolar concentration and sample volume (18 µL). Once normalized, 5 µL aliquots of each sample were pooled as final library. The quantification of the final library was determined using a qPCR with a KAPA library quantification kit for Illumina platforms (Kapa Biosystems, Woburn, MA, USA) and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). Additionally, amplicon product size was assessed using an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA).

2.5 16S rDNA Sequencing

The final pooled library was diluted to 20 nM in HT1 Buffer and subsequently denatured in fresh 0.2 N NaOH for a concentration of 12 pM. The resulting library and PhiX control v3 (Illumina, Carlsbad, CA, USA) were then further diluted to 6 pM and combined (20% w/v PhiX control v3). The combined library and PhiX solution were loaded onto a MiSeq v2 (500 cycles) reagent cartridge (Illumina, Carlsbad, CA, USA). Resulting sequences were uploaded to BaseSpace (Illumina, San Diego, California, USA), NCBI Sequence Read Archive (Project Accession), and Github (Lab repository).

2.6 Bioinformatic Analyses

Bioinformatic analyses were performed using the Quantitative Insights into Microbial Ecology 2 (QIIME2) pipeline (version 2021.11; Bolyen et al., 2018), Within the QIIME2 pipeline, microbiome main effects were considered significant with a $P \leq 0.05$, and a pairwise difference of $Q \leq 0.05$, with each statistical comparison. Demultiplexed sequencing reads were retrieved by downloading the FASTQ files from the Illumina BaseSpace website. Demultiplexed

sequencing reads were imported into QIIME2-2021.11 using Casava 1.8 paired-end demultiplexed format (via qiime tools import), then filtered and denoised in DADA2 via q2-dada2 (Callahan et al., 2016). Using the mafft plugin, the operational taxonomic units (OTU's) were aligned, and a phylogenetic tree was produced utilizing fasttree2 (via q2-phylogeny; Price et al., 2010). Once the phylogenetic tree was generated, SILVA (99% OTUs full-length sequences) differentiating at 95% confidence (via q2-feature-classifier), was used to identify the aligned OTUs (Bokulich et al., 2018a). Using the q2-diversity feature, α- and β-diversity were evaluated. From the generated metrics, main effects and interactions were examined for α-diversity using ANOVA (via q2-longitudinal; Bokulick et al., 2018b) and β- diversity using ADONIS (Anderson et al., 2001). Moreover, pairwise comparisons were analyzed using Kruskal Wallis for α-diversity metrics, Shannon's Diversity Index and Pielou's Evenness (Shannon, 1948; Pielou, 1966; Pielou's Evenness; 1992), and ANOSIM for β-diversity metrics comparisons, Bray Curtis and Weighted Unifrac is (Bray and Curtis, 1957; Lozupone and Knight, 2005; Lozupone et al., 2007). Lastly, the analysis of microbiomes composition (ANCOM) was used to evaluate compositional variation from the various agar plates to identify different abundant features within the paired populations (via q2-composition; Mandal et al., 2015). When generating relative abundance taxa bar plots, anything less than 1% was represented as other for the taxa.

3. Results

3.1 Effect of species and media on microbial diversity recovered from media

In the current study**,** interactions between *Campylobacter* spp. and media type on diversity and microbiota composition were analyzed. Using ANOVA to delineate main effect and interactions, there was no interaction observed between species and media for alpha diversity

metrics, but there was a main effect of species and media (Pielou's Evenness and Shannon Diversity; **Table 1**). However, when using ADONIS to delineate beta diversity main effect and interactions, there was a significant interaction of species and media type on the Bray Curtis and Weighted Unifrac of the pooled colonies from inoculated rinsates ($P < 0.05$; **Table 2**).

3.2 Impact of species on the microbiota recovered from media

During the study, three *Campylobacter* spp. were used, *C. jejuni, C. coli* and *C. lari*. After the enumeration of each species on mCCDA, Campy-Cefex, and TSA, colonies were pooled, and DNA was extracted and sequenced from the different media. Initially, the pooled colonies sequenced from agar plates were examined for their alpha diversity and pairwise contrasts for significant effects between the species. Using an ANOVA, the main effects were seen using the Pielou's Evenness and Shannon Diversity metric ($P < 0.05$; **Table 1**; **Figure 1**). For Pielou's Evenness, main effect was observed for species under the Pielou's Evenness metric ($P < 0.05$; **Table 1**). Pairwise differences among the species were explored using Kruskal-Wallis. When analyzed, differences were observed with *C. jejuni* and *C. coli,* as well as *C. jejuni* and *C. lari* ($P < 0.001$; $Q < 0.001$). The greatest evenness was observed with *C. jejuni* followed by *C. lari*, then *C. coli*. Additionally, under Shannon's Diversity, a main effect of species was detected with pairwise differences being delineated between *C. jejuni* and *C. lari* ($P < 0.001$; $Q < 0.001$) as well as *C. jejuni* and *C. coli* ($P < 0.001$; $Q < 0.001$). Pooled colonies from *C. jejuni* inoculated poultry samples exhibited a greater richness than *C. coli* and *C. lari*.

When beta metrics were compared, differences among the three *Campylobacter* spp. inoculated in the poultry rinsate samples were elucidated using Bray Curtis and Weighted Unifrac (**Figure 2**). Using ADONIS, an interaction of species and media was significant ($P < 0.05$; **Table 2**). Pairwise differences between the species were explored using ANOSIM.

Differences in Bray Curtis and Weighted Unifrac were observed between all pairs (P< 0.05; Q < 0.05; **Supplemental Table 3**; **Table 2**).

The microbial compositional profiles were generated using the median relative abundance of the taxa throughout the study at both the phyla and genus level. On the phyla level, *Campilobacterota* was the main taxa present among all three *Campylobacter* strains (**Figure 3**). Additionally, other phyla were present at much lower levels, such as *Firmicutes, Bacteriodata*, and *Proteobacteria* (**Figure 3a)**. When investigated at the genus level, numerous distinguishable taxa were identified, including Campylobacter, *Pseudomonas*, *Lactobacillus, Enterococcus, Clostridia* and *Staphylococcus*. In addition, when using ANCOM to determine the significantly different taxa among different *Campylobacter* spp. inoculated in poultry rinsates, at the phyla level, *Campilobacterota* was significantly different (P< 0.05; W= 14). At the genus level *Campylobacter, Lachnospiraceae,* and *Oscillospiraceae* were significantly different (P < 0.05; W= 218, 209, and 201, respectively).

3.3 Impact of media on the microbiota recovered from media

The main effect and interactions were explored for alpha diversity metrics. The main effect of media was significant for Pielou's Evenness and Shannon Diversity (P < 0.05; **Supplemental Table 2**). Pairwise differences were observed in Pielou's Evenness and Shannon Diversity with differences being detected between the pooled colonies from Cefex with TSA and TSA and mCCDA (P < 0.001; Q < 0.001; **Figure 4**). For both metrics, the Shannon's Entropy and Pileous Evenness among TSA was numerically the largest, followed by Campy-Cefex, then mCCDA.

The main effect of media and an interaction between media and species was detected using Bray Curtis and Weighted Unifrac (**Table 2**; **Figures 5**). Moreover, there were differences

between TSA and mCCDA (P = 0.001; Q = 0.002) and TSA and Campy-Cefex (P = 0.001; Q = 0.002) for the Bray Curtis and Weighted Unifrac metrics. As seen with alpha diversity metrics, TSA was numerically the greatest, followed by Campy-Cefex then mCCDA, among Bray Curtis and Weighted Unifrac metrics.

Mean taxa bar plots were generated for the overall microbial composition of poultry rinsates inoculated with *Campylobacter* on the different media. In addition, ANCOM was utilized to determine the significantly different abundant taxa among the microbiota at the phylum and genus levels (**Figure 6**; P < 0.05). Overall, 99.00 and 99.43% of the taxa at the genus level were identified as *Campilobacterota* for Campy-Cefex and mCCDA media. However, TSA plates yielded an overall prevalence of 97.5% *Staphylococcus* and 2.5% *Campilobacterota*. When ANCOM was utilized, there were two main taxa at the phyla level among the media, *Campilobacterota* and *Firmicutes* (W = 14 and 13, respectively). When the mean taxa at the genera level were examined, numerous taxa were present on the media after being plated with inoculated poultry rinsates samples, including *Bacillus, Lactobacillus, Staphylococcus, Anicobacter,* and *Enterococcu*s. However, when using ANCOM, the only significant difference occurring among the different media was *Staphylococcus* (W = 219; **Figure 6B**).

4. Discussion

4.1 Microbial diversity between *Campylobacter* species isolated from media

In the current study, *Campylobacter*, *Pseudomonas*, *Lactobacillus,* and *Clostridia* were among the most represented taxa at the genus level recovered from the *Campylobacter* spp. respective plating media. The ANCOM revealed that *Campylobacter, Oscillospiraceae,* and *Lachnospiracea*e were significantly abundant taxa among the plates for the three species.

However, the corresponding *C. jejuni* inoculated poultry rinsates in the current study was primarily comprised of *Campylobacter*, *Oscillospiraceae, Lachnospiraceae, Enterococcaceae, Clostridiaceae*, *Faecalibacterium,* and *Bacteroides* (approximately 70%). The relative abundance values of *Streptococcus*, *Enterococcaceae,* and *Lactobacillales* among the microbiota of *C. jejuni* were more abundant taxa than *C. coli* and *C. lari* poultry rinsates samples. Under diversity metrics Pileou's Evenness and Shannon's Entropy, a significant difference was observed for *C. jejuni* plates compared to the other species. When comparing *Campylobacter* spp. with each other, Bray Curtis and Weighted Unifrac metrics revealed a significant difference among the three, with *C. jejuni* being different from *C. coli* and *C. lari* plate colonies. These results suggest a potential interaction occurring between *C. jejuni* and the microbial ecology of poultry rinsates, impacting both the diversity and the taxa present.

The poultry environment is a niche that *C. jejuni* is known to be well adapted for inhabiting (Hakeem and Lu, 2021). Although, there have been some reports of *C. coli* and *C. lari* infections caused by poultry contamination, they are seldom reported as frequently as *C. jejuni* (Wagenaar et al., 2013). More recently, *C. col*i and *C. lari* have been observed to be present more frequently in dairy products and vegetables (Facciola et al., 2017). It is possible that the different *Campylobacter* spp. have their own unique metabolic requirements and thus thrive in different environments (Stokes et al., 2022). As such, after *C. jejuni* was inoculated in the poultry rinsates, interactions with competitors and adverse effects of antimicrobials used throughout processing may have allowed for a more diverse microbial ecology compared to the other *Campylobacter* spp. Therefore, in the current study, the microbial diversity differences may have been possible due to the relationship between *C. jejuni* and the poultry environment. When *C. jejuni* was inoculated into the poultry rinsate matrix, it resulted in the recovery of multiple

microorganisms, as compared to the other *Campylobacter* spp. used in the study, *C. coli* and *C. lari*.

Campylobacter has a specific environmental niche required for survival and its metabolism is poorly understood (Hilbert et al., 2010; Stahl et al., 2012). Likewise, each species has its unique metabolic requirements. However, past research and performance standards were usually focused on *C. jejuni* because it is the leading cause of human disease (Butzler, 2004; Wagenaar et al., 2013). As such, in the poultry processing facility, whole carcass processing intervention strategies used on the whole carcass are targeted for *C. jejuni*. It is possible that these strategies may be less effective on *C. coli* and *C. lari* (Zhao et al., 2010). As a result, *C. coli, C. lari* and other competing microorganisms may be more equipped to withstand interventions used on the poultry carcass used during processing. Additionally, the antimicrobials focused on *C. jejuni* and other pathogenic foodborne pathogens prominent in poultry such as *Salmonella* could create a niche where other microorganisms present in the poultry environment will proliferate, potentially creating a more diverse rinsate microbial community.

4.2 Media effect on microbial diversity of *Campylobacter* colonies

In the U.S., the USDA-FSIS recommends using of Campy-Cefex for *Campylobacter* sampling of detection and enumeration from poultry products (USDA-FSIS, 2021). However, mCCDA is recommended by the Bacteriological Analytical Manual (BAM) protocol, designed by the Food and Drug Administration (FDA) protocol for *Campylobacter* isolation (FDA, 2001). Current problems with these methods are that they are labor intensive requiring approximately 24 to 48 h of enrichment before results (USDA-FSIS, 2021). However, such methods can be problematic because they may not be sensitive enough. *Campylobacter* is known to require

relative restrictive growth conditions. Additionally, under stressful conditions such as cold temperatures and aerobic environments, *Campylobacte*r can be viable but non-culturable, making it difficult to get precise results due to the possibility of generating false negatives (Kim et al., 2019). The purpose of this study was to elucidate the efficacy of selective plating for *Campylobacter* isolation in poultry rinsates. This was accomplished by analyzing the microbial community of selective media (Campy-Cefex and mCCDA) and non-selective (TSA) media from poultry rinsate samples.

The mode of action for selective media relies on antimicrobials to select for the microorganism of choice (Al-blooshi et al., 2021). In a diverse microbial matrix such as poultry rinsate samples, some microorganisms may be resistant to the antibiotics used in plating media, enabling them to grow on plating media considered to be selective (Davies and Davies, 2010). However, it is possible that these same selective antibiotics can also support microorganisms that have a similar metabolism to *Campylobacter* or perhaps exhibit a synergistic effect, allowing them grow in the presence of *Campylobacter* (Wang et al., 2017). Therefore, in the present study, all plated colonies were pooled for sequencing. It is important to note that *C. coli* had no colonies on mCCDA agar for all poultry rinsate samples. *Campylobacter* cells were apparently VBNC, as *Campylobacter* DNA was picked up from all mCCDA plates and sequenced.

The mCCDA media consists of bacteriological charcoal, casein hydrolysate, sodium deoxycholate, ferrous sulfate and sodium pyruvate which are required for *Campylobacter* to grow; but is also supplemented with cefoperazone and amphotericin B as an antimicrobial to counter competing microorganism (Kiess et al., 2010). Campy-Cefex is composed of Brucella agar, ferrous sulfate, sodium bisulfite and sodium pyruvate and utilizes lysed horse blood, as well as cefoperzone and cycloheximide as antibiotics to eliminate competitive non-

Campylobacter organisms (Oyrazabal et al., 2005). The only antibiotic shared by mCCDA and Campy-Cefex is cefoperazone, which is added at similar rates of 32 mg/L and 33 mg/L, respectively. In the current study, *Clostridacea, Bacteroides, Staphylococcus*, and *Campylobacter* were among the most represented taxa at the genus level recovered from the plates, but only *Staphylococcus* was significantly different among the three media types, mCCDA, Campy-Cefex, and TSA. However, the unselective TSA was primarily composed of *Staphylococcus* with a relative abundance value above 90%.

In this study, TSA was used to recover microorganisms potentially present along with *Campylobacter* spp. Since, TSA is considered a non-selective medium, with a nutrient content designed to support growth of a wide range of microorganisms, differences were expected compared to selective media. Established by the USDA-FSIS, TSA determines the abundance of indicator organisms in the whole carcass rinsate. The results indicate that *Staphylococcus* is potentially in competition with *Campylobacter* as it has a relative abundance value above 90%. Previous research has shown that *Staphylococcus* spp. are prevalent in poultry (Syed et al., 2020; Szafraniec et al., 2022). It has been shown that poultry can be at a higher risk for *Staphylococcus* contamination over swine and cattle because the skin of poultry is left intact on most cuts, while the skin is removed from swine and cattle (Hanning, et al., 2012). As such, poultry meat can be considered a major reservoir for *Staphylococcus*. Additionally, the lack of selective antimicrobials used in making TSA likely supports more *Staphylococcus* on the TSA medium compared to Campy-Cefex and mCCDA.

Some studies have evaluated the efficacy of different agar plates for their ability to isolate *Campylobacter* from poultry samples. In a study by Gonsalves et al. (2016), they evaluated plating on Campy-Cefex and mCCDA for isolation and identification and direct plating of *C.*

jejuni and *C. coli* samples from the broiler slaughter process (Gonsalves et al., 2016). The results from this study showed that significant differences occurred between the Campy-Cefex and mCCDA plates used for cell recovery in the analysis of pre-chiller carcass samples, where Campy-Cefex yielded higher *Campylobacter* cell numbers. However, despite resulting in higher isolation of colonies from the Campy-Cefex, no *C. coli* was recovered in pre- and post-chiller carcass samples, although it was recovered from mCCDA. The authors concluded that mCCDA and Campy-Cefex are efficient for isolating *C. jejuni* from poultry carcass samples, but *C. coli* is variable and inconsistent with other matrices. However, in a study by Oyarzabal et al. (2005), they evaluated 240 broiler carcass rinse samples by enumerating *Campylobacter* on Campy-Cefex, mCCDA, Karmali and CLA (Campy-Line agar) agar plates. The results of this study demonstrated that Campy-Cefex yielded the highest efficacy for *Campylobacter* enumeration (Oyarzabal et al. 2005). However, there was no significant differences among the other selective media used throughout the study. In the present study, we looked at the microbial diversity of selective media Campy-Cefex and mCCDA. The results demonstrated no significant differences between Campy-Cefex and mCCDA for alpha and beta metrics regardless of *Campylobacter* spp. sample used. These results suggest that both selective media, Campy-Cefex and mCCDA have equal efficacy for *Campylobacter* isolation.

5. Conclusions

Among *Campylobacter* spp., *C. jejuni* is the most prevalent, and it is primarily associated with poultry meat infections. However, campylobacteriosis is also caused by *C. coli* and *C. lari*. As a result, poultry processing has steps such as scalding, chiller tanks, and the use of antimicrobials in place to reduce *Campylobacte*r in products. The USDA-FSIS recommends whole carcass rinses as a sampling method for *Campylobacter* prevalence in poultry after

interventions have been used to reduce pathogenic microbial load. Therefore, in the study we wanted to elucidate the microbial diversity of poultry rinsates inoculated with either *C. jejuni*, *C. coli* and *C. lari*. The present study indicates that poultry rinsates inoculated with *C. jejuni* have a greater richness and evenness that that of *C. coli* and *C. lari*. It is unclear if the difference in richness and evenness was based on solely on species. Research has shown that background microorganisms can have select responses to a novel species introduced into their environment (Ratzke and Gore, 2018). In the present study, chemical interventions used during the poultry process could prompt a microbial community that competes with *C. jejuni*, but not as competitive for *C. coli* and *C. lari*. As a result, more research is needed to understand the effect of poultry chemical interventions and how they impact microbial diversity as well as to uncover if *C. jejuni* is part of a truly more diverse and even community compared to *C. coli* and *C. lari* by conducting a more competitive microbial ecology study. Additional research would ensure microbial community differences are not caused by interactions with background microbiota.

Ultimately for detection, the USDA-FSIS recommends multiple media for *Campylobacter* isolation. However, the medium chosen may affect the recovery of *Campylobacter* spp. based on its mechanism of action for providing growth. The current study elucidated the microbial community of recommended media for *Campylobacter* isolation. The results suggested that there is a significant difference between selective media Campy-Cefex and mCCDA compared to the non-selective media TSA for alpha and beta diversity from pooled colonies sequenced by 16S rDNA. No pairwise differences were detected for alpha and beta diversity metrics between mCCDA and Campy-Cefex. The results demonstrated that mCCDA and Campy-Cefex are effective for *Campylobacter* isolation. In addition, mCCDA is a blood free medium, as opposed to Campy-Cefex that has laked horse blood. As such, mCCDA is less labor

intensive to make and easier to maintain. Laked horse blood is also costly and must be considered for large *Campylobacter* sampling projects (Oyazabal et al., 2005; Baron et al., 2013). Therefore, the results from this study suggest mCCDA can be used as an alternative to Campy-Cefex plating for *Campylobacter* detection. It is important to note that the poultry rinsate samples were artificially inoculated with *Campylobacter* spp.

As such, elucidating the microbial community of selective and non-selective media of *Campylobacter* spp. in poultry rinsate samples may allow for a more comprehensive microbial population profile of the microbial ecology associated with poultry carcasses and the processing environment (Feye et al., 2020; Ricke et al., 2022). It is also possible that enrichment medium can play a part in impacting the selectivity of plate media. In the current study, the USDA recommended 2xBFBB was used an enrichment for *Campylobacte*r. However, after 20 h enrichment, though 2xBFBB was able to grow *Campylobacter*, TSA plates revealed *Staphylococcus* was also abundant. The results suggest that 2xBFBB is effective for *Staphylococcus* growth in poultry rinsates. Therefore, future studies should consider using TSA under *Campylobacter* preferred growth conditions when sampling poultry products to uncover the enrichment efficacy and selectivity of the media being used.

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Tables

Table 1. Main effects and interactions using ANOVA of the α-diversity metrics of the rinsates.

		Pielou's Evenness			Shannon's Entropy		
	Df[1]	SS[2]	F-value[3]	P-value[4]	SS[2]	F-value[3]	P-value[4]
Media	2	1.126	18.07	18.07	18.07	27.74	< **0.001**
Species	2	1.012	41.05	41.05	41.05	24.94	< **0.001**
Media×Species	4	0.078	2.682	2.682	2.682	0.964	0.432

[1]Df degrees of freedom, maximum number of logical independent values
[2]SS variance of set of scores
[3]F-Value main effect differences between populations
[4]P-Value main effect differences

Table 2. Main effects and interactions using ADONIS of the β-diversity metrics of the poultry rinsates.

		Weighted Unifrac					Bray Curtis				
	Df[1]	SS[2]	MS[3]	F-value[4]	R^2	P-value[6]	SS[2]	MS[3]	F-value[4]	R^2	P-value[6]
Species	2	1.112	0.556	8.631	0.105	**0.001**	11.55	5.773	34.12	0.341	**0.001**
Media	2	2.918	1.459	22.63	0.276	**0.001**	3.451	1.725	10.20	0.102	**0.001**
Species×Media	4	0.688	0.171	2.667	0.064	**0.015**	3.637	0.909	5.374	0.108	**0.001**
Residual	70	4.512	0.064	8.631	0.426	0.001	11.84	0.169	34.12	0.350	0.001
Total	78	8.610	NaN	NaN	1.000	NaN	30.48	NaN	NaN	1.000	NaN

[1]Df degrees of freedom, maximum number of logical independent values
[2]SS variance of set of scores
[3]MS average sum of squares
[4]F-Value main effect differences between populations
[5]R^2 variance for dependent variable that's explained by independent variables
[6]P-Value main effect differences

Figures

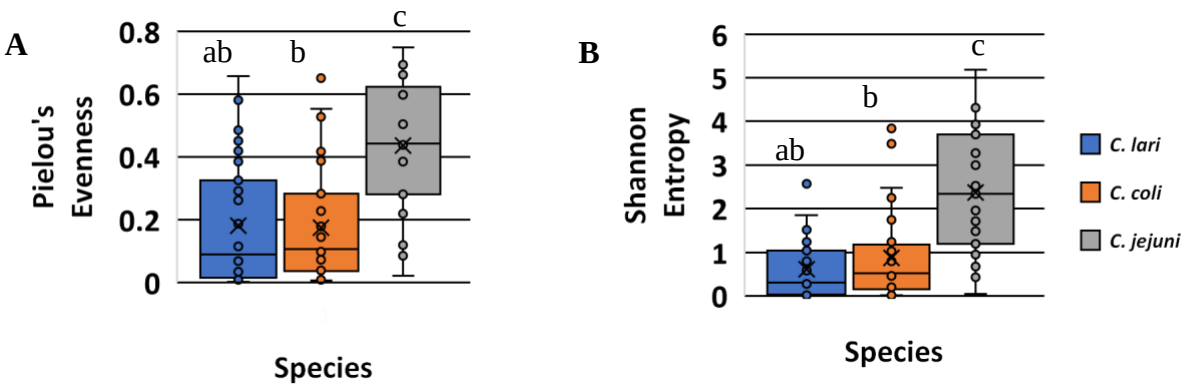


Figure 1. Effect of species on the Pielou's Evenness (A) and Shannon's Entropy (B). Alpha diversity metrics represented as a box plot. Main effects of species were determined using ANOVA ($P < 0.05$). Pairwise differences between species were determined using Kruskal-Wallis, with significance indicated by (a-c).

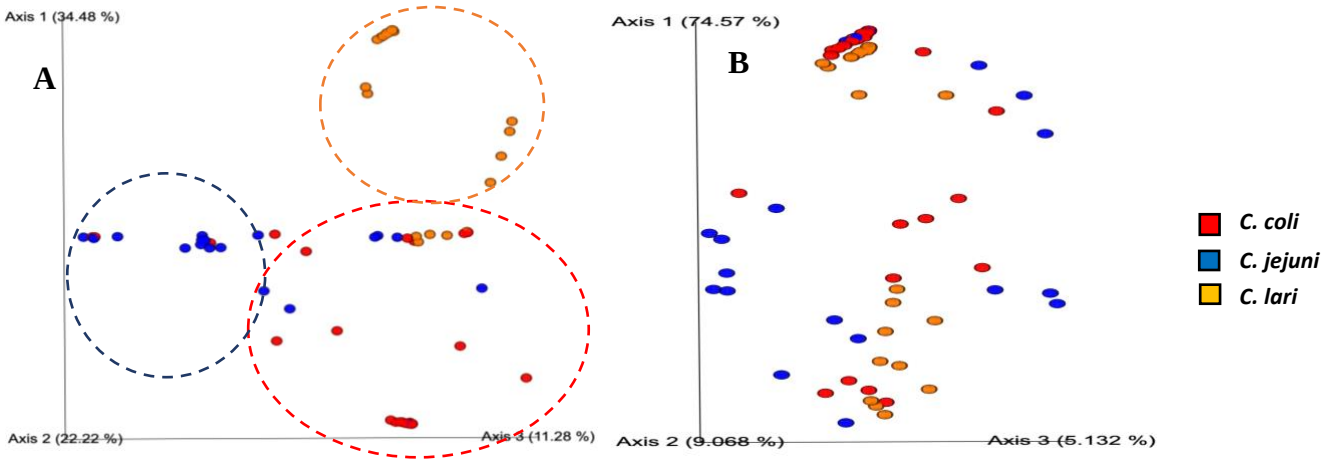
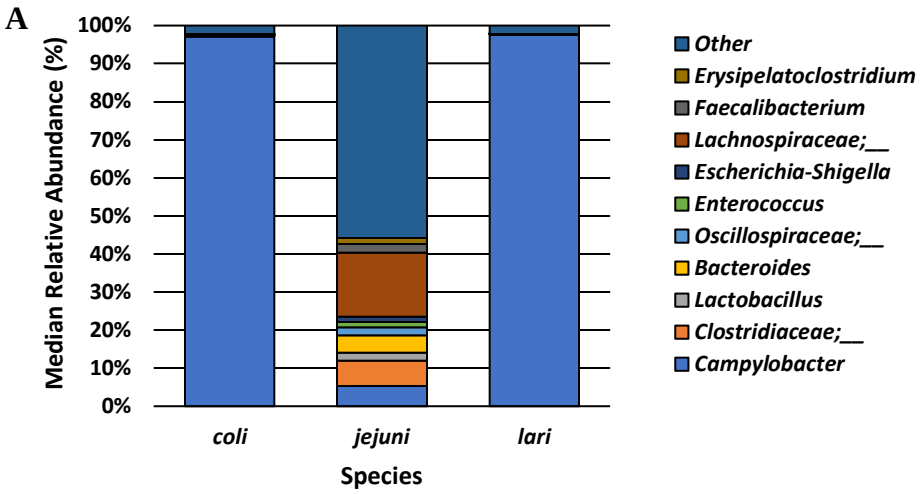


Figure 2. The effect of Campylobacter spp. on Bray Curtis (A) and Weighted Unifrac (B). Beta diversity metrics represented as a PCOA plot. Main effects of species were determined using ADONIS ($P < 0.05$; $Q < 0.05$). Pairwise differences between species were determined using ANOSIM.



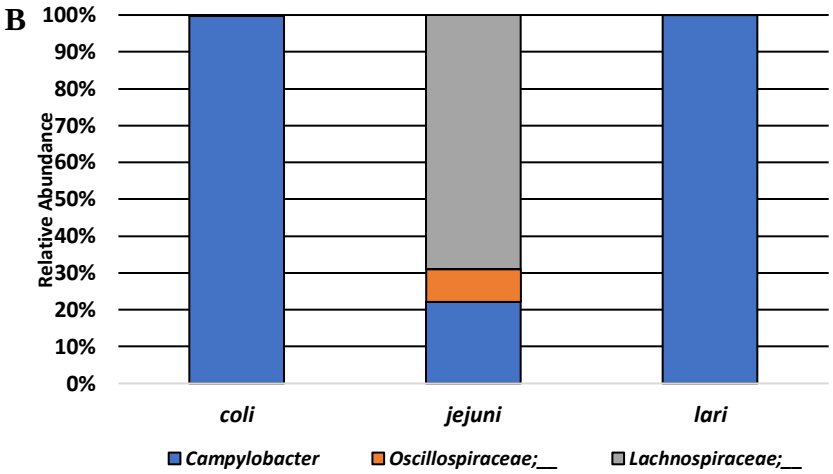


Figure 3. Median Taxa (A) & significantly different taxa (B) using ANCOM at the genus levels between species. The effect of *Campylobacter* species from inoculated post-chill rinsates with 2x BFBB. ANCOM was used to compare taxa recovered on the genus level, and significant differences were observed for *Campylobacter*, *Oscillospiraceae* and *Lachnospiraceae* ($P < 0.05$; W= 218, 209 and 201).

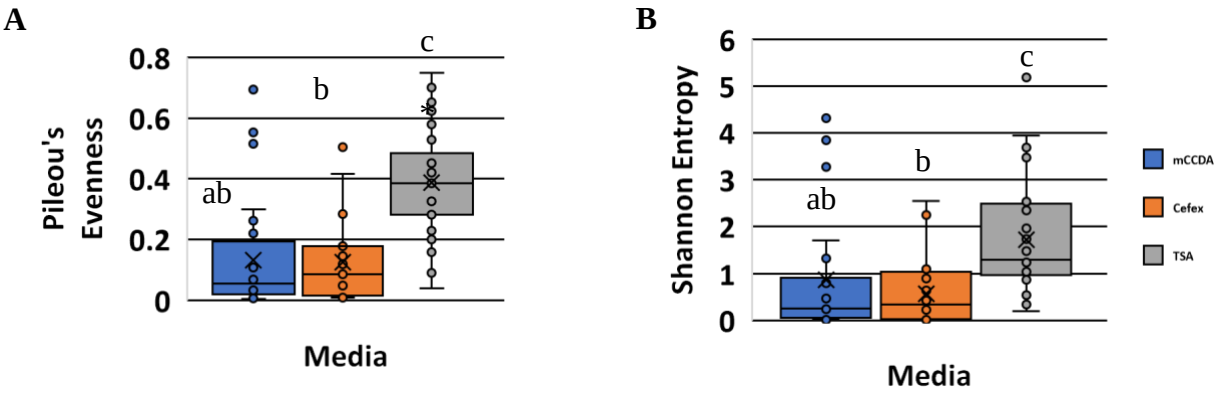


Figure 4. The effect of media on Pielou's Evenness (A) and Shannon's Entropy (B). Alpha diversity metrics represented as a box plot. Main effects of media were determined using ANOVA ($P < 0.05$). Pairwise differences between media were determined using Kruskal-Wallis, with significance indicated by (a-c).

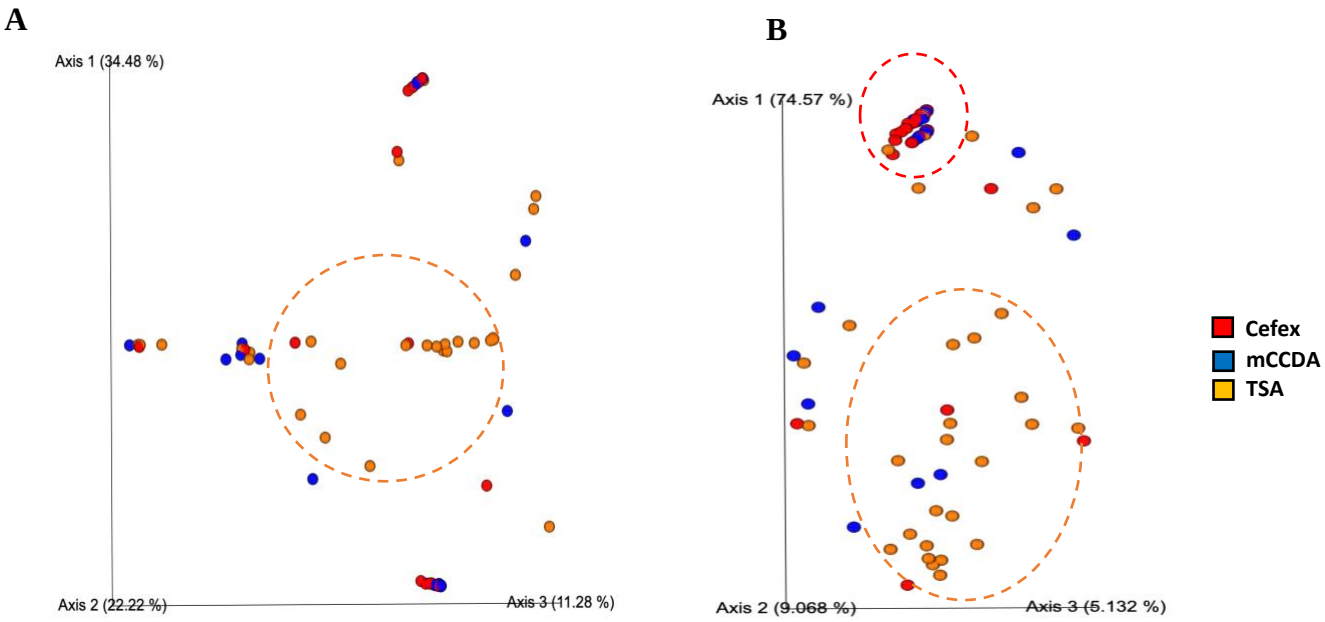


Figure 5. The effect of media on the Bray Curtis (A) and Weighted Unifrac (B). Beta diversity metrics represented as a PCOA plot. Main effects of media were determined using ADONIS ($P < 0.05$; $Q < 0.05$). Pairwise differences between media were determined using ANOSIM.

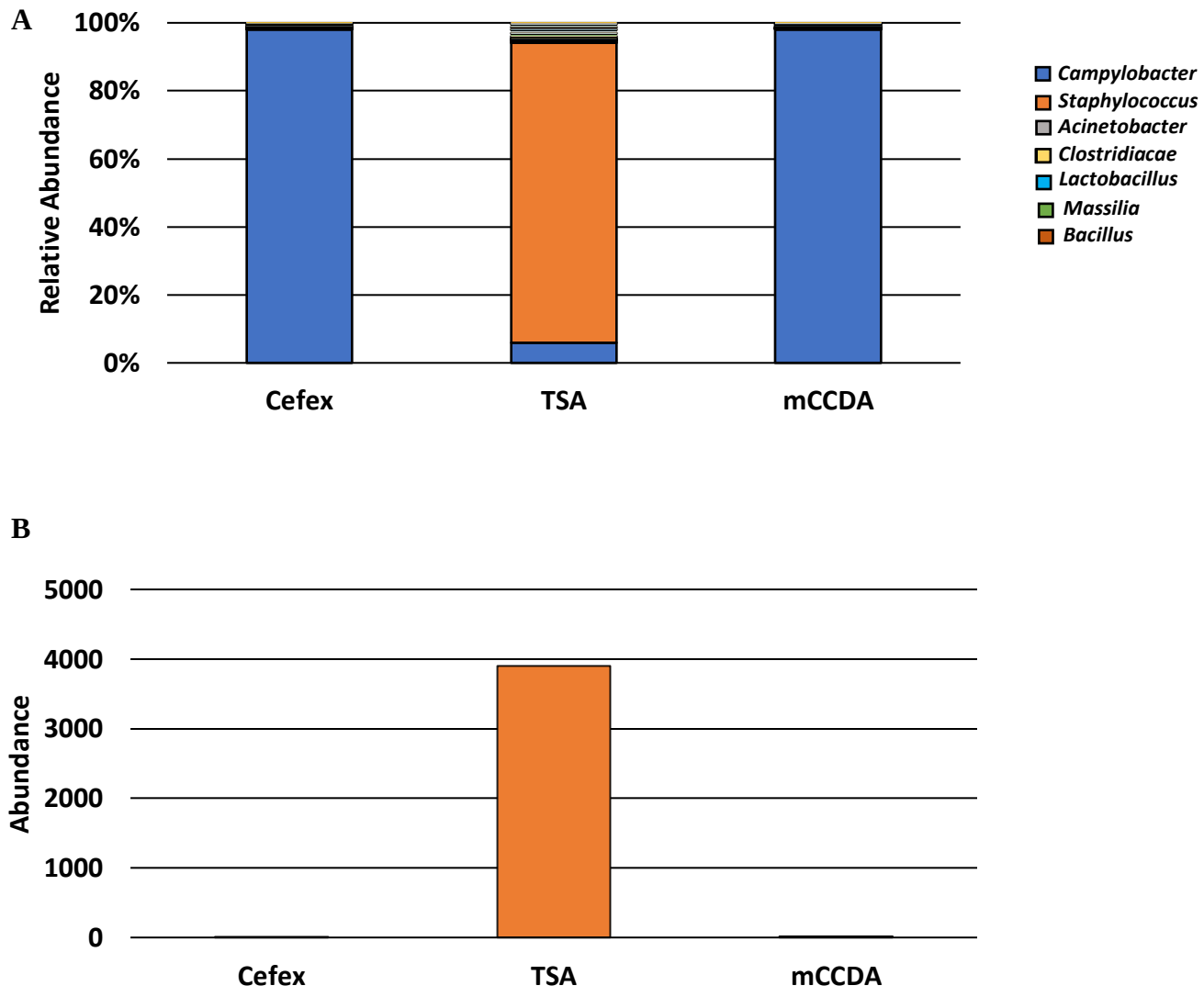


Figure 6. The median taxa (A) and significant taxa using ANCOM (B) between media types. The effect of media from inoculated post-chill rinsates with 2x BFBB. ANCOM was used to compare taxa recovered on the genus level, and significant differences were observed for *Staphylococcus (*$P < 0.05$; W=219).

Supplemental Tables

Supplemental Table 1. Impact of *Campylobacter* species on the Pielou's Evenness and Shannon's Entropy of *Campylobacter* inoculated poultry rinsates using Kruskal Wallis.

		Pielou's Evenness			Shannon's Entropy		
Group 1	Group 2	H[1]	P-value	Q-value	H	P-value	Q-value
coli (n=34)	j*ejuni* (n=19)	14.74	**< 0.001**	**< 0.001**	14.52	**< 0.001**	**< 0.001**
coli (n=34)	*lari (*n=35)	0.560	0.454	0.454	1.102	0.294	0.294
jejuni (n=19)	*lari* (n=35)	19.77	**< 0.001**	**< 0.001**	15.81	**< 0.001**	**< 0.001**

[1] H index test statistic for Kruskal-Wallis

[2] P-Value main effect differences

[3]Q-Value main effect differences with false discovery rate

[4]Bolded values are those with significant P and Q values

Supplemental Table 2. Impact of media type, Campy-Cefex, mCCDA, and TSA, used to detect *Campylobacter* from inoculated poultry rinsates on the Pielou's Evenness and Shannon's Entropy using Kruskal Wallis.

		Pielou's Evenness			Shannon's Entropy		
Group 1	Group 2	H[1]	P-value[2]	Q-value[3]	H[1]	P-value[2]	Q-value[3]
Cefex (n=34)	TSA (n=19)	22.37	**< 0.001**	**< 0.001**	17.13	**< 0.001**	**< 0.001**
Cefex (n=34)	mCCDA	0.006	0932	0.932	0.007	0936	0.936
TSA (n=19)	mCCDA	18.44	**< 0.001**	**< 0.001**	16.02	**< 0.001**	**< 0.001**

[1]H index test statistic for Kruskal-Wallis

[2] P-Value main effect differences

[3]Q-Value main effect differences with false discovery rate

Bolded values are those with significant P and Q values

Supplemental Table 3. Pairwise differences of Bray Curtis and Weighted Unifrac between species using ANOSIM.

		Bray Curtis			Weighted Unifrac		
Group 1	Group 2	R[1]	P-value[2]	Q-value[3]	R[1]	P-value[2]	Q-value[3]
coli (n=34)	*jejuni* (n=19)	0.390	**0.001**	**0.001**	0.276	**0.002**	**0.002**
coli (n=34)	*lari* (n=35)	0.742	**0.001**	**0.001**	0.121	**0.001**	**0.002**
jejuni (n=19)	*lari* (n=35)	0.698	**0.001**	**0.0015**	0.220	**0.001**	**0.002**

[1]R index test statistic for dissimilarity between groups
[2]P-Value main effect differences
[3]Q-Value main effect differences with false discovery rate
Bolded values are those with significant P and Q values

Supplemental Table 4. Pairwise differences of Bray Curtis and Weighted Unifrac between media types using ANOSIM.

		Bray Curtis			Weighted Unifrac		
Group 1	Group 2	R[1]	P-value[2]	Q-value[3]	R[1]	P-value[2]	Q-value[3]
Cefex (n=34)	TSA (n=19)	0.233	**0.001**	**0.002**	0.418	**0.001**	**0.002**
Cefex (n=34)	mCCDA	-0.015	0.715	0.715	-0.008	0.642	0.642
TSA (n=19)	mCCDA	0.226	**0.001**	**0.002**	0.362	**0.001**	**0.002**

[1]R index test statistic for dissimilarity between groups
[2]P-Value main effect differences
[3]Q-Value main effect differences with false discovery rate
Bolded values are those with significant P and Q values

Comparison of Growth Kinetics for Pure Culture *Campylobacter jejuni, coli* and *lari* Enriched in Blood Free Bolton Broth

Aaron R. Bodie and Steven C. Ricke*
[1]Meat Science and Animal Biologics Discovery Program, Dept. of Animal and Dairy Sciences, University of Wisconsin, Madison, WI, 53706, USA

*Corresponding Author:
Steven C. Ricke
Meat Science and Animal Biologics Discovery
Dept. of Animal and Dairy Sciences,
University of Wisconsin,
Madison, WI, 53706, USA
Email: sricke@wisc.edu

Abstract

Currently, culture-based methods used for *Campylobacter* enumeration from the environment remain challenging because it is difficult to isolate and identify species within diverse communities. Microbial growth kinetics is a crucial tool that can be used to optimize detection and quantification methods because it provides quantitative information on *Campylobacter's* growth response in specific environments. *Campylobacter* growth kinetic parameters can be used to refine the sensitivity and efficiency of microbial growth-based methods. Therefore, the aim of this study was to construct growth curves for *C. jejuni*, *C. coli,* and *C. lari* in pure culture and calculate growth kinetic parameters for each *Campylobacter* species in the same environmental conditions. *Campylobacter jejuni*, *C. coli* and *C. lari* were grown over 48 h. Initial concentration of *C. jejuni*, *C. coli,* and *C. lari* were approximately 8.24, 9.01, and 9.26 Log_{10} CFU/mL. After incubation, a 10-fold serial dilution was performed to achieve 4.00 Log_{10} CFU/mL. A 1.00 mL aliquot of each *Campylobacter* species' 4.00 Log_{10} CFU/mL inoculum was added to 14.50 mL of BFBB into respective 15 mL Hungate tubes (N = 45 total samples; n = 5 species per biological replicate per species; 3 species; 15 total per species). After dilutions and sample preparation, initial experimental concentrations were 1.90, 2.51, and 2.76 Log_{10} CFU/mL, respectively. Absorbance measurements were taken in 45 min intervals over 24 h. Optical density readings were plotted versus time to generate the corresponding growth curves and calculate growth kinetic parameters for comparison among species. In pure culture, *C. coli* exhibited the fastest doubling time at 1 h 01 min, while *C. jejuni* and *C. lari* doubling durations were under 1 h 30 min. The exponential phase duration was no later than 5 h for all species, while *C. jejuni* had the longest lag phase at 15 h and 20 min. The growth kinetics variation in the three species of *Campylobacter* illustrates the importance of

flexibility in designing culture growth conditions for optimizing detection based on minimal bacterial concentrations. This study provides kinetics and estimates to shorten enrichment times necessary for low concentration *Campylobacter* detection.

Key words: *Campylobacter*, Kinetics, Enrichment, Prediction, Media

1. Introduction

Campylobacter is the world's leading cause for gastroenteritis (Galanis, 2007; WHO, 2020). This zoonotic microorganism can be isolated from several animal species; however, it represents a severe public health problem in poultry production (Horrocks et al., 2009; Wagenaar et al., 2013). *Campylobacter* spp. are fastidious microorganisms that require a microaerophilic atmosphere containing 5% O_2, 10% CO_2, and 85% N_2. Since *Campylobacter* requires specific growth conditions, isolation can be difficult as cells appear to be much more sensitive to environmental conditions than other major foodborne pathogens such as *Salmonella* and *Escherichia coli* (Mihaljevic et al., 2007). Research has shown that environmental stressors, including suboptimal nutrient conditions, pH, osmotic stress, ambient air composition, and temperatures, can derail *Campylobacter's* metabolism resulting in the cells entering shock and eventually death (Jackson et al., 2009). In addition, *Campylobacter*'s ability to change its physiological and metabolic biological characteristics can interfere with the sensitivity and specificity of culture-dependent methods. When stressed, *Campylobacter* spp. possess the ability to switch to a viable but non-culturable state (VBNC), making it unable to detect phenotypically (Lv et al., 2020). Ultimately, these physiological and metabolic characteristics are not fully understood, therefore detection and enumeration of *Campylobacter* remain challenging.

Molecular-based methods have been used as an alternative to conventional culture-dependent methods such as plating for *Campylobacter* detection and enumeration. Molecular methods are based on the detection of *Campylobacter* spp. specific genomic DNA to identify *Campylobacter* cells. However, one of the main drawbacks to these methods are that they are not sensitive enough to detect low concentrations of *Campylobacter* in samples (Lauri and Mariani, 2009). Molecular-based methods such as real time polymerase chain reaction (RT-PCR) and

impedance-based biosensors still rely on microbial growth, typically requiring a culture-based enrichment step prior to conducting the molecular assay. This approach is recommended because most molecular methods have a limit of detection and require the concentration of *Campylobacter* cell population to be above a certain threshold before the organism can be consistently detected (Chik et al., 2018; Ferone et al., 2020). Ideally, optimizing enrichment for detection can shorten the enrichment time leading to a more rapid detection method. If methods could detect near the exact time point when samples initially achieve the limit of detection, enrichment times can be shortened and potentially increase precision of the assay. Currently, growth-based methods typically use endpoints to ensure concentrations are reached to achieve the appropriate limit of detection. However, determining the earliest time frame when the endpoint of maximum growth is achieved, requires assessing growth response over time and calculating the doubling times for the bacterial cell population. In literature, only minimal growth kinetic information is available for *Campylobacter* spp. growth (Riss and Moravec, 2004). In addition, there is the potential for physiological variation among *Campylobacter* spp. which could impact their respective growth responses and result in different growth kinetic assessments. The variation of species growth becomes an important consideration when attempting to develop universal detection protocols to accommodate multiple *Campylobacter* spp. potentially present in food and environmental samples. Therefore, further development and optimization of these growth-based systems require a better understanding of the growth kinetic parameters of individual *Campylobacter* spp.

Growth kinetic assessments can provide insight into the dynamic response of biological systems and predict phase changes based on time, environment, and nutrient availability. Predictive models for *Campylobacter* spp. cell growth and gauging growth kinetic parameters

(e.g., growth curve, time of doubling, time of lag phase, and specific growth rate) can be essential tools to improve detection and quantification methods. With growth prediction models, identifying growth rates will help to calculate respective phases in *Campylobacter* growth for predicting the earliest times to detect individual species of *Campylobacter*. Therefore, the aim of this study was to develop growth curves for *Campylobacter jejuni*, *coli*, and *lari* under the same growth conditions recommended by the International standard organization (ISO). Blood free Bolton broth (BFBB) is a widely accepted selective enrichment medium used for *Campylobacter* detection. In addition**,** the current protocol of the International Organization for Standardization (ISO) and the former protocol of the United States Department of Agriculture and Food Safety and Inspection Services (USDA-FSIS) recommend BFBB for enrichment of *Campylobacter* spp. therefore, it was the enrichment medium used for growth kinetic determinations in this study.

2. Material and Methods

2.1 Inoculant preparation

Three *Campylobacter* species, *C. jejuni* American Type Culture Collection® (ATCC®) 700819, *C. coli* ATCC® BAA – 1061 and *C. lari* ATCC® BAA1060, were used for this study. To isolate colonies, frozen stock cultures (-80 °C) of each *Campylobacter* species were streak plated on modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA; Himedia, Mumbai, India). Plates were incubated at 42 °C for 48 h using the Anoxomat system (Advanced Instruments, Norwood, MA, USA) to create a microaerophilic (5% O_2, 10% CO_2, and 85% N_2) environment. After incubation, plates were examined to ensure they exhibited *Campylobacter* colony phenotypic characteristics. Following confirmation, one colony from each *Campylobacter* species mCCDA plate was inoculated into 10.00 mL of BFBB respectively. After inoculation,

pure culture *Campylobacter* spp. samples were incubated for 48 h at 42 °C, under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) using the Anoxomat system.

2.2 Enrichment optimization by Growth Kinetics

After incubation of each *Campylobacter* species, a 10-fold dilution series was performed to obtain a concentration of 4.00 Log_{10} CFU/mL. A 14.5 mL aliquot of BFBB was added into each 15 mL Hungate tubes (Bellco, Vineland, NJ, USA), and the tubes were sealed with rubber stoppers and screw tops (N = 45 total samples; n = 5 species per biological replicate per species; 3 species; 15 total per species). Using a 15 mL syringe (Thermo Fischer Scientific, Waltham, MA, USA), a puncture was made through the rubber stopper of the bottles to add 1.00 mL of the 4.00 Log_{10} CFU/mL. Respective *Campylobacter* spp. was added to each Hungate tube by inserting the needle into the rubber stoppers of the tubes. Initial concentrations were approximately 1.90 Log_{10} CFU/mL (*C. jejuni)*, 2.51 Log_{10} CFU/mL (*C. coli*) and 2.76 Log_{10} CFU/mL after incubation volumes were finalized. Samples were incubated in a water bath (Thermo Fischer Scientific, Waltham, MA, USA), set at 42 °C with minimal headspace to create a microaerophilic environment.

2.3 Growth Kinetic Measurements

Campylobacter spp. growth responses were measured by absorbance turbidity of each sample at $OD_{600\ nm}$ using a Spectronic 20 (ThermoFisher Scientific, Waltham, USA). Absorbance was taken in 45 min intervals over 24 h for all samples. Growth curve measurements from plotting the growth responses were used to calculate growth kinetic parameters (Maximum OD, Lag phase, Exponential phase, Specific Growth rate and Doubling time) for each *Campylobacter* species (*C. jejuni*, *C. coli* and *C. lari*). Maximum optical density (MOD) is the highest optical density achieved, where no further OD increases are detected (Ricke et al., 1994;

Ricke and Schaefer, 1996; Froelich et al., 2002). Once MOD was achieved, the growth culture was considered to have reached stationary phase where no further detectable growth occurs. In a bacterial culture, lag phase is defined as the period where growth is not detectable before exponential growth occurs. Lag phase is calculated using the equation L = t - (lnx - lnxi)/p, where L is lag phase (h), p is growth rate, x is initial OD value, and xi is OD value measured at time t between inoculation and the establishment of the logarithmic (log) phase (Lodge and Hinshelwood 1943; Pirt, 1975; Li et al., 2000; Li and Ricke, 2002). Exponential phase was calculated using the natural log OD growth measurements (Ricke and Schaefer, 1991). Exponential phase was defined as the period of time when bacterial cells initiated cellular division through binary fission and subsequently increased exponentially by doubling (Pirt, 1975; Wang et al., 2015). In this study, exponential phase was determined as the initiation of growth, until microbial growth was no longer occurring exponentially. The specific growth rate is the rate of increase in cell population per unit of biomass concentration. The specific growth rate (μ) was estimated for each species using the slope from plotting the natural logarithm of the exponential growth portion against time (Ricke and Schaefer 1991). Lastly, doubling time was calculated. Doubling time of a population is defined as the time required for the bacterial population to double in cell numbers (Pirt, 1975). Throughout the study, doubling time was calculated using the formula: ln2/specific growth rate (μ). After 24 h, a RT- PCR was conducted (Hygiena, New Castle, DE, USA), to ensure specificity of respective *Campylobacter* spp. cultures after enrichment.

2.4 Statistical Analysis

All statistical analyses for this study were performed in JMP 14.0 (SAS institute Inc, Cary, North Carolina, USA). Linear regressions were plotted, and equations were generated

using a General linear regression model to analyze absorbance $OD_{600\ nm}$ values against time for each *Campylobacter* spp. growth kinetic responses. Using the linear regression plots, MOD, lag phase, exponential phase, doubling time, and specific growth rate were calculated for each *Campylobacter* species (*C. jejuni*, *C. coli* and *C. lari*). Each set of calculations generated from growth measurements were analyzed using a One-Way ANOVA to compare between species, with pairwise comparisons investigated under a Tukey's HSD.

3. Results

The aim of this study was to develop pure culture growth curves for *Campylobacter* spp. (*C. jejuni, C. coli,* and *C. lari*) under what are considered favorable conditions in a specific enrichment media recommended by USDA-FSIS and routinely used in the poultry processing industry. The initial concentrations of *C. jejuni*, *C. coli,* and *C. lari* before dilutions were 8.24, 9.01, and 9.26 Log_{10} CFU/mL, respectively. After dilutions concentrations were estimated at 1.90, 2.51 and 2.76 Log_{10} CFU/mL, respectively. Using the 45-min intervals $OD_{600\ nm}$ readings obtained during enrichment and time, a growth curve was constructed for *C. jejuni, C. coli*, and *C. lari* (**Figures 1A, 2A, and 3A**). Growth curves were subsequently transformed into the natural log $OD_{600\ nm}$ readings versus time to calculate growth kinetic parameters for each *Campylobacter* spp. as a function of time (**Figures 1B, 2B, and 3B**). Using the natural log growth curve, growth kinetic measurements were calculated for each *Campylobacter* species (**Table 1**). Additionally, the RT-PCR revealed all biological replicates were specific to their species after 24 h enrichment.

Maximum optical densities were analyzed based on the optical density measurements from the growth curves. The MOD was considered the highest OD reading achieved after logarithmic growth had occurred without further increases in OD values (Ricke et al., 1994;

Ricke and Schaefer, 1996). The MOD for each species was 1.00 (*C. jejuni*), 0.99 (*C. coli*), and 1.05 (*C. lari*). An ANOVA was conducted to compare MOD means among the three species. When compared, no difference was detected among the three species ($P > 0.05$; **Table 1**). From these same growth plots, lag phase responses were calculated and estimated for each *Campylobacter* spp. Numerically, *C. lari* had the shortest lag phase at 9 h and 27 min, followed by *C. coli* at 11 h and 15 min and *C. jejuni* at 15 h and 20 min. An ANOVA revealed that there was a difference among the three *Campylobacter* species for lag phase duration time (**Table 1**). Additionally, when compared, differences were observed for all comparisons (*C. jejuni* and *C. coli*, *C. jejuni* and *C. lari*, *C. coli* and *C. lari*; $P < 0.05$; **Supplemental Table 3**).

Using the natural log, exponential growth phase plots, time was extrapolated using R^2 to identify the timepoint that initiation of growth occurred until the respective culture ceased doubling (**Figures 1B, 2B, and 3B**). The exponential phase duration for each species is as follows, *C. jejuni* 5 h, *C. coli* 4 h 45 min, and *C. lari* 4 h and 15 min (**Figures 1C, 2C, and 3C**). Using an ANOVA, mean differences were detected among the three species ($P < 0.05$; **Supplemental Table 3**). Pairwise differences were then examined to assess statistical significance among the *Campylobacter* species. Differences were observed among *C. jejuni* and *C. coli*, as well as *C. jejuni* and *C. lari*. *C. jejuni* lag phase was 45 min longer than both *C. coli* and *C. lari* lag phase duration.

Doubling time is a metric used to estimate the time it takes for *Campylobacter* cell to double in concentration (Pirt, 1975). Using time and plotting the natural log of $OD_{600\ nm}$ absorbance readings, doubling time was calculated and analyzed for each *Campylobacter* species used throughout the study. Results from the study suggest that *C. coli* had the most rapid

doubling time at 1 h and 01 min, followed by *C. lari* at 1 h and 03 min and finely *C. jejuni* at 1 h and 22 min (**Table 1**).

Lastly, specific growth rates were calculated for each *Campylobacter* species. When growth rates were compared among the three species, no differences were detected based on ANOVA (Table 1). Numerically, *C. coli* exhibited the most rapid specific growth rate (0.70 h^{-1}), followed by *C. lari* (0.66 h^{-1}), and finally *C. jejuni* (0.53 h^{-1}).

Using the initial concentration of *Campylobacter* spp. in the samples and growth kinetic parameters, concentration estimates were calculated during exponential phase (**Table 2 – 4**). Based on the projected cell concentration calculations, *C. lari* would have the highest concentration of 3.96 Log_{10} CFU/mL after 4 h 15 min exponential phase, while *C. coli* was 3.72 Log_{10} CFU/mL during the same period. *C. jejuni* had a longer exponential phase of 5 h and had the lowest projected concentration of *Campylobacter* at 3.96 Log_{10} CFU/mL.

4. Discussion

Understanding the mechanisms that govern *Campylobacter* growth kinetics are critical for designing enrichment protocols for molecular-based detection and quantification for *Campylobacter*. However, *Campylobacter* growth rates are difficult to assess not only because of the stringent conditions in which the microorganism grows, but also because genetic differences exist among species (Meinersmann et al., 2002; Silvan and Martinez-Rodriguez, 2019). Research has shown that unlike other foodborne pathogens such as *Salmonella* and *E. coli*, *Campylobacter* lack sigma factor RpoS, which controls the genetic regulatory switch to change from one growth phase to another based on metabolism (Turonova et al., 2017). Currently, the metabolic mechanism that regulates changes in the growth phases have not yet been identified in this fastidious microorganism (Fields et al., 2016; Turonova et al., 2017).

Therefore, a better understanding of *Campylobacter* growth responses under optimal conditions and estimated time for phase changes remain necessary for the development of routine detection and enumeration of nongrowth-based methods such as PCR, Mass Spectrometry Fluorescent Staining, and Laser Excitation**.**

Generating detailed growth curves provide the means to predict the timepoints more precisely when *Campylobacter* will potentially shift its respective growth phases. The present study generated plots of growth responses for *C. jejun*i, *C. coli* and *C. lari* in BFBB. All species were grown under the same conditions in Hungate tubes with low atmospheric head space to create a microaerophilic environment to support growth. Each species underwent a 24 h growth period resulting in absorbance readings that could be measured every 45 min. After 21 h, all species were in stationary phase (**Figures 1 – 3**). Using the exponential growth phase, the *C. coli* growth curve was the best fit curve of the three species, with an R^2 of 0.977 however, all curves were greater than 0.95 (**Figure 1C, 2C, and 3C**). The linearity of the corresponding transformed OD values among species shows that the three species are relatively similar in their growth rates and thus would accommodate a universal enrichment time for reaching limit of detection in a molecular detection assay.

Additionally, using the individual curves made for each species, growth kinetic parameters (MOD, Lag phase, Exponential phase, Specific Growth rate, and Doubling time) were compared among the three *Campylobacter* spp. Comparisons for growth rates are needed for *Campylobacter* detection methodologies to differentiate fast versus slow growing strains of *Campylobacter*. The critical issue is to delineate any differences when exponential specific growth rates are initiated versus differences in lag phase. If specific growth rates are dramatically different, then culture enrichment step may need to be adjusted to optimize growth. However, if

only the stationary phases are different, estimates must ensure *Campylobacter* spp. inocula concentrations are uniform prior to the exponential growth for starting the enrichment incubation. Uncovering these differences beforehand ensures that sufficient enrichment time is given to reach the endpoints needed to achieve the limit of detection using culture-based and molecular methods. Therefore, differences in *Campylobacter* species growth kinetic parameters under the same conditions are needed to determine whether incubation conditions can be universal for *Campylobacter* species. Assessing the variation of growth kinetics and differences among growth kinetic-based parameters between these *Campylobacter* species will determine whether data collected with species can also be useful for predicting the growth of other species for future development. In a study by Wagely et al., (2014), the authors aimed to characterize the metabolic diversity of both *C. jejuni* and *C. coli* using a multi genome analysis and Biolog phenotyping to determine differences in carbon source utilization. The authors were able to identify a core set of carbon sources utilized by both species as well as group of sources that are differentially utilized by a diverse panel of *C. coli* strains (Wagely et al., 2014). Their study demonstrated the different utilization patterns for metabolic compounds of different *Campylobacter* spp. These differences may well support the observation in the present study, that *C. jejuni*, *C. coli*, and *C. lari* were significantly different for the corresponding lag phase time periods.

In the present study, *C. jejuni*, *C. coli*, and *C. lari* were significantly different for the lag phase. Previous research has shown that when isolated from food or the environment, stress factors can inhibit growth initiation of *Campylobacter*, causing changes growth kinetics such as lag phase and doubling time (Mihaljevic et al., 2007; Bui et al., 2012; Cameron et al., 2012). The lag time duration is dependent on two factors, the concentration of cells that survived stress prior

to enrichment and the ability of individual *Campylobacter* spp. to recover from stress (Lanzl et al., 2020). While some of the mechanisms associated with recovery mechanisms remain unknown, a comparison of *Campylobacter* strain growth rates does allow a means to take this into account to further optimize growth conditions for molecular assays. In the present study, *C. jejuni* had a longer lag time at 15 h and 20 min, however all species exhibited lag phase times longer than 9 h. When compared among species, there were significant differences among *Campylobacter* species and within species, as well as a significant difference between *C. jejuni* and *C. lari* for lag time prior to initiation of exponential growth. In contrast, in a study by Haigh and Ketley, (2011), they compared *C. jejuni* growth using two different methods, a test tube incubator and 96 microtiter well plate spectrophotometer instrument. When lag phase was calculated for both methods, it was between 4 to 5 h (Haigh and Ketley, 2011). Differences in lag phase of *Campylobacter* between the two studies are likely attributable to the method of generating growth. In the present study, small Hungate tubes were used with a small surface area. Research has shown that *Campylobacter* grows more slowly when incubated with small surface areas, and, in turn adjusting to the more unfavorable growth conditions (Phung et al., 2021). Therefore, more research is needed to conduct additional comparative studies utilizing different growth methods and their impact on *Campylobacter* kinetics.

The doubling time of *Campylobacter* is the period it takes for cells to double in concentration at a constant growth rate. When doubling times were analyzed for each species, *C. coli* had the shortest doubling time at 1 h 01 mins, followed by *C. lari* with 1 h 03 min and then *C. jejuni* at 1 h and 22 min. No differences were observed among the species when compared, suggesting they all double the number of cells the same under these experimental conditions. In contrast to several studies, the doubling times from our study were more rapid. In a study by

Axelsso-Olosffon et al., (2007), they assessed the potential of a novel method (ACC, *Acanthamoeba-Campylobacter* Coculture method) to compare growth kinetics of *Campylobacter* spp. by using six strains of *C. jejuni* and one strain each of *C. coli*, and *C. lari*. All the strains tested were successfully enriched by the ACC method. *Campylobacter* cells were harvested, suspended in Peptone Yeast Glucose medium, and then diluted to a final concentration of approximately 10^3 CFU/ml. Confluent cultures of *A. polyphaga* were grown at the bottom of a 12-well plate in 1.9 ml of Peptone Yeast Glucose medium and inoculated with 100 µl of the bacterial stock solutions in triplicate. After a 72 h grow out, the doubling times under exponential growth were 2.03 h for *C. jejuni*, 1.45 h for *C. coli*, and 1.25 h for *C. lari* (Axelsso-Olosffon et al., 2007). Likewise, in a study by Battsby et al. (2016), the authors characterized Campylobacter growth in enrichment broths (Bolton broth and brain heart infusion broth) by assessing doubling times. To accomplish this, overnight inocula cultures of *C. jejuni* were inoculated into the respective broths (Bolton broth or brain heart infusion broth) and the cultures were diluted fivefold to 10^{-5} in 9 ml maximum recovery diluent. The inocula were subsequently transferred to 99 mL of to provide an inoculum with approximately 1.00 $\log_{10}$ CFU/mL. Absorbance readings were taken every 3 h over a 93 h grow out time period. They observed mean doubling times of 2.1 and 2.2 h in Bolton broth and brain heart infusion broths incubated at 42 °C, respectively. As with differences in previous research on lag times it also appears that differences in numerical means observed among doubling times across the different studies is primarily due to different growth systems used. Variation of environmental conditions vary, potentially causing the difference in growth phases among the different research studies (Bronowski et al., 2014; Teen Teh et al., 2019).

The growth kinetics of different *Campylobacter* species provides information as a guide to optimize enrichment for molecular growth-based methods. The growth response plots generated in this study can be used as a predictive model for *Campylobacter jejuni, coli*, and *lari* growth in BFBB over 24 h. For molecular growth-based methods, rapid reliable testing is needed. Therefore, a predictive model with growth kinetic parameters can be used to decrease enrichment time for detection analyses in these systems. Typically, endpoints are used when bacteria are in stationary phase for detection. However, using the information generated from the predictive model in this study can potentially shorten enrichment time by using growth kinetic parameter estimates to predict the earliest timepoint when cell concentrations reach the limit of detection. This should improve the accuracy, efficiency, and precision of future growth-dependent detection methods. In the present study, *C. jejuni* has the longest doubling time and slowest specific growth rate. Using this growth kinetic parameters, the earliest *C. jejuni* can potentially initiate bacterial cell doubling after 15 h 20 min. Concentrations entering the stationary phase are estimated to be 3.11 Log_{10} CFU/mL for *C. jejuni*, 3.72 Log_{10} CFU/mL for *C. coli*, and 3.96 Log_{10} CFU/mL for *C. lari* using the growth kinetic parameters. This is critical because several molecular methods have been shown to have a limit of detection above 3.00 Log_{10} CFU/mL. Bartella et al. (2013), developed a multiplex RT-PCR assay to detect *Campylobacter*, *Salmonella*, and *Shigella*, in stool samples. The results from this study showed their RT-PCR assay to have a limit of detection of 10^3. In addition, Reis et al. (2018), compared various molecular methods for *Campylobacter* detection and identification in broiler chicken carcasses. Rinsates were collected and analyzed using a RT-PCR, conventional PCR, and automated Enzyme Linked Fluorescent Assay (ELFA). Results revealed the limit of detection was 10^4 to 10^6 using the conventional PCR method, while the RT-PCR and ELFA have a limit of

detection of 10^2. Jainonthee et al. (2022), also reported the detection limit of 10^3 CFU/mL in enriched meat samples using a Loop-mediated isothermal amplification (LAMP) assay for *Campylobacter jejuni* detection. Additionally, the current industry standards for *Campylobacter* spp. enrichment from food samples takes approximately 24 to 48 h (FDA, 2001; USDA-FSIS, 2022). Based on their protocol, samples are subsequently plated on Campy-Cefex and characterized in a range of 25 to 250 colonies (1.4 to 2.4 Log_{10} CFU/mL). These studies suggest that low level concentration *Campylobacter* in samples will need to be enriched for detection. Consequently, using the growth kinetic parameters in this study, *Campylobacter* enrichment can be reduced to approximately 20 h and 20 min for detection on selective plates and molecular growth-based methods from samples of at least 1.90 Log_{10} CFU/mL (*C. jejuni)*, 2.51 Log_{10} CFU/mL (*C. coli*) and 2.76 Log_{10} CFU/mL (*C. lari*).

5. Conclusions

This study provides data on the growth of *C. jejuni*, *C. coli* and *C. lari* in pure culture and BFBB. BFBB was used because it is the recommended enrichment medium by the USDA and FDA. The purpose of the growth kinetics is to use it to assess growth kinetic parameters for further detection methodology applications. One limitation of this study is that it was conducted in a pure culture enrichment medium and not in the presence of a mixed culture which would be expected of samples derived from food products. Enrichment media provides the appropriate conditions for *Campylobacter* to grow selectively and favorably. Analyzing species differences under various growth conditions for the respective microbial growth responses is vital because predictive models should allow for universal recovery of target bacteria from samples of different food and environmental matrices. Therefore, growth kinetics in this study can only be used as a model for mixed culture samples. The significant differences among species growth

kinetic parameters suggest new methods should provide species specific assays to improve accuracy. Finally, if the detection assay time is to be further shortened, development of enrichment media and/or incubation conditions that deceases the lag phase time substantially and allow *Campylobacter* to enter exponential growth sooner would be a considerable improvement.

The resulting growth curves plotted in this study were used to calculate growth kinetic parameters. These kinetic measurements can be used to predict time points for incubation of samples that permit standard protocol parameters for employing molecular detection and quantitation methods. Defining growth kinetic responses should improve time efficiency, precision, and accuracy for the development of any future *Campylobacter* molecular-based methods. More specifically, these results will provide a standardized time for reaching the limit of detection for each *Campylobacter* spp. and provide the means to designate a universal time to accommodate all three species.

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Tables

Table 1. Means and main effect difference of growth kinetic parameters using an ANOVA analysis under a linear regression standard least square model.

Species	*C. jejuni*	*C. coli*	*C. lari*	Effect
MOD	1.00 ± 0.05 OD	.99 ± 0.11 OD	1.05 ± 0.09 OD	P = 0.150
Exponential Phase	5 h ± 54 min	4 h 15 min ± 29 min	4 h 15 min ± 59 min	P = 0.488
Lag Phase	15 h 20 min ± 30 min	11 h 15 min ± 17 min	9 h 27 min ± 15 min	P < 0.001
Doubling Time	1 h 22 min ± 8 min	1 h 01 min ± 5 min	1 h 03 min ± 3 min	P= 0.124
Specific growth rate	0.53 ± 0.10 h^{-1}	0.70 ± 0.10 h^{-1}	0.66 ± 0.06 h^{-1}	P= 0.141

P-Value main effect differences of mean between the three species

Table 2. *C. jejuni* concentration estimates during 5 h ± min exponential phase using initial concertation and doubling time.

Concentration	1.90 Log_{10} CFU/mL	2.20 Log_{10} CFU/mL	2.51 Log_{10} CFU/mL	2.81 Log_{10} CFU/mL	3.11 Log_{10} CFU/mL
Time of Doubling	15 h 20 min ± 30 min	16 h 42 min ± 8 min	18 h 04 min ± 8 min	19 h 26 min ± 8 min	20 h 48 min ± 8 min

Table 3. *C. coli* concentration estimates during 4 h 15 min exponential phase using initial concertation and doubling time.

Concentration	2.51 Log_{10} CFU/mL	2.81 Log_{10} CFU/mL	3.11 Log_{10} CFU/mL	3.41 Log_{10} CFU/mL	3.72 Log_{10} CFU/mL
Time of Doubling	11 h 15 min ± 17 min	12 h 16 min ± 5 min	13 h 17 min ± 5 min	14 h 18 min ± 5 min	15 h + 19 min ± 5 min

Table 4. *C. lari* concentration estimates during 4 h 15 min exponential phase using initial concertation and doubling time.

Concentration	2.76 Log_{10} CFU/mL	3.06 Log_{10} CFU/mL	3.36 Log_{10} CFU/mL	3.66 Log_{10} CFU/mL	3.96 Log_{10} CFU/mL
Time of Doubling	9 h 27 min ± 17 min	10 h 30 min ± 3 min	11 h 33 min ± 3 min	12 h 36 min ± 3 min	13 h 42 min ± 3 min

Figures

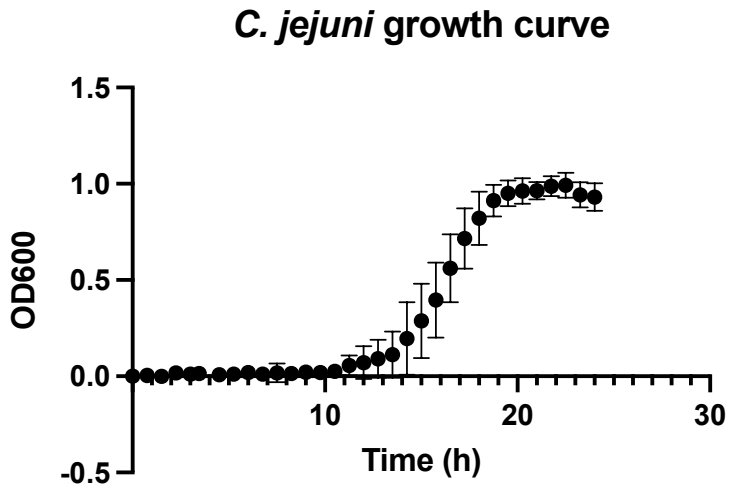

Figure 1A. *C. jejuni* growth curve. Absorbance readings from spectrometer on the Y-axis, Time on X-axis.

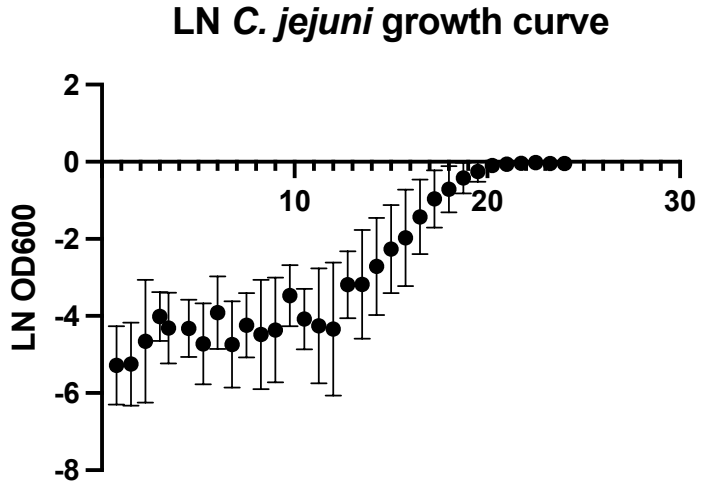

Figure 1B. *C. jejuni* growth curve. Natural log of absorbance readings from spectrometer on the Y-axis, Time on X-axis.

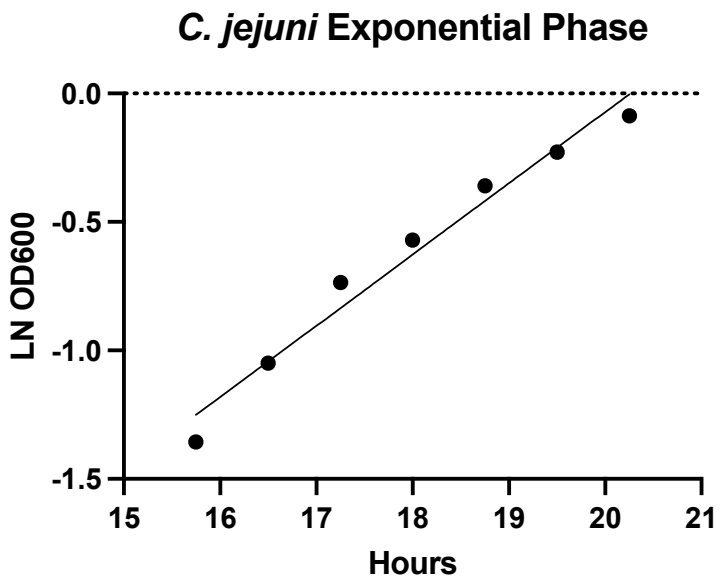

Figure 1C. *C. jejuni* exponential phase. Natural log of absorbance readings from spectrometer on the Y-axis, Time on X-axis. Y = 0.529x – 11.211.

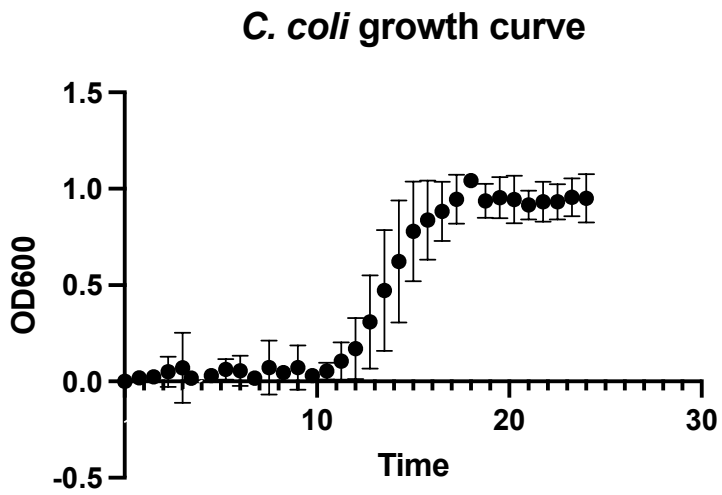

Figure 2A. *C. coli* growth curve. Absorbance readings from spectrometer on the Y-axis, Time on X-axis.

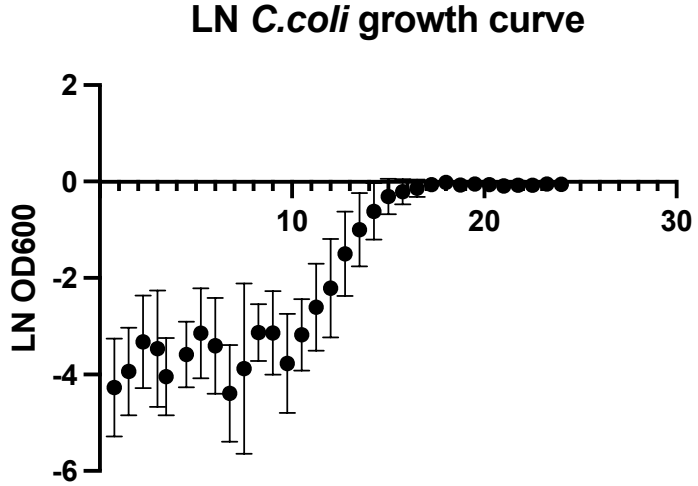

Figure 2B. *C. coli* growth curve. Natural log of absorbance readings from spectrometer on the Y-axis, Time on X-axis.

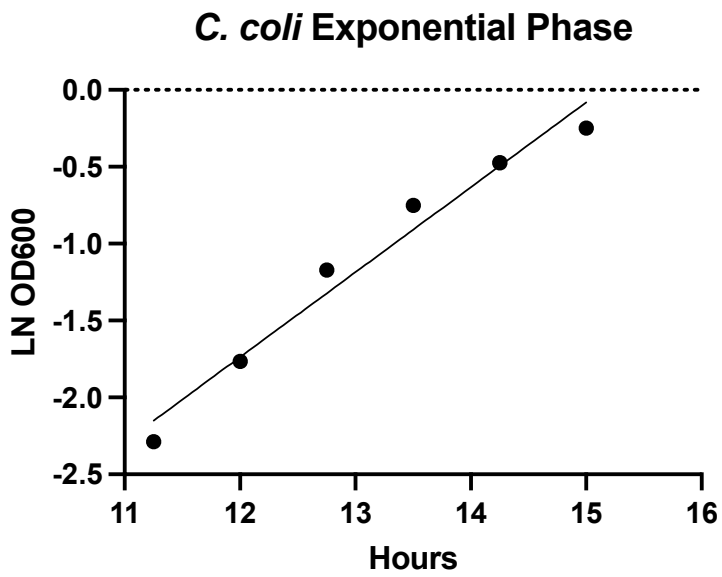

Figure 2C. *C. coli* exponential phase. Natural log of absorbance readings from spectrometer on the Y-axis, Time on X-axis. Y = 0.696x – 10.632.

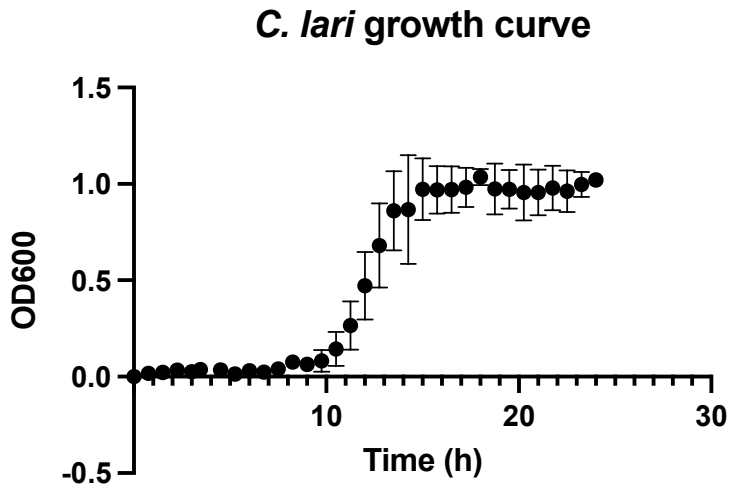

Figure 3A. *C. lari* growth curve. Absorbance readings from spectrometer on the Y-axis, Time on X-axis.

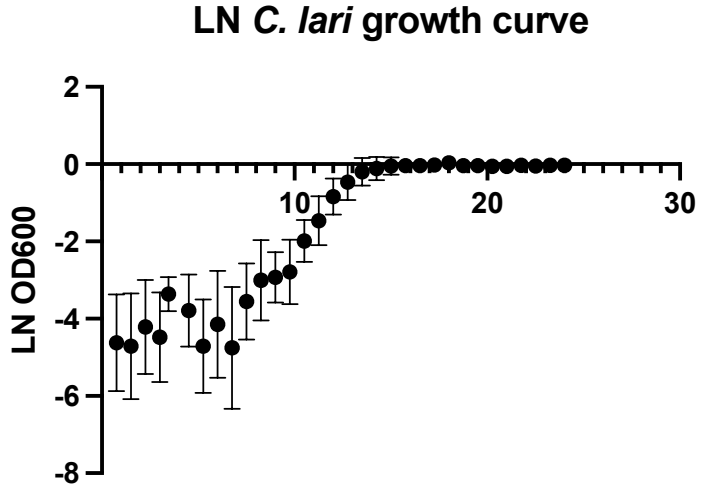

Figure 3B. *C. lari* growth curve. Natural log of absorbance readings from spectrometer on the Y-axis, Time on X-axis.

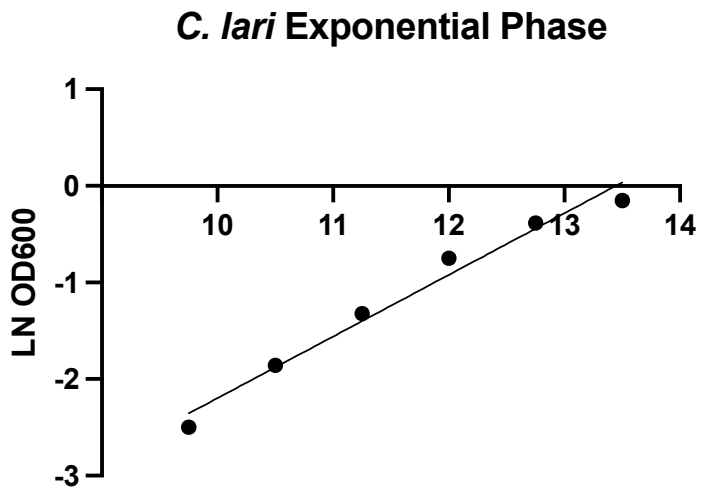

Figure 3C. *C. lari* growth curve. Natural log of absorbance readings from spectrometer on the Y-axis, Time on X-axis. Y = 0.662x – 8.770.

Supplemental Tables

Supplemental Table 1. Statistical pairwise comparison of MOD between *Campylobacter* species separated by Turkeys HSD.

Group 1	Group 2	P-Value
C. jejuni	*C. coli*	0.977
C. jejuni	*C. lari*	0.252
C. coli	*C. lari*	0.176

P-Value main effect differences

Supplemental Table 2. Statistical pairwise comparison of Exponential Phase between *Campylobacter* species separated by Turkeys HSD.

Group 1	Group 2	P-Value
C. jejuni	*C. coli*	0.464
C. jejuni	*C. lari*	0.732
C. coli	*C. lari*	0.865

P-Value main effect differences

Supplemental Table 3. Statistical pairwise comparison of Lag Phase between *Campylobacter* species separated by Turkeys HSD.

Group 1	Group 2	P-Value
C. jejuni	*C. coli*	**0.001**
C. jejuni	*C. lari*	0.001
C. coli	*C. lari*	1.00

P-Value main effect differences

Supplemental Table 4. Statistical pairwise comparison of Doubling Time between *Campylobacter* species separated by Turkeys HSD.

Group 1	Group 2	P-Value
C. jejuni	*C. coli*	0.140
C. jejuni	*C. lari*	0.183
C. coli	*C. lari*	0.965

P-Value main effect differences

Supplemental Table 5. Statistical pairwise comparison of Specific growth rate between *Campylobacter* species separated by Turkeys HSD.

Group 1	Group 2	P-Value
C. jejuni	*C. coli*	0.146
C. jejuni	*C. lari*	0.242
C. coli	*C. lari*	0.879

P-Value main effect differences

Development of a Rapid PCR Method Utilizing Shortened Enrichment Time for the Enumeration of *Campylobacter jejuni, C. coli,* and *C. lari* in Poultry Post-Chill Whole Bird Carcass Rinses

Aaron R. Bodie[1] Dana K. Dittoe[1], Savannah F. Applegate[2], Tyler P. Stephens[2], and Steven C. Ricke[1]

[1]Meat Science and Animal Biologics Discovery Program, Dept. of Animal and Dairy Sciences, University of Wisconsin, Madison, WI, 53706, USA

[2] Hygiena, 2 Boulden Circle, New Castle, DE 19720

Corresponding Author:
Dr. Steven C. Ricke
Meat Science and Animal Biologics Discovery Program
Department of Animal and Dairy Sciences
1933 Observatory Drive
Madison, WI 53706
TEL: (608) 262-7328
Email: sricke@wisc.edu

Abstract

Campylobacter is one of the leading causes of gastroenteritis in the United States and poultry serve as one of its primary reservoirs. Currently, the culture plate method using selective agar such as Campy-Cefex agar and Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) is accepted as the preferred methodology for quantification of *Campylobacter* spp. among poultry products. However, because of the specific nature of *Campylobacter*, this methodology is not sensitive, which can lead to skewed detection and quantification results. Therefore, methods for detecting and quantifying *Campylobacter* are needed to improve consumer food safety. The objective of the current study was to develop a rapid quantification method for *Campylobacter* species (CampyQuant™) in post-chill poultry rinsates using the BAX® System Real-Time PCR Assay for *Campylobacter*. The specificity and sensitivity for the detection of *C. jejuni, C. coli,* and *C. lari* in pure culture were determined. The BAX® System Real-Time PCR Assay consistently detected and identified each species 100% of the time with an enumeration range of 4.00 Log to 9.00 Log_{10} CFU/mL. Enrichment time parameters for low-level concentrations (0.00, 1.00, and 2.00 Log_{10} CFU/mL) of *Campylobacter* using the BAX® System Real-Time PCR Assay were elucidated. It was determined that an enrichment time of 20 h was needed to detect at least 1.00 Log_{10} CFU/mL of *Campylobacter* spp. using the BAX® System Real-Time PCR Assay for *Campylobacter*. As a result, time of detection, detection limits, and enrichment parameters were used to develop the CampyQuant™ linear standard curve using the detected samples from the BAX® System Real-Time PCR Assay to quantify levels in post chill poultry rinsates. A linear fit equation was generated for each *Campylobacter* species using the cycle threshold (CT) from the BAX® System Real-Time PCR Assay to estimate pre-enrichment of 1.00 to 4.00 Log_{10} CFU/mL of rinsates detected. The statistical analyses of each equation yielded an R^2 of 0.93, 0.76, and 0.94 with a Log_{10} RMSE of 0.64, 1.09, and 0.81 from

C. jejuni, *C. coli*, and *C. lari*, respectfully. The study suggests that the BAX® System Real-Time PCR Assay for *Campylobacter* is a rapid, accurate, and efficient alternative method for *Campylobacter* enumeration.

1. Introduction

As *Campylobacter* is among the leading causative agents of gastroenteritis in the US, it is considered a major foodborne pathogen of public health interest. *This* zoonotic microorganism can be isolated from a wide range of food animals; however, it represents a critical food safety problem for poultry production (Wagenaar et al., 2013). Currently, more than 25 species of *Campylobacter* have been identified, and some species, such as *C. jejuni*, *C. lari,* and *C. coli,* are responsible for most human campylobacteriosis infections (Kaakoush et al., 2015; CDC, 2019). The members of this genus are thermophilic, microaerophilic bacterium with optimal temperatures for growth between 37 to 45 °C (Hazeleger et al., Hill et al., 2017). Research has shown that the microorganism grows best in an environment with 5% O_2, 10% CO_2, and 85% N_2 (Facciolà et al., 2017). Due to the microaerophilic nature of *Campylobacter*, it is assumed that they are less resilient as they appear to be very sensitive to environmental conditions compared to other foodborne pathogens such as *Salmonella* and *Staphylococcus aureus* (Mihaljevic et al., 2007).

The primary source of human campylobacteriosis originates from poultry products. Approximately 20 to 30% of human infections are linked to the mishandling and consumption of broiler meat, while 50 to 80% may be attributed to the chicken, which is a reservoir for *Campylobacter* (Di Giannatale et al., 2019). Over recent years, there has been a 12% increase in *Campylobacter* cases reported between 2015 through 2017 (Geissler et al., 2017). The increase in *Campylobacter* cases can in part be attributed to the fact that the epidemiology of campylobacteriosis in poultry is still poorly understood (Brena et al., 2016). As a result, rapid and reliable detection, and quantification of *Campylobacter* directly from poultry samples remain a challenge.

Currently, the identification and quantification of *Campylobacter* spp. rely on culture-based methods and phenotyping (Vizzinni et al., 2019). These conventional methods include selective plate enumeration, optical microscopy, and fluorescence optical density measurements that lead to an elevated level of specificity; however, these methods do possess disadvantages. When culturing *Campylobacter* spp., in stressful environments, cells can become viable but non-culturable (VBNC), resulting in false reporting. Detection and quantitation can be time-consuming, requiring prolonged incubation periods, which may take several days to retrieve the results (Papić et al., 2017). Additionally, selective media variation and the increasing emergence of antibiotic-resistant *Campylobacter* spp. can contribute to inaccurate results (Luangtongkum et al., 2009).

Molecular assays such as real-time polymerase chain reaction (RT-PCR) provide an alternative to microbiological methods of *Campylobacter* detection and quantification. The real-time PCR Assay is a rapid and specific nucleic acid amplification method for the detection with advantages in terms of turnaround time, specificity, and sensitivity. The BAX® System Real-Time PCR Assay for *Campylobacter* is a commercial PCR assay system approved by the Association of Official Agricultural Chemist (AOAC) International for the detection of *Campylobacter* in food and environmental samples. The BAX® System amplifies an approximately 400 base pair species-specific genomic region (Zhang et al., 2004). The BAX® System Real-Time PCR Assay is specific to the multiplex detection and quantification of *C. jejuni*, *C. lari*, and *C. coli*. The assay does not amplify sequences of other *Campylobacter* species nor react with non-*Campylobacter* bacteria prominent with poultry samples, such as *Salmonella*, *Escherichia coli*, and *Lactobacillus* spp. (Hygiena, 2022). The BAX® System Real-Time PCR Assay minimizes problems common to phenotypic and antibody-based methods, such as cross-

reaction with related organisms (Silbernagel et al., 2003). Thus, it provides for specific screening of *Campylobacter* in poultry samples (Silbernagel et al., 2003).

Therefore, the objective of this study was to develop and optimize a rapid quantification method for *Campylobacter* species (CampyQuant™) in post-chill poultry rinsates using the BAX® System Real-Time PCR Assay for *Campylobacter*. As such, the parameters of the BAX® System Real-Time PCR Assay were examined using various enrichment media. After determining optimal enrichment media, the detection limit was determined to mitigate enrichment parameters and establish a detection limit for each *Campylobacter* species using the BAX® System Real-Time PCR Assay for *Campylobacter*. Lastly, the media comparison and enrichment time of detection parameters were used to develop a standard linear curve using the detected samples from the BAX® System Real-Time PCR Assay. The development of this standard curve, in turn, allows the BAX System to perform as a potential quantification method for *C. jejuni, C. coli,* and *C. lari* in poultry rinsates.

2. Methods and Materials

2.1 Inocula Preparation

Campylobacter species *C. jejuni* American Type Culture Collection® (ATCC®) 700819, *C. coli* ATCC® BAA – 1061™, and *C. lari* ATCC® BAA – 1060™ were used for this study. The ATCC® strains utilized in the current study were chosen due to the extensive data available on their ecology, functionality, and genetic and biochemical characteristics. To obtain precultures for the growth experiments, frozen stock cultures (-80 °C) of each *Campylobacter* species (*C. jejuni*, *C. coli,* and *C. lari*) were plated on modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA; Himedia, Mumbai, India) and incubated under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) using the Anoxomat system (Advanced Instruments, Norwood, MA,

USA) at 42 °C for 48 h. After incubation, one colony was inoculated in 10 mL of Blood Free Bolton Broth (BFBB; Criterion, Hardy Diagnostics, Santa Maria, CA, USA), followed by incubation under microaerophilic conditions at 42 °C for 48 h using the Anoxomat system. The cell density of the inoculum used throughout the study was 9.00 Log_{10} CFU/mL, as determined by spread plating on mCCDA plates.

2.2 Enumeration range, Specificity, and Sensitivity of *Campylobacter* species using BAX® System Real-Time PCR Assay

After 48 h of growth, 1 mL of *Campylobacter jejuni, coli*, and *lari* cultures were individually transferred to 19 mL of fresh 2× BFBB with 2× the antibiotic supplement (Cefoperazone 40 mg/L, Vancomycin 40 mg/L, Trimethoprim 40 mg/L, and Cycloheximide 50 mg/L) and Buffered Peptone Water (BPW) and incubated under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) using one aerobic pack sachet (Thermofisher Scientific, Waltham, Massachusetts, USA) for 24 h. Following the 24 h incubation, a 10-fold dilution to 10^{-6} was performed on each medium using 9 mL of the respective medium and 1 mL of the 24 h *Campylobacter* species inoculum. Samples were spread plated using 100 µL of diluent on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) to determine the concentration of *Campylobacter*. Additionally, *Campylobacter* in all media samples and dilutions was detected using the BAX System using the BAX® System Real-Time PCR Assay for *Campylobacter* (KIT2018, Hygiena, Camarillo, California, USA) according to the manufacturer's instructions. The experiment was repeated three times with three technical replications per medium (N = 54 total number of samples, n = 3 total biological replicates, species = 3, trial = 3). A standard curve was obtained using the genomic DNA extracted from the dilutions (1:10) of the pure culture samples and the CT values from the BAX system.

2.3 Preparation of commercial poultry rinsate

Poultry post-chill rinsates were obtained from a commercial poultry producer in the southeastern United States and shipped via overnight courier to the Meat Science and Animal Biologics Discovery (MSABD) building at the University of Wisconsin- Madison (UW-Madison, Madison, WI, USA) in an insulated shipping cooler with frozen gel packets (ULINE, Pleasant Prairie, Wisconsin, USA). Upon arrival at MSABD, rinsates were stored at -20 °C until use.

No more than 24 h prior to the onset of the experiment, rinsates were thawed and combined to create bulk rinsates to reduce the variation between biological rinsate samples. Additionally, prior to the study, the bulk rinsate was screened for the presence of *Campylobacter* on the BAX system to determine if there was a significant presence of indigenous *Campylobacter*. At the same time, the bulk post-chill rinsate was plated in quadruplicate on mCCDA, and Tryptic Soy Agar (TSA) plates with duplicate plates were incubated under either aerobic conditions at either 37 °C for 24 h or microaerophilic conditions at 42 °C for 24 h. These incubation methods allowed for the determination of the indigenous presence of *Campylobacter* or competing microorganisms. After incubation, Gram stains were performed on the bulk rinsate and isolated colonies from the aerobically incubated TSA plates. The pH of the bulk rinsate was determined with a SympHony pH meter and probe (VWR International, Radnor, PA, USA) to determine if the rinsates needed to be neutralized to a pH of 7 since many poultry processors use processing aids that alter the pH and this could impact downstream microbiological inferences (Wages et al., 2022). All bulk homogenate rinsates in the current study had a pH of 7 ± 0.2 and did not require neutralization using sodium thiosulfate, sodium bicarbonate, and soy lecithin (Wages et al., 2022).

2.4 Rinsate Time of Detection Sample Preparation

From the bulk post-chill poultry rinsates, 30 mL were aliquoted into 24 oz sterile Whirl-Pak (Nasco, Fort Atkinson, WI, USA) bags and inoculated at a targeted 0.00, 1.00, and 2.00 Log_{10} CFU/mL of *C. jejuni* (ATCC® 700819™), *C. coli* (ATCC® BAA-1061™), or *C. lari* (ATCC® BAA-1060™) (N = 216 total number of samples; k = 3 number of treatments; n = 24 number of samples per treatment group per timepoint; 3 timepoints; 8 per bio replicate; 1 non-inoculated sample/enrichment time; **Supplemental Figure 1**). Subsequently, 30 mL of pre-warmed (42 ˚C) 2× BFBB with 2× the antibiotic supplement was added to each sample and incubated at 42 ˚C for either 16, 18, or 20 h, respectively (United States Department of Agriculture Food Safety and Inspection Service USDA-FSIS, MLG 2021). When this research was performed, 2× BFBB with 2× antibiotic supplements was recommended by the USDA-FSIS to enrich *Campylobacter* in poultry rinsates (MLG 41.05). Per USDA-FSIS, samples were incubated under microaerophilic conditions using one anaerobic pack sachet (ThermoFisher Scientific, Waltham, Massachusetts, USA) inside a 7 L anaerobic box (Mitsubishi, New York City, New York, USA). At each enrichment time, samples were removed from the incubator. *Campylobacter* presence and quantity were determined on the BAX system using the BAX® System Real-Time PCR Assay for *Campylobacter* (KIT2018, Hygiena, Camarillo, California, USA) according to the manufacturer's package insert, with 8 technical replicates per biological replicate (n = 3).

2.5 Quantification Sample Preparation

After elucidating the enrichment time parameters for the limit of detection using the BAX® System Real-Time PCR Assay, a standard curve was developed for each species. For each serovar, *C. jejuni* (ATCC® 700819™), *C. coli* (ATCC® BAA – 1061™), or *C. lari* (ATCC® BAA

– 1060™), 30 mL of the bulk post-chill whole bird carcass rinsates were aliquoted into 24 oz Whirl-Pak bags (N = 39). Samples were either uninoculated (n = 1) or inoculated with 1.00, 2.00, 3.00, or 4.00 Log_{10} CFU/mL of (n = 3 at each inoculation level) of *Campylobacter* spp. Pre-warmed (42 ˚C) 2× BFBB (30 mL) with 2× antibiotic supplements were enumerated on Campy-Cefex. Campy-Cefex plates were used throughout the study to retain consistency with regulatory and industry standards (USDA-FSIS, 2021). Samples were incubated under microaerophilic conditions using one anaerobic pack sachet (ThermoFisher Scientific, Waltham, Massachusetts, USA) inside a 7 L anaerobic box (Mitsubishi, New York City, New York, USA) at 42 ˚C for 20 h and tested in quintuplet with the BAX® System Real-Time PCR system. Plated samples were incubated for 48 h using the Anoxomat system (Advanced Instruments, Norwood, MA).

2.6 Lysate Generation and DNA Amplification Using BAX® System Real-Time PCR

The generation of a lysate and subsequent amplification of the genomic DNA of the inoculated samples was performed using the BAX® standard protocols for poultry rinsates (Hygiena, 2021). After the 20 h incubation, 5 µL of the samples were run on the BAX® System Real-Time PCR to obtain a Cycle Threshold (CT) value. A linear regression curve was generated using CT values detected from the PCR and spiked sample concentration.

2.7 Statistical Analyses

Pure culture media comparison: All statistical analyses for this study were performed in JMP 14.0 (SAS Institute Inc, Cary, North Carolina, USA). Among the pure cultures, each dilution series of the sample was statistically analyzed using linear regression analysis to determine if there was a relationship between CT value and *Campylobacter* detection in the BAX® System Real-Time PCR. After linear regression, a Mann-Whitney U test was used to determine significant differences within species and the detection limit between different media for each

species in the BAX® System Real-Time PCR. Quantification metric parameters including specificity, sensitivity, efficiency, accuracy, positive and negative likelihood ratios (PLR, NLR), prevalence, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each species and media. The specificity of the PCR was calculated using the equation: Specificity = 1- false positives/ true negatives + false positives. The sensitivity of the PCR was calculated using the equation: 1- false negatives/true positives + false negatives. The efficiency of the PCR was calculated using the equation: Efficiency = $-1+10^{(-1/\text{slope})}$. The accuracy of the PCR was calculated using the equation: (sensitivity)*(prevalence) + (specificity)*(1 - prevalence). When calculating likely ratios, the PLR followed the equation: sensitivity/(1-specificity), whereas the negative likelihood ratio NLR equation used was: 1-sensitivity/specificity. Prevalence was subsequently calculated by the equation; positive samples/total samples were tested. In addition, predictive values of the PCR were calculated by the following equations, PPV: True positives/ (true positives + false positives). The last quantification metric calculated was NPV with the equation: True negatives/ true negatives + false negatives). The main effect and interaction of media and species were evaluated for the BAX system quantification metric parameters such as sensitivity, accuracy, prevalence, NLR, and NPV using a Mann-Whitney U test. Other metric parameters were not statistically analyzed because the numerical values were the same, resulting in no differences. Further analysis was done on quantification metric parameters to determine differences between *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*) in 2× BFBB and BPW and between the enrichment media in *Campylobacter* species. Pairwise differences were analyzed using χ^2 analysis. Significant differences were considered at $P < 0.05$.

Enrichment time of detection: To determine the appropriate time needed for *Campylobacter* detection in the BAX® System Real-Time PCR, three time points were used, 16, 18, and 20 h. PCR quantification metric parameters sensitivity, specificity, PLR, NLR, prevalence, PPV, NPV, and accuracy were calculated for each species and time point. Furthermore, the quantification metrics were analyzed for main effect and interactions using a Mann-Whitney U test. Pairwise differences comparing significances between the time (16, 18, and 20 h) of *Campylobacter jejuni*, *coli*, and *lari* and the species *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*) at 16, 18, and 20 h of enrichment were evaluated using a χ^2 Analysis. The significant differences were considered at $P < 0.05$.

Validation and standard curve development: In poultry rinsates, each range of *Campylobacter* species, 1.00 to 4.00 Log_{10} CFU/mL of sample, was statistically analyzed using a linear regression model to determine the relationship with CT values from the BAX® system and plate counts. Additionally, PCR quantification metric parameters, sensitivity, accuracy, prevalence, NLR, and NPV, were analyzed among CampyQuant™ and Campy-Cefex plates. *Campylobacter* rinsates samples quantified using Campy-Cefex and CampyQuant™ main effects and comparisons were explored for sensitivity, accuracy, prevalence, NLR, and NPV using a Mann-Whitney U test. Pairwise differences were determined using χ^2 analysis. An ANCOVA test was run to investigate if there is a significant different linear slope between *C. jejuni*, *C. coli*, and *C. lari* after 20 h of enrichment in poultry rinsate samples. The significant differences were considered at $P < 0.05$.

3. Results

3.1 Pure culture media comparison

The first study aimed to determine the best fit media for *Campylobacter* detection and enrichment with the BAX® system. Initially, the concentration limit, sensitivity, and specificity of the BAX® System Real-Time PCR Assay for *Campylobacter* detection of *C. jejuni, C. coli,* and *C. lari* were evaluated for each of the media, BBFB or BPW. The BAX® system could detect *Campylobacter* regardless of the media used (**Table 1**). Additionally, using the designed primers and PCR assay of the BAX® system, the amplification accuracy of all media was above 89%, with *C. coli* in 2× BFBB being the most accurate at 96.2% accuracy (**Table 1**). The prevalence of *Campylobacter* using BAX® System Real-Time PCR Assay was higher when *Campylobacter* was enriched in 2× BFBB than BPW for all species.

Three biological replicates per inoculation level were analyzed under a linear regression model for the quantification metric parameters. All quantification parameters were evaluated for each *Campylobacter* species individually in 2× BFBB and BPW. The BAX® system could differentiate between the different *Campylobacter* species (**Table 2**). Interaction and main effects of the enrichment media and *Campylobacter* species between quantitative metric parameters were compared (**Table 2**). No interactions were shown between the *Campylobacter* species and media for all quantification parameters ($P > 0.05$) except accuracy ($P < 0.05$; T**able 2**). Within the interaction of enrichment media and *Campylobacter* species on the accuracy of detection, the accuracy of detection of *Campylobacter* was highest among *C. jejuni* and *C. coli* enriched in 2× BFBB (93.8 and 96.3%) compared to *C. jejuni*, *coli*, and *lari* enriched in BPW (81.9, 79.0, and 84.0%). The accuracy of detection of *C. lari* when enriched in 2× BFBB was not different than when enriched in BPW (89.8 and 84.0%).

Additionally, the main effect of species and media were statistically analyzed using a Mann-Whitney U test for quantification performance parameters (T**able 2).** There was no effect

of *Campylobacter* species on the sensitivity, accuracy, prevalence, NLR, and NPV of BAX® System Real-Time PCR Assay ($P > 0.05$). However, there was an effect of media type for all quantification quality parameters ($P < 0.05$; **Table 2**). The highest sensitivity was observed among *Campylobacter* species enriched in 2× BFBB (93.3, 96.3, and 88.0 %, respectively) compared to BPW (*C. jejuni* 76.0, *C. coli* 64.0 and *C. lari* 80.0 %). Moreover, BPW enrichments had the lowest accuracy compared to 2× BFBB for *Campylobacter* species.-Furthermore, media effect on PCR prevalence was observed. *Campylobacter* species enriched in BPW (*C. jejuni* 73.1, *C. coli* 61.5 and *C. lari* 74.9 %) had a lower prevalence than those enriched in 2× BFBB (90.0, 87.2 and 84.6 %, respectfully). There was an effect of media on the NLR, with *Campylobacter* species enriched in BPW having higher NLR than enriched in 2× BFBB. Among BPW, *C. coli* had the highest NLR at 36.0 %, followed by *C. jejuni* at 24.0 %, then *C. lari* at 20.0 %. Within 2× BFBB, *C. lari* had the highest NLR percentage at 12.0 %, followed by *C. jejuni* at 6.7 % and *C. coli* at 4.0 %.

Additionally, the main effect and pairwise differences were analyzed for quantification metrics between the *Campylobacter* species (*C. jejuni*, *C. coli,* and *C. lari*) in 2× BFBB and BPW and between the enrichment media (2× BFBB and BPW) for *C. jejuni*, *C. coli,* and *C. lari* (Supplemental Table 2-3). There was no effect of *Campylobacter* species within either 2× BFBB and BPW when analyzed individually ($P > 0.05$; Supplemental Table 2). When comparing media differences within *C. jejuni*, differences between media types were seen in sensitivity, accuracy, prevalence, and NLR ($P < 0.05$; Supplemental Table 3). The means from 2× BFBB were greater for sensitivity (93 and 76%), accuracy (94.0 and 81.5 %), prevalence (89.0 and 75.0 %), NLR (67.0 and 24.0 %) and NPV (38.9 and 15.0%) than that of BPW when only comparing media within *C. jejuni* (Table 2). Within *C. coli*, sensitivity and accuracy were different between media

types ($P < 0.05$; Supplemental Table 3). The means for sensitivity was 54.0 % higher in 2× BFBB than BPW, while accuracy was 17.3 % higher in 2× BFBB than BPW (Table 2). There were no differences between the quantification parameters due to enrichment types within *C. lari* ($P > 0.05$).

Pure culture samples were analyzed to determine the initial concentration for the BAX® System Real-Time PCR system's detection limits after 24 h enrichment. The detection limit for *Campylobacter* enriched in 2× BFBB using the BAX® System Real-Time PCR Assay was 3.03 Log_{10} CFU/mL for *C. jejuni*, 2.93 Log_{10} CFU/mL for *C. coli*, and 3.13 Log_{10} CFU/mL for *C. lari*. Moreover, detection limit concentrations were numerically higher when enriched in BPW for *C. jejuni* (5.60 Log_{10} CFU/mL), *C. coli* (5.50 Log_{10} CFU/mL), and *C. lari* (4.50 Log_{10} CFU/mL; Supplemental Table 1). Using the CT values from biological and technical replicates at each inoculation, linear regression standard curves were developed for *C. jejuni*, *C. coli*, and *C. lari* (**Figure 1-3**). The R^2 for the inoculated *Campylobacter* in 2× BFBB was greater than 0.94, indicating an optimal fit of the curve to the data. Significant differences between the slopes were identified when compared between media for each species ($F < 0.05$; **Figure 1-3**).The *C. jejuni* curve exhibited the highest slope (-2.90), followed by *C. coli* (-3.32), then *C. lari* (3.42).The results indicated the BAX® System Real-Time PCR system was sensitive and specific for the detection of *C. jejuni*, *C. coli*, and *C. lari* with an enumerable range of 4.50 to 9.00 Log_{10} CFU/mL in pure culture enrichment with 2× BFBB.

3.2 Enrichment time of detection

To determine the detection time for low concentrations (0.00, 1.00, and 2.00 Log_{10} CFU/mL), *Campylobacter* poultry rinsate samples were enriched for either 16, 18, or 20 h using 2× BFBB. Prior to the study, a bulk rinsate was made from multiple rinsates of whole carcass

birds with a pH of 7.04. At each time point, poultry rinsates inoculated with the respective *Campylobacter* species were analyzed for prevalence means of detection in the BAX® System Real-Time PCR Assay (**Table 3**). *C. jejuni* inoculation levels were determined to be 0.58, 1.58, and 2.58 Log_{10}CFU/mL. After 16 h of enrichment, *C. jejuni* was detected at 18, 70, and 100% among rinsates inoculated at 0.58, 1.58, and 2.58 Log_{10} CFU/mL of *C. jejuni*, respectively (**Table 3**). After 18 h of enrichment, the prevalence increased to 73, 100, and 100% for each inoculation level. *C. coli* inoculation levels were determined to be 0.17, 1.17, and 2.17 Log_{10} CFU/mL (**Table 3**). After 16 h and 18 h of enrichment, *C. coli* was only detected in 2.17 Log_{10} CFU/mL at 58 and 100%. However, at 20 h, the detectable percentages were 5, 92, and 100%, respectively (**Table 3**). Lastly, *C. lari* had an inoculation level of 0.03, 1.03, and 2.03 Log_{10} CFU/mL (**Table 3**). After 18 h of enrichment, 1.03 and 2.03 were detected at 33 and 66% (**Table 3**). After 20 h enrichment, inoculation levels of 1.00 and 2.00 Log_{10} CFU/mL increased to 100% detection, respectively. At each time point, quantification metrics such as sensitivity, specificity, PLR, NLR, prevalence, PPV, NPV, and accuracy were calculated for each *Campylobacter* species' numerical means (**Table 3**) with the main effect and interaction of time and species being explored (**Table 4**). Additionally, the effect of time (16, 18, and 20 h) within each *Campylobacter* species and the effect of *Campylobacter* species within 16, 18, and 20 h of enrichment was determined (Supplemental Table 4-5).

There was an interaction effect of species and time on sensitivity (**Table 4**). The sensitivity of detection of *C. jejuni* enriched for 20 h (94.4%) was different from the sensitivity of all other species and enrichment times. The lowest sensitivity was seen among *C. coli* at 16 and 18 h (14.6 and 25.7 %) and *C. lari* at 18 h (13.9 %), which were all statistically the same, yet different from the other enrichment times and species. Sensitivity differences within time and

species were also determined. Within time (16, 18 and 20 h), sensitivity differences were observed for each species, *C. jejuni*, *C. coli*, and *C. lari* ($P < 0.05$). *C. jejuni* had the highest sensitivity at 16, 18 and 20 h. *C. lari* had the lowest sensitivity at 16 h of enrichment. *C. coli* had the lowest sensitivity at 18 and 20 h (**Table 4**). Within species (*C. jejuni*, *C. coli* and *C. lari*) sensitivity, the only difference between species was seen at 20 h of enrichment. After 20 h of enrichment, *C. jejuni* was the only species to achieve above 90 % sensitivity (94.4 %) which was significantly greater than *C. coli* and *C. lari* (63.9 and 72.2 %) after 20 h enrichment.

An interaction was also observed between species and time for accuracy of the BAX® PCR for detection ($P < 0.05$). Accuracy was lowest for each species at 16 h compared to 20 h for all species, with *C. coli* enriched for 16 h being the lowest of all treatments (3%). The highest accuracy was seen with *C. jejuni* after 20 h enrichment (78 %) followed by 18 h enrichment (71.4 %) which were not different from one another, but different from the other treatments. When comparing enrichment time differences within *C. jejuni* for accuracy, differences between time was shown. The mean for *C. jejuni* enriched for 16 h was 35.3 % lower than the highest mean accuracy for *C. jejuni* at 20 h. Additionally, within *C. coli* and *C. lari*, differences were shown with enrichment time, for accuracy. As enrichment time progress, the accuracy for *C. coli* and *C. lari* detection gradually increased, with their lowest at 16 h and highest after 20 h. When comparing *Campylobacter* species differences within enrichment times, a difference was shown at 18 and 20 h for accuracy on the BAX® System Real-Time PCR (**Supplemental Table 5**). At 18 h, *C. jejuni* had the greatest accuracy mean and was 44.1 % higher than *C. lari* and 62.8 % higher than *C. coli*. At 20 h, detection of *C. jejuni* had the greatest accuracy, followed by *C. lari* at 50.4 % and *C. coli* at 39.2 %.

The prevalence means of the BAX® System Real-Time PCR assay had a main effect of enrichment time and species but not an interaction of these variables. The prevalence of detected *Campylobacter* at 20 h (73.8 %) was greater than prevalence detected at 18 h (55.1%) and 16 h (33.0 %). The prevalence of *Campylobacter* was higher for *C. jejuni* at all timepoints compared to *C. coli* and *C. lari* species. Therefore, differences within species and time were analyzed. Within *Campylobacter* species, enrichment times were statistically different for all species individually (*C. jejuni*, *C. coli* and *C. lari*) for prevalence (**Supplemental Table 4**). *C. jejuni* had the highest prevalence after 16, 18, and 20 h of enrichment compared to the other species and enrichment times. In addition, *C. lari* had a higher prevalence after 16, 18, and 20 h compared to *C. coli* for each timepoint. However, within enrichment times, a difference was only seen at 20 h between *C. jejuni*, *C. coli* and *C. lari* for prevalence (**Supplemental Table 5**). *C. jejuni*, had the highest prevalence mean at 20 h with 90.7 %, followed by *C. lari* with 69.3 % and *C. coli* with 61.3 %.

Furthermore, an interaction was seen between species and time on the NLR for detection of *Campylobacter* spp. using the BAX® System Real-Time PCR assay (**Table 4**). The lowest NLR mean calculated was observed among *C. jejuni* inoculated rinsates after 20 h of enrichment (5.6 %) which was not different than that of *C. jejuni* inoculated rinsates at 18 h of enrichment (13.9%). The highest NLR calculated was *C. lari* at 16 h enrichment (86.1 %) followed by *C. coli* at 16 h (85.1 %), which were not different from those inoculate with *C. coli* enriched for 18 h (74.3%). Within *Campylobacter* species, differences were seen between enrichment times for all species individually (*C. jejuni, C. coli* and *C. lari;* **Supplemental Table 4**). Numerically, as enrichment time increase, the NLR decreases, with the lowest shown at 20 h. Within species, after 16 h, *C. lari* had the largest NLR (86.1%); however, *C. coli* had the largest NLR for 18 and

20 h (74.3 and 36.1 %). Moreover, within enrichment time, differences were seen between *Campylobacter* species for 20 h enrichment (**Supplemental Table 5**). At 20 h, *C. coli* had the largest NLR at 36.1 %, followed by *C. lari* at 27.7 % and *C. jejuni* at 5.6%.

When the NPV of the BAX® System Real-Time PCR was analyzed, an interaction was seen between *Campylobacter* species and time. The lowest NPV was seen with *C. lari* at 16 h (5.1 %), which was statistically different from that of *C. jejuni* enriched at 18 and 20 h of enrichment. However, the largest NPV was *C. jejuni* after 20 h enrichment (44.4 %), and this combination was statistically different from all other groups. Additionally, *C. jejuni* at 16 h, *C. coli* 16, 18 and 20 h, along with *C. lari* 18 and 20 h, were all statistically the same, regardless of their respective NPV. When differences were explored within *Campylobacter* species, enrichment times were only different for *C. jejuni* for NPV As enrichment time increased, so did the NPV of *C. jejuni*, which increased 6.70 % from 16 to 18 h and 27.40 % from 18 to 20 h. All *C. jejuni* NPV means were the highest at each timepoint out of the three species. Within enrichment times for NPV using the BAX® System Real-Time PCR assay, a difference of the *Campylobacter* species was seen at 20 h (**Supplemental Table 5**). At 20 h, *C. jejuni* and *C. lari* were not different in NPV with a 2.3 % difference. However, *C. jejuni* was 31.8 % higher than *C. lari* at 20 h.

In summation, 20 h enrichment, resulted in the highest mean calculated for sensitivity, accuracy, prevalence, NLR and NPV for all *Campylobacter* species, in contrast to 16 and 18 h enrichment (**Table 4**). When investigating pairwise differences between the *Campylobacter* species at 20 h enrichment, differences were seen for all quantification metric parameters ($P < 0.05$; Supplemental Table 5). After 20 h enrichment 2× BFBB, *C. jejuni* displayed the highest values for sensitivity (94.4 %), accuracy (78.1%), prevalence (90.0 %) and NPV (44.4 %; **Table**

4). The largest NLR value was 36.1 %, shown from *C. coli* after 20 h of enrichment in 2×BFBB. These results suggest that after 20 h of enrichment in poultry rinsates, *Campylobacter* could be detected from low concentrations, 1.00 and 2.00 Log_{10} CFU/mL.

3.3 Validation and standard curve development

After determining the enrichment time needed for low concentrations of *Campylobacter* to be detected in the BAX® System Real-Time PCR system was 20 h of incubation, a CampyQuant™ standard curve was developed for *Campylobacter* quantification in poultry rinsates using a linear regression model. The pH of the bulk poultry rinsate was 7.03 before the inoculant of each species was added. The initial concentrations of *C. jejuni*, *C. coli,* and *C. lari* before dilutions were 9.01, 9.13, and 9.05 Log_{10} CFU/mL, respectively. The specificity of the BAX® System Real-Time PCR Assay primers probe set for the detection of *C. jejuni, C. coli,* and *C. lari* in poultry rinsates was tested at each inoculation level (1.00 to 4.00 Log_{10} CFU/mL; n = 13 number of samples per inoculation level), all of which were correctly identified (**Table 5**).

Standard curve predicted values. A linear fit equation was generated for each *Campylobacter* species using the CT values from the BAX® System to estimate pre-enrichment Log_{10} CFU/mL of rinsates. The linear equation was used to estimate pre-enriched *Campylobacter* levels to mimic the logarithmic growth of the bacteria. For *C. jejuni,* CT values from biological and technical replicates at each inoculation level resulted in a standard curve linear equation with a Log_{10} RMSE of 0.64 and R^2 of 0.93 (**Figure 4**). When the linear regression curve was utilized to estimate inoculated CFU/mL concentration in the inoculated rinsates samples, the PCR reported mean estimates of 1.18 Log_{10} CFU/mL (95% CI; 1.10 to 2.17), 1.89 Log_{10} CFU/mL (95% CI; 1.74 to 2. 04), 2.92 Log_{10} CFU/mL (95% CI; 2.83 to 3.02) and 4.08 Log_{10} CFU/mL (95% CI; 3.93 to 4.24), respectively for each targeted inoculation of 1.00, 2.00, 3.00 and 4.00

Log_{10} CFU/mL **(Table 5**). The paired CFU/mL Campy-Cefex estimate of the respective inoculated samples were 1.64 Log_{10} CFU/mL (95% CI; 1.10 to 2.17), 2.18 Log_{10} CFU/mL (95% CI; 1.72 to 2.18), 3.05 Log_{10} CFU/mL (95% CI; 2.98 to 3.13) and 4.03 Log_{10} CFU/mL (95% CI; 3.91 to 4.24; **Table 5**).

A standard linear equation was developed from rinsates with *C. coli* with a log RMSE of 1.09 and R^2 of 0.76 (**Figure 5**). This equation was then utilized to estimate the *C. coli* inoculated CFU/mL concentrations in the rinsate samples prior to enrichment. The BAX® System Real-Time PCR reported mean estimates of 1.21 Log_{10} CFU/mL (95% CI; 0.77 to 1.73), 2.09 Log_{10} CFU/mL (95% CI; 1.76 to 2.24), 2.74 Log_{10} CFU/mL (95% CI; 1.87 to 2.86) and 3.90 Log_{10} CFU/mL (95% CI; 3.72 to 4.10), respectively for each targeted inoculation of 1.00, 2.00, 3.00 and 4.00 Log_{10} CFU/mL. The paired CFU/mL Campy-Cefex estimate of the respective inoculated samples were 1.26 Log_{10} CFU/mL (95% CI; 0.91 to 1.56), 1.74 Log_{10} CFU/mL (95% CI; 0.81 to 3.29), 3.23 Log_{10} CFU/mL (95% CI; 2.904 to 3.554) and 4.13 Log_{10} CFU/mL (95% CI; 3.29 to 4.96) (**Table 5**).

The standard curve developed from the enriched *C. lari* inoculated rinsate samples had a Log_{10} RMSE of 0.81 and an R^2 of 0.94 (**Figure 6**). Using this developed curve, *C. lari* pre-enrichment concentrations reported mean estimates of 1.17 Log_{10} CFU/mL (95% CI; 1.15 to 1.24), 1.87 Log_{10} CFU/mL (95% CI; 1.72 to 2.02), 2.92 Log_{10} CFU/mL (95% CI; 2.75 to 3.09) and 4.09 Log_{10} CFU/mL (95% CI; 3.98 to 4.20), respectively for each targeted inoculation of 1.00, 2.00, 3.00 and 4.00 Log_{10} CFU/mL **(Table 5**). The paired Log_{10} CFU/mL Campy-Cefex estimate of the respective inoculated samples were 0.70 Log_{10} CFU/mL (95% CI; 0.21 to 1.18), 1.50 Log_{10} CFU/mL (95% CI; 0.735 to 2.27), 2.68 Log_{10} CFU/mL (95% CI; 2.28 to 3.15) and 3.55 Log_{10} CFU/mL (95% CI; 2.82 to 4.28 Log_{10} CFU/mL) (**Table 5**).

Also, using CampyQuant™, an ANCOVA test was used to investigate if there was a significant difference between the linear slopes of curve generated from *C. jejuni*, *C. coli,* and *C. lari* after 20 h of enrichment in poultry rinsate samples. The results of the ANCOVA exhibited significant differences in detection between the three *Campylobacter* species standard curve ($F < 0.05$; **Table 5**). *C. coli* linear slope was the highest at -1.89, followed by *C. jejuni* with a linear slope of -2.28. The lowest linear slope was *C. lari* with -3.03.

Predicted *Campylobacter* spp. levels from each quantification method. The main effect and subsequent interaction of quantification method and species was explored at individual inoculation levels ($P > 0.05$; **Table 5**). When evaluating the interaction of quantification method and species at 1.00 Log_{10} CFU/mL ($P < 0.05$), *C. jejuni* detection in Campy-Cefex (1.64 Log_{10} CFU/mL) was significantly higher than when *C. jejuni* detected with CampyQuant™ (1.18 Log_{10} CFU/mL) or *C. lari* detected with either CampyQuant™ or Campy-Cefex (1.18 and 0.700 Log_{10} CFU/mL). No difference in detection of *C. jejuni* on Campy-Cefex (1.64 Log_{10} CFU/mL), *C. coli* on CampyQuant™ (1.212 Log_{10} CFU/mL), or *C. coli* on Campy-Cefex (1.26 Log_{10} CFU/mL).

Interactions were also observed at 2.00 Log_{10} CFU/mL between species and quantification method. *C. jejuni* enumerated on Campy-Cefex plates (2.18 Log_{10} CFU/mL) was significantly higher than *C. jejuni* quantified using CampyQuant™ (1.89 Log_{10} CFU/mL), *C. coli* quantified on Campy-Cefex plates (1.74 Log_{10} CFU/mL), and *C. lari* quantified on both CampyQuant™ (1.88 Log_{10} CFU/mL) and Campy-Cefex plates (1.50 Log_{10} CFU/mL). Moreover, there was no statistical difference between quantification of *C. jejuni* on Campy-Cefex plates (2.18 Log_{10} CFU/mL) and *C. coli* enumeration using CampyQuant™ (2.09 Log_{10} CFU/mL).

When results from 3.00 Log_{10} CFU/mL were analyzed for interactions, *C. coli* quantified on Campy-Cefex plates (3.23 Log_{10} CFU/mL), was the highest concentration seen and significantly different from *C. coli* using CampyQuant™ (2.74Log_{10} CFU/mL) and *C. lari* using either CampyQuant™ (2.92 Log_{10} CFU/mL) or Campy-Cefex plates (2.68 Log_{10} CFU/mL). No differences were seen for quantification at 3.00 Log_{10} CFU/mL between *C. coli* using Campy-Cefex plates and *C. jejuni* enumerated using either CampyQuant™ (2.93 Log_{10} CFU/mL) or Campy-Cefex plates (3.05 Log_{10} CFU/mL).

At 4.00 Log_{10} CFU/mL, the highest concentration used in the study, an interaction was observed between species and quantification methods. *C. coli* quantified in Campy-Cefex (4.13 Log_{10} CFU/mL) was significantly higher than *C. coli* with CampyQuant™ (3.90 Log_{10} CFU/mL) and *C. lari* enumerated using Campy-Cefex plates (3.55 Log_{10} CFU/mL). When compared to C. *coli* in Campy-Cefex, no differences were observed against *C. jejuni* quantified with either CampyQuant™ or Campy-Cefex (4.08 and 4.03 Log_{10} CFU/mL) and C. *lari* quantified using CampyQuant™ (4.09 Log_{10} CFU/mL)

Additionally, the effect of quantification method (CampyQuant™ vs. Campy-Cefex) was explored within each individual *Campylobacter* spp. (*C. jejuni, coli, lari*), at each inoculation level (**Supplemental Table 6**). At 1.00 Log_{10} CFU/mL of targeted inoculation, there was an effect of quantification method within *C. jejuni* where CampyQuant™ (1.64 Log_{10} CFU/mL) was higher than Campy-Cefex (1.18 Log_{10} CFU/mL). Also, at 1.00 Log_{10} CFU/mL, there was an effect of method on *C. lari* predicted levels where CampyQuant™ (1.18 Log_{10} CFU/mL) was higher than Campy-Cefex (0.70 Log_{10} CFU/mL). At 2.00 Log_{10} CFU/mL of targeted inoculation, differences between quantification methods were detected with *C. lari*, where CampyQuant™ (1.88 Log_{10} CFU/mL) was higher than Campy-Cefex (1.50 Log_{10} CFU/mL). At 3.00 Log_{10}

CFU/mL, there were differences of *C. jejuni* between the methods used where Campy-Cefex (3.05 Log_{10} CFU/mL) was higher than CampyQuant™ (2.93 Log_{10} CFU/mL). There were no differences based on quantification method used regardless of species at a targeted inoculation of 4.00 Log_{10} CFU/mL.

Performance parameters. Quantification metric parameters (sensitivity, specificity, PLR, NLR, prevalence, PPV, NPV, and accuracy) were calculated for each *Campylobacter* species (**Table 6**). Main effect and interaction of the quantification method (CampyQuant™ vs. Campy-Cefex) and *Campylobacter* species on the sensitivity, accuracy, prevalence, NLR, and NPV were analyzed (**Table 7**). Using the Mann-Whitney U test, no interactions were seen between the quantification method and *Campylobacter* species ($P > 0.05$; **Table 7**). A main effect was seen between the three *Campylobacter* species for NPV ($P < 0.05$; **Table 7**). *C. jejuni* had the highest NPV (CampyQuant™ 34.4 %; Campy-Cefex 30.5 %) among species, followed by *C. lari* (CampyQuant™ 26.1 %; Campy-Cefex 27.8 %) and lastly *C. coli* (CampyQuant™ 20.6 %; Campy-Cefex 15.9 %). was greater than prevalence detected at 18 h (*C. jejuni* 82.7%, *C. coli* 33.3 % and *C. lari* 49.3 %). In addition, when looking within individual detection methods, there was a main effect of species within Campy-Cefex ($P < 0.05$; **Supplemental Table 7**). The NPV of *C. lari* was 27.7% compared to that of 30.6 and 30.6% among *C. jejuni* and *C. lari* when using Campy-Cefex for quantification (Supplemental **Table 7**). The effect of method used on the quantification metrics was also explored within each species (*C. jejuni*, *coli*, and *lari*). No differences were observed for quantification metrics between methods used (Supplemental Table 8). Campy- Cefex was numerically higher for sensitivity, accuracy, and prevalence (94.5, 82.5 and 87.2 %), compared to CampyQuant™ (88.3, 74.7 and 84.1 %). However, CampyQuant™ was larger for NLR and NPV (11.7 and 34.4 %), opposed to Campy-Cefex (5.5 and 30.5 %).

4. Discussion

In 2016, the USDA-FSIS proposed the performance standards for *Campylobacter* and *Salmonella* in an effort to mitigate pathogen presence on poultry carcasses. Currently, prevalence-based data is used to determine process controls. However, little insight is provided. In addition, regulatory baselines and the literature have, for the most part, utilized direct plating to estimate *Campylobacter* concentrations in poultry products. Currently, there is limited published literature evaluating the efficacy of various enrichment media in combination with assay performance for detecting *Campylobacter* in food matrices. Preharvest and postharvest poultry research on *Campylobacter* and *Salmonella* have primarily relied on culture-based methods such as ISO 10272 (International Organization for Standardization, 2017; Ricke et al., 2019; Chaney et al., 2021; O'Bryan et al., 2022). This study aimed to develop and optimize a rapid quantification method for *Campylobacter* (CampyQuant™) in post-chill poultry rinsates using the BAX® System Real-Time PCR Assay. To use real-time PCR for quantitative measurements and ensure accurate quantification, PCR efficiency metric parameters must be assessed. Therefore, the limit, linear range, amplification efficiency, sensitivity, specificity, likelihood ratios, prevalence, predictive values, and accuracy of the BAX® System Real-Time PCR Assay for *Campylobacter* detection were determined.

In the present study, media 2× BFBB and BPW were used to elucidate the detection parameters of the BAX® System Real-Time PCR Assay. Preliminary results indicated that the BAX system could detect *Campylobacter* in the respective media used. Numerically, rinsates enriched in 2× BFBB had the lowest detection limit for all *Campylobacter* species in the BAX® System Real-Time PCR (*C. jejuni*: 3.03; *C. coli*: 2.93; *C. lari*: 3.13 Log_{10} CFU/mL). The method of the International Organization for Standardization for the detection of *Campylobacter* spp. in

food (ISO 10272-1:2017) recommends the use of 2× BFBB. Main effect differences between media 2× BFBB and BPW were observed for all quantification metric parameters analyzed. To differentiate media differences between *Campylobacter* species, PCR quantification metrics analyzed were further examined. When means were compared, 2× BFBB was statistically different from BPW for sensitivity, accuracy, prevalence, and NLR for *C. jejuni*, while pairwise differences were also detected in *C. coli* assays for sensitivity and accuracy. There was no effect of media on these parameters for *C. lari* between BFBB and BPW. Similar results were seen in a study by Solis-Soto et al. (2011). Solis-Soto et al. (2011) used various enrichment broths, including Preston's, Bolton's broth, Blood Free Enrichment Broth (BFEB), and Modified-BFEB (M-BFEB) were used to determine the recovery rate of *Campylobacter* spp. Solis-Soto et al. (2011) indicated that *Campylobacter* could be recovered from all enrichment broths; however, Bolton's broth resulted in the greatest detection sensitivity by consistently allowing the detection of 10 *C. jejuni* cells. *Campylobacter* cell recovery was followed by BFEB, M-BFEB, and Preston, with the lowest sensitivity of 10^3 cells (Solis-Soto et al., 2011).

In the current study, BPW and 2× BFBB were utilized. As BPW is a non-selective media, it has sufficient nutrients to allow multiple organisms present to grow. However, it is possible that non-neutralized antimicrobials used throughout processing in the rinsates can hamper *Campylobacter* recovery (Wages et al., 2022). Moreover, competition between competing organisms during enrichment will likely inhibit *Campylobacter* growth. Therefore, the current study suggested that 2× BFBB be used as the best-fit medium for *Campylobacter* detection. Using 2× BFBB as the enrichment broth, the BAX® System Real-Time PCR Assay appeared to be highly specific for each primer set for the detection of *C. jejuni* (n = 15*), C. coli* (n = 15), and *C. lari* (n = 15), with 100 % specificity for each. Therefore, 2× BFBB was used for the

enrichment time of detection and validation studies to remain consistent with industry standards (USDA-FSIS, 2021).

Although interventions are in place to reduce *Campylobacter* and other foodborne pathogens, it does not eliminate them entirely. Broiler chickens, a natural reservoir for *Campylobacter*, typically harbor 10^5 to 10^8 in their gastrointestinal tract when harvested for processing (Facciolà et al., 2017). Currently, there are no *Campylobacter* detection limitations on poultry products in the United States. However, the European Union (EU) allows less than 3.00 Log_{10} CFU/g limit of *Campylobacter* on poultry carcasses (Emanowicz et al., 2021). For the development of quantitation parameters, 3.00 Log_{10} CFU/mL was used for the detection limit of *Campylobacter* in poultry rinsate. Therefore, a pre-enrichment step is needed to detect and quantify the pathogen. As a result, further research was performed to determine the appropriate time of enrichment for low-level *Campylobacter* to grow for detection in BAX® System Real-Time PCR.

In a poultry rinsate inoculated with *Campylobacter*, the detection limit was 3.90, 4.50, and 3.80 Log_{10} CFU/mL in the BAX® System Real-Time PCR Assay for *C. jejuni*, *C. coli*, and *C. lari*, respectively. The detection limit of the BAX® System Real-Time PCR Assay was less effective for poultry rinsate samples than for pure culture (*C. jejuni*: 3.03; *C. coli*: 2.93; *C. lari*: 3.13 Log_{10} CFU/mL). The higher detection limit of poultry rinsates are likely because foodborne pathogens such as *Campylobacter* are generally in lower abundance on processed poultry carcass microbiota and thus require propagation for detection. In addition, competitor organisms, loss of template during DNA extraction, and inhibitors present in the rinsate could reduce the detection efficiency (Zhang et al., 2013). Similar results were seen in a study by Zhang et al. (2013). Using

their multiplex PCR for *Campylobacter* detection, they reported a limit of detection of 4.3 CFU/g in pure culture but 10^3 CFU/g in cecal contents.

Additionally, the poultry rinsates consisted of a wide range of microorganisms, which could influence *Campylobacter* growth and biochemical interactions in media, potentially supporting the development of non-*Campylobacter* colonies. Ricke et al. (2019) suggested that different poultry matrices could induce biochemical changes to *Campylobacter* and actively interfere with the sensitivity and specificity of certain isolation and detection methods. These results further support the difficulties associated with *Campylobacter* detection using either molecular-based or culture-based methods in complex matrices (Zhang et al., 2013; Ricke et al., 2019).

According to the USDA-FSIS 2021 method of isolation, *Campylobacter* can require up to 48 h of enrichment before it is detectable. In this study, the BAX® System Real-Time PCR Assay for *Campylobacter* yielded 100 % detection in all samples after 20 h incubation in poultry rinsates for concentrations of 2.00 Log_{10} CFU/mL (Table 3). The results from the study demonstrate that the BAX® System Real-Time PCR Assay for *Campylobacter* detection appears to be precise and accurate compared to the culture enumeration method. Additionally, using the developed detection parameters of the BAX® System Real-Time PCR Assay, such as the limit of detection and enrichment time, CampyQuant™ was developed for potential quantification of *Campylobacter jejuni, coli,* and *lari* in poultry rinsates.

To accomplish CampyQuant™ development, the detected *Campylobacter* concentrations from the poultry rinsate samples and CT values from the BAX® System Real-Time PCR Assay were used to develop standard curves with an enumerable range of 1.00 to 4.00 Log_{10} CFU/mL. In poultry rinsates, the BAX® System Real-Time PCR Assay was 100 % specific for the

detection of *Campylobacter* levels of 1.00 to 4.00 Log_{10} CFU/mL after 20 h enrichment. The log RMSE evaluates the standard deviation of the data and illustrates the utility of the CT values for estimating the respective *Campylobacter* concentrations. From these results, using the CampyQuant™ for *C. jejuni*, *C. col*i, and *C. lari* quantification, there was approximately 0.64, 1.09, and 0.81 Log_{10} CFU/mL variation in rinsate samples quantified between 1.00 to 4.00 Log_{10} CFU/mL after 20 h enrichment, respectively. While the variability of each standard curve fluctuated, it is likely the variability of the spread plating, which is still considered an acceptable standard, is potentially subject to phenotypic-based assumptions (Keegstra et al., 2017). However, when evaluated, CampyQuant™ estimates with 95 % confidence intervals reached the targeted inoculated levels of each *Campylobacter* species. As observed, the paired evaluation of the CampyQuant™ and the Campy-Cefex plating method on inoculated samples produced comparable estimates with confidence intervals bracketing the targeted inoculated levels of *Campylobacter*.

Quantification parameters such as sensitivity, accuracy, prevalence, NLR, and NPV were evaluated between the quantification method (CampyQuant™ vs. Campy-Cefex) and the *Campylobacter* species for main effects and interactions. There was no main effect for the quantification method or *Campylobacter* species. Additionally, no interaction was seen between the quantification method and species for quantification parameters. Rinsate sample concentration estimates were also compared between quantification methods between species. Main effects and interactions were found, therefore differences within each species, were analyzed between quantification methods at each inoculation level individually. Within *C. jejuni*, there was a difference between using CampyQuant™ and Campy-Cefex for 1.00 and 3.00 Log_{10} CFU/mL. There was no difference for levels 2.00 and 4.00 Log_{10} CFU/mL.. However, when

quantifying *C. coli*, there was no difference shown between the CampyQuant™ and Campy-Cefex plating. Within *C. lari*, differences were seen between CampyQuant™ and Campy-Cefex for 1.00 and 2.00 Log_{10} CFU/mL. Although no significant differences were found when quantifying *C. coli* with CampyQuant™ compared to Campy-Cefex plating, CampyQuant™ does lend some advantages for *Campylobacter* detection and quantification. *Campylobacter* exhibits unique physiological and metabolic biological characteristics that can depend on growth conditions among other factors (Ricke et al., 2019). Some of these characteristics can be altered due to environmental stress, which can cause *Campylobacter* cells to be viable but non-culturable (Kim et al., 2021). In the present study, it is possible that cells could be viable but non-culturable on Campy-Cefex plates. This could lead to underestimates of *Campylobacter* colonies on Campy-Cefex plates.

In addition, the conventional culture method in this study is that selective media is not able to specify differences between different *Campylobacter* species just by visually examining the colony morphology. The specificity of the assay by the species-specific amplification of DNA was 100 %, as all *Campylobacter* samples were detected as such in the present study. Moreover, the cells on the Campy-Cefex plates may not be as specific as the RT-PCR. In a study by Kim et al. (2017), they compared sequences from poultry carcass rinsates from Campy-Cefex plates. The colonies recovered from the plates revealed a significant range of non-*Campylobacter* bacteria, such as *Oscillospira, Acinetobacter, Enterococcus,* and *Bacillus* (Kim et al., 2017). This shows the potential for false- positives to occur with some culture methods.

Nevertheless, molecular methods for *Campylobacter* detection and quantification do have some concerns. Traditional DNA-based PCR can detect the genomic DNA of dead or nonviable *Campylobacter* spp. However, the detection of these organisms, which would be expected not to

be capable of causing disease, should be considered false-positive samples (Ricke et al., 2019). However, the growth of viable cells will dominate the nonviable cells during enrichment. The free genomic DNA from the dead cells is denatured from the DNAase enzymatic activity from the enriched *Campylobacter* cells (Jung et al., 2017; He et al., 2019). This process allows for potential dead cells to be excluded from the detection signal of the PCR.

Slopes of species-specific CampyQuant™ curves were explored for significant differences. The ANCOVA test revealed a significant difference between the species of *Campylobacter* (F-Value of 0.023). Differences were expected because each *Campylobacter* species has a unique metabolic requirement (Yeow et al., 2020). These unique metabolic characteristics of each *Campylobacter* species will have specific interactions with the poultry rinsate, which can alter the detection in the RT-PCR. Wagley et al., (2014) studied the different carbon substrate utilization patterns of *Campylobacter* species. It was revealed that *C. coli* and *C. jejuni* differed in their ability to utilize propionic acid as a carbon source in a culture medium.

Additionally, these differences in metabolism can also result in growth rate differences. In a study by Olsson et al. (2007), they compared growth rates of different *Campylobacter* species after 48 h. This study revealed that *C. jejuni, C. coli,* and *C. lari* exhibited significantly different exponential phases for each other (Olsson et al., 2007). The metabolic requirement and growth kinetic differences between species can cause the differences observed in the standard curve slopes. Therefore, each *Campylobacte*r species should be quantified with its respective specific CampyQuant™ standard curve.

5. Conclusions

In summary, the BAX® System Real-Time PCR Assay detected *Campylobacter* in all post-chill rinsate samples through an enumerable range of 1.00 to 4.00 Log_{10} CFU/mL. The

results demonstrate that the BAX® System Real-Time PCR Assay is a more rapid and sensitive method for detecting low levels of *Campylobacter* in poultry rinsates. With the ability to differentiate and quantify individual *Campylobacter* species, further risk assessment studies can be conducted. In addition, this study utilized post-chill rinsates collected from a single source, which may represent potential limitations with different rinsate microbiome compositional profiles that may influence the quantitation parameters. Testing across different processing samples and conditions is likely needed to determine the ultimate utility of the method proposed in the current study.

This study suggests that the BAX® System Real-Time PCR Assay also has the potential to quantitate *Campylobacter* spp. in poultry rinsates. Based on the results in the current study, 20 h enriched poultry rinsate samples can be quantified using the CampyQuant™ standard curves for *C. jejuni*, *C. coli*, and *C. lari*. With current industry standards, this method can potentially provide the poultry industry with a rapid and accurate quantitative method for *Campylobacter* enumeration to ensure that process controls are working adequately to provide safe products to consumers.

Conflict of Interest Statement

S.F. Applegate and T.P. Stephens are employed by Hygiena (2 Boulden Circle, New Castle, DE). Although Hygiena funded the research, all authors except for S.F. Applegate and T.P. Stephens declare that this research was conducted without any commercial relationships that can produce a potential conflict of interest.

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Tables

Table 1. The sensitivity, specificity, efficiency, Positive Likelihood Ratio (PLR), Negative Likelihood Ratio (NLR), Prevalence, Positive Predictive Value (PPV), Negative Predictive Value (NPV), Accuracy of the detection of *Campylobacter* species between media, Blood free Bolton broth (2× BFBB), and Buffered peptone water (BPW) when using BAX® System Real-Time PCR Assay[1,2].

Media		*C. jejuni*	*C. coli*	*C. lari*
2× BFBB	Sensitivity	93.0 % (88.0 to 99.0)	96.0 % (86.0 to 100.0)	88.0 % (71.0 to 100.0)
	Specificity	100.0 %	100.0 %	100.0 %
	Efficiency	121.0%	100.0 %	96.0%
	PLR	100.0 %	100.0 %	100.0 %
	NLR	6.7 %	4.0 %	12.0 %
	Prevalence	89.7 %	87.2 %	84.6 %
	PPV	100.0 %	100.0 %	100.0 %
	NPV	38.9 %	55.5 %	27.8 %
	Accuracy	94.0%	96.2%	89.4%
BPW	Sensitivity	76.0 % (66.0 to 86.0)	64.0 % (42.0 to 100.0)	80.0 % (80.0 to 100.0)
	Specificity	100.0 %	100.0 %	100.0 %
	Efficiency	107.0 %	371.0 %	93.0 %
	PLR	100.0 %	100.0 %	100.0 %
	NLR	24.0 %	36.0 %	20.0 %
	Prevalence	73.1 %	61.5 %	76.9 %
	PPV	100.0 %	100.0 %	100.0 %
	NPV	14.5 %	11.2 %	16.7 %
	Accuracy	81.8%	79.7%	84.0 %

[1]Significance for the main effect of species separated by media is presented in **Supplemental Table 2.**

[2]Significance for the main effect of media separated by *Campylobacter* species is presented in **Supplemental Table 3.**

Table 2. Main effect and interaction of the enrichment media and *Campylobacter* species between the sensitivity, accuracy, prevalence, negative likelihood ratio (NLR), negative predictive value (NPV), and specificity of CampyQuant™ BAX® System Real-Time PCR Assay. [1,2]

	C. jejuni		*C. coli*		*C. lari*		Effects		
Media	2× BFBB	BPW	2× BFBB	BPW	2× BFBB	BPW	Interaction	Media	Species
Sensitivity	93.3	76.0	96.3	64.0	88.0	80.0	0.102	< **0.0001**	0.277
Accuracy	93.8[a]	81.9[bc]	96.3[a]	79.0[c]	89.8[ab]	84.0[bc]	**0.024**	< **0.0001**	0.855
Prevalence	90.0	73.1	87.2	61.5	84.6	74.9	0.273	**0.0022**	0.366
NLR	6.7	24.0	4.0	36.0	12.0	20.0	0.063	**0.003**	0.57
NPV	38.9	14.5	55.6	11.2	27.9	16.7	0.312	**0.009**	0.578

[1]Significance was determined using the nonparametric Mann-Whitney U test.
[2]Different letters denote pairwise differences (a-c).

Table 3. Results of sensitivity, specificity Positive Likely Ratio (PLR), Negative Likely Ratio (NLR), Prevalence, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Accuracy of the BAX® System Real-Time PCR Assay for the detection of each *Campylobacter* species between media, enrichment times 16, 18, and 20 h in 2× Blood free Bolton broth (2× BFBB)[1,2].

Species	Inoculated Log_{10} CFU/mL	16 h	18 h	20 h
C. jejuni	0.58	18.0 %	73.0 %	93.0 %
	1.58	70.0 %	100.0 %	88.0 %
	2.58	100.0 %	100.0 %	100.0 %
	Sensitivity	66.7 %	86.1 %	94.4 %
	Specificity	100.0 %	100.0 %	100.0 %
	PLR	100.0 %	100.0 %	100.0 %
	NLR	33.3 %	13.9 %	5.60 %
	Prevalence	64.0 %	82.7 %	90.7 %
	PPV	100 %	100 %	100 %
	NPV	10.7 %	17.0 %	44.4 %
	Accuracy	42.7 %	71.2 %	85.6 %
C. coli	0.17	0.0 %	0.0 %	5.0 %
	1.17	0.0 %	5.0 %	92.0 %
	2.17	58.0 %	100.0 %	100.0 %
	Sensitivity	18.1 %	34.7 %	65.3 %
	Specificity	100.0 %	100.0 %	100.0 %
	PLR	100.0 %	100.0 %	100.0 %
	NLR	81.9 %	65.3 %	34.7 %
	Prevalence	17.3 %	33.3 %	61.3 %
	PPV	100.0 %	100.0 %	100.0 %
	NPV	6.81 %	6.00 %	10.4 %
	Accuracy	3.0%	8.6%	39.2 %
C. lari	0.03	0.0 %	0.0 %	21.0 %
	1.03	0.0 %	33.0 %	100 %
	2.03	42 %	63 %	100 %
	Sensitivity	13.9 %	31.9 %	72.2 %
	Specificity	100.0 %	100.0 %	100.0 %
	PLR	100.0 %	100.0 %	100.0 %
	NLR	86.1 %	68.1 %	27.8 %
	Prevalence	21.3 %	49.3 %	69.3 %
	PPV	100.0 %	100.0 %	100.0 %
	NPV	5.1 %	6.8 %	12.6 %
	Accuracy	1.2 %	27.3 %	50.4 %

[1]Significance for the main effect of time separated by *Campylobacter* species is presented in **Supplemental Table 4.**

[2]Significance for the main effect of *Campylobacter* species separated by time is presented in **Supplemental Table 5.**

Table 4. Main effect and interaction of the time and *Campylobacter* species on the sensitivity, accuracy, prevalence, negative likelihood ratio (NLR), and negative predictive value (NPV) of CampyQuant™ BAX® System Real-Time PCR Assay .[1,2]

	C. jejuni			*C. coli*			*C. lari*			Effects		
Time	16h	18h	20h	16h	18h	20h	16h	18h	20h	Interaction	Time	Species
Sensitivity	66.7^{cd}	86.1^{ab}	94.4^{a}	14.6^{e}	25.7^{e}	63.9^{cd}	13.9^{e}	53.0^{d}	72.2^{bc}	**0.004**	**< 0.0001**	**< 0.0001**
Accuracy	42.7^{bc}	71.4^{a}	78.1^{a}	3.0^{d}	8.6^{d}	39.2^{bc}	1.2^{d}	27.3^{c}	50.4^{b}	**0.010**	**< 0.0001**	**< 0.0001**
Prevalence	64.0	82.7	90.7	17.3	33.3	61.3	21.3	49.3	69.3	0.161	**< 0.0001**	**< 0.0001**
NLR	33.3^{bc}	13.9^{de}	5.6^{e}	85.1^{a}	74.3^{a}	36.1^{bc}	86.1^{a}	47.0^{b}	27.7^{cd}	**0.0004**	**< 0.0001**	**< 0.0001**
NPV	10.7^{bc}	17.0^{b}	44.4^{a}	6.8^{bc}	6.0^{bc}	10.4^{bc}	5.1^{c}	6.8^{bc}	12.6^{bc}	**< 0.0001**	**< 0.0001**	**< 0.0001**

[1]Significance was determined using the nonparametric Mann-Whitney U test.
[2]Different letters denote pairwise differences (a-c).

Table 5. Log_{10} CFU/mL estimates from Campy-Cefex and CampyQuant™ for each species**.** Main effect and interactions of the quantification method (CampyQuant™ vs. Campy-Cefex) and *Campylobacter* species on each specific Log_{10} CFU/mL estimates (1.00 to 4.00 CFU/mL). An ANCOVA test was used to investigate a significant different linear slope between the species of *Campylobacter* (F-Value = 0.023).

	C. jejuni		*C. coli*		*C. lari*		Effects		
Log_{10} CFU/mL[1]	CampyQuant™	Campy-Cefex	CampyQuant™	Campy-Cefex	CampyQuant™	Campy-Cefex	Interaction	Method	Species
1.00	$1.18^{b} \pm 0.16$	$1.64^{a} \pm 0.16$	$1.22^{ab} \pm 0.10$	$1.260^{ab} \pm 0.12$	$1.18^{b} \pm 0.16$	$0.70^{c} \pm 0.10$	**0.002**	0.913	**0.001**
2.00	$1.89^{bc} \pm 0.05$	$2.18^{a} \pm 0.05$	$2.09^{ab} \pm 0.06$	$1.74^{c} \pm 0.05$	$1.88^{bc} \pm 0.05$	$1.50^{d} \pm 0.05$	**<0.001**	**<0.001**	**0.002**
3.00	$2.93^{ab} \pm 0.04$	$3.05^{ab} \pm 0.10$	$2.74^{b} \pm 0.09$	$3.23^{a} \pm 0.09$	$2.92^{b} \pm 0.09$	$2.68^{b} \pm 0.09$	**0.001**	**0.100**	0.639
4.00	$4.08^{ab} \pm 0.08$	$4.03^{ab} \pm 0.05$	$3.91^{b} \pm 0.07$	$4.13^{a} \pm 0.05$	$4.09^{ab} \pm 0.07$	$3.55^{c} \pm 0.05$	**<0.001**	**0.005**	**<0.001**

Table 6. Results of quantification metric parameters: sensitivity, specificity Positive Likely Ratio (PLR), Negative Likely Ratio (NLR), Prevalence, Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Accuracy of the BAX® System Real-Time PCR Assay for the detection of each *Campylobacter* species

Method	Performance Test	*C. jejuni*	*C. coli*	*C. lari*
CampyQuant™	Sensitivity	88.3 %	83.3 %	86.6 %
	Specificity	100.0 %	100.0 %	100.0 %
	Positive Likely Ratio	100.0 %	100.0 %	100.0 %
	Negative Likely Ratio	11.7 %	16.7 %	13.3 %
	Prevalence	84.1 %	79.4 %	82.5 %
	PPV	100.0 %	100.0 %	100.0 %
	NPV	34.4 %	20.6 %	24.4 %
	Accuracy	74.1 %	66.1%	71.5 %
Campy-Cefex	Sensitivity	94.4 %	80.6 %	83.3 %
	Specificity	100.0 %	100.0 %	100.0 %
	Positive Likely Ratio	100.0 %	100.0 %	100.0 %
	Negative Likely Ratio	5.6 %	19.4 %	16.7 %
	Prevalence	87.2 %	74.4 %	76.9 %
	PPV	100.0 %	100.0 %	100.0 %
	NPV	30.6 %	30.6 %	27.7 %
	Accuracy	82.5%	60.0%	64.5%

[1]Significance for the main effect of *Campylobacter* species separated by quantification method is presented in **Supplemental Table 7.**

[2]Significance for the main effect of the quantification method separated by *Campylobacter* species is presented in **Supplemental Table 8.**

Table 7. Main effect and interaction of the quantification method (CampyQuant™ vs. Campy-Cefex) and *Campylobacter* species on the sensitivity, accuracy, prevalence, negative likelihood ratio (NLR), and negative predictive value (NPV).[1]

	C. jejuni		*C. coli*		*C. lari*		Effects		
Method	Campy Quant™	Campy-Cefex	Campy Quant™	Campy-Cefex	Campy Quant™	Campy-Cefex	Interaction	Method	Species
Sensitivity	88.3	94.5	83.3	80.5	86.7	83.3	0.411	1.000	0.079
Accuracy	74.7	82.5	66.5	60.0	71.7	64.5	0.415	0.7046	0.074
Prevalence	84.1	87.2	79.4	74.3	82.5	76.9	0.433	0.405	0.083
NLR	11.7	5.5	16.7	19.5	15.0	16.6	0.475	0.863	0.073
NPV	34.4	30.5	20.6	15.9	26.1	27.8	0.733	0.527	**0.020**

[1]Significance was determined using the nonparametric Mann-Whitney U test.

Figures

Curve development – *C. jejuni* – 2× Blood free Bolton broth vs BPW

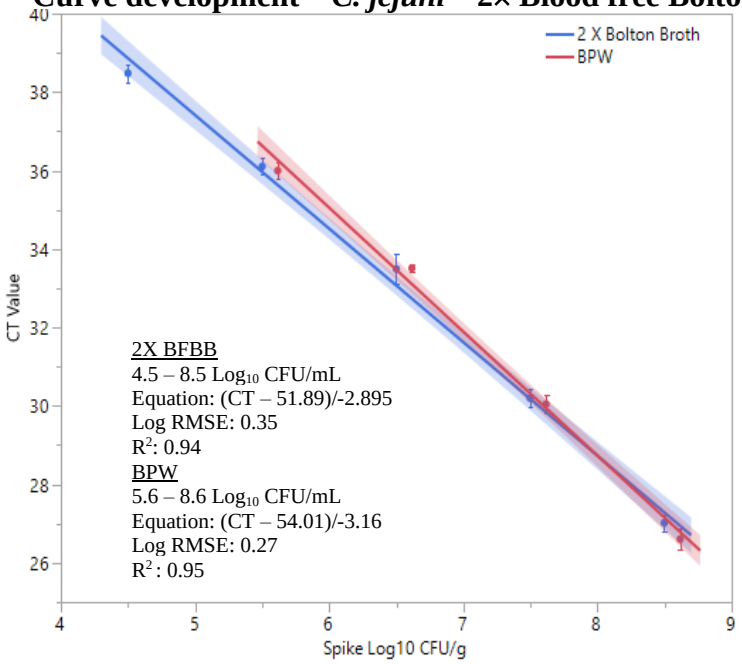


Figure 1. The linear curve of CT values from the BAX® System Real-Time PCR Assay on the Y-axis, Spiked Log_{10} CFU/mL concentration of the samples on the X-axis. 2× BFBB in the blue and BPW in the red. Mann-Whitney U test, P = 0.032, resulting in a significant difference between BFBB and BPW detection for *C. jejuni.* ANCOVA revealed a significant difference between slopes from BB linear curve and BPW between media (F= 0.001).

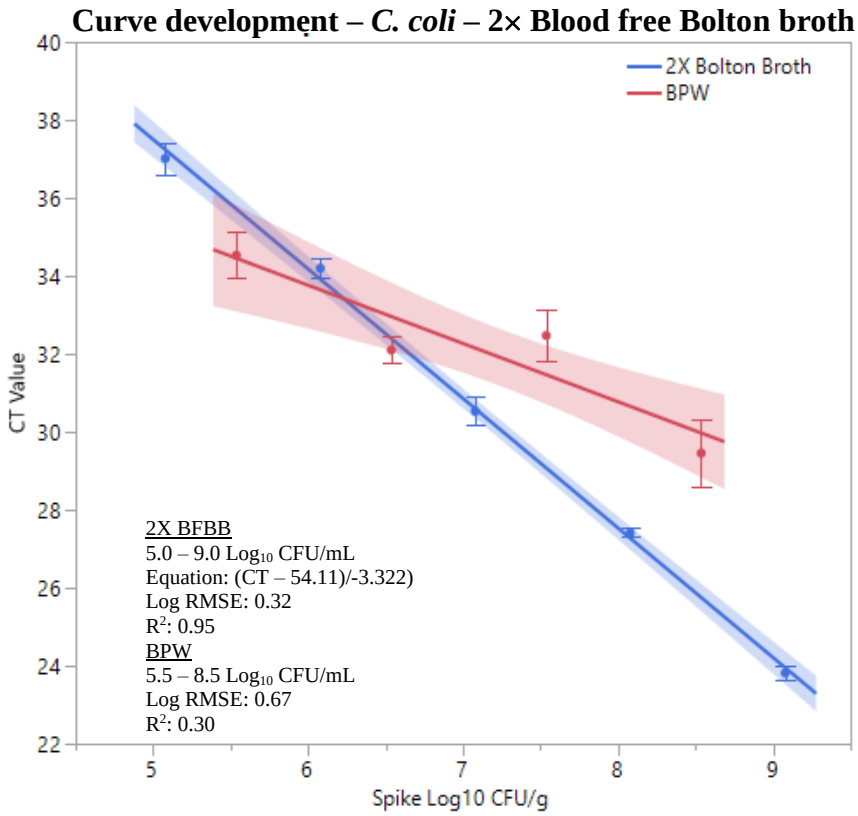


Figure 2. The linear curve of CT values from the BAX® System Real-Time PCR Assay on the Y-axis, Log_{10} CFU/mL concentration of the samples on the X-axis. 2× BFBB in the blue and BPW in the red. Mann-Whitney U test, P = 0.038, results in a significant difference between 2× BFBB and BPW detection for *C. coli.* An ANCOVA revealed a significant difference between the slopes of the linear curve from *C. coli* detection in BB and BPW (F = 0.004).

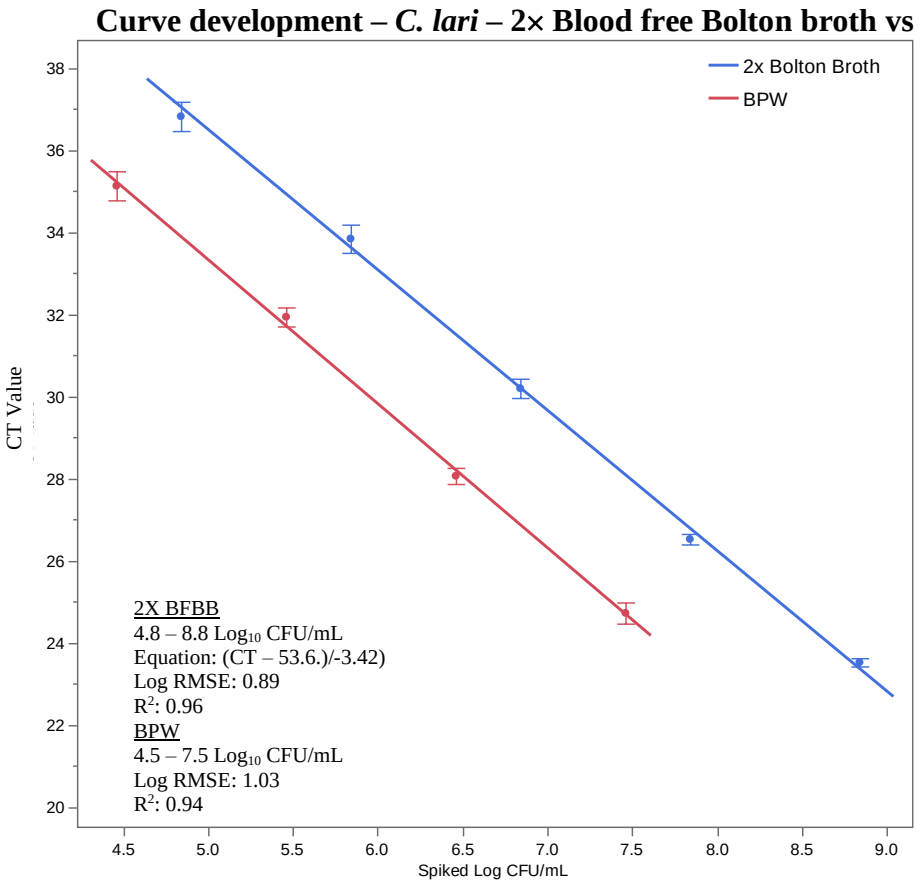


Figure 3. The linear curve of CT values from the BAX® System Real-Time PCR Assay on the Y-axis, Spiked Log_{10} CFU/mL concentration of the samples on the X-axis. 2× BFBB in the blue and BPW in the red. Mann-Whitney U test, P = 0.034, results in a significant difference between BFBB and BPW detection for *C. lari.* ANCOVA revealed a significant difference between slopes from BB linear curve and BPW between media (F= 0.001).

***C. jejuni*– CampyQuant 20 h enrichment – 1.00 to 4.00 CFU/mL**

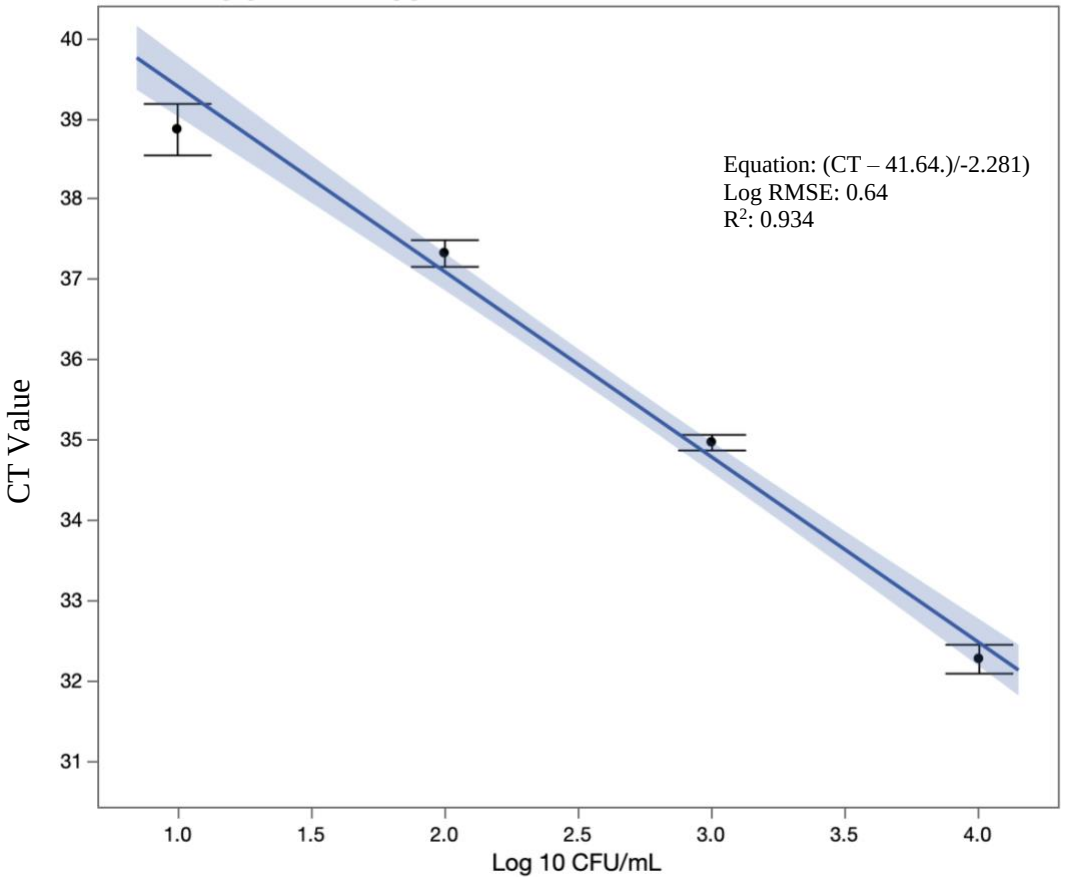


Figure 4. Poultry rinsate standard curve developed for *C. jejuni* with enumerable ranges 1.00 to 4.00 Log_{10} CFU/mL. Y= 41.64 – 2.281 x X. RMSE = 0.64 R^2= 0.934.

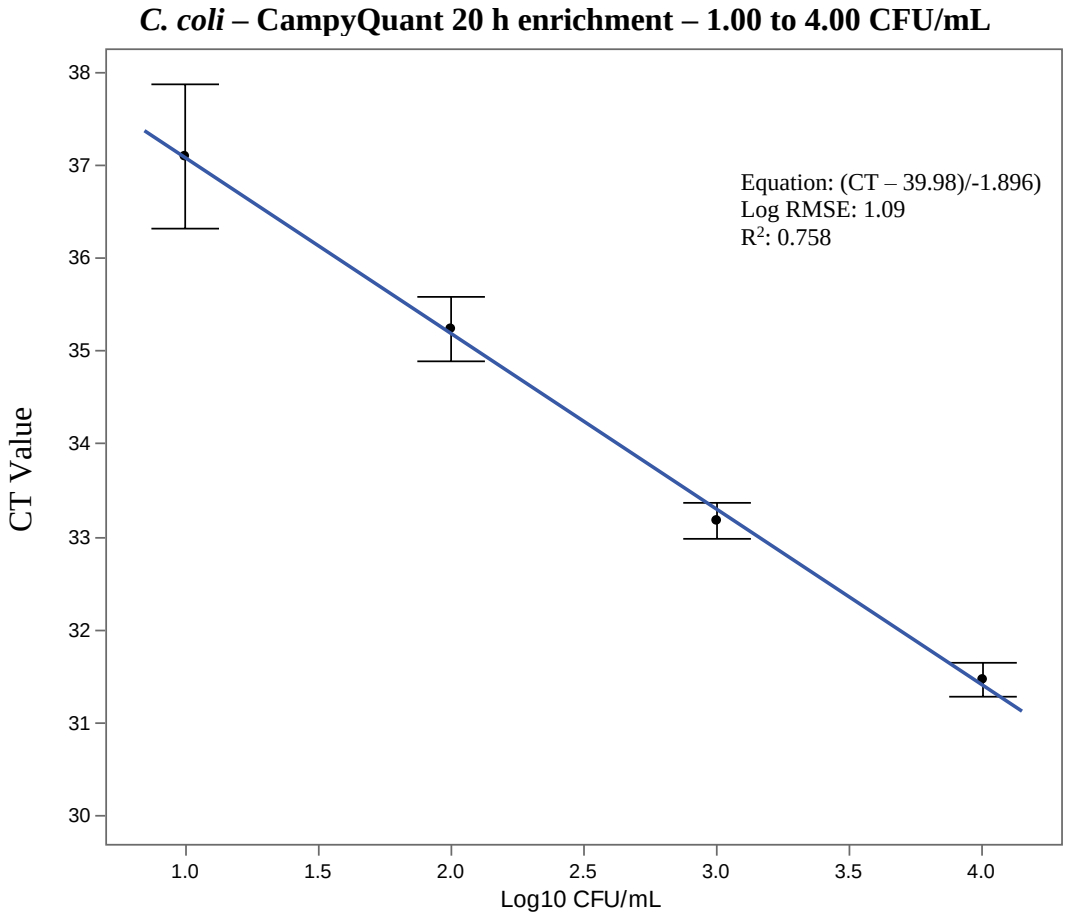


Figure 5. Poultry rinsate standard curve developed for *C. coli* with enumerable ranges 1.00 to 4.00 Log_{10} CFU/mL. Y= 39.98 – 1.896 x X. RMSE = 1.09 R^2= 0.758.

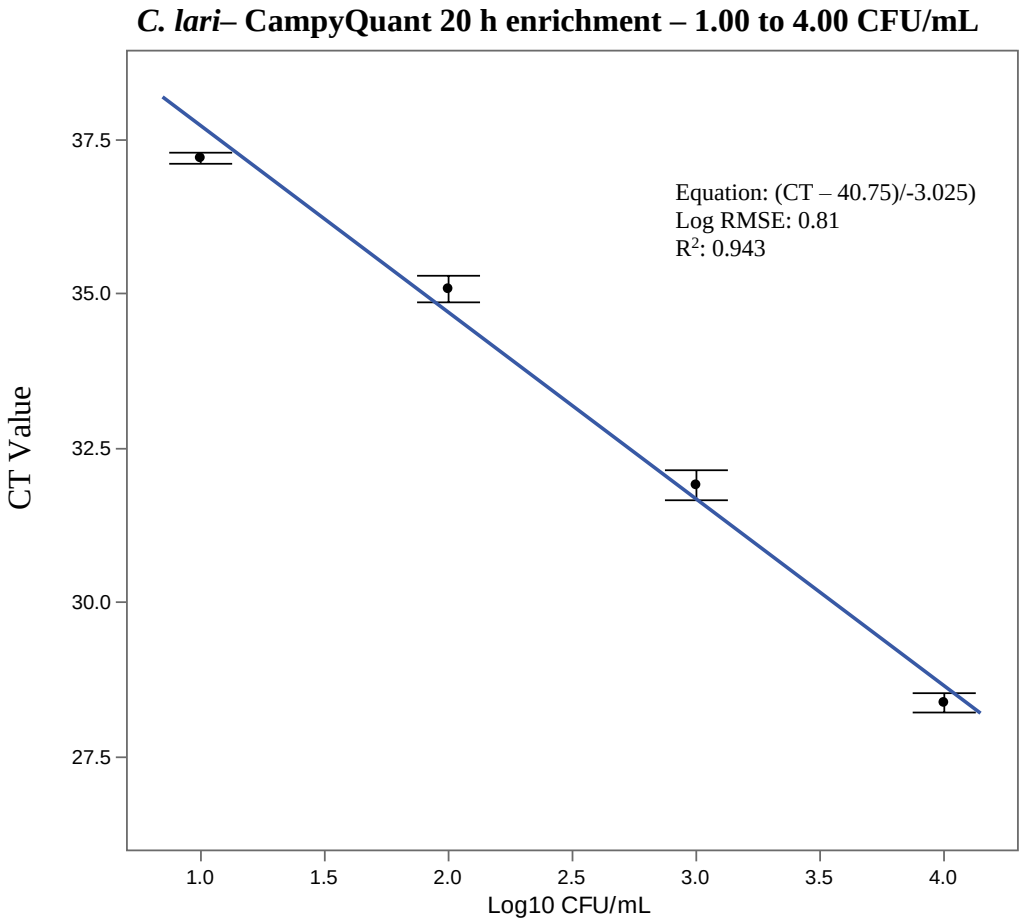


Figure 6. Poultry rinsate standard curve developed for *C. lari* with enumerable ranges 1.00 to 4.00 Log_{10} CFU/mL. Y= 40.75 -3.025 x X. RMSE = 0.81. R^2 = 0.943.

Supplemental Data

Supplemental Table 1

	2× Bolton Broth	Buffered Peptone Water
C. jejuni	3.03 Log_{10} CFU/mL	5.60 Log_{10} CFU/mL
C. coli	2.93 Log_{10} CFU/mL	5.50 Log_{10} CFU/mL
C. lari	3.13 Log_{10} CFU/mL	4.50 Log_{10} CFU/mL

Plate counts of initial *Campylobacter* species concentration from growth and dilution in 2× Bolton broth and Buffered peptone water on mCCDA.

Supplemental Table 2. Statistical significance between the sensitivity, accuracy, prevalence, negative likelihood ratio (NLR), negative predictive value (NPV), and specificity between the species in either media, 2× blood-free Bolton broth (2× BFBB) or buffered peptone water (BPW).[1]

	2× BFBB	BPW
Sensitivity	P = 0.167	P = 0.074
Accuracy	P = 0.167	P = 0.064
Prevalence	P = 0.549	P = 0.074
NLR	P = 0.167	P = 0.074
NPV	P = 0.459	P = 0.064
Specificity	P = 1.000	P = 1.000

[1]Significance was determined using the nonparametric Mann-Whitney U test.

Supplemental Table 3. Statistical significance between the sensitivity, accuracy, prevalence, negative likelihood ratio (NLR), negative predictive value (NPV), and specificity between the enrichment media, 2× blood-free Bolton broth (BB), or buffered peptone water (BPW) for *Campylobacter jejuni*, *coli*, and *lari*.[1]

	C. jejuni	*C. coli*	*C. lari*
Sensitivity	P = 0.046	P = 0.050	P = 0.114
Accuracy	P = 0.046	P = 0.050	P = 0.114
Prevalence	P = 0.046	P = 0.077	P = 0.114
NLR	P = 0.050	P = 0.114	P = 0.114
NPV	P = 0.453	P = 0.101	P = 0.114
Specificity	P = 1.000	P = 1.000	P = 1.000

[1]Significance was determined using the nonparametric χ^2 analysis.

Supplemental Table 4. Statistical significance between the sensitivity, accuracy, prevalence, negative likelihood ratio (NLR) negative predictive value (NPV) between the time (16, 18, and 20 h) of *Campylobacter jejuni*, *coli*, and *lari*.[1]

	C. jejuni	*C. coli*	*C. lari*
Sensitivity	P = **0.045**	P = 0.034	P = 0.027
Accuracy	P = 0.027	P = 0.034	P = 0.027
Prevalence	P = 0.027	P = 0.034	P = 0.038
NLR	P = **0.027**	P = 0.034	P = 0.027
NPV	P = 0.033	P = 0.102	P = 0.061

[1]Significance was determined using the nonparametric χ^2 analysis.

Supplemental Table 5. Statistical significance between the sensitivity, accuracy, prevalence, negative likelihood ratio (NLR) negative predictive value (NPV) between the *Campylobacter* spp., *jejuni*, *coli*, and *lari*, at 16, 18, and 20 h of enrichment.[1]

	16 h	18 h	20 h
Sensitivity	P < 0.054	P = 0.061	P = **0.030**
Accuracy	P < 0.059	P = **0.047**	P = **0.027**
Prevalence	P = 0.050	P = 0.061	P = **0.006**
NLR	P = 0.054	P = 0.062	P = **0.030**
NPV	P = 0.840	P = 0.060	P = **0.030**

[1]Significance was determined using the nonparametric χ^2 analysis.

Supplemental Table 6. Statistical comparison of CampyQuant™ vs. Campy-Cefex of each Log concentration (1.00 to 4.00 Log_{10} CFU/mL), within each species (*C. jejuni*, *coli*, and *lari*).[1]

Log_{10} CFU/mL	*C. jejuni*	*C. coli*	*C. lari*
1.00	**P = 0.001**	P = 0.469	**P < 0.001**
2.00	P = 0.173	P = 0.590	**P < 0.001**
3.00	**P < 0.001**	P = 0.061	P = 0.092
4.00	P = 0.739	P = 0.057	P = 0.230

[1]Significance was determined using the nonparametric χ^2 analysis.

Supplemental Table 7. Statistical comparison of performance criteria between the species of *Campylobacter* (*C. jejuni*, *coli*, and *lari*) when using CampyQuant™ or Campy-Cefex.[1]

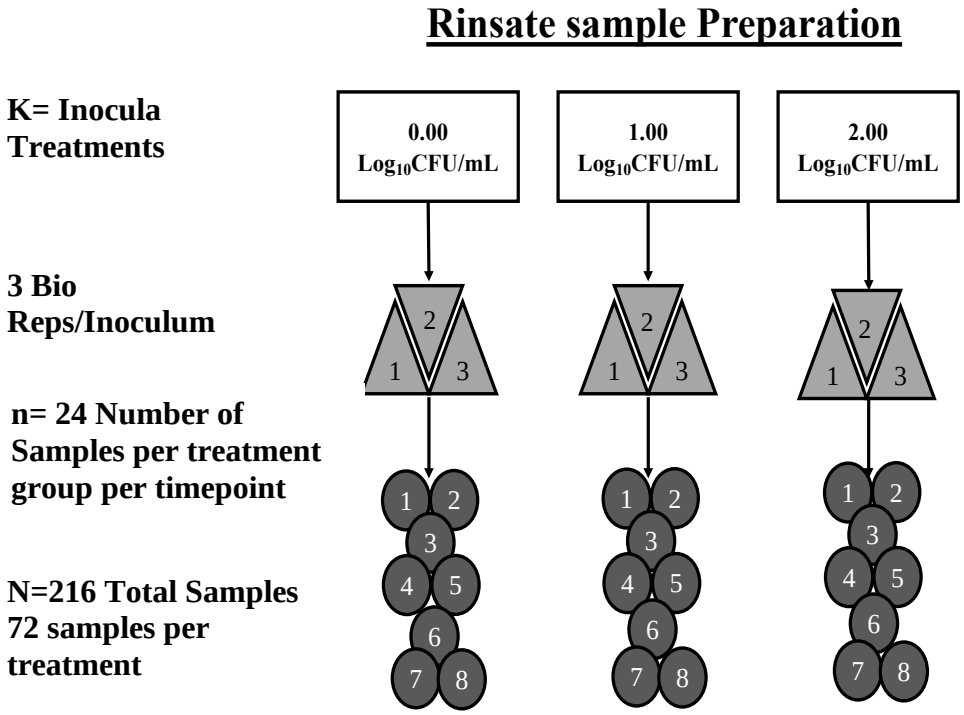
	CampyQuant™	CampyCefex
Sensitivity	P = 0.591	P = 0.089
Accuracy	P = 0.552	P = 0.090
Prevalence	P = 0.591	P = 0.089
NLR	P = 0.591	P = 0.079
NPV	P = 0.329	P = **0.049**

[1]Significance was determined using the nonparametric χ^2 analysis.

Supplemental Table 8. Statistical comparison of performance criteria between the *Campylobacter* quantification methods utilized in the current study, CampyQuant™ vs. Campy-Cefex, used to detect *C. jejuni, coli*, and *lari*.[1]

	C. jejuni	*C. coli*	*C. lari*
Sensitivity	P = 0.268	P = 0.376	P = 0.825
Accuracy	P = 0.825	P = 0.268	P = 0.825
Prevalence	P = 0.268	P = 0.268	P = 0.268
NLR	P = 0.289	P = 0.369	P = 0.825
NPV	P = 0.817	P = 0.268	P = 0.361

[1]Significance was determined using the nonparametric χ^2 analysis.



Supplemental Figure 1. Rinsate sample preparation for *Campylobacter* time of detection. Three timepoints, 16, 18, and 20 h; Three biological replicates per inoculation level at each timepoint; 72 samples each timepoint, 216 total samples.

CONCLUSION

Campylobacter is the number one cause of gastroenteritis, with raw poultry products as the main vehicle of transmission to humans (Eberle, and Kiess, 2012). Currently, prevalence-based data is used to determine process control during poultry processing. The preferred culture-based plating method is used for the detection of *Campylobacter*. Problems with this method exist, such as complications with plate sensitivity, unable to identify species, long wait time for results and it does not provide information on the level and/or risk of *Campylobacter* contamination. (Silva et al., 2011; Frano-Duarte et al., 2019). Therefore, this dissertation research focuses on steps needed to develop a practical RT-PCR assay for rapid detection and quantification of *Campylobacter* spp. in post chill- poultry rinsates.

To do this, a deep dive was taken into *Campylobacter'*s literature to understand the mechanism of action for detection in culture-based and molecular methods. Next, the best fit medium was designated for future development. To do this, we must uncover the microbial community of various media for *Campylobacter* detection. Over time, many selective and enrichment media have been developed for *Campylobacter*. Yet, they predominantly focus on C. *jejuni.* Limited research exists that explores the selectivity of colonies obtained from selective media. As such, the microbial community was characterized pooling colonies from selective and non-selective media of post-chill rinsates artificially inoculated with *C. jejuni, C. coli* or *C. lari*. The study revealed that *Campylobacter, Lachnospiraceae,* and *Oscillospiraceae* were significantly different taxa among the three *Campylobacter* spp. When examining among the three media, *Staphylococcus* was significantly different. The results prove that the media used, and *Campylobacter* spp. can influence the microbial diversity and composition of inoculated enriched poultry rinsates.

This study provides kinetics and estimates for shortening enrichment time for faster detection of *Campylobacter* spp. based on pure culture cultivation. Next, growth curves and kinetics were obtained for *C. jejuni*, *C. coli*, and *C. lari* in pure culture using blood free Bolton broth. The data suggest that low levels of *Campylobacter* spp. can be detected over 3.00 Log_{10} CFU/mL after 20 h and 20 min. The data indicates that to optimize rapid detection methods for *Campylobacter* spp., growth conditions must be flexible to enrich all species present. The last objective was to develop and validate a rapid enumeration method for Campylobacter spp. in post-chill poultry rinsates using the CampyQuant™ BAX® System Real-Time PCR Assay. Combining knowledge from research about *Campylobacter* literature (**Chapter 1**), looking at the microbial composition of selective media to elucidating the best media for validation, based on selectivity (**Chapter 2**) and assessing growth kinetics for rapid enrichment for detection (**Chapter 3**), CampyQuant™ BAX® System Real-Time PCR Assay was developed. Using CampyQuant™, after 20 h of enrichment, poultry rinsate samples can be detected. Furthermore, using the generated linear fit equations for pre-enriched sample, poultry rinsate samples can be quantified between 1.00 to 4.00 Log_{10} CFU/mL (**Chapter 4**). The study suggests that this is a rapid, sensitive, and efficient alternative method for *Campylobacter* detection and quantification.

The research in this dissertation meets some of the immediate food safety issues in the industry for *Campylobacter* detection and quantitation. First, it provides information on *Campylobacter*'s growth in pure culture. This can provide information necessary for future detection and quantification methods. Secondly, there is a need for rapid detection methods in the food industry. Current methodologies are tedious and time consuming. The research presented in this thesis provides a more rapid method optimizing enrichment. Lastly, a level of

risk can be associated with prevalence-based data. The CampyQuant™ BAX® System Real-Time PCR Assay can differentiate among *C. jejuni*, *C. coli* and *C. lari* in poultry rinsate samples, with a quantification range from 1.00 to 4.00 Log_{10} CFU/mL.

This research is directly applicable to the food industry because the CampyQuant™ BAX® System Real-Time PCR Assay uses the USDA-FSIS materials for detection and quantification. In the future, continued refinement, particularly of the enrichment conditions should lead to a more routine and rapid assay for multiple species of *Campylobacter*.

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Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A., and Teixeira, P. (2011). *Campylobacter* spp. as a Foodborne Pathogen: A Review. Front. Microbiol. 27:200. doi: 10.3389/fmicb.2011.00200

APPENDIX

Comparison of media for the detection of *Campylobacter jejuni* using a commercial RT-PCR system

Aaron Bodie[1], Peter Rubinelli[1], April Englishbey[2], Tyler Stephens[2] and Steven C. Ricke[1]*
[1]Department of Food Science and Center for Food Safety, University of Arkansas,
2650 Young Avenue, Fayetteville, AR, USA 72704
[2]Qualicon Diagnostics LLC, A Hygiena Company, New Castle, DE

Abstract
Campylobacter jejuni is a major cause of diarrhea and foodborne gastroenteritis worldwide. Rapid and accurate detection of *C. jejuni* is needed to improve surveillance throughout food processing plants. The purpose of the current study was to compare the limit of detection for *C. jejuni* grown in different media, using a commercial real time polymerase chain reaction (RT-PCR). *C. jejuni* (10^6 CFU/mL) was inoculated and grown for 24 h under microaerophilic conditions in either a selective medium, Mueller-Hinton Broth (MHB); enrichment medium, blood free Bolton's broth 2x (BFBB2x); or Buffered Peptone Water (BPW). The experiment was repeated three times with three technical replications per medium. After 24 h, samples were serially diluted to 10^{-5}, 10 µL was spot plated in quadruplet on Modified Charcoal-Cefoperazone-Deoxycholate Agar (MCCDA), and subsequently incubated under microaerophilic conditions for 24 h at 42 °C. RT-PCR was performed in duplicate using the BAX Q7 RT-PCR system. Data was statistically analyzed using a linear regression and considered significant when $P \leq 0.05$. Slopes of the linear regressions of each media was compared using an analysis of covariance. The BAX Q7 RT-PCR system was able to detect *C. jejuni* regardless of media used. BPW had the highest limit of detection (6.19×10^{-3} CFU/mL), while MHB and BFBB2x reach a lower limit at 1.85×10^{-4} CFU/mL and 6.16×10^{-4} CFU/mL respectively. Using the designed primers and PCR assay of the BAX Q7 RT-PCR system, the amplification efficiency of all media is above 90%, while standard curves developed have an $R^2 > 0.95$. The results of this

study suggest that media, MHB, BFBB2x and BPW, were effective in detecting *C. jejuni* using the BAX- Q7 RT-PCR system. This RT-PCR assay and system will provide rapid and sensitive approach for the detection of *C. jejuni* for poultry processors and food safety personnel.

Key Words: *C. jejuni*, RT-PCR, detection, media

Methods and Materials

Bacterial strain and culture conditions

The following strains were used in this study; *Campylobacter* subsp. *jejuni* ATCC 700819 a whole genome sequenced strain, Strains were stored at -80°C in Tryptic Soy Broth (Ward's Science, Ontario, Canada) containing 20% (v/v) glycerol. To obtain cultures for the growth experiments, frozen stock cultures of each organism was streak plated from the -80°C vials on Modified Charcoal-Cefoperazone-Deoxycholate Agar (MCCDA; Himedia, Mumbai India), under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 42°C for 24 h. After incubation, a single colony *Campylobacter jejuni* was inoculated into 20mL of Mueller Hinton Broth (MHB; Neogen, Lansing, Michigan), 2x Bolton's blood free broth (BFBB2x; Neogen, Lansing, Michigan) and Buffered Peptone Water (BPW; ThermoScientific, Waltham, MA, USA). Followed by incubation under microaerophilic conditions at 42 °C with constant shaking at 100 RPM for 24 h.

2.2 Detection curves and detection limits of *Campylobacter* in pure cultures by BAX Q7-RT system

The detection limits of the BAX System were assessed from pure culture of the *Campylobacter* strains grown. At the end of the incubation period, all *Campylobacter* species samples were 10-fold serial diluted from initial inoculation through 10^{-7} in MHB, BFBB2x, and BPW. All dilutions at the 10^{-7} to 10^{-4} were plated on mCCDA plates. Additionally, all pure culture *Campylobacter* samples and dilutions were performed in the BAX Q7-RT PCR using the *Campylobacter* species assays by following the manufacturers' instructions to detect *C. jejuni*, diluted in MHB, BFBB2x and BPW. 5uL of each sample was added to cluster tubes with 200 ml of prepared BAX System lysis reagent. Lysis was performed by heating the tubes for 20 min at

37 °C and 10 minutes at 95 °C and then cooling the tubes at 4 °C for at least 5 minutes. Lysate was used to hydrate a PCR tablet the BAX System assays. PCR tubes were loaded into the BAX System Q7 instrument and run according to the procedure described in the BAX System user guide. All samples were examined in five replicates. The results were analyzed with BAX Q7 software (version 2.8). Detection curves of the BAX RT systems were generated based on the PCR threshold cycle (CT) values. Presumptive positive *Campylobacter* colonies were confirmed by phenotypical characteristics. Each dilution was plated using the dot plate method onto MCCDA plates and incubated for 24 h in microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 42 °C. All samples were plated in duplicates and counted to determine the number of *Campylobacter* present.

Statistical Analysis

Each dilution series of media was statistically analyzed using a linear regression analysis in R, this will show if there is any relationship with dilution series and *C. jejuni* detection in the BAX Q7 PCR. After linear regression, an ANOVA was used to determine if there are any significant difference in the detection limit of different media in the BAX Q7 PCR. This study was performed on three separate occasions.

Results

The CT values generate from BAX Q7 after 24 timepoint were evaluated to develop linear-fit curve equations, R^2, and Root Mean Square Errors (RMSE). The linear equation was used to estimate pre-enriched *Campylobacter* levels to mimic the logarithmic growth of bacteria. The R^2 evaluates the percentage of dependent variable variation a linear model explains and can be observed when CT value variation depends on the enrichment time and known bacterial inoculation level. The RMSE evaluates the standard deviation of the data and describes how well the CT values estimate the *C. jejuni* concentration. Each equation developed was evaluated based

upon the statistical parameters of the R^2 (> 0.80), Log RMSE (< 0.60), and enumerable range (4.00 – 8.00 Log_{10} CFU/mL(g)). Developed linear fit equations that did not meet these statistical parameters caused inaccurate estimations compared to known spike levels. Therefore, the importance of meeting statistical parameters throughout the development and verifications of enrichment protocols and linear fit equations is vital to create a rapid tool for quantification.

There was a significant difference for detection and enumerable ranges between MHB BFBB2x and BPW. MHB had the lowest limit of detection of 2.56 Log CFU/mL, followed by BFBB 2x at 2.93 Log CFU/mL then BPW at 3.03 Log CFU/mL. Additionally, enumerable ranges for MHB and BFBB2x were 5 log CFU, whereas BPW was only a 4 Log range (**Figure 1 to 3**). When compared, all standard curves using the BAX RT-PCR estimates had a larger enumerable range compared to curves using plate count estimates.

Conclusion

The results of this study suggest that media, MHB, BFBB2x and BPW, were effective in detecting *C. jejuni* using the BAX RT-PCR system. Standard curves using the BAX RT-PCR system will provide rapid and sensitive approach for the detection of *C. jejuni* in pure culture.

Figures

MHB Standard Curve with plate counts

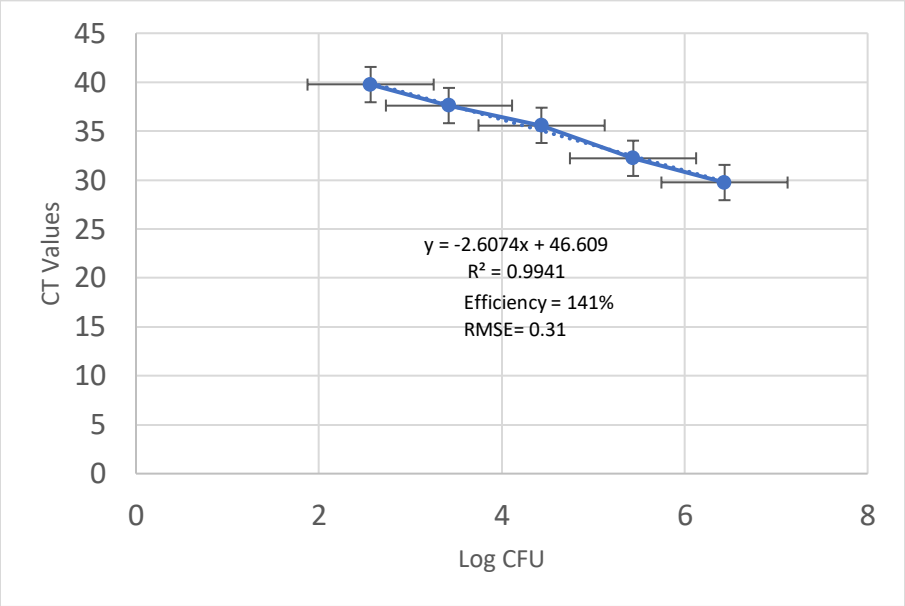

Figure 1a. *C. jejuni* dilution samples in Muller-Hinton Broth. X axis Log CFU/mL plate count and Y axis is CT values from BAX RT-PCR.

MHB Standard Curve BAX

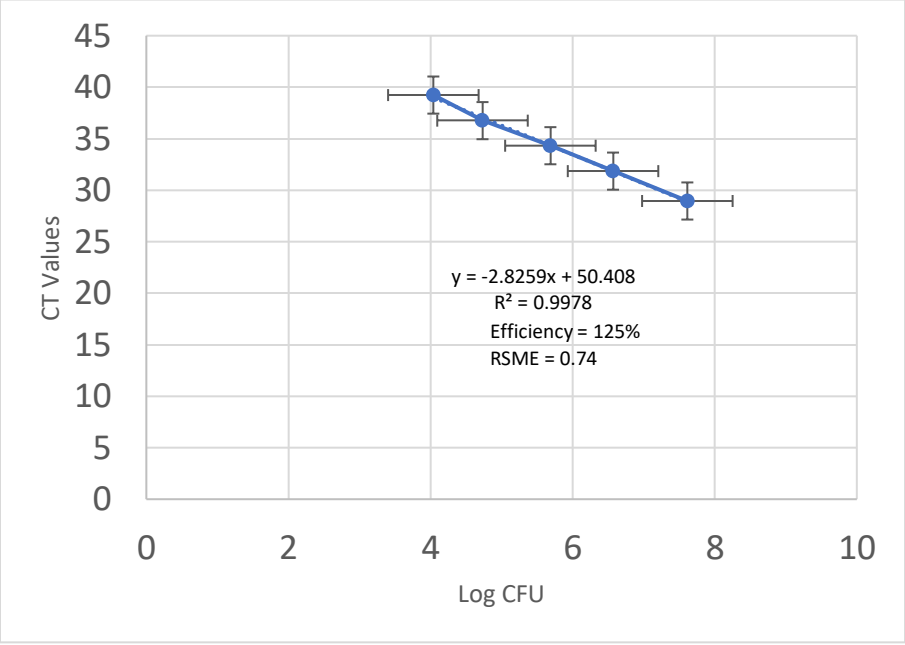

Figure 1b. *C. jejuni* dilution samples in Muller-Hinton Broth. X axis Log CFU/mL from BAX RT-PCR and Y axis is CT values from BAX RT-PCR.

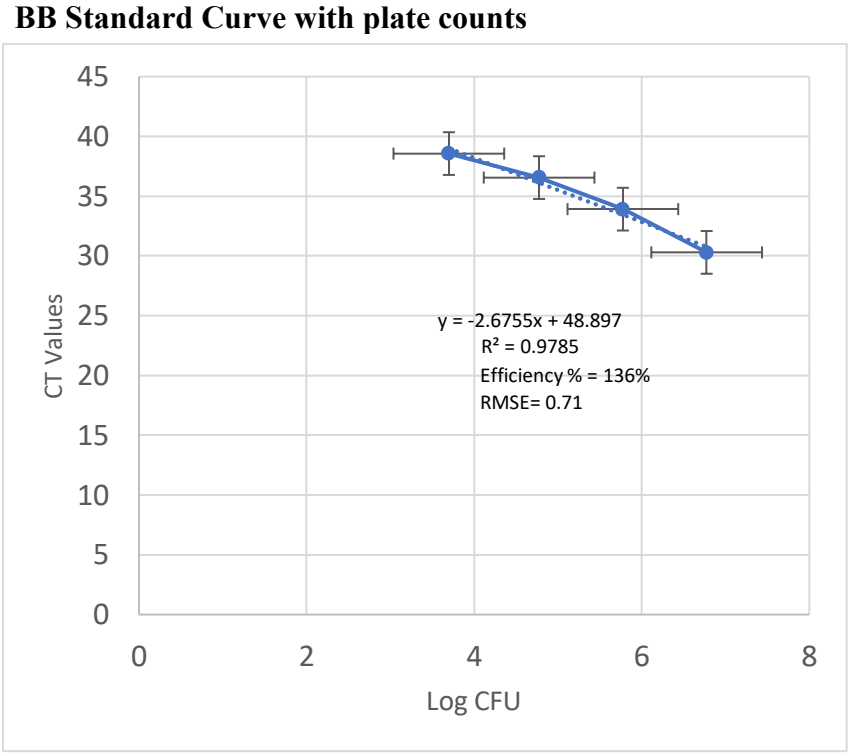

Figure 2a. *C. jejuni* dilution samples in Bolton Broth. X axis Log CFU/mL plate count and Y axis is CT values from BAX RT-PCR.

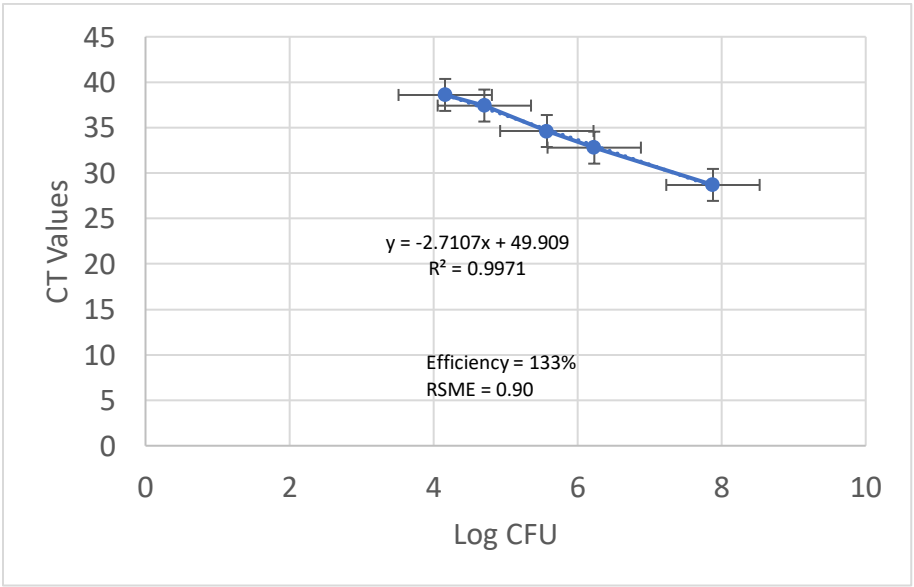

Figure 2b. *C. jejuni* dilution samples in Bolton Broth. X axis Log CFU/mL from BAX RT-PCR and Y axis is CT values from BAX RT-PCR.

BPW Standard Curve with plate counts

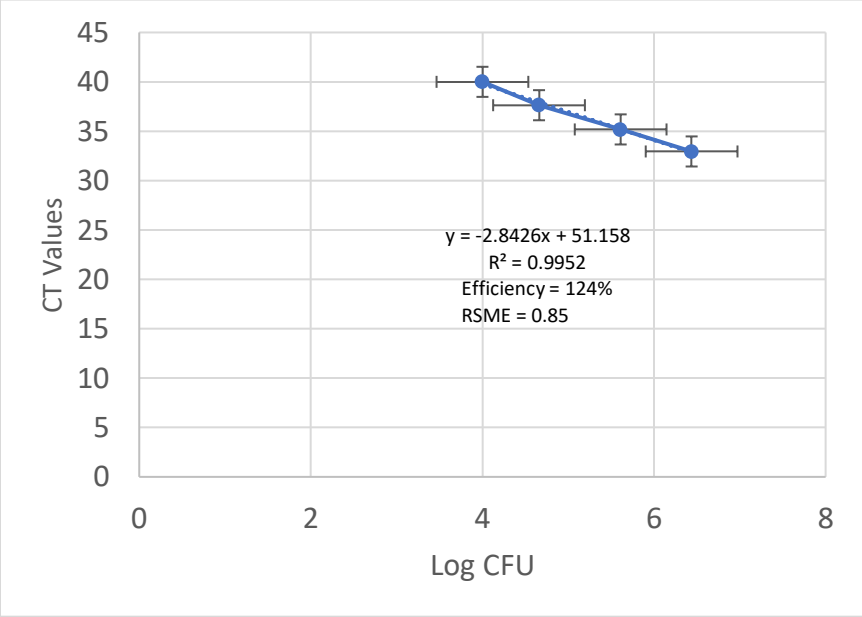

Figure 3a. *C. jejuni* dilution samples in Buffered Peptone Water. X axis Log CFU/mL plate count and Y axis is CT values from BAX RT-PCR.

BPW Standard Curve BAX

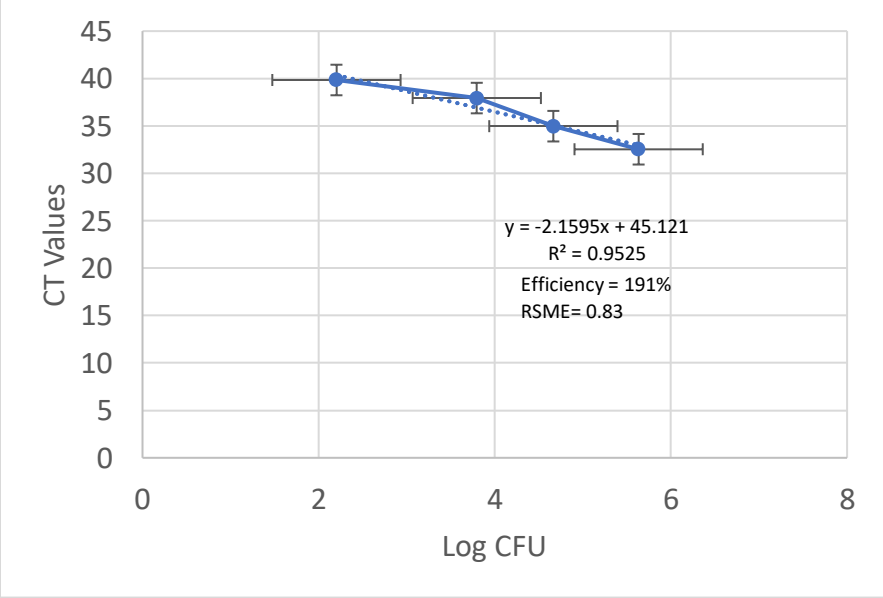

Figure 3b. *C. jejuni* dilution samples in Buffered Peptone Water. X axis Log CFU/mL from BAX RT-PCR and Y axis is CT values from BAX RT-PCR.

Curriculum Vitae

EDUCATION

Doctor of Philosophy in Animal and Dairy Sciences, Food Safety
August 2022
University of Wisconsin-Madison

Meat Science & Animal Biologics Discovery
Advisor: Dr. Steven C. Ricke
Dissertation: "Detection and Quantification of *Campylobacter* using commercial RT-PCR"

Master of Science in Food Science
August 2017
University of Arkansas

Dale Bumpers College
Advisor: Dr. Steven C. Ricke
Thesis: "Controlling *Listeria monocytogenes* in Ready- to-eat frankfurters"

Bachelor of Arts in Biology
August 2016
University of Arkansas

Bachelor's degree of Arts in Biology
Department of Biology, Fulbright College of Arts and Sciences

Project Title: "Construction of sub-unit vaccines for the control of *Campylobacter* in Chickens"
Advisor: Dr. Steven Ricke and Dr. Peter Rubinelli

PROFESSIONAL EXPERIENCE

Graduate Research Assistant
University of Wisconsin-Madison
August 2017- Present

Advisor: Dr. Steven Ricke
Meat Science & Animal Biologics Discovery

Project leader for various research studies while aiding in service center with company projects, preparation, and submission of manuscripts.

Assisting with the experimental design and data analysis.

Communicate directly with various internal and external partners to understand, define, and communicate progress on projects.

Mentor other undergraduate students while helping with general lab management and upkeep and assist lab manager in developing IBC protocols.

Work directly with the food industry for product development of *Campylobacter* quantification using molecular techniques.

Live Production/Meat Production
Varsity Meats
August 2020- Present

Manager: Dillion Walker
Meat Science & Animal Biologics Discovery

Responsible for HACCP validation and regulatory of USDA inspected meats for various products sold at Varsity meats.

Assisted in the making of different meat products for sale at Varsity meats such as summer sausage and bacon.

Ensure product quality ensures all finished product meets or exceeds requirements.

Proficient in bird, cattle and pork handling and processing throughout the harvest process.

Teachers Assistant
University of Wisconsin-Madison
August 2021 – December 2021

Supervisor: Dr. Jim Claus
Intro to Meat Technology
Taught all lab classes as well as taught a few lectures during the semester. Help mentor students with meat science studies and homework.

Laboratory Technician
Center for Food Safety
January 2014- December 2016

Supervisor: Dr. Peter Rubinelli
University of Arkansas
Position duties included projects and help with general lab management and upkeep.

SKILLS AND TECHNIQUES MASTERED

- **Standard molecular biology techniques**: DNA extractions, RNA extractions, Next Generation Sequencing, Agarose Gel Electrophoresis, PCR, qPCR
- **Statistical Data Analysis**: R, JMP, SAS, ArcGIS
- **Excellent microbiological techniques**: bacterial plating and dilutions, anaerobic chamber use, media and buffer preparation, survival assays and analysis (growth curves), differential biochemical testing, BSL-2 level foodborne pathogens
- Plating Procedures and Interpretation of rapid aerobic count plates, E. coli/Coliform count plates, Enterobacteriaceae counts plates
- Knowledge and training of pathogen reduction and testing: *Salmonella, Listeria, Campylobacter* and *E.coli*
- Proficient in chemical techniques: titrations, liquid chromatography, solvent extraction, and acid hydrolysis
- Grant Writing

- Research and Development
- Bird (poultry) handling and processing

PUBLICATIONS

- **Bodie AR**, Dittoe DK, Feye KM, Knueven CJ, Ovall C, Ricke SC (2022) Comparison of ready-to-eat "organic" antimicrobials, sodium bisulfate, and sodium lactate, on *Listeria monocytogenes* and the indigenous microbiome of organic uncured beef frankfurters stored under refrigeration for three weeks. PLoS ONE 17(1): e0262167. doi: 10.1371/journal.pone.0262167

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- **Bodie A.R.**, Micciche A.C, Atungulu G.G, Rothrock M.J Jr and Ricke S.C (2019). Current trends of rice milling byproducts for agricultural applications and alternative food production systems. *Front. Sustain. Food Syst.* 3:47. doi: 10.3389/fsufs.2019.00047

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- Rothrock, M.J Jr, Micciche, A.C, **Bodie A.R** and Ricke SC (2019). *Listeria* Occurrence and Potential Control Strategies in Alternative and Conventional Poultry Processing and Retail. *Front. Sustain. Food Syst.* 3:33. doi: 10.3389/fsufs.2019.00033

- Rothrock, M.J, Davis, M.L, Locatelli A, **Bodie A.R**, Mcintosh TG, Donaldson J.R and Ricke S.C. (2017). *Listeria* Occurrence in Poultry Flocks: Detection and Potential Implications. Front. Vet. Sci., 11 August 2017 | https://doi.org/10.3389/fvets.2017.00125

SYMPOSIUM PRESENTATIONS

- **Bodie, A.R.**, S.A. Kim, J. Atchely, C. Knueven, and S.C. Ricke. 2017. Potential antimicrobials for controlling *Listeria monocytogenes* in hotdogs. 9th Annual Arkansas Association Protection Educational Conference, Holiday Inn, Springdale, AR., Sept. 19-21

- **Bodie, A.R.**, D. Dittoe, K. Feye, C. Knueven, and S.C. Ricke. 2019. Comparison of bisulfate of soda and sodium lactate on refrigerated organic frankfurters over three weeks for controlling *Listeria monocytogenes*. 11th Annual Arkansas Association Protection Educational Conference, Fayetteville Town Center, Fayetteville, AR., Sept. 18-20.

- **Bodie, A.R.**, K. Feye, A. Englishbey, T. Stephens, and S.C. Ricke. 2019. PCR Detection efficacy of *Campylobacter* utilizing various media types. 11th Annual Arkansas

Association Protection Educational Conference, Fayetteville Town Center, Fayetteville, AR., Sept. 17-18.

- **Bodie, A.R**., D.K. Dittoe, S.F. Applegate, T.P. Stephens, and S.C. Ricke. 2022. Quantification of *Campylobacter jejuni*, *coli*, and *lari* in poultry post-chill whole bird carcass rinses utilizing shortened enrichment time and PCR. International Poultry Scientific Forum, Atlanta, GA, Jan. 24-25.

ACADEMIC SCHOLARSHIPS, AWARDS AND HONORS

- Hygiena Travel Scholarship, 2021
- USDA, Poultry Facilities Food Defense Workshop, 2016
- Arkansas Association for Food Protection 3rd Place Winner, Poster Competition, 2017
- Bahamas National Merit Scholarship, 2017
- Bahamas National Merit Scholarship, 2018
- Jones Hamilton Travel Scholarship, 2018
- University of Arkansas Graduate Travel Grant, 2018

PROFESSIONAL AFFILIATIONS

- Badger Poultry Science Club
- University of Arkansas Food Science Club
- International Association of Food Protection- Member
- Arkansas Association of Food Protection- Member
- Poultry Science Association- Member
- Caribbean Students Association
- Kappa Alpha Psi Fraternity Incorporated

REFERENCES

Dr. Steven Ricke	sricke@wisc.edu
Dr. Si Hong Park	sihong.park@oregonstate.edu
Dr. Jeff Sindelar	jsinderlar@wisc.edu
Dr. Kathleen Glass	kglass@wisc.edu
Dr. Vanessa Leone	valeone@wisc.edu
Dr. Griffiths Atungulu	atungulu@uark.edu