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PREVALENCE AND CONTROL OF VIABLE *CAMPYLOBACTER* ON CHICKEN BREASTS

A Thesis in

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by

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ABSTRACT

Since 2014, *Campylobacter* has been the leading bacterial cause of foodborne illness, resulting in billions in economic losses each year and straining public health. Chicken, the most consumed meat in the US, is the primary source of *Campylobacter* infection in humans, accounting for 50 – 90% of all cases. To survive food processing stressors like oxidative and cold stress, *Campylobacter* enters a viable but nonculturable (VBNC) state, where cells remain intact (viable) but cannot grow in conventional culture media (nonculturable). This presents a food safety challenge since growth in selective media, which only determines the culturable cells, is the standard method for food safety monitoring and surveillance. Recently, culture-independent detection methods like viability quantitative polymerase chain reaction (qPCR) have been developed to detect both culturable and nonculturable viable cells.

In the first project, we tested retail chicken breasts (n = 211) for *Campylobacter* spp. using the gold-standard culture-based methods and viability qPCR. Culture-based enrichment yielded isolates for 16 samples, with whole genome sequencing identifying isolates from five samples as *C. jejuni*, eight samples as *Acinetobacter* spp., one as *Micrococcus luteus*, and one as *Escherichia coli*, resulting in 1.9% prevalence on retail chicken breast. Isolate from one sample could not be resuscitated for whole genome sequencing. The *Campylobacter* concentrations on five enrichment-positive chicken breast samples appeared to have been below the limit of detection of direct plating (60 CFU per 325 g sample) and viability qPCR (3,200 – 5,000 cells per 325 g sample) methods.

In the second project, we evaluated the antimicrobial activity of peroxyacetic acid (PAA), an antimicrobial commonly used in poultry processing to control foodborne pathogens post-slaughter. PAA acts as an antimicrobial by inducing oxidative stress, which can inactivate *Campylobacter* but may also induce a VBNC state. To assess to what extent PAA inactivates

Campylobacter on chicken meat, we treated *Campylobacter*-inoculated chicken breasts with 500 ppm PAA for 10 seconds via spray or immersion and quantified culturable cells via direct plating and viable cells via viability qPCR. Immediately after the 10-second PAA treatment, immersion reduced the viable *Campylobacter* population by in 0.25 ± 0.16 $\log_{10}$ ($p = 0.99$) and the culturable population by 0.81 ± 0.10 $\log_{10}$ ($p < 0.001$), while spray reduced the viable population by 0.19 ± 0.16 $\log_{10}$ ($p = 1.00$) and the culturable population 0.51 ± 0.10 $\log_{10}$ ($p = 0.11$). This demonstrated that PAA induced 0.56 ± 0.18 $\log_{10}$ CFU/mL (immersion) and 0.32 ± 0.19 $\log_{10}$ CFU/mL (spray) into a VBNC state immediately after treatment. Post-treatment, the samples were stored at 4°C and were sampled after one hour and 24 hours. PAA did not exert significant additional antimicrobial effects during post-treatment storage. Exposure to aerobic and cold stress had a stronger impact on viability than PAA itself, as samples treated with PAA by spraying had 0.37 ± 0.23 log ($p = 0.98$) lower and 0.03 ± 0.24 log ($p = 1.00$) lower *Campylobacter* counts 1-hour and 24-hours post-treatment, while the reduction was 0.07 ± 0.28 log and 0.08 ± 0.22 ($p = 1.00$) for PAA-immersed samples. Culture-based quantification overestimated PAA's antimicrobial activity by 0.56 ± 0.18 $\log_{10}$, 1.01 ± 0.32 $\log_{10}$ and 0.86 ± 0.37 $\log_{10}$ immediately after, one hour, and 24 hours post-immersion treatment, while for spray, it overestimated antimicrobial activity by 0.32 ± 0.19 $\log_{10}$, 0.43 ± 0.28 $\log_{10}$, and 0.72 ± 0.40 $\log_{10}$, respectively. These findings suggest that previous studies using culture-based methods may have overestimated PAA's antimicrobial efficacy due to VBNC.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1

Literature Review

1.1 *Campylobacter*

Campylobacter spp. are Gram-negative, microaerophilic, and thermophilic bacteria, which makes them well suited to reside in the gastrointestinal tract of poultry, where they find their natural niche. They have a polar flagellum on one or both ends of the cell, allowing them to move rapidly in thick mucosal lining to infect hosts. The *Campylobacter* genus is large and diverse, consisting of 49 species (Costa & Iraola, 2019; Sneath et al., 1980). *C. jejuni* is the most significant species within this genus, as it is responsible for approximately 90% of human campylobacteriosis cases. Other species, including *C. coli* and *C. fetus*, also cause illness but at a substantially lower rates (CDC, 2024; Schielke et al., 2014). The term "*Campylobacter*" comes from the Greek words "kampylos" and "baktron", meaning "curved rod" due to characteristic spiral morphology of the viable *Campylobacter*. However, when exposed to stressors, this morphology can change to a coccoid shape.

1.1.1 Growth Requirements

C. jejuni is thermophilic, thriving between 37 - 42°C, with optimal growth at 42°C, the body temperature of poultry. Its growth declines significantly below 30°C (Jackson et al., 2009). However, at refrigeration temperatures, it remains culturable for longer periods than at 25 - 30°C (Jackson et al., 2009). *Campylobacter* is a microaerophilic pathogen that grows best in an atmosphere with reduced oxygen (2 - 10%) and an elevated carbon dioxide concentration (1 - 10%) (Kaakoush et al., 2007). In a study where *C. jejuni* was cultured in brain heart infusion

broth in aerobic and microaerobic conditions for 18 h, growth varied by initial cell density. At cell densities above 10^7 CFU/ml, it grew better aerobically; at cell densities lower than 10^5 CFU/ml it required microaerobic conditions, and at 10^5 - 10^6 CFU/ml, it grew similar under both conditions (Kaakoush et al., 2007*). Campylobacter* can undergo anaerobic respiration using final electron acceptors including fumarate, nitrate, and nitrite (Sellars et al., 2002), which helps it thrive in mostly anaerobic environments like the chicken gut. However, it cannot grow under entirely anaerobic conditions (Sellars et al., 2002; Véron et al., 1981), and settles instead in the mucus layer of the gastrointestinal tract where it can scavenge more oxygen than it could further down in the lumen.

The mucus layer is an optimal place for *Campylobacter* to grow due to an abundance of nutrients. While many other gut pathogens feed off glucose, *Campylobacter* cannot grow on glucose and other mono- and disaccharides, with only some strains being able to break down fucose (Burnham & Hendrixson, 2018; Hofreuter, 2014). Instead, it catabolizes amino acids, peptides, and organic acids, which are abundant in the gut, with serine, aspartate, glutamate and proline being preferentially used in that order (Guccione et al., 2008). Many of these nutrients serve as chemoattractants to identify optimal niches for *Campylobacter* to settle in (Hofreuter, 2014). However, *Campylobacter* metabolism is strain dependent due to a variable presence of metabolic genes and different chemoreceptors interacting differently with various nutrients (Hofreuter, 2014). Despite being a fastidious bacterium*, Campylobacter* demonstrates remarkable adaptability, withstanding many environmental conditions, like pH changes, oxidative stress and low nutrient availability (Chaveerach et al., 2003; Okada et al., 2023; Sanders et al., 2008).

1.1.2 Detection of Viable Cells

In the context of food safety, a food product is considered safe if it is free of viable foodborne pathogens. However, defining viability and death in bacteria is difficult, given the complexity of the concept of death at the cellular level. Hence, four main categories of bacterial viability status have been proposed: i. reproductive (i.e., growing) cells, ii. metabolically active but not actively growing cells, iii. intact cells without compromised membrane, and iv. permeabilized cells with a compromised membrane (i.e., dead cells) (Nebe-von-Caron et al., 2000). Nebe-von-Caron et al. (2000) include energy dependent (biosynthesis, pump activity, membrane potential) and energy independent (enzyme activity) mechanisms within metabolic activity (Nebe-von-Caron et al., 2000). Depending on the application, the first three states (reproductive, metabolically active, and intact cells) have been used to define "viable" bacteria. For instance, a viable cell can be detected by direct plating methods only if it *reproduces* and grows into a visible colony. Further, a viable cell can be detected by direct viable count only if it is *metabolically active*, while a cell must be *intact* to be detected as a viable cell using a viability quantitative polymerase chain reaction (qPCR). In the latter, permeabilized cells with compromised membranes are detected as non-viable cells. Stingl et al. (2015) and Nebe-von-Caron et al., (2000) suggest that the most accurate way to define bacterial death in the context of controlling foodborne pathogens is by monitoring membrane permeability, which is based on the premise that cells cannot recover from a severely compromised membrane (Nebe-von-Caron et al., 2000; Stingl et al., 2015). For example, a treatment with 5% hydrogen peroxide results in *Campylobacter* death, as determined by complete loss of culturability and membrane integrity (Krüger et al., 2014). Therefore, the term "intact and potentially infectious units (IPIU)" was suggested for viable cells with an intact membrane, since they can regain growth when

reintroduced into a favorable environment. This is also the definition of viability that was adopted in this thesis.

1.1.3 Viable but Nonculturable State

Many bacteria temporarily enter dormancy states, such as viable but nonculturable (VBNC), spore, or persister state, as a survival strategy when in unfavorable environments (McDonald et al., 2024). In various dormancy states, cells reduce or completely halt their metabolism, stop growing, and increase their resistance to stress. Cells in all dormancy states maintain their viability and resuscitate once conditions are suitable for their growth (McDonald et al., 2024). Sensors in the inner membrane detect sugars and amino acids which can trigger spore germination (Christie & Setlow, 2020; Zhou et al., 2019). Spores are made up of multiple protective layers, including a cortex and spore coat, which shield the cell from heat, chemicals and desiccation. Their low metabolic state and the large amount of dipicolinic acid stabilize DNA and proteins against damage (Wen et al., 2022). The spore is a highly genetically programmed state that some bacteria use to resist stressful conditions. However, there are also less elaborate systems that provide shorter term resistance to stressful conditions that can be used by bacteria. Two examples of these are the viable but nonculturable (VBNC) and persister cells.

First identified in *Campylobacter* in 1986, VBNC cells are defined as having intact membranes, low to no levels of metabolic activity, and exhibiting gene expression (Patrone et al., 2013; Rollins & Colwell, 1986). In this state, some *Campylobacter* cells transition from their notable helical and spiral morphology into a coccoid shape (Ikeda & Karlyshev, 2012). This coccoid shape has a thickened cell wall, which increases resistance to stressors and prolongs survival (Frirdich et al., 2019). However, VBNC cells are not able to divide on nutrient media and are not immediately able to regain this ability. They can resuscitate through *in vivo* passage, as

was shown for *Campylobacter* through the mouse and chick gut (Baffone et al., 2006; Cappelier et al., 1999), or by the reintroduction of favorable environments like a microaerobic or nutrient rich ones (Bovill & Mackey, 1997). The VBNC state can be induced by stressors including low nutrient environments, temperature, pH, and oxidative stress, but VBNC cells can also exist before exposure to stress (Ayrapetyan et al., 2015). On the other hand, persister cells are a slow-growing or nongrowing subpopulation that can survive antibiotic treatment without acquiring resistance (Ayrapetyan et al., 2015). They are genetically identical to the non-persister majority but have a drug-tolerant phenotype (Balaban et al., 2004). Like VBNC cells, they can exist randomly in cultures but are also induced by stressors including low-nutrient, temperature, pH, oxidative and antibiotic stress (Ovsepian et al., 2020; Wu et al., 2012).

Ayrapetyan et al. (2015) hypothesized that due to the mechanic similarity of these states, they exist at different points on a 'dormancy continuum', with VBNC cells entering a deeper state of hibernation (as evidenced by a more drastic ATP depletion) and requiring more time to recover after stress has been removed compared to persister cells (Ayrapetyan et al., 2015). In addition, persister cells show a higher level of metabolic activity, while cell division shuts down completely for VBNC cells. Detection methods differ as well, with VBNC cells detected using viability stains (see 1.3.2), persister cells identified through antibiotic tolerance assays, and spores distinguished by spore-specific stains (e.g., malachite green) (Harms et al., 2017; Kozuka & Tochikubo, 1991). One proposed distinction is that persister cells can resume growth on solid media after antibiotics treatment, while VBNC cells require up to 24 h of resuscitation before regaining culturability. In my thesis, I do not resuscitate any cells in a dormant state. Since we do not resuscitate dormant cells, we cannot experimentally distinguish persisters from VBNC cells. However, we will use the term VBNC going forward to characterize cells that did not regain culturability within 48 h of incubation on solid media.

Food safety monitoring of *Campylobacter* is typically conducted using standard microbiological methods that require growth in selective media (FDA, 2021; USDA FSIS, 2024). In this sense, many standard protocols used by food companies and food safety authorities rely on the "reproductive" cell definition of viability, potentially leading to false negative detection of VBNC *Campylobacter* in food systems. Reproductive cells of pathogens have the highest infectivity and exhibit full virulence, while metabolically active and intact cells are typically in survival mode expending little energy on virulence. Therefore, it is sensible to prioritize detecting growing cells in food products. However, VBNC *Campylobacter* also presents a food safety risk, given that it can regain its culturability and infectious potential, as demonstrated with resuscitation in embryonated eggs (Cappelier et al., 1999) and mouse intestine (Baffone et al., 2006). We can therefore hypothesize that upon entry into the human intestinal tract, VBNC cells may resume growth and cause campylobacteriosis.

During poultry processing, *Campylobacter* is exposed to multiple stressors, including oxidative stress from exposure to an aerobic environment, heat stress from scalding, cold stress from chilling, acid stress from antimicrobial sprays or dips, and osmotic stress from sodium chloride (NaCl) used as a preservative (Pokhrel et al., 2022). While these hurdles aim to kill pathogens, sublethal stress can induce a VBNC state, facilitating *Campylobacter* survival.

Oxidative Stress

C. jejuni can respire through both anaerobic and aerobic pathways, allowing it to adapt to environments with different oxygen levels. Rollins & Colwell first reported that *Campylobacter* can withstand prolonged aerobic exposure by entering a VBNC state (Rollins & Colwell, 1986). They also found that oxidative stress induces a morphological shift from a spiral to a coccoid form, which reverses upon removal of stress. This morphological transition has been further

supported by more recent studies (Chaisowwong et al., 2012; Klančnik et al., 2009; Oh et al., 2015). Exposure to aerobic conditions at 42°C increased the VBNC population, but antioxidant treatment reduced this effect, reinforcing the link between oxidative stress and VBNC induction (Oh et al., 2015). Similarly, at 4°C, the VBNC state developed more rapidly under aerobic conditions than in microaerobic environment (Yagi et al., 2022). Zhang & Lu (2023) further confirmed that all tested *C. jejuni* isolates entered a VBNC state within 24 hours when exposed to aerobic conditions at 37°C. These findings suggest that when *Campylobacter*-contaminated chicken gut contents are exposed to an aerobic atmosphere, a subpopulation of cells likely enters the VBNC state, evading detection by traditional culture-based methods.

Cold Temperature Stress

As a thermophilic pathogen, *C. jejuni* thrives between 37°C and 42°C, with its optimal growth occurring at the higher end of this range. Prolonged culture at 37°C largely led to cell death, with the remaining viable cells transitioning into VBNC (Yagi et al., 2022). Below 30°C, the growth rate of *C. jejuni* declines rapidly (Jackson et al., 2009), and after 48 h at 30°C, *C. jejuni* became non-culturable. However, at lower temperatures, the transition to VBNC occurred more gradually, taking up to 21 days at 4°C. *C. jejuni* exhibits strain-dependent prolonged culturability at 4°C, ranging from 12 days to 4 months, before eventually entering a VBNC state, where viability can be maintained for up to 7 months (Baffone et al., 2006a; Rollins & Colwell, 1986; Zhang & Lu, 2023). These findings suggest that cold temperatures not only extend survival but also promote VBNC induction over time.

As chicken products progress through slaughter and packaging, they are typically stored at 4°C for prolonged periods. While this prevents microbial growth, it likely inadvertently

induces *Campylobacter* into a VBNC state, potentially allowing persistence despite standard food safety controls.

Acid Stress

A hurdle approach combining multiple strategies to inhibit microbial growth is commonly used in meat processing to enhance antimicrobial effectiveness. One such hurdle is the application of antimicrobials like chlorine, which inactivates microorganisms by damaging nucleic acids, altering the membrane permeability, and acidifying the cell (see 1.2.3). *Campylobacter* can survive at pH levels above pH 4.5, with optimal growth occurring between pH 6.5 and 7.5 (Jackson et al., 2009). In acidic environments, *C. jejuni* enters a VBNC state, but can be resuscitated through passage in fertilized eggs (Chaveerach et al., 2003). Exposure to chlorine (25 ppm) also induced a VBNC state in 1 - 10% of the *C. jejuni* population that was not completely inactivated by the treatment (Zhang & Lu, 2023). This suggests that while chlorine effectively reduces viable *Campylobacter,* it may also contribute to the survival of a subpopulation in VBNC state. Beyond chlorine, other antimicrobials used in poultry processing, such as peroxyacetic acid, have not been thoroughly investigated for their effect on the viability of *C. jejuni,* as most studies use culture-based methods to determine their efficacy, which do not account for nonculturable cells (see Chapter 2).

Low Osmolarity Stress

Like acid treatment, sodium chloride treatment is commonly used post-processing to inhibit microbial growth on meat products. A slight decrease in osmotic pressure within bacterial cells can inhibit essential processes and induce a VBNC state (Jackson et al., 2009). When treated

with 4.5% NaCl, *C. jejuni* culturability decreased by 5.5 $\log_{10}$ within 12 h at 42°C, whereas at 4°C, culturability decreased by approximately 3 $\log_{10}$ over 14 days, suggesting that *C. jejuni* maintains culturability longer at lower temperatures when osmotically stressed (Doyle & Roman, 1982). Similarly, prolonged incubation in low osmolality (distilled water) at 4°C largely led to cell death, and culturability was completely lost by day 20, with around 30% of cells induced into a VBNC state (Yagi et al., 2022).

While individual stressors affecting *Campylobacter* viability have been studied, *Campylobacter* on chicken meat is usually exposed to simultaneous stress, such as oxidative and cold stress. Yagi et al. (2022) determined the effect of temperature (4°C vs. 37°C), nutrient availability (low nutrient vs. high nutrient), oxygen levels (aerobic vs. anaerobic vs. microaerobic), and osmotic pressure on VBNC induction, both individually and in combination. The fastest VBNC induction occurred within 25 days in a nutrient rich environment at 4°C under aerobic conditions (Yagi et al., 2022). These stressors are analogous to the environment *Campylobacter* encounters on packaged chicken meat in refrigerated storage. The rate of VBNC induction was strain dependent; hence more research is required to identify factors leading to strain-specific differences, such as aerotolerance.

Expression of Virulence Factors in VBNC

A large part of *Campylobacter*'s virulence is attributed to its ability to adhere and invade host cells in the intestine. Since entry into a VBNC state greatly reduces the metabolic activity, it is hypothesized that virulence factors expression is also diminished. Indeed, the transcription of virulence genes was shown to be down-regulated in *C. jejuni* in a VBNC state (Chaisowwong et al., 2012). Specifically, transcription of genes associated with host invasion was reduced in some strains when in a VBNC state, while those involved in iron transport and oxidative stress

protection remained actively transcribed (Santos et al., 2023). Despite this reduction in virulence gene transcription, *C. jejuni* in a VBNC state still expressed the CadF protein, a key adhesin that mediates bacteria-host interactions, and maintained its ability to adhere to intestinal epithelial cells *in vitro*, although with 26.9 - 40% reduced efficiency (Patrone et al., 2013). These findings indicate that *C. jejuni* may be able to persist in a host even in a dormant state until conditions favor resuscitation and infection.

Outside of the host, incubation at 4°C led to complete loss of culturability after 38 days, yet VBNC cells retained their ability to invade human epithelial cells *in vitro* (Chaisowwong et al., 2012). This suggests that while transcription of invasion-related genes is reduced, VBNC *C. jejuni* can still adhere to and invade host cells, posing a potential risk for transmission and infection even when non-culturable.

1.2 *Campylobacter* and Food Safety

The Center for Disease Control (CDC) estimated that 1.5 million people in the United States become ill from a *Campylobacter* infection annually (CDC, 2025), and *Campylobacter* has been the leading cause of reported bacterial foodborne illness in humans in the United States since 2014 and in Europe since 2005 (EFSA, 2024; Shah, 2024). This leads to a major economic burden. In 1997, a report estimated that between $1.3 - $6.2 billion per year are lost to campylobacteriosis in the US, and with cases rising in the last decade and set to continue rising due to climate change (Kuhn et al., 2020), this number has likely already significantly increased (Buzby & Roberts, 1997; Hoffmann et al., 2012; Kaakoush et al., 2015). This places a financial and public health burden on the public, since *Campylobacter* can be controlled effectively at the level of production yet is not. Despite technological advances and economic prosperity, one in six individuals contract foodborne illnesses annually in the United States, and approximately 3,000

die due to preventable foodborne illness(CDC, 2018). This poses food scientists with a challenge to accelerate progress towards improving the safety of food to protect consumers.

1.2.1 *Campylobacter* Prevalence and Transmission

Campylobacter is a zoonotic pathogen, with poultry and cattle, among others, serving as its commensal hosts. Chickens are the main source of human campylobacteriosis, accounting for 50 - 90% of cases (Rosner et al., 2017), while cattle and pigs are less frequent sources of human infections (Cody et al., 2019). Chickens and chicken meat are important vectors for transmission of *Campylobacter*, with chicken meat consumption being a particularly high-risk factor for infection. Other risk factors include dining out, handling raw meat alongside uncooked food, and contact with poultry, which places farm and slaughterhouse workers at higher risk (Igwaran & Okoh, 2019; Rosner et al., 2017). Most (97%) campylobacteriosis cases are sporadic (Ebel et al., 2016; Rosner et al., 2017), meaning they are not associated with an outbreak. These sporadic cases can result from consumption of undercooked chicken (cooked below 74°C/165°F as recommended by the USDA FSIS) or due to cross-contamination from other foods in the kitchen.

Campylobacter colonizes the broiler gut and is shed in the feces, contaminating the environment. *C. jejuni* can survive in broiler feces for up to six days, which makes the fecal-oral route a common transmission pathway (Sadek et al., 2023). The close quarters on broiler farms facilitate the rapid spread of *Campylobacter*, and, it has been reported that once introduced, *Campylobacter* can colonize the whole flock by the time of slaughter (Plishka et al., 2022). Positive flocks can also contaminate flocks that are negative for *Campylobacter* during transport and slaughter. The dose needed to colonize broilers is quite low, but once in the gut, *Campylobacter* can grow rapidly, with fecal droppings reaching concentrations as high as 6 - 10 $\log_{10}$ CFU/g (Battersby et al., 2016). Fecal runoff can then contaminate surface water, introducing

Campylobacter into the environment, leading to colonization of livestock and wildlife and persistence along the food chain in products like raw milk and meat. Consumption of contaminated water and food can also spread it to humans (Igwaran & Okoh, 2019).

The fact that chicken meat is the most consumed meat in the US, with more than 8 billion broilers slaughtered annually, and consumption projected to continue to increase into to the 2030s (USDA, 2024), highlights the significance of controlling *Campylobacter* in poultry. The projected increase in consumption is expected to increase consumers' exposure to *Campylobacter* unless more effective control strategies are implemented.

The prevalence of *Campylobacter* on chicken meat varies widely. In the US, studies reporting the prevalence on retail cuts of chicken range between 10 - 84% (Cui et al., 2005; Mollenkopf et al., 2014; Noormohamed & Fakhr, 2014; Price et al., 2007; Zhao et al., 2001), while retail carcasses have shown prevalence of up to 96% (Nannapaneni et al., 2005) and farmers market chickens up to 90% (Scheinberg et al., 2013).

Once ingested, *Campylobacter* passes through host's digestive tract and settles in the intestine where it colonizes the mucus layer of the ileum and colon. Here, if present in high enough numbers, it disrupts the normal activity of the epithelial cells by cell invasion and the production of toxins, or by the immune system's inflammatory response to infection (Ketley, 1997). This leads to human campylobacteriosis, with symptoms including diarrhea, cramping, abdominal pain, and fever (El-Saadony et al., 2023). More serious complications can also arise, such as pancreatitis, reactive arthritis and Guillain-Barré syndrome, which can be fatal (Kaakoush et al., 2015).

1.2.2 Control Strategies in the Poultry Industry

Antimicrobial interventions are employed along the poultry processing chain to meet the United States Department of Agriculture (USDA) standards. These interventions use hot water, cooling, and chemical antimicrobial applications to reduce the microbial load of the final product (Chowdhury et al., 2023).

Campylobacter's main introductory route to the food chain is through colonization of poultry at farms, which requires only a low dose to take hold in their gut, with estimates ranging between 35 – 10,000 CFU (Line et al., 2008). As *Campylobacter* grows, this can quickly lead to high levels in the gut, which remain stable as the chickens grow (Stern, 2008) and can contaminate meat during slaughter, ultimately leading to human exposure through food (Lu et al., 2021). The most common source of *Campylobacter* introduction into poultry farms is through horizontal environmental transmission, meaning from other livestock reared near the poultry farms and from hatcheries (Bull et al., 2006; Lu et al., 2021). This encompasses multiple sources, including contaminated water and feed, and the movement of farm workers. Therefore, there are preharvest practices that can be implemented to reduce the risk of broiler colonization and meat contamination.

Enhanced biosecurity on farms is considered the best method to prevent flock colonization with *Campylobacter* (Vandeplas et al., 2008). Biosecurity encompasses measures to prevent entry of the pathogen into the farm all the way to transport to the slaughterhouse. Biosecurity measures employed to prevent the spread of *Campylobacter* at the farm level include footbaths, frequent shoe changes, and improved cleaning and sanitation practices around nourishment areas for the chickens (Lu et al., 2021). However, while these measures can drastically reduce the prevalence of *Campylobacter*, they are costly to farmers (Siekkinen et al.,

2012). For biosecurity measures to be broadly adopted, they must be low-cost, easy to implement and proven effective.

In addition to biosecurity measures, broiler colonization with *Campylobacter* can be controlled by adding chlorine or organic acids into birds' drinking water (Jansen et al., 2014). Although this alone does not eliminate the risk of colonization, it can be used in combination with other measures, such as feed supplements, to improve the broiler gut health and reduce the fecal shedding of *Campylobacter*. Probiotics, prebiotics, organic acids, bacteriophages, and bacteriocins have been used as feed or water additives to control *Campylobacter* by promoting the growth of beneficial bacteria (Fonseca et al., 2024; Lu et al., 2021). While vaccination *in ovo* or of live chicks is another option, no vaccine currently exists that is effective for *Campylobacter* spp. due to its genetic diversity, and this is not yet seen as a fully effective strategy (Hermans et al., 2011; Pumtang-on et al., 2021).

Next in the processing chain is transport to the slaughterhouse, where *Campylobacter* transmission can occur between contaminated and uncontaminated flocks. During transport, flocks are stressed, leading to defecation and the spread of pathogens. A study from the UK reported that 23.5% of crates used for transportation of birds were contaminated (Allen et al., 2008), and that *Campylobacter* can survive for prolonged periods on these crates. Therefore, cleaning and sanitizing transportation equipment is crucial for preventing the spread of pathogens. However, they are difficult to clean and even those cleaned and disinfected are not completely free of *Campylobacter* (Hansson et al., 2005).

During slaughter, *Campylobacter* can be transmitted from broiler intestine to meat. Therefore, several slaughterhouse interventions aim to reduce microbial load on meat. Scalding is the first step in poultry meat processing where *Campylobacter* is significantly reduced, by approximately 2 logs (Berrang & Dickens, 2000). After scalding, feather plucking puts pressure on the carcasses, which leads to defecation and further contamination, and has been shown to

increase *Campylobacter* counts (Rasschaert et al., 2020). Subsequently, during evisceration, the intestine can be cut open, spilling contents and further increasing *Campylobacter* by 0.25 to 1.5 $\log_{10}$ (Rasschaert et al., 2020). Evisceration is considered as the highest risk step for *Campylobacter* contamination (Althaus et al., 2017). Once eviscerated, carcasses typically undergo chilling with cold water (1 - 2°C) for 30 - 60 min. This step results in the greatest reduction of *Campylobacter*, while the extent of reduction depends on the chilling duration (Rosenquist et al., 2006). There is typically a post-chill step following primary chilling, where the product is immersed or sprayed with a high concentration of an antimicrobial for a short time to further reduce microbial load and control pathogens.

1.2.3 Antimicrobial Interventions Used on Chicken Meat

Antimicrobials can be introduced during or after the meat chilling step. These antimicrobials must have documented efficacy (with a standard 1 $\log_{10}$ reduction), defined parameters for application concentration and contact time, and have no detrimental effects on product quality. Lastly, antimicrobial products must be competitively priced (Cano et al., 2021).

One of the most used antimicrobials in poultry processing is chlorine. However, high organic loads, high temperatures, prolonged application times, and pH fluctuations reduce its efficacy. It was has been reported that levels above the regulatory limit were required to inactivate pathogens attached to poultry skin (Bauermeister, Bowers, Townsend, & Mckee, 2008). Alternatives to chlorine include organic acids (e.g., acetic, formic, citric, lactic, propionic), acidified sodium chlorite, sodium hypochlorite and hydrogen peroxide (Cano et al., 2021). Some of these antimicrobials are more effective in post-chill immersion and spray applications, while others are more effective at lower concentrations in the chiller (Cano et al., 2021).

Recently, poultry processing establishments have employed commercial peroxyacetic acid (PAA) products, which are a mixture of peroxyacetic acid (PAA), hydrogen peroxide (HP), acetic acid, and water. Some PAA products also include sulfuric acid, 1- Hydroxyethylidene1,1-diphosphonic acid (HEDP), or dipicolinic acid (DPA) as stabilizers. The USDA has approved PAA for use on poultry carcasses and parts at a maximum concentration of 2,000 ppm (USDA FSIS, 2024). Chemical companies supply PAA specifically for poultry decontamination and the recommended application concentrations range depending on the intended application. PAA-based products usually include a mixture of PAA, acetic acid and hydrogen peroxide (Ebel et al., 2019). PAA has lower environmental impact than chlorine, as it decomposes into acetic acid and oxygen or hydrogen peroxide and does not result in toxic byproducts when exposed to high levels of organic matter. PAA reduces microbial load by oxidizing sulfhydryl and sulfur bonds in the cells. This permeates the cell wall and interferes with protein synthesis, causing the loss of membrane integrity (Oyarzabal et al., 2005). Its oxidizing and acidic properties make it effective against a broad range of microorganisms, from Gram-negative and Gram-positive organisms, to viruses, spores, and fungi (Kitis, 2004). Multiple studies have proven its superior efficacy to chlorine at reducing microbial load, as determined using culture-based methods. For example, chicken carcass immersion treatment with 200 ppm PAA was shown to be more effective compared to 50 ppm chlorine treatment at three different temperatures (4°C for 20 min, 15°C for min, and 22°C for 6 s). A one hour treatment with 30 ppm chlorine or 25 or 100 ppm PAA using immersion in a poultry chiller did not significantly reduce the *Campylobacter* populations, while treatment with or PAA at 200 ppm did, by 1.5 $\log_{10}$ CFU per sample (Bauermeister, Bowers, Townsend, & McKee, 2008). Chlorine is likely ineffective in chillers due to the high level of organic matter and long exposure time, while higher concentrations of PAA were not affected by organic matter.

Compared to antimicrobial treatments of whole carcasses at the chilling step, post-chill treatments are typically applied onto chicken parts and are shorter in time. Due to shorter exposure, higher concentrations of antimicrobials are applied to achieve similar pathogen reductions. PAA treatment is more effective than chlorine at decontaminating native and inoculated *Campylobacter* in prechill, chill, and postchill-like conditions at concentrations of 200 ppm or higher. Poultry carcasses immersed in PAA (400 and 1000 ppm) for 20 s reduced the population of *Campylobacter* by approximately 2 $\log_{10}$ CFU/mL at both concentrations, while chlorine treatment (40 ppm) resulted in less than 1 $\log_{10}$ reduction (Nagel et al., 2013). PAA (700 and 1000 ppm) was also proven more effective than chlorine (30 ppm) when applied by immersion of chicken drumsticks for 10, 20, and 30 s. Specifically, it significantly reduced the concentration of *Campylobacter* by more than 1.5 $\log_{10}$ CFU/mL at each application time and concentration, while chlorine reductions were similar to controls immersed in water (Zhang et al., 2019). Similarly, Chen et al. (2014) found that when skin-on chicken breast and thighs were immersed for 23 s at the same concentrations and then ground up, chlorine treatment did not differ from the water control. At the same time, PAA provided a reduction of around 1.5 $\log_{10}$ (Chen et al., 2014). Generally, longer exposure times result in greater reductions in *Campylobacter* (Kumar et al., 2020, Smith et al., 2015). However, while some reported similar efficacy of spray and immersion application, others found greater antimicrobial efficacy when PAA was applied by immersion (Kumar et al., 2020, Smith et al., 2015).

Individual changes in pH (8.2 - 11), time (10 s or 60 min) and PAA concentration (50 and 500 ppm) did not significantly impact the efficacy of PAA treatment on chicken wings inoculated with *C. coli*, and PAA treatment resulted in a reduction of *C. coli* populations by 2 – 2.5 $\log_{10}$ CFU/mL (Kataria et al., 2020).

To enumerate culturable *Campylobacter*, these studies rinsed chicken parts in a neutralizing solution after treatment, which halts the antimicrobial action of PAA, and plated

rinsates on standard media. However, during normal processing, PAA is not rinsed off chicken parts after spray or immersion but rather dissipates as the product moves through final processing and packaging steps. Walsh et. al (2018) determined that PAA levels on poultry dip below the limit of detection at 27.9 minutes, resulting in no long-term residues on the product (Walsh et al., 2018). Therefore, studies determining PAA efficacy should incorporate prolonged rest times to determine whether additional reductions are achieved within the post-treatment time (see Chapter 3).

1.3 *Campylobacter* Detection Methods

The USDA Food Safety and Inspection Service (FSIS) regulates *Campylobacter* on chicken by setting performance standards for poultry processing plants and regularly tests for *Campylobacter* on carcasses and cuts to determine whether they meet the standards in place. The current standards for chicken carcasses and chicken parts allow for 8 of 51 (15.7%) and 4 of 52 (7.7%) samples to test positive for *Campylobacter* using their isolation and identification methods, respectively. The evaluation is done over a moving window period of one year (52 weeks), and the sampling plan depends on the size and production volume (Cano et al., 2021; USDA, 2015).

1.3.1 Culture-based Detection Methods

The current guidelines for isolation and identification of *Campylobacter* from poultry sources in the US are the USDA FSIS Microbiology Laboratory Guidelines (MLG) 41.09, and the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) Chapter 7 (FDA, 2021; USDA FSIS, 2024). NOTE: FSIS intends to update the *Campylobacter* performance

standards; therefore, although FSIS continues to test product for *Campylobacter*, it does not assess whether establishments meet the performance standards (FSIS, 2025). In addition to these methods, the ISO 10272-1:2017 protocol outlines the method for isolation and enumeration of *Campylobacter* from poultry (FSIS, 2025). These are all culture-based methods that use conditions and media selective for *Campylobacter* to support its growth and recovery and employ antibiotics to inhibit the growth of non-targeted species. Once putative *Campylobacter* colonies are isolated, they are confirmed using biochemical tests or molecular methods for species identification. Compared to the FDA BAM and USDA FSIS MLG protocols, the ISO standard includes a quantitative method (detection method C), in addition to the enrichment method, which allows for the determination of *Campylobacter* concentration in a tested food product.

1.3.2 Molecular Detection Methods

Molecular detection methods provide a more comprehensive assessment of *Campylobacter* prevalence compared to traditional culture-based methods outlined above, especially when detecting nonculturable cells. Culture-based methods rely on bacterial growth and can therefore underestimate the true bacterial load when nonculturable cells are present (Magajna & Schraft, 2015). Molecular methods can identify viable and nonculturable cells by determining their membrane integrity, which provides a more accurate depiction of the infectious microbial load (Josefsen et al., 2010).

For fresh *Campylobacter* cultures, Magajna and Schraft detected similar counts of viable and culturable cells. However, culturable cell counts decreased substantially during incubation for up to 60 days, while viable cells remained relatively stable (Magajna & Schraft, 2015). This discrepancy highlights a critical limitation of culture-based methods. While such methods are effective for detection of *Campylobacter* in fresh samples such as fecal droppings or carcasses

sampled immediately post-slaughter (Josefsen et al., 2010), they often underestimate the number of sub-lethally injured cells introduced by food processing stressors such as oxidative and cold stress (Jasson et al., 2007; Ritz et al., 2007).

These stressors can render *Campylobacter* cells VBNC, making them challenging to resuscitate and detect using growth-dependent methods, since they often cannot divide in conventional culture media. As a result, traditional, growth-dependent, culture-based methods fail to capture the true potential of the foodborne *Campylobacter* population. To address this, alternative approaches have been developed to detect all intact and putatively infectious units (IPIU) of *Campylobacter* in food matrices.

Much research has focused on molecular based methods like quantitative polymerase-chain-reaction (qPCR) as a promising alternative to culture-based methods. However, qPCR alone amplifies DNA from both live and dead cells, as it does not differentiate between membrane-intact and membrane-compromised cells. To overcome this limitation, DNA-intercalating dyes, such as propidium monoazide (PMA) can be used. These dyes selectively bind DNA in membrane-compromised cells, forming covalent bonds upon light-activation, which prevents DNA amplification during PCR (Nocker et al., 2006). This theoretically eliminates the signal from dead cells, leaving only DNA from viable cells to be amplified. For this approach to be effective, the dye must be passively excluded from viable cells to avoid underestimating those with low or no metabolic activity.

Propidium monoazide (PMA) and ethidium monoazide (EMA) are widely used in membrane permeability assays. Both dyes enter membrane-compromised cells and bind to DNA after light activation, preventing amplification. However, EMA has significant drawbacks for viability studies. Krüger et al. (2014) and Jernaes and Steen (1994) demonstrated that the EMA exclusion from intact cells depends on active efflux, with more metabolically active cells showing higher efflux activity (Jernaes & Steen, 1994; Krüger et al., 2014). When they added an efflux

inhibitor, EtBr (an EMA analogue) permeability increased in intact cells regardless of their metabolic state. This limitation renders EMA unsuitable for differentiating between viable and dead cells in samples containing VBNC cells, as it could misclassify VBNC cells as dead. They also showed that EMA permeability into *Campylobacter* cells was highly dependent on the cell suspension medium, the incubation time and the metabolic state of the cells. At the same time, PMA was passively excluded from viable *Campylobacter*, regardless of these factors (Krüger et al., 2014; Nocker et al., 2006). While Krüger et al. (2014) successfully excluded PMA from viable *Campylobacter* at all incubation times and temperatures tested, dead cell signal reduction was optimal at 30°C for 15 min with a concentration of 20 µM (Krüger et al., 2014). Therefore, to identify IPIU cells from a food matrix, PMA is an appropriate dye.

Methods involving PMA treatment followed by a qPCR have been effective at quantifying viable *Campylobacter* cells in various suspension matrices, including chicken rinsates (Josefsen et al., 2010a; Lv et al., 2020a; Okada et al., 2023). However, PMA concentrations and outcomes vary among studies. For instance, Josefsen et al. (2010) inoculated chicken rinses with heat inactivated *C. jejuni* (2 – 6 $\log_{10}$), confirmed the lack of viable cells through culture-based quantification, and performed PMA-qPCR to determine whether the dead cell signal was absent. They showed that 10 µg/mL (~ 20 µM) PMA completely inhibited the qPCR signal from dead cells of *C. jejuni* up to 6 $\log_{10}$ CFU/mL (Josefsen et al., 2010). In contrast, Pacholewicz et al. (2013) performed the same assay and reported incomplete inhibition of dead cells at 4.1 $\log_{10}$ CFU/mL in a chicken rinse. Above this concentration the dead cell signal was reduced compared to the control that did not receive PMA treatment, but was not completely absent (Pacholewicz et al., 2013). Lv et al. (2020) observed similar variability, finding complete dead cell signal inhibition at 6 $\log_{10}$ CFU/mL of dead *C. jejuni* cells using 100 µM PMA, but only partial reduction using 20 µM (> 2 $\log_{10}$ CFU/mL reduction) (Lv et al., 2020). For *C. coli*, a concentration of 50 µM PMA did not fully eliminate the dead cell signal at any concentration

tested (2 - 6 $\log_{10}$ CFU/mL), indicating that PMA may be less permeable to *C. coli* (Duarte et al., 2015).

Further innovations, such as two-round PMA treatment and PMAxx (a more reactive PMA derivative), have improved dead cell signal inhibition. Okada et al. (2022) determined that adding the PMA Enhancer developed by Biotium significantly inhibited the dead cell signal by a ΔCt difference of around 9 cycles compared to PMAxx without the enhancer. They found that a single round treatment of treatment with PMAxx at 25 µM did not completely inhibit the dead cell signal in a bacterial suspension at a concentration of 5.7 $\log_{10}$ CFU/mL, while two round-treatment did. Two-round treatment was also effective in a chicken rinse matrix spiked with 4.7 $\log_{10}$ CFU/mL of *C. jejuni,* as it completely inhibited the dead cell signal (Okada et al., 2022). Differences in inhibition of the dead cell signal could be due to the variations in membrane composition of the different strains tested, and the degree of damage caused by the various stress factors, all of which could impact dye permeability (Fittipaldi et al., 2012).

PMA-qPCR methods are consistent with culture-based enumeration when most cells are culturable but recover significantly more when cells have entered a VBNC state. Josefsen (2010) evaluated their PMA-qPCR against a culture-based enumeration method and qPCR using chickens naturally contaminated with *Campylobacter*. They determined that the viability-qPCR correlated well with the culture-based method, with 42 chicken samples testing positive by culture, 45 by viability qPCR, and 48 by qPCR without PMA treatment. Using this method, Pacholewicz (2013) analyzed chicken carcass rinse samples inoculated with live *Campylobacter* and naturally contaminated samples from various processing stages. They found no significant difference between methods for the inoculated samples, likely because these were fresh cultures with few to no dead or VBNC cells. In naturally contaminated rinse samples, they found that the PMA-qPCR recovered significantly more *Campylobacter* than culture-based method (Pacholewicz et al., 2013). Similarly, Duarte (2015) enumerated 26 neck skin samples from

naturally contaminated broiler carcasses using a PMA-qPCR and direct plating on mCCDA (Duarte et al., 2015), and found that the *Campylobacter* concentrations determined by the PMA-qPCR were higher than those determined by culture-based method. The discrepancy between the inoculated and naturally contaminated can be attributed to fewer VBNC cells in the freshly prepared inoculum compared to naturally contaminated chicken samples, where cells have been exposed to oxidative stress for extended time.

All previously mentioned culture-based methods have low detection limits (below 1 $\log_{10}$ CFU/g), while the limit of detection among the viability-qPCR methods varies. The method developed by Josefsen et al. has the limit of quantification (LOQ) of 100 CFU/mL (2 $\log_{10}$ CFU/mL), and the PCR assay was approved for the detection of *Campylobacter* from chicken carcasses and cuts, cloacal swabs, and boot swabs, and with the addition of the PMA treatment is more selective for viable cells. The limit of quantification of another qPCR method is 210 CFU/g broiler meat (~ 1.72 $\log_{10}$ CFU/mL), which is comparable with Josefsen (2010) (Duarte et al., 2015). Lv (2020) developed a PMA-qPCR specific for *C. jejuni*, with an LOD of 3.52 $\log_{10}$ CFU/g for *C. jejuni* VBNC detection on chicken breasts; this LOD is higher than that of the Josefsen method (Lv et al., 2020). While this method successfully detected and quantified VBNC *C. jejuni* cells (Lv et al., 2020), it was developed specifically for *C. jejuni*, and is therefore unsuitable for detection of *C. coli* and *C. lari*.

The need for robust controls in PMA-qPCR (viability qPCR) assays is crucial to confirm efficient PMA crosslinking of dead and viable cells. Stingl (2015) recommended using dead cell samples with known membrane permeability properties to assess PMA cross-linking efficiency and reliably quantify *Campylobacter* (Stingl et al., 2015). By quantifying the number of dead cells that PMA did not permeate, the efficacy of dead cell reduction could be applied to the sample cells. This also eliminates the need for a complete reduction of a dead cell signal, which, except for Josefsen et al. (2010), was not achieved on samples containing over 4 $\log_{10}$ CFU/mL

dead *Campylobacter* (Duarte et al., 2015; Josefsen et al., 2010a; Lv et al., 2020a; Pacholewicz et al., 2013). Additionally, Pacholewicz et al. (2019) introduced an internal sample process control (ISPC) consisting of a known number of dead *C. sputorum* cells to monitor the dead cell signal reduction through PMA treatment and to offset DNA loses during DNA extraction and sample processing (Pacholewicz et al., 2019). The ISPC was added directly to a 1 ml aliquot of a rinse sample, which then underwent PMA staining and qPCR. *C. sputorum* was chosen because it shares key properties (amplicon size, target copy number per chromosome) with thermophilic *Campylobacter*. However, it can be distinguished by the intervening sequence (IVS) in its 16S rRNA gene sequence. It has also not been isolated from poultry and is therefore unlikely to contribute to false-positive results.

Building on these advancements, Stingl et al. (2021) developed and validated a multiplex viability qPCR through an interlaboratory, international ring trial, combining thermophilic *Campylobacter* spp. detection in meat rinses, ISPC monitoring, and internal amplification controls (IACs) (Stingl et al., 2021). Having previously developed the ISPC, they adapted the method for routine use in laboratories by lyophilizing the ISPC and developing a single triplex qPCR which targets thermophilic *Campylobacter*, the ISPC, and the internal amplification control (IAC).

Many food components, including fats and proteins, can inhibit PCR (Rossen et al., 1992) by affecting the cell lysis, nucleic acids, and even inactivating DNA polymerase during amplification. For example, one study comparing a viability qPCR to culture-based method screened chicken meat samples for the presence of *Campylobacter* and found that the culture-based method detected more positive results than the PMA-qPCR. They lacked an IAC to account for false negatives, so it is possible that the chicken matrix interfered with DNA amplification in some samples (Okada et al., 2023). Therefore, IACs are added into PCR to prevent false negative results caused by such inhibitors. A 125-bp sequence from *Nicotiana tabacum* is used as the IAC

target in the validated multiplex qPCR. The triplex qPCR developed by Stingl et al. (2019) performed similar to their two previously developed duplex qPCRs up to a concentration of 4.7 $\log_{10}$ CFU/mL of *Campylobacter*, and the limit of quantification was 2.3 $\log_{10}$ CFU/mL, which is similar to previously developed methods (Josefsen et al., 2010b; Lv et al., 2020). Through the interlaboratory ring trial, they determined this method was more reliable and reproducible than the reference CFU counting method. Therefore, this method and a reference culture-based method were adopted in this thesis research.

While PMA-qPCR remains the most extensively validated method for enumerating viable *Campylobacter* cells, alternative molecular approaches, such loop mediated isothermal amplification (LAMP) have shown promise. Peterson et al. (2012) developed LAMP assay for VBNC *C. jejuni,* achieving specificity for *C. jejuni* with a limit of detection of around 3 $\log_{10}$ CFU/mL, which is higher than the qPCR methods developed by Josefsen (2010) and Stingl (2021). In addition, they designed primers to target the hippuricase-encoding gene (*hipO*), which is highly specific to *C. jejuni,* meaning that this gene is predominantly present in *C. jejuni* and is not commonly found in other *Campylobacter* species (LaGier et al., 2004). This makes it unsuitable for detecting all thermophilic *Campylobacter* from food samples, of which *C. coli* and *C. lari* are the next most prevalent after *C. jejuni*. Nevertheless, qualitative LAMP assays using primers that target the conserved 16S rRNA sequences (Babu et al., 2020) or include multiple primers sets targeting different species (Yamazaki, 2013), could be coupled with a PMA treatment to detect viable thermophilic *Campylobacter* spp.

1.4 Statement of the Problem

This study had two aims. The first was to determine the prevalence and levels of retail chicken breast contamination with *Campylobacter*, and the second was to assess the antimicrobial efficacy of peroxyacetic acid spray and rinse against viable *Campylobacter* on chicken breasts.

To recover cells in a VBNC state, recent studies have developed molecular methods to enumerate viable cells from poultry products, including a validated viability qPCR method. While their efficacy has been shown for inoculated chicken, it remains unclear whether molecular methods are useful for the enumeration of low-level contamination like what is commonly found on retail chicken breasts. Studies have also determined the prevalence of viable and VBNC *Campylobacter* on retail chicken breasts using direct plating and viability qPCR methods. Therefore, we aimed to compare their efficacy at recovering *Campylobacter* and to determine the prevalence of native (uninoculated) VBNC *Campylobacter* on retail chicken breasts. We hypothesized that the culture-based and molecular methods would not show the same recovery of cells, suggesting the presence of VBNC *Campylobacter*.

Antimicrobials like peroxyacetic acid (PAA) are used during post-slaughter decontamination interventions to control the growth of pathogens such as *Campylobacter*. Previous studies have solely used culture-based recovery methods to determine the efficacy of PAA at reducing *Campylobacter* concentrations on chicken meat. Using both direct plating and viability qPCR recovery methods we aimed to compare the culturable and viable *Campylobacter* counts on chicken breasts after PAA treatment. We hypothesized that application of peroxyacetic acid through spray and immersion would reduce the culturable cell count of *Campylobacter* by inducing a subpopulation into a VBNC state.

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Chapter 2

Low Prevalence and Concentrations of *Campylobacter* Detected on Retail Chicken Breasts

2.1 Introduction

The Center for Disease Control estimates that 1.5 million people in the US become ill from a *Campylobacter* infection every year (CDC, 2025). In fact, campylobacteriosis is the most frequently reported bacterial food-borne illness in humans (Shah, 2024). This illness imposes a significant economic burden, with annual losses in the US estimated at approximately $2 billion (Hoffmann et al., 2012). *Campylobacter* is a Gram-negative, microaerophilic, and thermophilic bacterium, and it finds its natural niche in the chicken gut. It is most associated with raw or undercooked poultry, but it can also thrive in other animal guts, such as pigs or cows. The symptoms of campylobacteriosis in humans include diarrhea, cramping, abdominal pain, and fever (El-Saadony et al., 2023). More serious post-infection complications can arise, such as pancreatitis, reactive arthritis and Guillain-Barré syndrome, which can be fatal (Ruiz-Palacios, 2007).

Campylobacter can spread quickly within flocks via fecal contamination when fecal droppings exhibit high concentrations of *Campylobacter*, from 10^6 to 10^{10} CFU/g (Battersby et al., 2016; Berndtson et al., 1996; Rudi et al., 2004). Flocks that are negative for *Campylobacter* can also be contaminated by positive flocks during transport and slaughter. *Campylobacter* is often highly prevalent on retail chicken meat, with some studies reporting a prevalence of up to 76% (Guyard-Nicodème et al., 2015). A recent study modeled dose-response curves and found that consuming 10 CFU *Campylobacter* in liquid resulted in a 100% predicted infection probability, whereas approximately 1,000 CFU were required for young adults consuming solid foods to reach the same probability (Abe et al., 2021).

Given the high prevalence and low infectious dose of *Campylobacter*, quantitative detection in poultry meat is crucial for improving pathogen control and reducing consumer exposure. However, detection methods need to account for both culturable and viable but non-culturable (VBNC) cells. *Campylobacter* encounters stressors such as oxygen and cold temperatures outside the chicken gut, which can impair its ability to grow on standard microbiological media by causing a VBNC state (Yagi et al., 2022). This state is characterized by reduced metabolic activity, increased stress tolerance, and decreased culturability (W. Hazeleger et al., 1994; Ikeda & Karlyshev, 2012).

There is a reliance on culture-based detection, and gold-standard methods, including the USDA Microbiology Laboratory Guidelines (MLG) and the FDA Bacteriological Analytical Manual (BAM), use enrichment, followed by pathogen isolation (FDA, 2021b; USDA FSIS, 2024). These methods may underestimate *Campylobacter* prevalence on retail meat by detecting only culturable cells, thereby underestimating the viable bacterial load. This is particularly relevant for retail chicken meat, which undergoes prolonged exposure to low temperatures and oxidative stress conditions known to induce a VBNC state in *Campylobacter* (Yagi et al., 2022). VBNC *C. jejuni* has been resuscitated through inoculation into embryonated chicken eggs (Cappelier et al., 1999) and *in vivo* in mice (Baffone et al., 2006), suggesting that failure to detect VBNC cells may lead to a underestimation of the pathogen's infectious potential.

In recent years, numerous molecular and non-culture-based detection methods have been developed to address limitations in *Campylobacter* detection from food sources, which present a unique challenge. Several viability qPCR assays using DNA-intercalating dyes such as propidium monoazide (PMA) have been designed to block amplification of DNA from dead cells, ensuring only viable cells are detected (Josefsen et al., 2010b; Lv et al., 2020b; Okada et al., 2022; Stingl et al., 2021). Stingl et al. (2021) developed and validated a multiplex viability qPCR specifically for detecting thermophilic *Campylobacter* spp. from meat rinses (Stingl et al., 2021).

Despite recent advancements in viability qPCR, its application to uninoculated retail chicken breasts has not been studied, leaving a gap in understanding the prevalence of viable *Campylobacter* on the most consumed chicken meat cut in the United States (USDA, 2017). Therefore, the effectiveness of a viability qPCR method for *Campylobacter* detection at the retail stage remains unclear.

This study compared culture-based methods, including the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) Microbiology Laboratory Guidelines (MLG) 41.09 and the ISO 10272-1:2017 protocols which outline *Campylobacter* isolation and enumeration from poultry, to the recently validated viability qPCR (ISO, 2022b; Stingl et al., 2021; USDA FSIS, 2024). The goal was to determine the level of *Campylobacter* on retail chicken breasts and to investigate whether culture-based enumeration recovers the same amount of native *Campylobacter* from retail chicken breasts as viability qPCR, designed to detect all viable cells.

2.2 Materials and Methods

2.2.1 Sample Collection and Processing

A total of 211 retail boneless, skinless whole chicken breasts were collected from seven grocery stores and two farmer's markets (bone in, skin-on whole chicken breast) in State College, Pennsylvania between October 2023 and July 2024. The following formula was used to determine the sample size: $N = Z^2 P (1-P)/(D^2)$, where $Z = 1.96$ at a 96% confidence interval, D is the tolerated margin of sampling error (5% was used), and P is the estimated prevalence of *Campylobacter* (Admasie et al., 2023). A previous study determined the prevalence of *Campylobacter* on skinless chicken breast to be 4.0%, resulting in a minimum sample size of 59

(Mujahid et al., 2023). Samples were immediately transported from the grocery store to the lab where they were stored at refrigeration temperatures (4°C) and processed within 24 h. On average, eight samples with different processing plant numbers were collected and processed weekly. For each sample processed, the establishment number on the package was used to identify the processing facility in the FSIS Meat, Poultry and Egg Product Inspection Directory (USDA, 2025). To prepare the chicken rinsate, 325 g of each sample was aseptically cut, weighed, and added to a homogenization bag (Whirl-Pak, Chicago, IL) pre-filled with 1,625 mL of sterile buffered peptone water (BPW; Neogen, Lansing, MI). A sample was then hand-massaged and shaken for one minute to allow for bacterial detachment.

2.2.2 Enrichment

Chicken rinsates were enriched for *Campylobacter jejuni/coli/lari* by following the USDA FSIS MLG 41.07 protocol (USDA, 2022). Briefly, 30 mL of chicken rinsate were added to a homogenization bag pre-filled with 30 mL Hunt Medium (Remel, Thermo Scientific, Waltham, MA) and incubated at 42°C for 24 h in microaerobic conditions (5% O_2, 10% CO_2, 75% N_2). After enrichment, each sample was streaked using a 10 µL sterile loop, in duplicates, onto Campy-Cefex Agar (Neogen, Lansing, MI) and incubated at 42°C for 48 h in microaerobic conditions. After 24 h enrichment, the plates were checked for typical *Campylobacter* colonies. The reference strain *C. jejuni* ATCC 33560 was used as a positive control by inoculating a colony in 30 mL of BPW, then following the same enrichment and isolation steps. Any colonies grown on the agar were Gram-stained and Gram-negative colonies were substreaked onto modified Charcoal Cefoperazone Deoxycholate (mCCD) agar (Oxoid, Waltham, MA). A representative colony was selected from a plate, and DNA was extracted using the GeneJet Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA), and tested using a multiplex PCR to confirm

Campylobacter (FDA, 2021b; Wang et al., 2002). DNA from all Gram-negative colonies was also whole genome sequenced using Nanopore as outlined below.

2.2.3 Direct Plating

In parallel to enrichment, rinsates prepared for enrichment were also used for *Campylobacter* quantification by following the ISO 10272-1:2018 (ISO, 2022). The rinsates were serially diluted and 100 µL of dilutions were plated in duplicate on both Campy-Cefex and mCCD agars using a spiral plater (Interscience, Woburn, MA) in the exponential setting. Two negative controls consisting of sterile PBS were plated before and after plating samples to control for spiral plater contamination. The Campy-Cefex plates were incubated at 42°C for 24 h, and the mCCDA plates were incubated at 42°C for 48 h, both in microaerobic conditions. Two negative controls for each medium were incubated alongside samples to confirm media sterility.

2.2.4 PMA Treatment and Viability qPCR

Chicken rinsates were treated with propidium monoazide (PMA), followed by DNA extraction and viability qPCR by following the protocol from Stingl et al., 2021. First, two 1-mL aliquots of the chicken rinsate were prepared - one for treatment with PMA and one without. Internal sample process control was prepared using the *C. sputorum* lyophilizate (German Federal Institute for Risk Assessment, Berlin, Germany), aliquoted and stored at -80°C until PMA treatment, at which point an aliquot was thawed and diluted with BPW and Peptone Water-blue (PW-blue) (0.05% bromophenol blue in BPW) to obtain the working solutions $ISPC_{high}$ and $ISPC_{low}$. One milliliter of BPW was added to the five controls. Samples and controls not treated with PMA were placed on ice for two min and 10 µL $ISPC_{low}$ was added to each sample, followed

by centrifugation at 16.000 x g for five min at 4°C (Galaxy 20R, VWR International, Radnor, PA). Supernatants were discarded, and the cell pellets were frozen at -20°C until DNA extraction.

Ten microliters of $ISPC_{high}$ were added to the PMA-treated samples and controls at room temperature (20°C), followed by vortexing. Then, 2.5 µL of PMA (20 mM) was added and samples were incubated in a thermomixer (Multi-Therm, Benchmark Scientific, Sayreville, NJ) for 15 min at 30°C under shaking at 700 rpm in the dark, covered with aluminum foil. Once the incubation was completed, the tubes were transferred to the PMA-Lite 2.0 LED Photolysis Device (Biotium, Fremont, CA) and crosslinked for 15 min in the dark. After cross-linking, samples were placed on ice for two min in the dark, followed by the addition of 10 µL $ISPC_{low}$. The samples were then centrifuged at 16.000 x g for five min at 4°C (Galaxy 20R, VWR International, Radnor, PA), supernatants were discarded, and the cell pellets were stored frozen at -20°C until DNA extraction.

DNA extraction was performed following the GeneJet Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA) protocol and extracted DNA was kept frozen at -20°C until the qPCR was performed. Quantitative PCR was carried out in a triplex reaction using Platinum Taq DNA Polymerase (Invitrogen, Thermo Scientific, Waltham, MA; 5 U/µL), targeting DNA of thermophilic *Campylobacter*, the ISPC, and the internal amplification control (IAC). The PCR cycling program consisted of an initial denaturation step for three min at 95°C, then 45 cycles of 15 s at 95°C, 60 s at 60°C, and 30 s at 72°C (Stingl et al., 2021).

2.2.5 DNA Extraction, Nanopore Sequencing, and Genome Analysis

For Nanopore whole genome sequencing, a loopful of a representative Gram-negative colony was substreaked onto mCCDA and incubated at 42°C for 48 h in microaerobic conditions. Genomic DNA was then extracted using the GeneJet Genomic DNA Purification Kit (Thermo

Scientific, Waltham, MA), and extracted DNA was kept frozen at -20°C until sequencing. The concentration of extracted DNA was quantified using a Qubit (Qubit 4, Invitrogen, Thermo Scientific, Waltham, MA). DNA concentration was adjusted to 400 ng per 7.5 µL for each sample. Libraries were prepared using the Rapid Barcoding Library Preparation Kit (Oxford Nanopore Tech, cat. No. SQK-RBK004, Oxford, United Kingdom) by following manufacturer's instructions and one isolate was sequenced per each flow cell (FLO-MIN106). Each isolate was sequenced for 48 hours using a MinION Mk1C sequencer (Oxford Nanopore Tech, Oxford, United Kingdom), and the base-calling was carried out in real time using the fast model. The generated fastq files were concatenated and quality of reads was assessed using FastQC in GalaxyTrakr (Galaxy Version 0.73) (Gangiredla et al., 2021). Sequencing reads were assembled using Flye (Galaxy Version 2.9.1) (Lin et al., 2016) and the read quality was checked using Quast (Galaxy Version 5.2.0) (Gurevich et al., 2013). GTDBtk (Galaxy Version 2.2.2) was used to identify taxonomic species. Subsequently, assemblies were analyzed using ABRicate (Galaxy Version 1.0.1) to detect antimicrobial resistance gene sequences. Lastly, genomes were queried via PubMLST to determine multilocus sequence typing (MLST) sequence types (STs). Genomes that had an undetermined ST were submitted to PubMLST for new allele and/or ST definition (Jolley et al., 2018).

2.3 Results and Discussion

From October 2023 to July 2024, 211 chicken breasts were sampled and analyzed for *Campylobacter* spp. to determine prevalence and to compare the performance of the culture-based and viability qPCR methods. Based on the processing establishment number listed on a packaging label, we determined that samples were processed in at least 18 processing plants. Among these, 50 of the 211 samples were processed in plants in Delaware, eight in Georgia, 11 in

Illinois, 19 in Kentucky, three in Maryland, 45 in North Carolina, 38 in Pennsylvania (including both the farmer's market samples), and 21 in Virginia (Fig. 2.1). The processing plant number was not listed on the packaging for fifteen of the analyzed samples. Based on the FSIS Meat, Poultry and Egg Product Inspection Directory records, all plants from which our samples were sourced were poultry processing plants, with some also including poultry slaughter or other meat processing (FSIS, 2025).

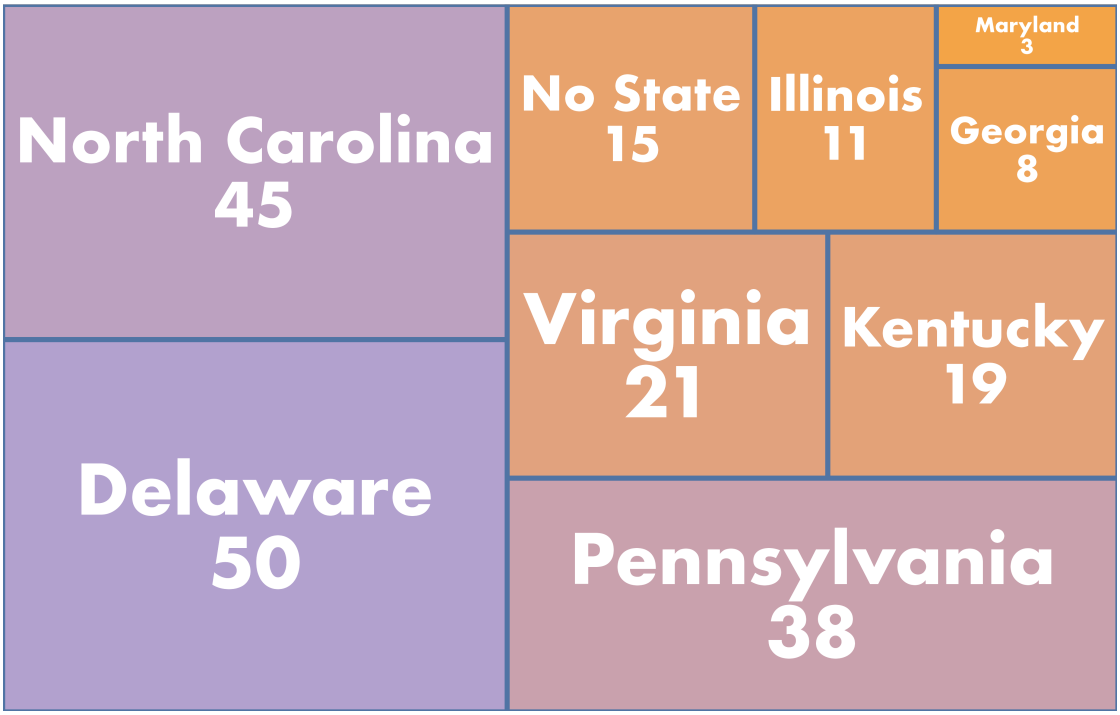

Figure 2.1. Relative number of retail chicken breast samples included in this study that had been processed in each state.

Enrichments of 16 chicken breast samples produced visible colonies upon streaking enrichments onto Campy-Cefex agar plates and incubating them at the prescribed conditions. Colonies from all 16 samples were Gram negative and colonies from five had a typical *Campylobacter* spp. morphology observed using a light microscope. However, none of the tested samples produced colonies when rinsates were plated directly onto Campy-Cefex and mCCDA

and incubated at prescribed conditions. Consistent with the results of direct plating, viability qPCR produced a negative results for all tested samples. *Campylobacter* enrichment method can detect as few as 12 cells per 325 g sample. In contrast, direct plating and viability qPCR have a higher limit of detection (60 CFU and 3200 - 5000 cells per 325 g chicken sample depending on the level of organic matter, or 0.18 CFU/g and 9.8 – 15.4/g, respectively). This suggests that the 16 samples that tested positive using enrichment and negative using quantitative methods were likely contaminated with fewer than 60 CFU per sample. This indicates that while enrichment was able to detect *Campylobacter* at low concentrations, the bacterial load was below the limit of detections for both quantitative methods. This highlights their limitations in the detection of low-level *Campylobacter* contamination.

Experimental human infection studies have estimated the infectious dose for *Campylobacter* to be between 500 and 800 cells (5 – 8 cells/g considering a 100 g serving size) (Black et al., 1988; Robinson, 1981). However, while lower doses have not been tested, a dose-response model found that 1 – 10 CFU can cause infection and 100 – 1,000 CFU have between a 60 – 100% chance of causing infection in young adults consuming solid food (Abe et al., 2021). Culture-based methods have a detection limit well below this dose (e.g., 0.04 cells/g chicken sample for enrichment) and can detect low-level contamination and can therefore be useful for assessing the positivity rate. In contrast, the limit of detection for viability qPCR is by approximately two orders of magnitude higher, which makes it less suitable for detection of very low-level contamination. However, it can detect viable *Campylobacter,* including VBNC cells, and provides a quantitative estimate of the contamination level, which can be used to assess compliance with regulation based on the quantitative limits. For example, in the European Union, Regulation (EU) 2017/1495 outlines microbiological criteria and the regulatory limit of 1,000 CFU/g for *Campylobacter* on broiler carcasses and requires that 40 out of 50 tested samples meet this criterium to comply with the regulation (EFSA, 2017). When quantitative limits are in place,

viability qPCR may offer a more accurate quantification by accounting for VBNC *Campylobacter*, which may regain infectious potential in a human host.

Nine of the 16 isolates were PCR positive using *Campylobacter* spp. specific primers, and eight were PCR positive using primers specific to the *C. jejuni* species. Among these, four showed intense bands in the gel electrophoresis, while all others produced faint bands. One isolate which produced a strong band in the *C. jejuni* confirmation PCR showed no band for the 23S rRNA amplicon. To mitigate false positive confirmation, 15 of these 16 isolates were whole genome sequenced using Nanopore. One isolate did not grow after subculturing and was therefore not sequenced. Based on the taxonomic classification using GTDBtk, five of the 15 isolates were confirmed as *C. jejuni,* eight were *Acinetobacter* spp. (three *Acinetobacter baumannii*, three *Acinetobacter seifertii*, two *Acinetobacter nosocomialis*), one *Micrococcus luteus*, and one *Escherichia coli*, which suggested poor specificity of the confirmation PCR method.

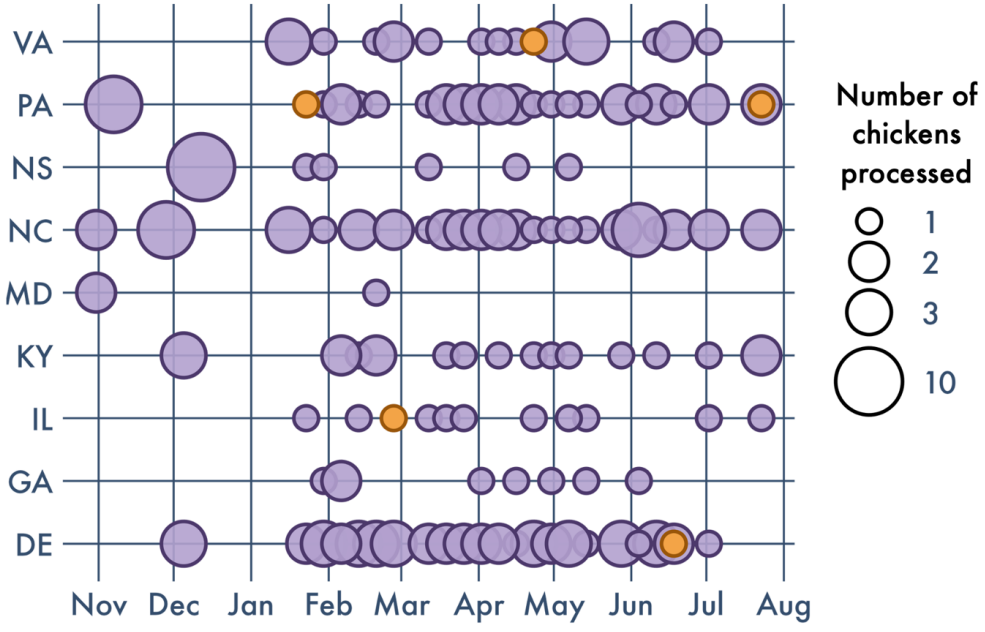

Figure 2.2. Number of chicken breast samples processed from the end of October 2023 to July 2024. The orange circles indicate isolates that were determined to be *C. jejuni* by WGS while the purple circles were not.

The five *C. jejuni* isolates were obtained from chicken breast samples processed in a Pennsylvania facility in January, Illinois facility in February, Virginia facility in April, Delaware facility in June, and from a farmer's market sample in July (Fig. 2.2). This resulted in the overall prevalence of *Campylobacter* spp. on retail chicken breast of 1.9% (n = 209).

Once *Campylobacter* leaves its natural niche in the chicken gut, it spreads through fluids and feces, attaching to the skin, which provides a more protective environment than exposed muscle tissue (Davis & Conner, 2007). As a result, contamination levels and prevalence rates are generally higher on chicken skin than on meat, making direct comparisons with studies on other cuts other than boneless, skinless breasts, difficult (Hansson et al., 2015; Luber & Bartelt, 2007). For example, *Campylobacter* prevalence was significantly higher on chicken carcasses (90%, n = 120) and legs (85.1%, n = 121) compared to skinless chicken breasts (53.3%, n = 120) (Guyard-Nicodème et al., 2015). Similarly, a study using a previous version of the same MLG (41.05) found *Campylobacter* in 4.0% of boneless, skinless chicken breasts, while the prevalence was higher (7.5%) in bone-in, skin-on breasts (Mujahid et al., 2023). Their samples, sourced from metro areas across the US (n = 499) in January and February, likely yielded a higher prevalence (compared to our 1.9%) due to seasonal variation, sample sourcing differences, and the use of a different enrichment method. These factors highlight the importance of considering sample type, methodology, and seasonality when comparing *Campylobacter* prevalence across studies. Our study focused exclusively on skinless chicken breasts, which may partially explain the lower *Campylobacter* prevalence compared to studies examining different chicken cuts, where prevalence ranges from 3.2% for boneless, skinless tenders to 95% for skin-on wings (Mujahid et al., 2023; Poudel et al., 2022; Sasaki et al., 2023; Walker et al., 2019a; Zendehbad et al., 2015).

A study comparing *Campylobacter* prevalence on fresh or frozen whole skin-on chicken from farmers' markets and supermarkets in Pennsylvania found that 52% (n = 50) of the retail chicken samples tested positive for *Campylobacter*. However, only one of these samples had a

quantifiable level of contamination. While their reported prevalence is higher, likely due to the presence of skin, the overall contamination level aligns with our findings, as all positive samples in our study had *Campylobacter* below the level of quantification.

During our sampling period, the FSIS collected national-scale prevalence data on *Campylobacter* in chicken meat and the reported prevalence from October 1, 2023, to September 30, 2024 was 20.63% (n = 7,206). FSIS samples raw chicken parts, including legs, breasts and wings. However, since this dataset does not specify the exact distribution of cuts tested, direct comparisons with our results are challenging. Given that multiple skin-on cuts are included, the higher prevalence observed in this dataset is expected.

Previous studies have demonstrated seasonal variation in *Campylobacter* prevalence, with higher prevalence in warmer months compared to cooler months (Hinton et al., 2004; Willis & Murray, 1997). While it is possible that temperature directly affects the increases in human cases, more likely it serves as a marker for increase in human behavior that increase the risk of infection, like consumption of barbecued meat or swimming (Mughini Gras et al., 2012; Rosner et al., 2017). Additionally, a model showed the risk of getting *Campylobacter* infection from chicken meat is lower during the winter months (Xu et al., 2023). In our study, two samples tested positive in winter, one in spring and one in summer. However, we sampled the fewest number of chickens in fall (n = 16) and summer (n = 40), and the most in spring (n = 87) and winter (n = 68). This inconsistency makes it difficult to draw definitive conclusions about seasonality based on our data, and a similar number of samples would need to be collected each season to draw conclusions based on seasonality.

The isolated *C. jejuni* strains were submitted to the *Campylobacter jejuni/coli* multilocus sequence typing (MLST) database (Jolley et al., 2012) on PubMLST (Jolley et al., 2018). The five isolates represented novel sequence types (STs), including ST 14450, ST 14451, ST 14452, ST 14453 and ST 14454, none of which were assigned to a clonal complex. Two isolates (80,

195) carried *blaOXA-61* (80) and *blaOXA-184* (195), and *tet(O)* genes that had been associated with ampicillin and tetracycline resistance (Yan et al., 2023). One isolate (137) carried just *blaOXA-61*. None of the *Campylobacter* isolates carried mutations or genes associated with resistance to fluoroquinolones and macrolides, which are used for the treatment of campylobacteriosis.

A Finnish study identified ST 45 (33.0%), ST 677 (9.5%) and ST 267 (5.5%) as the most prevalent *Campylobacter* sequence types in chicken pre-slaughter (n = 380) (Llarena et al., 2015). In a seven year study subtyping *Campylobacter* from commercial broiler chickens, the most prevalent ST was ST 353 (Berrang et al., 2023). Since our *Campylobacter* isolates were assigned novel STs and not placed into existing clonal complexes, they are likely not similar to the most prevalent genotypes reported.

In addition to *Campylobacter*, the enrichment broth and Campy-Cefex agar supported growth of off-target species of *Acinetobacter* in eight samples, *Micrococcus luteus* and *E. coli*. These species included *Acinetobacter baumannii*, *Acinetobacter seifertii*, and *Acinetobacter nosocomialis*. Three isolates (two *A. nosocomialis* and one *A. seifertii*) along with *Micrococcus luteus* were recovered from chicken breasts processed at the same plant in Georgia between April and June. Additionally, three *Acinetobacter* isolates (one *A. baumannii* and two *A. seifertii*) originated from chicken breasts processed at a Delaware plant between February and March. One *A. baumannii* isolate was collected from chicken processed at a plant in Virginia in April, and *Campylobacter jejuni* was isolated from chicken breast processed at the same facility two weeks later. The last *A. baumannii* isolate was recovered from chicken processed in a plant in Virginia in May. The *E. coli* isolate was obtained from chicken processed in a plant in Pennsylvania in April. Although some isolates were isolated from the same processing facilities, none of them were closely related as determined by percent relatedness using kSNP, with the lowest pairwise SNP difference being 248 (Gardner et al., 2015). This indicates that these isolates are not

genetically related and hail from distinct lineages. The eight *Acinetobacter* isolates had undetermined sequence types, and were therefore submitted to PubMLST for typing using the Pasteur scheme (Diancourt et al., 2010; Jolley et al., 2018). Of the three *A. baumannii* isolates, one was assigned ST 203 and one ST 869. Of the three *A. seifertii* isolates one was ST 2746 and one ST 2747. One of the *A. nosocomialis* isolates was assigned ST 359. One isolate per species was not assigned a ST due to insertions or deletions (indels) in the sequence. All strains isolated in this thesis are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject ID PRJNA1233267 (see table 2.1).

Table 2.1. Isolate number, name, species, sequence accession number, sequence type, and state the chicken was processed in for 15 isolates that grew on *Campylobacter* selective enrichment plates.

Isolate number	Isolate name	Species	Sequence Accession Number	MLST[a]	State
44	PS03104A	*Campylobacter jejuni*	SRR32610261	14450	PA
57	PS03188A	*Acinetobacter seifertii*	SRR32610260	2746	DE
67		*Acinetobacter baumannii*	SRR32610254	203	DE
80	PS03105A	*Campylobacter jejuni*	SRR32610253	14451	IL
87	PS03189A	*Acinetobacter seifertii*	SRR32610252	2747	DE
121		*Acinetobacter baumannii*	SRR32610251	869	VA
124	PS03190A	*Acinetobacter seifertii*	SRR32610250		GA
132		*Escherichia coli*	SRR32610249		PA
137		*Campylobacter jejuni*	SRR32610248	14452	VA
146	PS03194A	*Micrococcus luteus*	SRR32610247		GA
158		*Acinetobacter nosocomialis*	SRR32610259		GA
163		*Acinetobacter baumannii*	SRR32610258		VA
174		*Acinetobacter nosocomialis*	SRR32610257	359	GA
195		*Campylobacter jejuni*	SRR32610256	14453	DE
204		*Campylobacter jejuni*	SRR32610255	14454	PA

[a] Multi-locus sequence type (MLST) determined through submission to PubMLST.

The three *Acinetobacter* species isolated in this study belong to the *Acinetobacter calcoaceticus-baumannii* complex (Acb), a group of six phenotypically similar pathogens associated with nosocomial infections such as pneumonia and bacteremia (Nemec et al., 2015). *Acinetobacter baumannii* is of particular concern due to its increasing antibiotic resistance and persistence in hospital environments, making infections exceptionally difficult to treat (Wareth et al., 2019).

Multi-drug resistant *A. baumannii* is among the six most critical nosocomial pathogens in the US, according to the Infectious Diseases Society of America (ISDA), with approximately 45,000 clinical infections annually and an ICU mortality rate of up to 50% (Wei et al., 2023). It is also part of the 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), a group of bacteria responsible for the majority of nosocomial infections in the US and known for their extensive antimicrobial resistance mechanisms (Miller & Arias, 2024; Pendleton et al., 2013).

Carbapenem-resistant *A. baumannii* (CRAB) is a major concern for hospital-acquired infections, as carbapenems were once the standard treatment for critical infections, but increased usage has driven resistance (Jiang et al., 2022). This led to CRAB being designated as a critical-priority pathogen by the WHO, highlighting the urgent need for new antibiotics (Tacconelli et al., 2018). The primary mechanism of carbapenem resistance in *A. baumannii* is the production of OXA-type β-lactamases, with five major groups (OXA-23-, OXA-24/40-, OXA-51-, OXA-58-, and OXA-143-like) being most common. The three *A. baumannii* isolates in this study carried sequences of *blaOXA-203*, *blaOXA-259*, and *blaOXA-78*, all of which have been associated with resistance to carbapenems (Evans & Amyes, 2014; Kafshnouchi et al., 2022). Both *blaOXA-78* and *blaOXA-203* encode enzymes within the intrinsic OXA-51-like enzyme group, commonly found in *A. baumannii* and *A. nosocomialis,* which confer baseline carbapenem resistance. However, their clinical significance depends on expression levels and the presence of additional

resistance mechanisms. The gene *blaOXA-259* is less well characterized but is known to be an intrinsic β-lactamase gene that also confers resistance to carbapenems. It was previously reported in a community-acquired carbapenem-resistant *A. baumannii* strain also carrying the *blaOXA-72* gene (Jia et al., 2019).

Although *Acinetobacter* is rarely associated with enteric disease, it has been suggested as a potential foodborne pathogen. Its role in foodborne illness is often overlooked due to its association with more severe infections and its frequent co-occurrence with well-known enteric pathogens. However, multiple studies have isolated *Acinetobacter* species from the feces of children with diarrhea, and these isolates exhibited cytotoxicity in cell cultures, suggesting a possible link to illness (Amorim & Nascimento, 2017). Unlike many foodborne bacteria, *Acinetobacter* can persist on dry surfaces commonly found in food processing environments and hospitals, form biofilms (Cerqueira & Peleg, 2011), and compete with established foodborne pathogens like *E. coli* and *Salmonella* (Damaceno et al., 2015). Given these characteristics, standardized methods for the isolation and identification of *Acinetobacter* from food should be developed, and its potential role as a foodborne pathogen warrants further investigation.

Furthermore, the ability of *Actinetobacter* spp. to survive under conditions selective for *Campylobacter* raises additional concerns regarding foodborne surveillance and detection. While *Acinetobacter* spp. are primarily aerobes with an optimal growth temperature of 30 - 37°C, *Acinetobacter* spp. has demonstrated the ability to grow under conditions designed for *Campylobacter* spp., as observed in our study. It has previously been isolated from poultry carcass rinses, retail meat products, surface water, and dairy cattle manure storage tanks under similar conditions (Cha et al., 2021; Fernando et al., 2016; Oyarzabal et al., 2005). Thus, its growth under microaerobic conditions (5% O_2, 10% CO_2, 75% N_2) at a 42°C, in the presence of multiple antibiotics, is not unexpected. The resistance of *Acinetobacter* to the antimicrobials present in selective media and its growth in these media may suppress *Campylobacter* growth,

potentially leading to underestimation of *Campylobacter* prevalence. *Acinetobacter* is intrinsically resistant to cephalosporins, carbapenems, and penicillins, so if contamination persists, addition of others such as tigecycline and aminoglycosides to selective media might aid in effectively isolating *Campylobacter* (Eliopoulos et al., 2008).

Lastly, the *E. coli* isolate that grew in the Hunt enrichment and Campy-Cefex agar carried *aph(3")-Ib, aph(6)-Id, blaTEM-1B, dfrA14* and *sul2* resistance genes associated with resistance to streptomycin, kanamycin, ampicillin, trimethoprim and sulfisoxazole, respectively (Poirel et al., 2018). This was the only isolate whose predicted phenotype was resistant to trimethoprim, an antibiotic added into *Campylobacter* enrichment media. In addition, a *sitABCD* homologue was detected in this isolate, which is significant as *sitABCD* was identified in avian pathogenic *E. coli* and was associated with iron and manganese transport, which contributes to oxidative stress resistance (Sabri et al., 2006). The presence of *sitABCD* in this isolate indicates a role in metal ion acquisition that may increase survival under the *Campylobacter* selective conditions. *E. coli* can grow in media selective for *Campylobacter*, like Campy-Cefex, Bolton media and mCCDA, all containing cefoperazone (Chon et al., 2012; W. C. Hazeleger et al., 2016; Kim et al., 2019).

This study provides insight into the prevalence of culturable *Campylobacter* on retail chicken breasts and demonstrates a lack of specificity of gold-standard enrichment methods for detecting *Campylobacter* from chicken meat. We were unable to determine the level of viable or culturable *Campylobacter* since the quantitative methods were not able to detect the low-level contamination of *Campylobacter* in any samples, highlighting the limitations in current detection approaches. The lack of specificity of the enrichment method led to the isolation of off-target bacteria, including the clinically relevant *Acinetobacter* spp., which demonstrates their persistence in poultry environments and raises concern about its transmission as a foodborne pathogen.

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Chapter 3

Effect of Peroxyacetic Acid Treatment on *Campylobacter* Culturability and Viability in Chicken Breasts

3.1 Introduction

Campylobacter is the leading cause of bacterial foodborne illness, and chicken is its main reservoir for transmission to humans (Rosner et al., 2017; Shah, 2024). Despite biosecurity measures at the farm level, ensuring that broilers are free of *Campylobacter* before entering the processing chain is challenging due to horizontal transmission and subsequent colonization and rapid growth in the poultry gut (Cody et al., 2019; Sadek et al., 2023). Therefore, control measures are introduced at the slaughterhouse to reduce microbial load on carcasses (Vandeplas et al., 2008). Once broilers have been eviscerated, carcasses move to a chill step, where cold water (1 – 2 °C) is used to reduce microbial load and inhibit microbial growth (Rosenquist et al., 2006). Low concentrations of antimicrobials can be added to the water to further reduce microbial load. At a post-chill step, higher concentrations of antimicrobials are typically applied to the carcass or chicken parts using spraying or immersion, without further rinsing (Cano et al., 2021).

The most commonly used antimicrobials approved for post-chill application on chicken carcasses are chlorine and peroxyacetic acid (PAA) (Wideman et al., 2016). While chlorine efficacy decreases in the presence of high organic loads, PAA remains effective at sufficiently high concentrations (Su et al., 2022). As a result, PAA has become a preferred choice in poultry processing facilities and is widely used post-chill to reduce microbial load through its oxidizing and acidic properties.

Studies have demonstrated that PAA effectively reduces culturable *Campylobacter* on chicken meat (Cano et al., 2021; Gonzalez et al., 2021; Kataria et al., 2020; Kumar et al., 2020; Laranja et al., 2023; Park et al., 2017; Smith et al., 2015). For example, at 200 ppm for 60 s,

immersion resulted in 1.42 $\log_{10}$ reduction of *C. jejuni,* whereas spraying reduced counts by 0.61 $\log_{10}$ (Smith et al., 2015). This was corroborated by another study, where 30-second immersion at 250 ppm PAA reduced *Campylobacter* by more than 1 $\log_{10}$, while a higher concentration (500 ppm) was required to achieve a similar reduction with spray application (Kumar et al., 2020). Similarly, a 5 s immersion at 550 ppm reduced culturable *Campylobacter* by over 2 logs on chicken wings, while a 4 s spray achieved less than 1 $\log_{10}$ reduction (Gonzalez et al., 2021). Notably, after a 24 h of storage at 4°C, the reductions remained consistent relative to untreated controls.

Higher PAA concentrations further enhance its efficacy. Immersion at 1200 ppm was shown to lead over 2 $\log_{10}$ reduction of culturable *C. coli* cells (Park et al., 2017). Despite the variability in experimental conditions, immersion seems to achieve a greater *Campylobacter* reduction on chicken meat compared to spray application, and PAA concentrations above 200 ppm were shown to consistently reduce *Campylobacter* by over 1 $\log_{10}$, with higher concentrations yielding greater reductions.

The studies discussed above used culture-based methods to recover *Campylobacter* cells after PAA treatment, even though oxidative and acidic stress can induce a viable but nonculturable (VBNC) state in *Campylobacter*. In this state, *Campylobacter* fails to grow on selective media but maintains its membrane integrity and may regain its infectivity under favorable conditions, such as in a host's gut (Baffone et al., 2006c; Yagi et al., 2022a; Zhang & Lu, 2023). Therefore, PAA interventions must assess not only reductions in culturable *Campylobacter*, but also in viable populations to ensure irreversible inactivation. To accurately determine the load of viable cells, viability qPCR methods have been developed using DNA-intercalating dyes to differentiate live and dead cells (Josefsen et al., 2010b; Lv et al., 2020a; Okada et al., 2022; Stingl et al., 2021). When used alongside culture-based methods, viability

qPCR helps quantify the proportion of *Campylobacter* population in the VBNC state, providing a more comprehensive assessment of antimicrobial effectiveness.

This study aimed to evaluate the efficacy of 500 ppm PAA in reducing *Campylobacter* on chicken breasts using immersion and spray application methods. Reduction in *Campylobacter* was quantified using both quantitative culture-based and viability qPCR methods to assess the effect of PAA on both culturability and viability.

3.2 Materials and Methods

3.2.1 Preparation of Inoculum

Three strains of *Campylobacter jejuni* were used to inoculate chicken breast samples, one of which was a human clinical isolate (PS00332, ST-50 CC) and two were chicken meat isolates (PS01840, ST-48 CC; PS01849, ST-353 CC). To prepare the inoculum, a loopful of each cryostock was streaked onto an mCCD agar plate (Oxoid, Waltham, MA) and incubated for 48 h at 42°C in microaerobic conditions (5% O_2, 10% CO_2, 75% N_2). Subsequently, one isolated colony was transferred into 40 ml Brucella broth (BD Biosciences, Franklin Lakes, NJ) and incubated for 48 h at 42°C in microaerobic conditions. After completed incubation, optical density of each culture was measured, and cultures were adjusted to 10^7 CFU/ml using an OD-CFU/ml curve. Cultures were then centrifuged at 3,000 *g* for 10 min. Supernatants were decanted, and the pellets were resuspended in 400 µL PBS, resulting in the final concentration of 10^9 CFU/ml. The adjusted cultures were combined in equal volumes and vortexed. The concentration of culturable cells in the inoculum cocktail was confirmed by plating serial dilutions prepared in buffered peptone water (BPW) onto mCCD agar plates (Oxoid, Waltham, MA). Inoculated plates were incubated at 42°C for 48 h in microaerobic conditions. The concentration of viable cells in

the inoculum was determined by propidium monoazide (PMA) treatment of the 10^{-5} dilution, followed by a viability qPCR, as described under the "PMA Treatment and Viability qPCR" below.

3.2.2 Sample Inoculation

Chicken breasts were purchased the day of the start of the experiment from a local grocery store. The chicken breasts were cut into 5 x 5 x 0.5 cm slices weighing approximately 25 g and were kept at 4°C in sterile Petri dishes until inoculation. A 100 µl aliquot of the inoculum was deposited onto the surface of a chicken slice with a micropipette and spread across the entire top surface with a sterile spreader. The inoculum was left to attach for 10 minutes at room temperature in a biosafety cabinet with the air flow on and the lid of the Petri dish off.

3.2.3 Peroxyacetic Acid Treatment

Peroxyacetic acid solution (22%) (Perasan MP-2C, Enrivotech, Modesto, CA) was diluted in chilled, sterile, deionized (DI) water to 500 ppm. The final concentration was confirmed using high-range PAA test strips (LaMotte, Chestertown, MD).

A total of six slices of chicken were prepared for each experiment and each experiment was repeated independently at least three times. In each experiment, two samples were treated with chilled 500 ppm PAA either through immersion (I) or spray (S), two were immersed or sprayed with sterile chilled DI water as immersion or spray controls (IC, SC), one was used as a negative control that was uninoculated and untreated (NC), and one was used as a positive control that was inoculated but untreated (PC). IC and SC were included as immersion or spraying controls, respectively, to quantify the *Campylobacter* reduction resulting from physical removal

during immersion or spraying, rather than inactivation by PAA. NC was included to assess the natural presence of *Campylobacter* on meat and to quantify the total aerobic mesophilic microbiota, while PC was used to evaluate the impact of non-PAA factors (e.g., exposure to air) on *Campylobacter* culturability and viability.

For the immersion treatment, sterile forceps were used to fully submerge each breast slice individually for 10 s in a 150 mL aliquot of pre-chilled PAA in a homogenization bag (Whirl-Pak, Chicago, IL). Freshly prepared PAA solution was used for treatment of each sample to prevent the reduction in antimicrobial efficacy due to increased organic load in the solution.

For spray treatment, a custom-enclosed spray apparatus was assembled using a sprayer wand (HDX, Home Depot, Atlanta, GA) and stainless-steel restaurant-style containers (6” depth, 12 ¾” length, 10 ½” width) (The Restaurant Store, Lancaster, PA). Before treatment, the spray apparatus was sanitized by running 70% ethanol through it for 10 s, followed by a 10 s flush with PAA. Using sterile forceps, breast slices were individually placed on a sterile mesh rack inside the pan and sprayed for 10 s on the inoculated side, depositing approximately 50 ml PAA per sample.

Samples were analyzed immediately after treatment, 1 h post-treatment, or 24 h post-treatment. Samples designated for 1 h and 24 h analysis were stored at 4°C in sterile Petri dishes with covered lids until processing. For analysis, samples were aseptically transferred to sterile homogenization bags (Whirl-Pak, Chicago, IL) containing 100 ml sterile neutralizing buffered peptone water (nBPW) supplemented with 0.1% sodium thiosulfate (Sigma, St. Louis, MO) to neutralize PAA and halt its antimicrobial activity. Samples were then hand-massaged and vigorously shaken for 1 minute to detach *Campylobacter* cells. The resulting suspension was serially diluted in sterile BPW, and 100 µl of each dilution (10^{-3} to 10^{-7}) were plated on mCCD agar plates (Oxoid, Waltham, MA) in duplicate, and incubated for 48 h at 42°C in microaerobic conditions. In addition to being plated on mCCD agar, the negative control was also plated on

standard plate count agar (SPCA) and lower dilutions (10^{-1} to 10^{-3}) were used. The SPCA plates were then incubated at 35°C for 48 h in aerobic conditions to enumerate the background microbiota present on the chicken and to assess the selectivity of mCCD agar for *Campylobacter*.

3.2.4 PMA Treatment and Viability qPCR

The samples underwent propidium monoazide (PMA) treatment, followed by DNA extraction and viability qPCR (Stingl et al., 2021). From each rinsate bag, two diluted rinsate samples (10 µl rinsate in 990 µl sterile BPW) were aliquoted into microcentrifuge tubes. Internal sample process controls, $ISPC_{high}$ and $ISPC_{low}$ were prepared and aliquoted in advance using the *C. sputorum* lyophilizate (German Federal Institute for Risk Assessment, Berlin, Germany), and were diluted in BPW and Peptone Water-blue (PW-blue) (0.05% bromophenol blue in buffered peptone water) on the day of PMA treatment. 1 ml sterile BPW was added to five control tubes, four without PMA treatment and one with PMA treatment. Samples and controls not undergoing PMA treatment were placed on ice for 2 min, then 10 µl $ISPC_{low}$ was added to each tube, followed by centrifugation at 16,000 x g for 5 min (4°C) (Galaxy 20R, VWR International, Radnor, PA). The supernatant was discarded, and the cell pellets were frozen at -20°C until DNA extraction. Ten microliters $ISPC_{high}$ were added to the PMA-treated samples and controls at room temperature (20°C), followed by vortexing to mix. Then, 2.5 µl of PMA (20 mM) were added and samples were incubated in a thermomixer (Multi-Therm, Benchmark Scientific, Sayreville, NJ) for 15 min at 30°C under shaking at 700 rpm in the dark, covered with aluminum foil. Once completed, the tubes were transferred to the PMA-Lite 2.0 LED Photolysis Device (Biotium, Fremont, CA) for PMA crosslinking for 15 min in the dark, covered with aluminum foil. After cross-linking, samples were placed on ice for 2 min in the dark, followed by the addition of 10 µl $ISPC_{low}$. The samples were then centrifuged at 16,000 x g for 5 min at 4°C (Galaxy 20R, VWR

International, Radnor, PA), the supernatant was discarded, and the cell pellets were frozen at -20°C until DNA extraction.

The GeneJet Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA) protocol was followed for DNA extraction, and extracted DNA was kept frozen at -20°C until the viability qPCR was performed. Viability qPCR was carried out in two duplex reactions, one targeting DNA of thermophilic *Campylobacter*, and either the ISPC or the internal amplification control (IAC). Platinum Taq DNA Polymerase (Invitrogen, Thermo Scientific, Waltham, MA; 5 U/µL) was in a PCR consisting of an initial denaturation step for 3 min at 95°C, then 45 cycles of 15 s at 95°C, 60 s at 60°C, and 30 s at 72°C.

3.2.5 Statistical Analysis

Microbial cell concentrations, as determined by colony counts and viability qPCR, were converted into $\log_{10}$ CFU/ml. The *Campylobacter* concentrations obtained for the PAA-treated samples (I or S) were subtracted from the *Campylobacter* concentrations for the respective rinsing controls (IC or SC) to determine the antimicrobial effect of PAA treatment without the contribution of physical rinsing. These differences were then subtracted from the respective inoculated but untreated control (PC) to determine the reduction in concentration resulting from PAA treatment alone.

A three-way ANOVA test was performed to examine the effect of interactions between the method (direct plating vs. viability qPCR), treatment (PC, I, S), and cold storage time post-treatment (0 h, 1 h, 24 h) on *Campylobacter* concentration. Interactions that were statistically significant ($p < 0.05$) were further examined using a Tukey's HSD to determine the significance of all pairwise comparisons.

3.3 Results and Discussion

3.3.1 A small but significant subpopulation of *Campylobacter* was in a non-culturable state prior to PAA treatment

We initially determined whether cells were in a VBNC state prior to inoculation onto the chicken by comparing the culturable and viable concentration of *Campylobacter* in the inoculum, as determined by dilution plating and viability qPCR. The concentration of viable cells (9.29 ± 0.57 $\log_{10}$ CFU/mL) was significantly higher ($p < 0.001$) than the concentration of culturable cells (8.76 ± 0.52 $\log_{10}$ CFU/mL), suggesting that 0.53 $\log_{10}$ CFU/mL were in a VBNC state before inoculation onto chicken breasts (Table 3.1). However, the margins of error (viable: 8.72 – 9.86 $\log_{10}$ CFU/mL; culturable: 8.24 – 9.28 $\log_{10}$ CFU/mL) overlap, so the culturable and viable differences should be interpreted with caution, as they may not be practically significant. Furthermore, we quantified culturable and viable *Campylobacter* after inoculation onto chicken breast (before PAA treatment) to assess the effect of environmental stressors (e.g., oxidative stress) on *Campylobacter* culturability. Immediately after inoculation, the population of culturable *Campylobacter* was 0.59 $\log_{10}$ CFU/mL lower ($p = 0.04$) compared to the viable *Campylobacter* (6.04 ± 0.14 $\log_{10}$ CFU/mL vs. 6.63 ± 0.35 $\log_{10}$ CFU/mL). This difference was similar to the difference between culturable and viable *Campylobacter* in the inoculum before inoculation on the chicken breasts (0.53 log), suggesting that inoculation onto chicken meat did not further induce VBNC in *Campylobacter*. The difference between viable and culturable cells on the untreated controls was insignificant when samples were tested 1 h or 24 h ($p = 0.99$) after inoculation, suggesting that prolonged cold storage did not further induce VBNC (see 3.3.2).

Table 3.*1*. *Campylobacter* concentrations (log_{10} CFU/mL; Mean ± SD) of inoculum and control.

Recovery Method	Inoculum	Untreated Control
culturable	8.76 ± 0.52[a]	6.04 ± 0.14[a]
viable	9.29 ± 0.57[b]	6.63 ± 0.35[b]

[a,b] Values followed by different letters (a, b) are significantly different ($p < 0.05$), as determined by the Tukey's HSD test.

3.3.2 Prolonged exposure to oxidative and cold stress significantly reduced *Campylobacter* viability on chicken breast

To further evaluate the impact of air exposure at 4°C on *Campylobacter* culturability and viability, we compared viable and culturable concentrations on untreated samples (PC) tested immediately after inoculation, after 1 h, and 24 h of incubation at 4°C under normal atmospheric (aerobic) conditions. The concentration of culturable *Campylobacter* on untreated controls decreased by 0.84 ± 0.21 log_{10} after 24 h at 4°C, compared to samples tested immediately after inoculation (6.04 ± 0.14 log_{10} CFU/mL vs. 5.20 ± 0.50 log_{10} CFU/mL; $p = 0.006$) (Fig. 3.1). Similarly, viable *Campylobacter* concentration decreased by 1.10 ± 0.19 log_{10} ($p < 0.001$) after 24 h (6.63 ± 0.35 log_{10} CFU/mL vs. 5.53 ± 0.39 log_{10} CFU/mL) (Fig. 3.2). This suggests that cold storage for 24 h resulted in both cell death (> 1 log) and the induction of VBNC state in a subpopulation of viable *Campylobacter*.

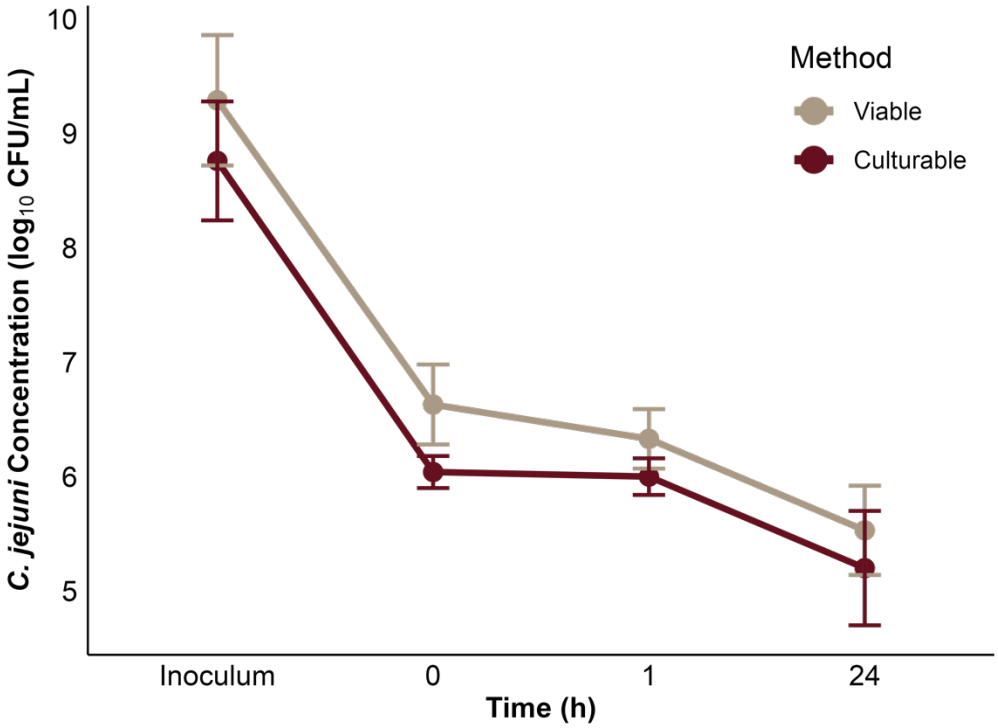

Figure 3.1. Concentrations of viable and culturable *Campylobacter* (log_{10} CFU/mL) in the inoculum and inoculated untreated control chicken breast samples (i.e., positive controls; PCs) tested immediately (0 h), 1 h and 24 h after inoculation.

Previous research demonstrated that *C. jejuni* strains cultured in Mueller-Hinton broth under aerobic conditions at 37°C fully entered VBNC within 24 h (Zhang & Lu, 2023). However, in our study, a subpopulation of *C. jejuni* retained culturability in all samples after 24 h, likely due to the lower storage temperature (4°C). *C. jejuni* has been shown to maintain culturability longer at refrigeration temperature, requiring more time to transition into non-culturable state compared to higher temperatures (Jackson et al., 2009). Our results indicate a similar decrease in culturable and viable cells, followed by the transition of a small subpopulation into the VBNC state within 24 h at 4°C. These differences may be attributed to our study using an inoculated *Campylobacter* cocktail on chicken breasts, whereas the previous study used *C. jejuni* cultured in

Muller-Hinton broth, highlighting potential matrix-dependent effects on VBNC formation. The chicken breast that we pipetted our inoculum onto could have provided protective niches to *Campylobacter* that impacted its transition into a VBNC state. In addition, Yagi et al. (2022) found that culture in MH broth induced a VBNC state quicker than a low nutrient medium (PBS), which is what our inoculum was resuspended in (Yagi et al., 2022).

3.3.3 A 10-second PAA treatment reduced *Campylobacter* culturability without significantly affecting its viability

To assess its antimicrobial and VBNC-inducing effect, we compared viable and culturable *Campylobacter* counts after 500 ppm PAA immersion and spray treatments. Immediately after the treatment, immersion and spray applications resulted in 0.25 ± 0.16 $\log_{10}$ ($p = 0.99$) and 0.19 ± 0.16 $\log_{10}$ ($p = 1.00$) reduction in viable *Campylobacter,* respectively, while culturablility was reduced by 0.81 ± 0.10 $\log_{10}$ ($p < 0.001$) for immersion and 0.51 ± 0.10 $\log_{10}$ ($p = 0.11$) for spray compared to the untreated control (Table 3.2). This suggests minimal antimicrobial effect, as the viable population remained largely unchanged, while the reduction in culturable cells was greater, particularly for immersion.

A significant difference ($p < 0.001$) was observed between culturable and viable *Campylobacter* counts immediately after immersion and spray treatment. Prior to treatment, the difference between untreated control culturable and viable counts was 0.59 ± 0.11 $\log_{10}$. After the treatment, the culturable/viable gap increased to 1.14 ± 0.15 $\log_{10}$ for PAA-immersed samples and 0.91 ± 0.15 $\log_{10}$ for PAA-sprayed samples. This indicates that 0.56 ± 0.18 $\log_{10}$ (immersion) and 0.32 ± 0.19 $\log_{10}$ (spray) of the population were induced into a VBNC state immediately after treatment, leading to an underestimation of viable *Campylobacter* when using culture-based methods. These findings highlight the risk of overestimating PAA's antimicrobial efficacy when

relying solely on culture-based quantification, as a subpopulation of intact, potentially infectious cells may persist in the VBNC state.

While previous studies have shown that PAA induces the VBNC state in *E. coli* and *Listeria monocytogenes* (Arvaniti et al., 2021, 2024; Truchado et al., 2021; Yin et al., 2023), this is the first study to demonstrate that PAA induces VBNC formation in *Campylobacter*, likely leading to the underestimation of viable cells when using culture-based methods. However, the reduction of culturable *Campylobacter* in this study was lower than previously reported. Kumar et al. (2020) observed a reduction from 1.16 ± 0.16 $\log_{10}$ to 1.23 ± 0.13 $\log_{10}$ in *C. coli* populations after 10 s PAA (500 ppm) treatment on chicken breasts, from a starting concentration of ~5 $\log_{10}$ CFU/g. A key limitation of our study is the high inoculum concentration (~7 $\log_{10}$ CFU/g) used to capture a broader range of reductions. This does not reflect the real-world contamination levels, which are significantly lower. A Japanese study found an average contamination level of ~26.3 CFU/g on retail chicken breasts, while an Australian study reported that 98% of tested retail chicken samples had < 4 $\log_{10}$ CFU per carcass (~ 5.62 CFU/g), and 10% had < 21 CFU total (Sasaki et al., 2023; Walker et al., 2019). It remains unclear whether PAA's antimicrobial efficacy at high contamination levels translates to its efficacy on naturally contaminated chicken with lower *Campylobacter* loads.

3.3.4 PAA treatment did not exert additional antimicrobial or VBNC-inducing effects during post-treatment cold storage

Previous research found that PAA residues become undetectable on poultry within 27.9 minutes post-application (Walsh et al., 2018), suggesting that the antimicrobial activity may persist for up to 27.9 minutes after treatment. In poultry processing, post-chill PAA-treated chicken is not rinsed before packaging and remains refrigerated until purchase. Thus, we

hypothesized that residual PAA could continue to exert oxidative stress and further reduce viable and culturable *Campylobacter* populations after treatment. To test this, PAA-treated chicken samples were stored at 4°C and analyzed 1 hour and 24 hours post-treatment. We found that PAA treatment did not continue to exert antimicrobial activity or induce VBNC state during post-treatment cold storage at 4°C. The untreated control showed no significant reduction in viable *Campylobacter* one hour post-treatment (0.30 ± 0.15 $\log_{10}$; $p = 0.99$) compared to immediately after inoculation. However, by 24 hours post-inoculation, viable *Campylobacter* on untreated controls decreased significantly (1.10 ± 0.19 $\log_{10}$, $p < 0.001$) compared to immediately after inoculation.

The reduction in viable *Campylobacter* due to PAA treatment 1 h and 24 h post-treatment was determined by subtracting both untreated control (PC) and rinsing control (SC or IC) analyzed at 1 h and 24 h post-treatment, respectively. At both 1-hour and 24-hours post-treatment, PAA did not achieve additional reduction of viable *Campylobacter*. Specifically, samples treated with PAA by immersion had 0.07 ± 0.28 $\log_{10}$ and 0.08 ± 0.22 $\log_{10}$ ($p = 1.00$) lower, and samples treated with PAA by spraying had 0.37 ± 0.23 $\log_{10}$ ($p = 0.98$) lower and 0.03 ± 0.24 $\log_{10}$ ($p = 1.00$) lower *Campylobacter* counts 1-hour and 24-hours post-treatment (Fig. 3.3). Although viable *Campylobacter* concentrations decreased over time in both treated and untreated samples after 24 h, the magnitude of reduction was greater in the untreated controls than in PAA-treated samples. This suggests that exposure to aerobic, low-temperature stress at 4°C had a stronger impact on viability than PAA treatment itself.

To determine whether the culturable counts aligned with the viable counts after treatment, we calculated the reduction due to PAA treatment for culturable *Campylobacter* and viable *Campylobacter*, then subtracted the viable reduction from the culturable reduction. The culture-based quantification overestimated antimicrobial activity of immersion by 0.56 ± 0.18 $\log_{10}$, 1.01 ± 0.32 $\log_{10}$ and 0.86 ± 0.37 $\log_{10}$ immediately after treatment, one hour and 24 h after

treatment, respectively, while for spray it was 0.32 ± 0.19 log_{10}, 0.43 ± 0.28 log_{10}, and 0.72 ± 0.40 log_{10}. These findings suggest that previous studies that used culture-based methods likely overestimated the PAA's antimicrobial effect due to its VBNC-inducing effect on *Campylobacter*. Across all time points, the average untreated culturable concentration was 5.82 ± 0.45 log_{10} CFU/mL while the average sprayed culturable concentration was 5.20 ± 0.59 log_{10} CFU/mL and the immersed culturable concentration was 4.98 ± 0.50 log_{10} CFU/mL. These are significant reductions of 0.62 ± 0.15 log_{10} ($p < 0.001$) and 0.84 ± 0.14 log_{10} ($p < 0.001$). This suggests that PAA treatment reduced the culturable population by inducing a VBNC state rather than killing *Campylobacter*, since the average viable cell reductions were minimal, at 0.17 ± 0.16 log_{10} ($p = 0.66$) and 0.09 ± 0.16 log_{10} ($p = 0.97$) for spray and immersion, respectively.

Our findings align with previous studies showing that while some sanitizers reduce culturability, their impact on viability is less pronounced. Zhang & Lu (2023) demonstrated that *Campylobacter* lost culturability immediately after chlorine (25 ppm) treatment, while viability remained stable over 24 hours (Zhang & Lu, 2023). After PAA treatment, the VBNC population also remained stable over 24 h in our study, however culturability was not completely lost. Similarly, Gonzalez et al. (2021) reported a 0.9 - 2.2 log_{10} reduction in *C. jejuni* culturability after 5 s exposure to 550 ppm PAA, with reductions persisting over 24 h (Gonzalez et al., 2021).

Our study observed comparable stable reductions in culturability after 10 s 500 ppm PAA spray, with reductions of 0.51 ± 0.10 log_{10} ($p = 0.11$), 0.80 ± 0.16 log_{10} ($p = 0.06$) and 0.75 ± 0.31 log_{10} ($p = 0.11$) immediately, one hour post-treatment, and 24 hours post-treatment, respectively. For immersion, the culturable reduction was 0.81 ± 0.10 log_{10} ($p < 0.001$), 0.95 ± 0.17 log_{10} ($p = 0.008$) and 0.78 ± 0.30 log_{10} ($p = 0.08$) immediately after, 1 h post-treatment and 24 h post-treatment. While these reductions remained consistent over time, only the immediate and 1 h post-treatment reductions due to immersion treatment significantly differed from the untreated

controls. These findings further support the idea that PAA's apparent antimicrobial effect is largely due to inducing a VBNC state rather than causing irreversible cell inactivation.

Table 3.2. *Campylobacter* concentrations (log_{10} CFU/mL; Mean ± SD) on chicken breasts immersed or sprayed with 500 ppm PAA for 10 s.

Post-treatment cold storage[a] (h)	Recovery Method	Untreated	Spray	Immersion
0	culturable	6.04 ± 0.14^{bcde}	5.53 ± 0.35^{ef}	5.23 ± 0.30^{f}
	viable	6.63 ± 0.35^{a}	6.43 ± 0.45^{ab}	6.37 ± 0.41^{abc}
1	culturable	6.00 ± 0.16^{bcde}	5.20 ± 0.36^{fg}	5.06 ± 0.37^{fg}
	viable	6.33 ± 0.26^{abcd}	5.96 ± 0.50^{bcde}	6.40 ± 0.63^{abc}
24	culturable	5.20 ± 0.50^{fg}	4.45 ± 0.58^{g}	4.42 ± 0.54^{g}
	viable	5.53 ± 0.39^{def}	5.50 ± 0.45^{ef}	5.61 ± 0.36^{cdef}

Values followed by different letters (a - g) are significantly different ($p < 0.05$), as determined by the Tukey's HSD test.

[a] Post-treatment cold storage refers to the time of samples storage at 4°C in aerobic conditions after completed PAA treatment. Samples were tested immediately after treatment (0 h), 1 h post-treatment (1 h), and 24 h post-treatment (24 h). The 1 h and 24 h post-treatment samples were stored at 4°C between treatment and analysis.

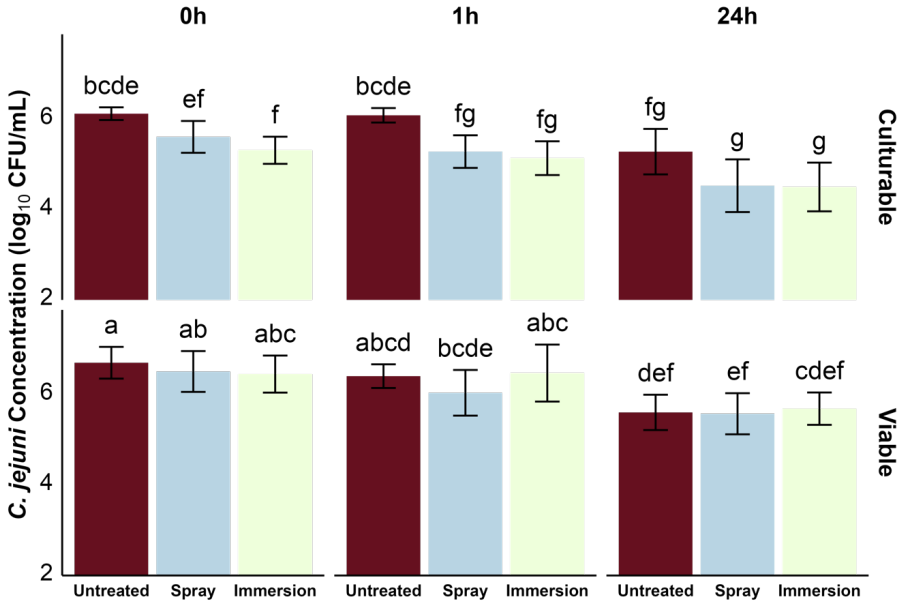

Figure 3.2. Viable and culturable *Campylobacter* concentrations (log_{10} CFU/mL) on chicken breast fillets either immersed in or sprayed with 500 ppm PAA for 10 s. Different significance letters (a-g) are significantly different by Tukey's HSD ($p < 0.05$).

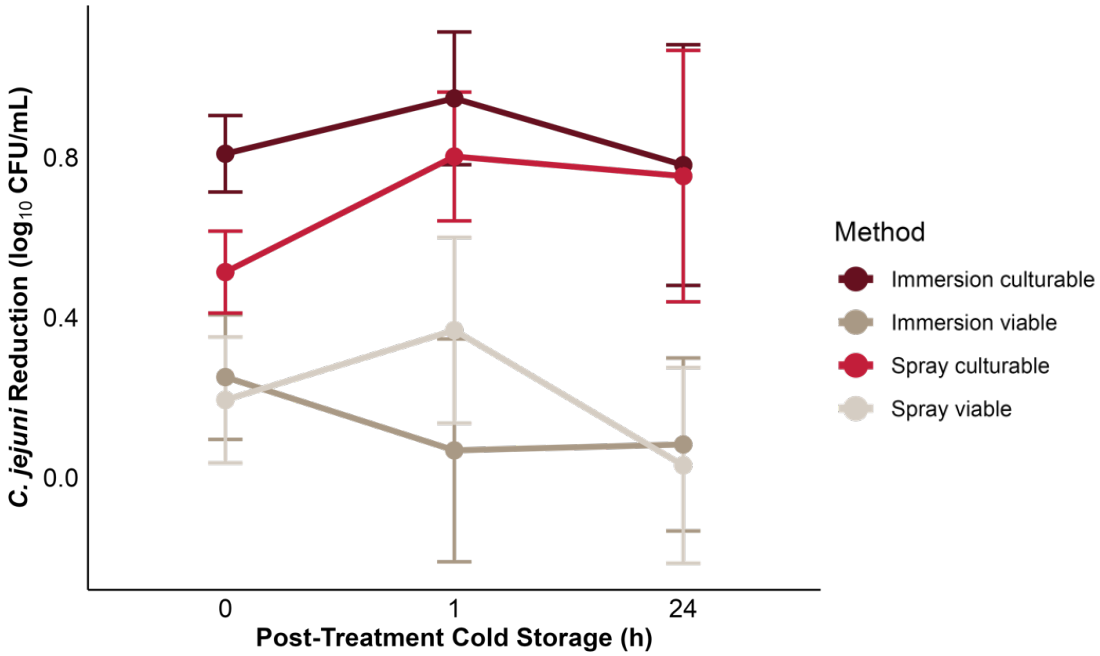

Figure 3.3. Viable and culturable *Campylobacter* reductions (log_{10} CFU/mL) due to PAA effect, after accounting for untreated control and rinsing control reductions.

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Chapter 4

Conclusions and Future Directions

The studies in this thesis provide insight into the prevalence of *Campylobacter* in retail chicken breasts collected between October 2023 and July 2024 and the antimicrobial efficacy of peroxyacetic acid against *Campylobacter* inoculated on chicken breasts.

Our findings show a low *Campylobacter* spp. prevalence of 1.9% on retail chicken breasts, which is consistent with previous studies that reported lower contamination prevalence in boneless, skinless cuts compared to skin-on chicken meat products. The negative results of testing chicken breast for *Campylobacter* using direct plating and viability qPCR suggest that the contamination levels in samples that tested positive by enrichment were low, given their 60 CFU and 3,200 – 5,000 cells per 325 g sample limit of detections, respectively. Notably, all isolated *Campylobacter* strains exhibited novel sequence types that could not be assigned to known clonal complexes, indicating their genetic distinctiveness compared to strains commonly isolated from chicken meat. A future study could sample meat cuts that are known to carry higher loads of *Campylobacter*, such as skin-on cuts, to be able to assess the discrepancy between viable and culturable *Campylobacter* on retail chicken. Studies aiming to decrease the limit of detection for quantitative detection methods to detect common low-level *Campylobacter* contamination would also be valuable for quantitative assessment of *Campylobacter* contamination on chicken meat.

In addition to *Campylobacter*, this study isolated *Acinetobacter baumannii*, *A. seifertii*, and *A. nosocomialis* from enrichments of eight samples. These species fall within the *Acinetobacter calcoaceticus-baumannii* complex, consisting primarily of opportunistic pathogens associated with nosocomial infections and increased antibiotic resistance. The presence of these species in retail chicken begs questions about their relatively understudied role in foodborne

transmission and requires further research into their prevalence, survival, and public health risks. In the future, methods for identifying and isolating *Acinetobacter* spp. from food sources such as chicken should be developed to determine their prevalence. Isolates from food sources could be further assessed by testing their antimicrobial resistance to clinically relevant antibiotics, determining their virulence potential, and investigating their transmission in the food systems. These steps would aid in assessing the potential role of *Acinetobacter* spp. as a foodborne pathogen.

In the third chapter, we studied the antimicrobial efficacy of PAA in *Campylobacter* and the limitations of culture-based methods in assessing antimicrobial efficacy. We found that a subpopulation of *Campylobacter* cells existed in a non-culturable state before treatment, as shown by higher recovery of viable compared to culturable cells in the inoculum. This discrepancy remained consistent after inoculation onto chicken and during 24 h storage at 4°C, showing that inoculation and cold storage did not induce additional cells into a VBNC state. However, while 24 h post-PAA-treatment cold storage at 4°C reduced both viable and culturable populations, the magnitude of reduction was greater for the untreated controls, indicating that oxidative and cold stress were more effective at killing *Campylobacter* after 24-hour post-treatment cold storage than the PAA treatment itself.

Immediately after PAA treatment, culturability was reduced significantly while viability was not, showing that it induced a subpopulation into a VBNC state rather than inducing cell death. We found that immersion and spray treatments had similar effects, in contrast to previous studies that found PAA immersion resulted in greater reductions of *Campylobacter*. During cold storage, PAA did not continue to induce VBNC or exert additional antimicrobial effects. This suggests that PAA's effect is primarily immediately upon application.

These findings have important implications for food safety, as gold-standard, culture-based methods may be overestimating PAA's antimicrobial effectiveness. Given the potential for

VBNC *Campylobacter* to regain infectivity, alternate quantitative detection methods would be beneficial for a more accurate assessment of risk associated with different levels of *Campylobacter* contamination, particularly in chicken parts that are known to carry higher loads of *Campylobacter* than chicken breast.

Future research should focus on the effects of PAA against natural contamination levels of *Campylobacter* on chicken meat, which would provide a more accurate assessment of PAA's antimicrobial efficacy. We hypothesize that because a subpopulation of native *Campylobacter* is likely already in a VBNC state due to processing stressors, they would be less susceptible to PAA treatment compared to a freshly cultured inoculum since they would have already undergone stress adaptations. In addition, to achieve a more comprehensive evaluation of antimicrobial interventions, we could incorporate viability-based detection methods such as viability qPCR into future challenge studies. This could prevent the overestimation of antimicrobial efficacy, provide accurate assessments of bacterial viability rather than just culturability, and provide better limits for food safety regulations. Lastly, while some research has focused on the resuscitation of VBNC *Campylobacter*, specifically through passage in embryonated eggs and mouse intestines, it is crucial to determine whether VBNC *Campylobacter* can regain viability and infectious potential in humans. *In vitro* human gut models could mimic passage through the gastrointestinal tract to identify whether VBNC resuscitation is possible in humans and to determine conditions that promote VBNC resuscitation.