

Evaluation of enumeration methods for *Campylobacter* and *Salmonella* in poultry

by

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(Under the Direction of Manpreet Singh)

ABSTRACT

Controlling *Salmonella* and *Campylobacter* in poultry requires robust quantification methods. This study compares enumeration methods in ground turkey, ground chicken, and chicken wings at varied inoculum levels (~8 log CFU/mL for high and ~5 log CFU/mL for low-level inoculation). *Salmonella* was quantified using conventional plating, miniaturized most probable number, and two PCR-based quantification assays. For *Campylobacter*, conventional plating, automated MPN, and PCR-based quantification assays were tested. ANOVA and Tukey's test revealed at high inoculation levels for *Salmonella*, mMPN resulted in the highest levels ($p \leq 0.05$), followed by PCR assay 1, plating, and PCR assay 2. At low levels, mMPN, PCR Assay 1 & 2 performed similarly ($p > 0.05$) with plating significantly lower. For *Campylobacter*, the plating was higher than automated MPN at high levels ($p \leq 0.05$), but PCR assay was significantly higher than both plating and automated MPN ($p < .0001$) at low levels. Traditional methods are reliable at higher contamination levels, whereas PCR-based precision is at low contamination levels.

KEYWORDS: *Salmonella*, *Campylobacter*, MPN, quantification, enumeration, prevalence

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DEDICATION

"To Guru Nanak and the eternal light of the Gurus, enshrined in Guru Granth Sahib Ji that holds the entire universe within. With gratitude for the divine wisdom and strength, and the words of Sant Maskeen Ji that carved my path, instilled patience and wisdom."

Whatever I am, whatever I will be, you will always remain my guiding force.

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CHAPTER 1

INTRODUCTION

Foodborne pathogens have been a significant public health challenge, causing over 48 million illnesses every year (Centers for Disease Control and Prevention [CDC], 2024), with *Salmonella* and *Campylobacter* being among the most common foodborne pathogens. *Campylobacter* is the leading cause of bacterial foodborne infections in the United States, with 11,926 reported cases resulting in 2,482 hospitalizations and 49 deaths. Following closely, *Salmonella* was reported in 8,454 cases, leading to 2,456 hospitalizations and 55 deaths. The incidence of *Campylobacter* has increased to 19% in 2022 compared to the 2016-2018 baseline, reaching 21.52 cases per 100,000 population; in the case of *Salmonella*, the incidence has remained relatively stable at 15.79 cases per 100,000 population. These pathogens significantly contribute to the overall burden of foodborne illnesses, with *Campylobacter* showing a concerning upward trend (U.S. Department of Agriculture[USDA], 2021). Poultry and poultry products have been recognized as significant reservoirs and sources of infection. According to the Centers for Disease Control and Prevention (CDC), over 1 million salmonellosis occur yearly due to *Salmonella*. Chicken and turkey are most associated among poultry, reporting 12,500 and around 43,000 foodborne *Salmonella* illnesses each year. With an increase in per capita consumption of poultry over time, the incidence and disease load of these pathogens has the potential to increase (Moller et al., 2022).

As per the United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) standards for *Campylobacter* in raw poultry products, the maximum acceptable percentage of positive tests for young chicken carcasses is 10.4% and 5.4% for turkey carcasses. Additionally, new regulations have been proposed by the USDA to reduce *Salmonella* in raw poultry products. The focus is on preventing contaminated products from entering the market. The proposal sets a limit of 10 colony-forming units (CFU)/mL or gram in the analytical portion for *Salmonella* in poultry products and targets specific serotypes of public health significance, such for raw comminuted chicken as Enteritidis, Typhimurium, and I,4,[5],12:I:-; and for raw comminuted turkey are Hadar, Typhimurium, and Muenchen. The proposed regulations also require poultry establishments to develop microbial monitoring programs to prevent contamination throughout the production chain (USDA, 2021).

The objective of Healthy People 2030 is to attain a 25% decrease in the national *Salmonella* illness rate, targeting no more than 11.5 cases per 100,000 population annually, compared to 15.3 cases per 100,000 population in 2016-2018. The FSIS has also adopted this objective to reduce *Salmonella* illnesses by 25% linked to FSIS-regulated products (USDA-FSIS, 2024). While there have been considerable efforts by regulatory agencies as well as implementation of stringent guidelines for poultry processing facilities, which have remarkably improved the control of foodborne pathogens like *Salmonella* and *Campylobacter*, complete control remains ever challenging (Ricke et al., 2019). One of the main reasons stems from the industry's heavy reliance on prevalence data for *Salmonella* and *Campylobacter* in poultry. This reliance on prevalence data rather than the number of pathogens in poultry products limits the ability to

make timely, data-driven decisions. As a result, it hampers efforts to prevent foodborne illnesses before the products are released into the market.

Numerous studies have established that although the prevalence of *Salmonella* can be low in some instances, it can still present a significant risk of causing illness (McEntire et al., 2014). This further reinstates the importance of quantification methods in addition to already employed prevalence methods to reduce the outbreaks and diseases related to poultry and poultry products (O'Bryan et al., 2022). Currently, the most employed enumeration methods are the most probable numbers (MPN) and traditional plating on selective and differential agars. Though these methods have been performing well and are relatively simpler, they are labor-intensive and take significant time to obtain results on bacterial load in the sample. Until then, the product is already in the market chain, posing a substantial public health risk. Though the MPN method can provide quantitative data, it is laborious and time-consuming, the requirement of a series of pre-enrichment and enrichment time periods, and the need for various types of selective media and optimum conditions for pathogens favorable growth -limits its nature of use in routine sampling and testing (Berghaus et al., 2013). Such limitations pose a challenge, especially in perishable food items like poultry, where time is the critical factor. These limitations highlight the need for assessing enumeration methods, which surpass the constraints posed by traditional culture-based methods and still generate precise and reliable data.

Rapid quantification methods like real-time PCR and automated most probable number (MPN) have significantly reduced the time compared to culture methods. With advancing technology, the limit of detection has been lowered, enabling the quantification of pathogens at lower levels and implementing corrective measures on a timely basis. Despite the numerous advantages

presented by rapid-quantification methods, there has been limited adoption of these on a large scale due to many reasons. The primary challenge mainly for the small-scale poultry processors is the large capital investment required by the need for advanced skilled labor as well to work with this technology. Another reason attributed to its low adoption is the limited amount of published data available that supports the accuracy as well as robustness of rapid quantification methods when compared to the widely adopted conventional culture-based methods.

This study was designed to investigate and compare traditional enumeration and automated rapid quantification methods for *Salmonella* and *Campylobacter* and compare their performance in poultry matrices (ground chicken, ground turkey, and chicken wing rinses). This study will provide a comprehensive understanding of the advantages and limitations of the methods and provide a robust comparison between conventional and rapid quantification methods.

Furthermore, it will aid in identifying dependable techniques for quantifying pathogen loads. The poultry industry can implement these methods to improve their data-driven decision-making in a timely manner.

REFERENCES

Centers for Disease Control and Prevention (CDC; 2018). *Estimates of foodborne illness in the United States*. Centers for Disease Control and Prevention. Available at <https://www.cdc.gov/foodborneburden/index.html>. Accessed on August 26, 2024

United States Department of Agriculture- Food Safety and Inspection Service (USDA-FSIS; 2021). FSIS Guidance Document for Controlling *Salmonella* and *Campylobacter* in Poultry (FSIS-GD-2021-0006, Issue. FSIS U.S. Department of Agriculture). Available at https://www.fsis.usda.gov/sites/default/files/media_file/2021-07/FSIS-GD-2021-0006.pdf. Accessed on August 26, 2024

Berghaus, R. D., Thayer, S. G., Law, B. F., Mild, R. M., Hofacre, C. L., & Singer, R. S. (2013). Enumeration of *Salmonella* and *Campylobacter* spp. in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. *Applied and environmental microbiology*, 79(13), 4106-4114.

McEntire, J., Acheson, D., Siemens, A., Eilert, S., & Robach, M. (2014). The Public Health Value of Reducing *Salmonella* Levels in Raw Meat and Poultry. *Food Protection Trends*, 34(6).

Moller, A., Leone, C., Kataria, J., Sidhu, G., Rama, E. N., Kroft, B., Thippareddi, H., & Singh, M. (2022). Effect of a carrageenan/chitosan coating with allyl isothiocyanate on microbial load in chicken breast. *LWT*, 161, 113397

O'Bryan, C. A., Ricke, S. C., & Marcy, J. A. (2022). Public health impact of *Salmonella* spp. on raw poultry: Current concepts and prospects in the United States. *Food Control*, 132, 108539

Ricke, S. C., Feye, K. M., Chaney, W. E., Shi, Z., Pavlidis, H., & Yang, Y. (2019). Developments in rapid detection methods for the detection of foodborne *Campylobacter* in the United States. *Frontiers in microbiology*, 9, 3280

CHAPTER 2

LITERATURE REVIEW

Impact of Foodborne Pathogens on Public Health

As reported by the Centers for Disease Control and Prevention (CDC), foodborne pathogens pose a significant public health challenge, causing 48 million illnesses (1 out of 6 Americans) and 3,000 deaths each year (CDC, 2024b). Foodborne illnesses can be acquired through various contamination routes, such as ingesting contaminated food and water, causing a wide range of symptoms, including varying degrees of fever and diarrheal illnesses, nausea, and vomiting. In many instances, these illnesses present symptoms resembling the common flu, leading to potential misinterpretation. The symptoms of these illnesses may remain dormant for weeks or even months, resulting in the under-recognition and misattribution of these conditions as foodborne illnesses. Especially in the case of high-risk populations, such as young, elderly, immunocompromised, and pregnant individuals, are particularly susceptible, with the potential for severe outcomes, prolonged illnesses, and neurological disorders (USDA, 2024). Despite ongoing efforts and the implementation of stringent regulatory measures, ensuring food safety for public consumption remains an increasingly intricate challenge.

Salmonella and *Campylobacter* are amongst the most common foodborne pathogens, posing significant risks to public health. Recent data from the Foodborne Diseases Active Surveillance Network (FoodNet) for 2023 indicates that *Campylobacter* is the leading cause of bacterial foodborne infections in the United States, with an estimated 11,926 reported cases, resulting in 2,482 hospitalizations and 49 deaths. Following closely, *Salmonella* was reported in 8,454 cases, leading to 2,456 hospitalizations and 55 deaths (USDA, 2021). The incidence rate of *Campylobacter* has increased by 19% compared to the 2016-2018 baseline, reaching 21.52 cases per 100,000 population, while the incidence of *Salmonella* has remained relatively stable at 15.79 cases per 100,000 population. These pathogens significantly contribute to the overall burden of foodborne illnesses, with *Campylobacter* showing a concerning upward trend (USDA, 2021). Poultry products, like turkey and chicken, are significant sources of *Salmonella* and *Campylobacter* infections. According to CDC, over 1 million human *Salmonella* infections occur yearly in the United States, with poultry being a leading source. Despite efforts to reduce contamination, these pathogens remain common in poultry, posing a continuing threat to public health. The USDA estimates approximately 125,000 chicken-associated and nearly 43,000 turkey-associated foodborne *Salmonella* illnesses annually. Similar economic impacts are likely for *Campylobacter*, including substantial healthcare costs, productivity losses, and effects on the poultry industry, such as recalls, increased regulatory scrutiny, and potential loss of consumer confidence (USDA, 2021). The USDA Food Safety and Inspection Service (USDA-FSIS) has established performance standards for *Campylobacter* in raw poultry products to address the ongoing threat of these pathogens. The maximum acceptable percentage of positive tests for young chicken carcasses is 10.4%, and for turkey carcasses, it is 5.4%. Also, the USDA

proposed new policies to reduce *Salmonella* in raw poultry products, focusing on preventing contaminated products from entering commerce. The proposal sets a limit of 10 colony-forming units (CFU)/ mL or g in the analytical portion for *Salmonella* in poultry products and targets specific serotypes of public health significance. The proposed *Salmonella* serotypes are Enteritidis, Typhimurium, and I,4,[5],12:I:- for chicken and for turkey are Hadar, Typhimurium, and Muenchen. The proposed regulations also require poultry establishments to develop microbial monitoring programs to prevent contamination throughout the production chain (USDA, 2021). The objective of Healthy People 2030 is to achieve a 25% reduction in the national case rate of *Salmonella* illness, aiming for no more than 11.5 cases per 100,000 population per year, compared to 15.3 cases per 100,000 population in 2016-2018. This reduction is necessary to meet the 2030 target. Additionally, the FSIS has also embraced this goal for foodborne illnesses linked to FSIS-regulated products to reduce *Salmonella* illnesses by 25% (USDA, 2024). Understanding the impact of foodborne pathogens such as *Salmonella* and *Campylobacter* on public health is crucial to establishing a national food safety system based on risk. These pathogens are mostly found in animal reservoirs (zoonotic diseases), environmental sources where they can survive in soil, water, or surfaces that can serve as potential sources of infection, or poultry house environments that serve as significant sources of pathogen transmission and infection. Humans often come in contact directly with infected animals or indirectly through vectors like insects, contaminated food, consumption of raw, undercooked meat, unpasteurized dairy products, contaminated produce, and water.

Salmonella

Pathogen Overview

Salmonella is a motile, non-spore-forming, gram-negative, rod-shaped bacterium in the Enterobacteriaceae family. *Salmonella*, previously known as *Bacillus choleraesuis*, was initially isolated in 1885 by Theobald Smith and Daniel Elmer from pigs infected with classical swine fever (Eng et al., 2015; Lewis, 2019). The current nomenclature system recommended by the World Health Organization (WHO) for research on *Salmonella* follows the Kauffmann-White scheme, supplement 2001 (no. 45). According to this system, the genus *Salmonella* consists of two species, *Salmonella enterica*, and *Salmonella bongori*, identified based on differences in 16S rRNA sequence analysis. These two species are divided into six subspecies based on biochemical and phylogenetic properties. *S. enterica* subsp. *enterica* is the most associated with human and animal infections within these species. Additionally, *Salmonella* is further classified into over 2600 serotypes based on the Kaufmann-White typing scheme, first published in 1934, which differentiates *Salmonella* strains by their surface and flagellar antigenic properties (U.S. Food and Drug Administration [FDA], 2022). The antigens on the bacterial cell surface used for differentiation include O, H, and Vi, which correspond to somatic or outer membrane antigens, flagellar antigens, and capsular antigens (Guibourdenche et al., 2010). *Salmonella* genus exhibits various metabolic traits, including its ability to reduce nitrates to nitrites, ferment glucose, and produce mannitol, sorbitol, catalase, and hydrogen sulfide (H₂S). Although it cannot produce oxidase, urease, and indole, it is unable to ferment lactose and sucrose (Sanderson & Nair, 2013). Further, these metabolic traits help us to identify and distinguish presumptive *Salmonellae* through a series of biochemical tests (Cosby et al., 2015). *Salmonella* has wide adaptability to varying temperature ranges, pH, water activity, and oxygen concentration that allows the pathogen to survive. The ideal temperature for *Salmonella* growth

is 35-40°C, although the pathogen can grow at temperatures between 2° and 54°C. The optimal water activity (aw) and pH for the growth of the pathogen are 0.94-0.99 and 6.5-7.5, respectively (Cosby et al., 2015).

Pathogenicity & Virulence

Salmonella species are highly adaptable and known for their ability to invade host cells, survive in hostile environments, and cause significant illness. The pathogen has the ability to invade, survive, and cause infection in its host and depends on the bacteria and host factors, primarily via the Type III Secretion System (T3SS), which acts like a needle-like structure and helps transfer bacterial effector proteins into the host cell. These effector proteins, such as SipA, SipB, SipC, and others, manipulate the host cell's cytoskeletal components and signaling pathways, leading to changes in the structure of the host cell, allowing the entry of bacterium inside the cell (Ibarra & Steele-Mortimer, 2009). The genes responsible for producing virulence factors in *Salmonella* are located on *Salmonella* Pathogenicity Islands (SPIs), amongst which SPI-1 and SPI-2 enable *Salmonella* to invade and survive in the host cells (Van Der Heijden & Finlay, 2012). In addition to that, the usage of fimbriae and flagella for attachment further aids the bacterium's entry through the epithelial barrier (Sun et al., 2007). Once the bacterium enters host cells, the specialized vacuole in *Salmonella*, called the *Salmonella*-Containing Vacuole (SCV), aids *Salmonella* in surviving and replicating in the host cell. In this environment, the bacterium employs effector proteins to prevent apoptosis and lysosomal fusion, promoting survival and replication (LaRock et al., 2015). *Salmonella* increases its survival rate inside the host cells by creating siderophores like *enterobactin* and *salmochelin* and by triggering the production of MgtC protein in response to low magnesium levels (Blanc-Potard & Groisman, 1997; Müller et

al., 2009). Additionally, *Salmonella* can form biofilms and become persistent, making it resistant to antibiotic treatments and potentially causing recurrent infections (Simm et al., 2014).

The development of *Salmonella* infections starts with consuming contaminated food or water.

After surviving in the environment, the bacteria move to the epithelium attached to the intestinal epithelia, causing. This causes inflammation and spreads throughout the body in cases of severe infection. (LaRock et al., 2015; Ibarra & Steele-Mortimer, 2009). *Salmonella* causes two significant illnesses, which are classified as nontyphoidal salmonellosis and typhoid fever.

Nontyphoidal salmonellosis is caused by types of *Salmonella* other than *S. Typhi* and *S.*

Paratyphi, usually causing temporary gastrointestinal symptoms. However, it can result in severe complications like bloodstream infections, especially in vulnerable populations. Typhoid fever, caused by *S. Typhi* and *S. Paratyphi* A, is a more severe illness affecting the whole body with a higher risk of death if not treated (Miller, 2000). Prolonged fever, gastrointestinal symptoms, and potential complications such as bloodstream infection and long-term gallbladder infection are typical characteristics of salmonellosis. *Salmonella virulence* is a complex process that involves various strategies, a Type III Secretion System (T3SS), and various effector proteins such as SipA and SipB, which play an important role in host-cell invasion and manipulation, as discussed above. Pathogens must penetrate host cells, acquire essential nutrients for survival, evade host immune responses, and establish an infection within the host. This makes it a significant pathogen of concern in the context of food safety.

Poultry as a Reservoir:

Salmonella contamination in poultry occurs through a complex interconnection of routes, forming a multifaceted network like cross-contamination occurring from intestinal contents of

infected birds during processing, handling of birds during slaughter, and personnel in poultry processing facilities (Cano et al., 2021). The predominant sources of *Salmonella* contamination within the poultry production process are the hatchery (48.5%), litter (25.4%), feces (16.3%), internal poultry house environment (7.9%), external poultry house environment (4.7%), feed (4.8%), chicks (4.7%), and drinker water, listed in order of prevalence (Wang et al., 2023). The prevalence may also vary based on product type, processing stage, and geographical location; for instance, 44.7% of chicken breast meat and 41% of drumsticks collected from various poultry establishments reported the prevalence of *Salmonella*. Berghaus et al. (2013) and Guran et al. (2017) further reported *Salmonella* concentrations of 2.57 and 2.32 log MPN/carcass for carcass rinses at pre-chill and post-chill stages of poultry processing, respectively. Comminuted poultry products, including mechanically separated chicken and turkey, tend to have higher prevalence rates of *Salmonella* than whole cuts. Similarly, a study by Mazengia et al. (2014) reported that out of 93 retail samples of chicken breasts, thighs, drumsticks, chicken wings, and ground chicken, 94% had *Salmonella* levels of less than 30 MPN/100g, and 6% had levels ranging between 30 and 240 MPN/100g. Chicken wings are another product susceptible to *Salmonella* contamination. It was reported that 94.6% of chicken liver samples had *Salmonella* levels ranging from 0.3 to 30 MPN/g, while 5.4% had 30 and 110 MPN/g levels (Jung et al., 2019). The prevalence of *Salmonella* also dramatically varies and depends on the processing chain. Many microbes, including *Salmonella*, can be released into the scald water from the birds' feathers and involuntary defecations during scalding and plucking. While elevated temperatures used in scalding can reduce microbial load, they may not eliminate bacteria attached to the skin (Hafez et al., 1997; James et al., 1992). The evisceration stage involves a series of automated equipment,

which can be another critical point for cross-contamination, as intestinal contents can leak onto the carcass and other surfaces, thus spreading *Salmonella* (Sarlin et al., 1998). Chilling broiler carcasses to $\leq 4^{\circ}\text{C}$ is intended to prevent the growth of *Salmonella*; however, using immersion chilling can increase the risk of cross-contamination as carcasses come in contact with each other in the chill tank, leading to flock-to-front transmission of *Salmonella* (Lillard, 1980; Sarlin et al., 1998)

Additionally, *Salmonella* can survive on refrigerated surfaces, posing a cross-contamination risk. For instance, *Salmonella* can detach from chicken skin and readily transfer to cutting boards when acid-treated (Jiménez et al., 2009). The moisture content of meat also plays a role in the attachment and transfer of *Salmonella* from carcasses to other surfaces when in fresh carcasses (De Boer & Hahné, 1990; Dickson, 1990).

Campylobacter

Pathogen Overview

Campylobacter spp. are gram-negative, slender, spiral rods characterized by a distinctive corkscrew-like darting motion (Silva et al., 2011). Its' unique growth conditions distinguish it from other foodborne bacterial pathogens, imposing specific limitations on the variety of food environments in which they can increase. *Campylobacter* requires special microaerophilic conditions to survive, and a very narrow temperature range between 37°C and 42°C presents an optimum temperature required for its growth and proliferation, although it can also survive at 7°C and perform vital cellular functions (Hazeleger et al., 1998). Theodor Escherich first documented the spiral-shaped bacteria in 1886 and named it *Cholera infantum* (Kist, 1986), isolating them from the stools of children suffering from diarrhea. The genus *Campylobacter* was

officially established in 1963 by Sebald and Véron based on its unique features, including a low DNA base composition, non-fermentative metabolism, and microaerophilic growth nature, distinguishing it from the original *Vibrio* spp. (Sebald & Veron, 1963).

The *Campylobacter* genus comprises at least 39 species and 16 subspecies, with 12 species recognized as pathogenic (Bhunia, 2018). The most notable pathogenic species include *C. jejuni*, *C. coli*, and *C. lari*—all of which are significant to public health and food safety due to their involvement in foodborne and zoonotic outbreaks (Bolton, 2015). These species are commensal pathogens in the gastrointestinal tracts (GIT) of wild and domestic animals, especially poultry. *C. jejuni* subsp. *Jejune* and *C. coli* are particularly noteworthy for causing a significant burden of gastrointestinal infections worldwide (Ammar et al., 2021). The infectious dose required to cause disease can be as low as 500 ingested cells, though the typical infectious dose is thought to be around 10,000 cells (Janssen et al., 2008). This variation in contagious dose can be attributed to factors such as the type of contaminated food consumed and the general health of the exposed individual (FDA, 2022). The growth conditions required for *Campylobacter* are unique compared to other foodborne bacterial pathogens, which imposes special restrictions on the variety of food conditions in which the species can proliferate. It is a commensal organism in various animal hosts like chicken. It shows the ability to multiply in the gastrointestinal tract of humans and utilizes the nutritional sources available in the host intestine (Hofreuter, 2014). Being a microaerophilic organism, it faces challenging conditions to proliferate in the host intestine, where the oxygen tension is lower than 5%. To overcome this, *Campylobacter* preferentially colonizes the mucus layer and the intestinal crypt near the epithelium, where oxygen tension is comparatively higher (Beery et al., 1988). Also, it uses a variety of electron

donors and acceptors besides oxygen, like nitrate, and obtains nitrogen from amino acids, peptides, and other nitrogen-containing compounds in the gut lumen (Sellars et al., 2002). The bacteria are chemoorganotrophic in nature, i.e., they utilize amino acids, or the tricarboxylic acid cycle intermediates as their primary energy source rather than carbohydrates like other foodborne pathogens (Garcia-Sanchez et al., 2018). Other metabolic traits of the pathogen include its ability to reduce nitrate, fumarate to succinate, oxidase-positive (except for *C. gracilis*), hydrolyze hippurate and indoxyl acetate, and inability to ferment and oxidize glucose or other carbohydrates. These metabolic traits are employed while performing different biochemical tests, such as hippurate hydrolysis, to differentiate between various species of *Campylobacter* (Debruyne et al., 2008).

Pathogenicity & Virulence

Campylobacter species colonized the gastrointestinal tract of the host, using various virulence factors to invade, survive, and cause disease. *Campylobacter* spp. possess a range of virulence factors, such as flagella-mediated motility, bacterial adherence to the intestinal mucosa, invasive capability, and toxin production, all of which significantly contribute to their pathogenesis and the onset of gastroenteritis (Dasti et al., 2010). The flagellum is essential for motility of the bacteria, allowing them to colonize and thrive within the epithelial cells of the host's gastrointestinal tract (GIT). In *C. coli*, the flagella consists of two main components encoded by FlaA and FlaB: flagellin A and B (Guerry et al., 1996). FlaA plays an essential role in invading the epithelial cells, followed by adherence and colonization of the gastrointestinal tract by producing invasion antigens (Cia) (Wassenaar et al., 1991). *Campylobacter* exhibits unique motility, particularly in adverse environments, due to its polar flagella and helical shape, which

facilitate corkscrew rotation (Ferrero & Lee, 1988). Chemotaxis, the process of motile bacteria moving toward favorable conditions, plays a crucial role in *Campylobacter* colonization. In poultry, *C. jejuni* uses chemotaxis to locate mucus-filled crypts in the ceca, which serve as primary colonization sites (Chang & Miller, 2006). Within 24 h of ingestion, *C. jejuni* can colonize and proliferate in the cecum (Coward et al., 2008; Smith, 2013).

After crossing the mucosal layer, *Campylobacter* spp. adhere to epithelial cells using outer membrane proteins such as CadF, which control adhesion by binding to fibronectin in the intestinal tract. This binding leads to the internalization of the bacterial cell through actin-mediated phagocytosis (Hofreuter et al., 2006). Once internalized, *Campylobacter* secretes cytolethal distending toxins (CDTs) encoded by the *cdtABC* operon. The *cdtA* and *cdtC* genes regulate toxin binding to the cell membrane, while *cdtB* encodes the functional unit that induces DNA damage by breaking double-stranded DNA, leading to apoptotic cell death (González-Hein et al., 2013). Additionally, *Campylobacter* produces other toxins, such as hepatotoxin and pore-forming hemolysin, which disrupt host protein production (Bhunia, 2018). *Campylobacter* spp. also possesses lipooligosaccharides (LOS), which protect the pathogen against the immune response. Sialylation of LOS can trigger an autoimmune response, producing antibodies that cause demyelination of nerve cells, potentially resulting in Guillain-Barré Syndrome (GBS) and Miller-Fischer Syndrome (MFS) (Louwen et al., 2008). The autoimmune response damages the peripheral nerves, blocking nerve impulses and causing paralysis (Young et al., 2007). Outside the natural reservoir, *Campylobacter* can transform into a nonculturable, coccoid form known as VBNC (viable but non-culturable). This form has an altered metabolism and can survive in hostile environments for extended periods, such as in water or under low-temperature conditions

(Rollins & Colwell, 1986). VBNC *Campylobacter* remains viable for up to seven months and can be re-cultured after recovery from this state, although some studies report variability in recovery success (Lázaro et al., 1999; Ziprin et al., 2003).

Poultry as a Reservoir

The consumption of poultry meat contaminated with *Campylobacter* spp. has been linked to many human campylobacteriosis cases. This can pose a risk when handling raw poultry during processing, further processing, or at home. In a survey conducted by USDA in 2023, *Campylobacter* was present in 16% of whole chicken carcasses, 16% of chicken parts, and 3% of comminuted chicken products (USDA-FSIS, 2023). In a study conducted from 2005-2011, *Campylobacter* spp. was isolated from 41% of retail broiler meat samples, including chicken breast, tenderloins, and thighs (Williams & Oyarzabal, 2012). Additionally, a prevalence study found that 54% of chicken livers collected from retail establishments in the Southeastern United States were contaminated with *Campylobacter*, indicating that chicken livers could be a potential source of *Campylobacter* infections in humans if mishandled or consumed undercooked (Berrang et al., 2018). Prevalence varied by processing stage, with 71.1% at post-pick, 64.4% at pre-chill, and a significant reduction to 1.1% post-chill (Thames et al., 2022). Another 2022 study found an overall *Campylobacter* prevalence of 25.4% in 414 poultry samples, with retail chicken samples showing a significantly higher prevalence (36.3%) compared to farm samples (18.5%) (Poudel et al., 2022). A meta-analysis of studies in South Korea revealed that duck meat had the highest *Campylobacter* prevalence at 70.46%, followed by chicken meat at 36.17% (Choi et al., 2023; Je et al., 2023). In Italy, a survey of 1,243 chicken meat samples found a *Campylobacter* prevalence of 17.38%, with higher contamination in portions with skin (21.80%)

compared to skinless portions (13.53%) (Di Giannatale et al., 2019). These studies consistently show that *Campylobacter* contamination is common in poultry products, with prevalence rates ranging from 15-70% depending on the product type and processing stage. The consumption of poultry meat contaminated with *Campylobacter* spp. has been linked to many human campylobacteriosis cases.

The increasing popularity of broiler meat consumption as an animal-based source of protein in the United States has led to increased production and, after that, caused a rising number of illnesses. *Campylobacter* is the leading cause of bacterial diarrheal illness in the US, causing more than 1.5 illnesses yearly (CDC, 2024a). As reported by the CDC, most infections occur due to the consumption of raw or undercooked poultry or cross-contamination during food handling and preparation. Zoonotic *Campylobacter* species naturally inhabit poultry, and *Campylobacter*, in particular, frequently colonizes the gut of broiler chickens. As a result, chicken meat products are believed to be the primary cause of campylobacteriosis in humans (Hermans et al., 2011).

According to estimates, broiler meat handling, preparation, and consumption account for 20 to 30% of human cases of campylobacteriosis, while the chicken reservoir may be responsible for 50 to 80% of *Campylobacter* infections in humans (European Food Safety Authority; [EFSA], 2013). Unique growth conditions required for *Campylobacter* growth limit the range of food environments in which species can thrive. *C. jejuni* is a commensal organism in a variety of animal hosts, particularly chickens. It can be commonly found in diverse environmental sources or refrigerated food products such as raw or undercooked poultry. It exhibits fastidious growth characteristics in vitro, and the atmospheric oxygen content and temperature highly impact its viability. It shows the ability to multiply in the GIT of humans and utilizes nutritional sources

available in the host's intestine (Hofreuter, 2014). Poultry is a primary source of *Campylobacter*, with the ceca being the primary site of colonization, where bacterial populations can reach 6 to 8 log colony-forming units (CFU) per g (Meade et al., 2009). In humans, the infection primarily occurs in the small intestine. Research has shown that passage through poultry increases the ability of *Campylobacter* to colonize and become more virulent in humans (Stern et al., 1992; Cawthraw et al., 1996).

Campylobacteriosis is caused by *C. jejuni* (75%), followed by *C. coli* (10%), and mixed infections of *C. coli/jejuni* (14%) (RKI, 2018). The infection usually has an incubation period of 2 to 5 days and starts with early symptoms like fever, headache, and muscle pain. This is then followed by acute enterocolitis, which is marked by watery and sometimes bloody diarrhea, cramp-like abdominal pain, and general malaise. Most cases resolve independently within 5 to 7 days, but severe cases can occur in YOPI (children, older adults, and people with weakened immune systems) (CDC, 2024a). In some instances, *Campylobacter* infections can result in post-infectious complications such as irritable bowel syndrome, reactive arthritis, and autoimmune diseases like GBS (Guillain-Barré syndrome) and MFS (Miller Fisher syndrome). GBS is a severe neurological condition that can cause paralysis, with a mortality rate of 2 to 3%, primarily due to respiratory failure (Molnar et al., 1982). These findings emphasize the continuous public health challenge presented by *Campylobacter* in poultry.

Need for *Salmonella* and *Campylobacter* Quantification and Rapid Enumeration Methods in the Poultry Industry

Methods for quantifying pathogens are essential for the poultry industry. Various traditional methods for pathogen testing have been used and employed. However, rapid, efficient methods

are necessary to maintain food safety standards when it comes to large sample size testing and shorter time frames to safeguard public health at large.

Prevalence methods only determine the presence or absence of pathogens. However, on the contrary, quantitative methods allow us to go one step further and determine the pathogen load, which is critical when making decisions for product disposition, public health, and safety standards for food products (Oscar, 2021). Numerous studies have established that although the prevalence of pathogens like *Salmonella* can be low in some instances, it still presents a significant risk of causing illness (McEntire et al., 2014). This highlights the importance of quantification methods in addition to already employed prevalence methods to reduce the outbreaks related to poultry and poultry products (O'Bryan et al., 2022). Historically, the poultry industry has heavily relied on most probable numbers (MPN) and traditional plating for determining the bacterial load in samples. Though these methods have been performing well, they are labor-intensive and take considerable time to give results. Until then, the product is already in the market chain, posing a substantial public health risk. Though the MPN method can provide quantitative data, it is laborious and time-consuming, thus limiting its use in routine sampling and testing (Berghaus et al., 2013).

With advancements in technology and molecular biology, there is an excellent development in rapid and accurate pathogen quantification methods. In the modern-day poultry industry, there has been an impetus for speed and efficiency from production to pathogen detection and control. With stringent food safety regulations, need for compliance, and consumer food safety, there is a need for reliable detection. Various methods like quantitative PCR (qPCR) have significantly impacted and improved pathogen detection by reducing the time limit providing results within a

few hours compared to traditional methods, which can take several days. The qPCR methods can quantify target genes in under two hours, delivering high accuracy and sensitivity, while traditional methods, which are known for their precision and ability to detect a wide spectrum of pathogens, cannot be entirely replaced.

Rapid quantification methods decrease the time between sample collection and result evaluation, enabling faster, data-driven decision-making. These methods have shortened or eliminated enrichment times, which aids in quick detection. This especially helps in the case of low-level contamination, which can pose a significant food safety risk if it is undetected. Although the initial investment in setting up these advanced techniques is higher overall, it results in significant cost savings, reduced labor costs, material costs, fewer product recalls, and reduced production downtime.

Adoption of rapid testing methods in the poultry industry not only helps in the reduction of detection time. Large datasets collected over time can be utilized by poultry processors to identify patterns in pathogen levels and risk factors, enabling them to uptake targeted preventive strategies and identify the critical control points (Franzo et al., 2023). However, most of the rapid methods still require validation and certification by organizations such as the International Association of Analytical Communities (AOAC); thus, to be considered effective, the new technology needs to be accurately validated for its speed, accuracy, and user-friendliness (Park et al., 2014). Additionally, adhering to performance standards set by regulatory agencies like United States Department of Agriculture- Food Safety and Inspection Service (USDA-FSIS), it is essential for the industry to adopt validated rapid measurement methods to ensure compliance and maintain safer food supply systems.

Conventional Plating

The direct plating method is the most common method employed for enumerating pathogens like *Campylobacter* and *Salmonella*, which involves direct inoculation onto the selective agar plates, which are further incubated at desired optimum temperature ranges. The presumptive positives are calculated as colony-forming units (CFUs) (Malorny et al., 2008). Various selective media are employed with specific addition of antibiotics to inhibit the growth of non-target microorganisms, making it easier to isolate and enumerate; they also sometimes contain differential indicators that can help us differentiate between the target bacteria and the background microflora, e.g., in XLT4, *Salmonella* colonies appear black due to hydrogen sulfite production as compared to non-*Salmonella* colonies and in reducing false positives (Miller et al., 1991).

Various media are used to enumerate *Salmonella* from food matrices. The most used laboratory media is Xylose Lactose Tergitol-4 (XLT4) agar, a selective medium containing sodium tetradecyl sulfate to inhibit background microflora. It also includes phenol red as a pH indicator, which changes color in response to the fermentation of xylose, lactose, sucrose, and lysine decarboxylation. *Salmonella* colonies appear black because they can reduce sodium thiosulfate to hydrogen sulfide (Miller et al., 1991). However, it is essential to note that atypical *Salmonella* strains that do not produce hydrogen sulfide may not be easily detected, necessitating additional differential media.

Similarly, Xylose-Lysine Deoxycholate (XLD) agar helps identify *Salmonella* through Xylose fermentation, decarboxylation of lysine, and hydrogen sulfide production. Incorporating supplements like tergitol, novobiocin, and cefsulodin also enhances the recovery of the targeted

pathogens. Brilliant green sulfa (BGS) is another employed selective differential medium that helps isolate *Salmonella*. It has a brilliant green dye that inhibits the growth of gram-positive bacteria, and some gram-negative species, such as *Shigella* and typical *Salmonella* colonies, appear pinkish-white or opaque, surrounded by a red halo (Line & Bailey, 2006). Similarly, R & F® *Salmonella* (Nontyphoidal) chromogenic plating medium isolates and identifies nontyphoidal *Salmonella* species. It incorporates 2-deoxy-D-ribose sugar, specifically metabolized by *Salmonella*, along with chromogenic substrates for visual differentiation and selective agents to impede non-*Salmonella* bacteria. Presumptive positive *Salmonella* colonies are exhibited as reddish-pink raised colonies, measuring 1.0-3.0 mm in diameter with a colorless ring after 20-24 h at 35°C. Only *Salmonella bongori* displays as a dark-blue colony, while one strain of *Escherichia coli* manifests as a red-pink raised color colony measuring 2.0 mm in diameter. (U.S. Department of Agriculture, 2021). In the case of *Campylobacter*, the direct plating method is endorsed by the USDA as a rapid alternative to the Most Probable Number (MPN) method, with an incubation temperature of 42°C in a microaerophilic atmosphere optimized for the growth of thermophilic *Campylobacter* (USDA, 2021). Several agars are employed for *Campylobacter* enumeration. Campy-cefex agar, being the most widely used, which is supplemented with 5% lysed horse blood, cefoperazone, and cycloheximide, is widely suggested by the USDA for *Campylobacter* enumeration from poultry carcass rinses. The lysed horse blood neutralizes toxic compounds produced in the presence of oxygen, enhancing the recovery of *Campylobacter* spp., while the selective agents inhibit the growth of fungi and other competing microflora (Line, 2001). Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) is also another selective agar used to enumerate *Campylobacter*. It is selective but non-differential,

which many times may lead to swarming of *Campylobacter* colonies, which can further lead to false counts. It also sometimes supports the growth of non-*Campylobacter* organisms like extended-spectrum β - β -lactamase (ESBL)-producing *Escherichia coli*, which further challenges accurate *Campylobacter* isolation (Andritsos et al., 2020; Andritsos et al., 2020). CHROMagar *Campylobacter* (CAC) is a selective and differential medium that allows the growth of *C. jejuni*, *C. coli*, and *C. lari*. It is blood-free and contains a chromogen that facilitates the development of purple colonies, inhibiting most non-*Campylobacter* organisms and improving enumeration accuracy (Sylte et al., 2018). Karmali agar is another blood-free selective media used for the enumeration of *Campylobacter* species, specifically *C. coli* and *C. jejuni*. This agar contains a nutrient base with selective agents like vancomycin and amphotericin B and antibiotics like potassium clavulanate for improved selectivity. *C. jejuni* colonies can be identified as grey, moist, and flat (Oyarzabal, 2005). Another comparatively newer selective media is Campy-line agar (CLA) which can be employed both as blood-free and with blood medium; it has a similar nutrient base as Campy-cefex with the addition of Triphenyl tetrazolium chloride (TTC) as it reduces the colorless tetrazolium salts to insoluble formazan compounds which further impart colors to the colonies at 200 mg/L and *Campylobacter* colonies to appear deep-red magenta. The deep-red colonies on a translucent background facilitate *Campylobacter* isolation and enumeration (Line, 2001). In the case of *Campylobacter*, various antibiotics are supplemented in the media to differentiate the growth of *Campylobacter* colonies from non - *Campylobacter* colonies because *Campylobacter* is a biochemically inert microorganism and cannot ferment common sugars like many other bacteria (Kiggins and Plastringe, 1958). As a result, traditional

chemical indicators used in the case of other bacteria are not effective for *Campylobacter* identification.

Streak plating is initially used to isolate pure cultures of the targeted pathogen by streaking the diluted sample across the selective agar surface. Further, spread plating is utilized for uniform bacterial colony distribution. Lower pathogen count samples may require increased plating volumes like 250 μ l in contrast to 100 μ l for improved detection range. Although conventional plating is cost-effective, it requires labor-intensive agar plate preparation and further dilution and plating followed by counting colonies after incubation. Traditional plating may not recover damaged/ injured bacteria or viable but nonculturable (VBNC) cells, potentially underestimating actual pathogen levels. Selective agar use with limited shelf-life and reduced selectivity upon prolonged storage can impact enumeration precision.

Most Probable Number (MPN)

McCrary (1915) first introduced a method that involved using Poisson distribution to count the number of organisms present in each unit of analyte. Traditionally, the most probable number (MPN) technique is employed to estimate bacterial populations when enumerating low levels of bacteria. In this method, triplicate or five replicates tubes are inoculated with a sample and then further diluted and subjected to an enrichment process, where primary enrichment is performed in non-selective media like Buffered Peptone Water (BPW) and followed by secondary enrichment in selective media like Rappaport-Vassiliadis (RV) or Tetrathionate (TT) broth for *Salmonella* (Mion et al., 2016). The presumptive positives are confirmed by plating on selective agars like Xylose Lysine Deoxycholate (XLD) (Wang et al., 2014). The MPN value is calculated based on the number of positive tubes in the dilution series, and the results are reported to MPN

per mL. MPN testing procedures are complex, demanding, and time-consuming. To address this, numerous studies have been conducted to reduce the sample volumes required for MPN tests while maintaining their sensitivity and specificity. A miniaturized version called mMPN is used, which retains the statistical foundation but reduces the material usage, labor, and time. The 96-well microplates methods is used, which allows for easy replication and efficient enumeration of pathogen. This also follows a two-step enrichment process, with primary enrichment in non-selective media like buffered peptone water (BPW) followed by secondary enrichment with transfer of 100 μ L enriched sample in selective media like modified semisolid Rappaport–Vassiliadis supplemented with novobiocin for selective enrichment (Colla et al., 2014). White edges on cluster tubes indicate presumptive positives on the tubes. This further undergoes confirmation step by streaking on selective chromogenic media like R & F® *Salmonella* (Nontyphoidal) plating medium in the case of *Salmonella*. The MPN method for *Campylobacter* involves a two-step process, which includes multiple dilutions and enrichment steps. The modified version of Blood-Free Bolton Broth (BFBB) supplemented with selective agents like sulfamethoxazole is used to help in pathogen recovery (Chenu et al., 2013). The miniaturized version of MPN (mMPN) has numerous benefits, such as being cost-effective, less labor-intensive, and easier to replicate whilst maintaining the accuracy of the traditional MPN.

Automated MPN

The BioMérieux TEMPO® system is a rapid enumeration tool based on an automated most probable number (MPN) principle used for the enumeration of *Campylobacter* and other indicator organisms (Owen et al., 2010). The system comprises a Tempo® MPN card specific to each microorganism, which is automatically filled using the Tempo® Filler. It involves utilizing

specific testing cards based on the pathogen being enumerated and contains a fluorescent growth transducer within each selective culture media, which helps interpret results as colony-forming units (CFU) per mL (Cayer et al., 2020). The MPN card is filled with a pre-prepared dilution of the food sample along with the reconstituted media in the glass vial. It uses cards with 48 wells of three different volumes representing three rows (225 μ L, 22.5 μ L, and 2.25 μ L); the difference in the volume of the sample in these rows represents the ten-fold dilutions between one row and the next. The system then calculates the initial bacterial concentration based on the number of wells exhibiting fluorescence; the MPN calculation method is applied to determine the microbial count (Owen et al., 2010). Post-filling, the cards are incubated as per the manufacturer's instructions. The microorganisms in the card lead to substrate reduction in the culture media and cause the appearance of a fluorescent signal, which is then detected by the second unit of this system, the reader unit of the system. This automated MPN method automates many processes like media preparation, handling of agar plates, and manual colony counting and reduces labor, material, and time required for enumeration of *Campylobacter*. These advantages make it a robust method and the preferred choice for detecting and quantifying *Campylobacter*, especially concerning large sample volumes (Taylor et al., 2020). Automated MPN has been investigated, and it proved to be an effective method for the detection of *Campylobacter* at various levels in poultry samples, including carcass rinses, neck skin, and cooked poultry meat, when compared to the traditional direct plating method (Taylor et al., 2020).

Real-Time Quantitative PCR

The demand for rapid, efficient, and reliable automated detection of foodborne pathogens remains significant in the food industry and regulatory agencies. Addressing these concerns,

Polymerase Chain Reaction (PCR) has emerged as a powerful tool in microbiological diagnostics over the last decade (Schrader et al., 2012). These methods are utilized for their capability to provide rapid and accurate detection mechanisms for pathogens. For a process to be called reliable, it must address some critical criteria, including high analytical and diagnostic accuracy, a high probability of detection, robustness incorporating an internal amplification control (IAC), minimal carryover contamination, and ease of use with accessible and user-friendly protocols for application and interpretation (Malorny et al., 2003). These criteria are met by second-generation PCR methodologies, specifically real-time PCR, which combines amplification and detection in a closed tube reaction, promising reliability in results and decreased contamination rates.

Various inhibitors can affect PCR, like foreign material from the food matrix and in-accuracies during isolation procedures. These inhibitors can be organic (like detergents, polysaccharides, and proteins) or inorganic (like calcium ions and salts), interfering with fluorescent probes and real-time PCR assay interpretation (Schrader et al., 2012). For accurate real-time PCR results, it is crucial to use different controls, such as competitive/non-competitive, internal/external, and process/amplification controls, to assess potential PCR inhibition (Schrader et al., 2012).

The real-time quantitative PCR (qPCR) allows for rapid quantification of target genes by monitoring the accumulation of PCR products during the amplification process, often in less than two hours. This method provides high accuracy and sensitivity, making it a valuable tool for pathogen detection. This technique involves amplification and simultaneous quantification of target DNA. The usage of specific DNA dyes and fluorescent probes is incorporated into the PCR mix. During the process of amplification, the enzyme Taq polymerase hydrolyzes the probe and separates the quencher dye from the fluorophore, which results in the emission of a

fluorescent signal (Löfström et al., 2015). The intensity of fluorescence increases with each PCR cycle and is measured at the end of each cycle, which generates a cycle threshold (CT) value. This CT value is further used to create a standard curve and then determine the unknown concentration of pathogen DNA in the sample (Chaney et al., 2022). A higher initial concentration of pathogens is signified by a lower CT value and vice versa. These results are based on the exponential amplification of initial DNA in the sample with the number of PCR cycles performed (Löfström et al., 2015).

In the case of *Salmonella*, the critical target gene utilized for this is *invA*, which can serve as a marker for rapid detection in foods of animal origin (González-Escalona et al., 2009). This gene works by encoding a protein located in the innermost bacterial membrane vital for intestinal cell invasion in the host (El-Sebay et al., 2017). The *invA* gene is situated on Pathogenicity Island 1 (SPI-1) of *Salmonella* spp. and encodes proteins for a type III secretion system crucial for the ability of *Salmonella* to invade host epithelial cells (Rahn et al., 1992). It is highly specific to most *Salmonella* serotypes, which makes it a dependable target for PCR-based detection methods (González-Escalona et al., 2009). The distinctive sequences of the gene ensure specificity to the *Salmonella* genus, underscoring its significance as a diagnostic tool (El-Sebay et al., 2017). PCR assays targeting the *invA* gene have undergone improvements to enhance detection efficiency and reliability, and the amplification conditions of these assays have been refined from previous protocols to improve sensitivity and specificity (Rahn et al., 1992). Quantifying *Salmonella* in real-time quantitative polymerase chain reaction (RT-qPCR) involves monitoring the real-time amplification of target DNA using fluorescent probes or dyes. The CT

value, which is inversely related to the initial quantity of *Salmonella* in the sample, is used to determine the number of colony-forming units (CFUs) present (AOAC International, 2021). Effective detection of *Salmonella* through PCR typically involves an enrichment step to elevate the number of target cells and improve detection sensitivity. For instance, the Salquant™ method (BAX® System) entails employing an enrichment step in BAX-MP media with novobiocin for 6-10 h. This facilitates the retrieval of impaired cells while restraining the growth of background microflora (Forgey, 2020). This step is pivotal for achieving precise quantification results and has demonstrated comparable outcomes to traditional methods, such as the Most Probable Number (MPN) method, but within a significantly shorter time (Chaney et al., 2022). Several alternative methods have been devised to eliminate the necessity for enrichment by utilizing techniques like centrifugation to concentrate cells. One such method is the BioMérieux Gene-Up Quant *Salmonella* method, which is a rapid and direct approach to detecting and quantifying *Salmonella* without an enrichment step (AOAC International, 2021). Recent advancements in these methods have led to the development of commercial RT-PCR-based kits, such as the BAX® System and BioMérieux's GENE-UP® QUANT *Salmonella*.

In the case of *Campylobacter*, there are various target genes for detection, like the *hipO* gene, which is responsible for encoding the enzyme Hippurate hydrolase specific to *C. jejuni* and commonly associated with human gastroenteritis. This gene provides clear differentiation of *C. jejuni* from other *Campylobacter* species due to high specificity (Linton et al., 1997). Multiple studies have compared PCR-based methods and conventional culture-based methods for detecting and illustrated the superiority of qPCR over standard direct plating on Campy-Cefex agar in detecting *C. jejuni* levels in water samples from scalding and chill tanks during poultry

processing. BAX® System Real-Time PCR Assay for *Campylobacter* is a rapid quantification method that utilizes RT-PCR to count *Campylobacter* in poultry rinses. It is distinguished by its reduced enrichment time of 18 h, enabling faster detection than conventional methods, which usually necessitate more extended enrichment periods (USDA, 2024).

REFERENCES

Centers for Disease Control and Prevention (CDC; 2024a). *Campylobacter Infections*. Available at <https://www.cdc.gov/campylobacter/about/index.html>. Accessed on August 26, 2024

Centers for Disease Control and Prevention (CDC; 2024b). *Foodborne Illnesses and Germs*. Available at <https://www.cdc.gov/foodborneburden/index.html>. Accessed on August 26, 2024

United States- Food and Drug Administration (US-FDA). *Bad Bug Book: Foodborne Pathogenic Microorganisms and Natural Toxins - Second Edition - Salmonella Species*. Available at <https://www.salmonellablog.com/salmonella-information/bad-bug-book-foodborne-pathogenic-microorganisms-and-natural-toxins-second-edition-salmonella/> .Accessed on August 26, 2024

U.S. Department of Agriculture, Food Safety and Inspection Service, (USDA-FSIS, 2021). *MLG 41.05. Isolation and Identification of Campylobacter jejuni/coli/lari from Poultry Rinse, Sponge, and Raw Product Samples*. Available at https://www.fsis.usda.gov/sites/default/files/media_file/2021-06/MLG-41.pdf .Accessed on August 26 ,2024.

U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS, 2024). *Salmonella Framework for Raw Poultry Products*. Available at

<https://www.federalregister.gov/documents/2024/08/07/2024-16963/salmonella-framework-for-raw-poultry-products> . Accessed on August 27,2024

U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS, 2021). *FSIS Guidance Document for Controlling Salmonella and Campylobacter in Poultry* (FSIS-GD-2021-0006, Issue. U.S. Department of Agriculture. Available at :

https://www.fsis.usda.gov/sites/default/files/media_file/2021-07/FSIS-GD-2021-0006.pdf

Accessed on August26,2024

U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS, 2024).

Foodborne Illness and Disease. U.S. Department of Agriculture. Available at:

<https://www.fsis.usda.gov/food-safety/foodborne-illness-and-disease>. Accessed on August 26, 2024

Ammar, A. M., El-Naenaeey, E.-S. Y., Abd El-Hamid, M. I., El-Gedawy, A. A., & Elmalt, R. M. (2021). *Campylobacter* as a major foodborne pathogen: A review of its characteristics, pathogenesis, antimicrobial resistance, and control. *Journal of Microbiology, Biotechnology and food sciences*, 10(4), 609-619.

Andritsos, N. D., Tzimotoudis, N., & Mataragas, M. (2020). Estimating the performance of four culture media used for enumeration and detection of *Campylobacter* species in chicken meat. *LWT*, 118, 108808.

AOAC International. (2021). *AOAC performance tested method 081201: BAX system real-time PCR assay for Salmonella*. Available at https://members.aoac.org/AOAC_Docs/RI/21PTM/21C_081201_DPS_ver3.pdf . Accessed on August 28,2022.

Beery, J., Hugdahl, M., & Doyle, M. (1988). Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Applied and environmental microbiology*, 54(10), 2365-2370.

Berghaus, R. D., Thayer, S. G., Law, B. F., Mild, R. M., Hofacre, C. L., & Singer, R. S. (2013). Enumeration of *Salmonella* and *Campylobacter* spp. in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. *Applied and environmental microbiology*, 79(13), 4106-4114.

Berrang, M., Meinersmann, R., Cox, N., & Thompson, T. (2018). Multilocus sequence subtypes of *Campylobacter* detected on the surface and from internal tissues of retail chicken livers. *Journal of Food Protection*, 81(9), 1535-1539.

Blanc-Potard, A.-B., & Groisman, E. A. (1997). The *Salmonella* selC locus contains a pathogenicity island mediating intramacrophage survival. *The EMBO journal*, 16(17), 5376-5385

Bolton, D. J. (2015). *Campylobacter* virulence and survival factors. *Food microbiology*, 48, 99-108.

Cano, C., Meneses, Y., & Chaves, B. D. (2021). Application of peroxyacetic acid for decontamination of raw poultry products and comparison to other commonly used chemical antimicrobial interventions: a review. *Journal of Food Protection*, 84(10), 1772-1783.

Cawthraw, S., Wassenaar, T., Ayling, R., & Newell, D. (1996). Increased colonization potential of *Campylobacter jejuni* strain 81116 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiology & Infection*, 117(1), 213-215.

Cayer, M.-P., Dussault, N., De Grandmont, M. J., Cloutier, M., Lewin, A., & Brouard, D. (2020). Evaluation of the Tempo® system: improving the microbiological quality monitoring of human milk. *Frontiers in Pediatrics*, 8, 494.

Chaney, W. E., Englishbey, A. K., Stephens, T. P., Applegate, S. F., & Sanchez-Plata, M. X. (2022). Application of a Commercial *Salmonella* Real-Time PCR Assay for the Detection and Quantification of *Salmonella enterica* in Poultry Ceca. *Journal of Food Protection*, 85(3), 527-533.

Chang, C., & Miller, J. F. (2006). *Campylobacter jejuni* colonization of mice with limited enteric flora. *Infection and Immunity*, 74(9), 5261-5271.

Chenu, J. W., Pavic, A., & Cox, J. M. (2013). A novel miniaturized most probable number method for the enumeration of *Campylobacter spp.* from poultry-associated matrices. *Journal of microbiological methods*, 93(1), 12-19.

Christensen, J. E., Pacheco, S. A., & Konkel, M. E. (2009). Identification of a *Campylobacter jejuni*-secreted protein required for maximal invasion of host cells. *Molecular microbiology*, 73(4), 650-662.

Colla, F., Mion, L., Parizotto, L., Rodrigues, L., Pilotto, F., Dickel, E., do Nascimento, V., & dos Santos, L. (2014). Miniaturized most probable number for the enumeration of *Salmonella* spp in artificially contaminated chicken meat. *Brazilian Journal of Poultry Science*, 16, 45-48.

Cosby, D. E., Cox, N. A., Harrison, M. A., Wilson, J. L., Buhr, R. J., & Fedorka-Cray, P. J. (2015). *Salmonella* and antimicrobial resistance in broilers: A review. *Journal of Applied Poultry Research*, 24(3), 408-426.

Coward, C., van Diemen, P. M., Conlan, A. J., Gog, J. R., Stevens, M. P., Jones, M. A., & Maskell, D. J. (2008). Competing isogenic *Campylobacter* strains exhibit variable population structures in vivo. *Applied and environmental microbiology*, 74(12), 3857-3867.

Dasti, J. I., Tareen, A. M., Lugert, R., Zautner, A. E., & Groß, U. (2010). *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms.

International Journal of Medical Microbiology, 300(4), 205-211.

De Boer, E., & Hahné, M. (1990). Cross-contamination with *Campylobacter jejuni* and *Salmonella* spp. from raw chicken products during food preparation. *Journal of Food Protection*, 53(12), 1067-1068.

Debruyne, L., Gevers, D., & Vandamme, P. (2008). Taxonomy of the family *Campylobacteraceae*. *Campylobacter*, 1-25.

Di Giannatale, E., Calistri, P., Di Donato, G., Decastelli, L., Goffredo, E., Adriano, D., Mancini, M. E., Galleggiante, A., Neri, D., & Antoci, S. (2019). Thermotolerant *Campylobacter* spp. in chicken and bovine meat in Italy: Prevalence, level of contamination and molecular characterization of isolates. *PLoS One*, 14(12), e0225957.

Dickson, J. S. (1990). Surface moisture and osmotic stress as factors that affect the sanitizing of beef tissue surfaces. *Journal of Food Protection*, 53(8), 674-679.

European Food Safety Authority (EFSA, 2013). Scientific Opinion on the public health hazards to be covered by inspection of meat (bovine animals). *EFSA Journal*, 11(6).

<https://doi.org/10.2903/j.efsa.2013.3266>

El-Sebay, N., Abu Shady, H., El-Rashed El-Zeedy, S., & Samy, A. (2017). InvA gene sequencing of *Salmonella Typhimurium* isolated from Egyptian poultry. *Asian J. Sci. Res*, 10(3), 194-202.

Eng, S.-K., Pusparajah, P., Ab Mutalib, N.-S., Ser, H.-L., Chan, K.-G., & Lee, L.-H. (2015). *Salmonella*: a review on pathogenesis, epidemiology, and antibiotic resistance. *Frontiers in Life Science*, 8(3), 284-293.

U.S. Food and Drug Administration (FDA, 2020). BAM Chapter 7: *Campylobacter*. Available at <https://www.fda.gov/food/laboratory-methods-food/bam-chapter-7-campylobacter>. Accessed on August 29, 2024

Ferrero, R. L., & Lee, A. (1988). Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. *Microbiology*, 134(1), 53-59.

Forgey, S. J. (2020). Development, verification, and validation of a RT-PCR based methodology for *Salmonella* quantification as a tool for integrated food safety management in poultry from live production to final product

Franzo, G., Legnardi, M., Faustini, G., Tucciarone, C. M., & Cecchinato, M. (2023). When Everything Becomes Bigger: Big Data for Big Poultry Production. *Animals (Basel)*, 13(11).

García-Sánchez, L., Melero, B., Díez, A. M., Jaime, I., & Rovira, J. (2018). Characterization of *Campylobacter* species in Spanish retail from different fresh chicken products and their antimicrobial resistance. *Food microbiology*, 76, 457-465.

González-Escalona, N., Hammack, T. S., Russell, M., Jacobson, A. P., De Jesús, A. J., Brown, E. W., & Lampel, K. A. (2009). Detection of live *Salmonella* sp. cells in produce by a TaqMan-based quantitative reverse transcriptase real-time PCR targeting *invA* mRNA. *Applied and environmental microbiology*, 75(11), 3714-3720.

González-Hein, G., Huaracán, B., García, P., & Figueroa, G. (2013). Prevalence of virulence genes in strains of *Campylobacter jejuni* isolated from human, bovine, and broiler. *Brazilian Journal of Microbiology*, 44, 1223-1229.

Guerry, P., Doig, P., Alm, R. A., Burr, D. H., Kinsella, N., & Trust, T. J. (1996). Identification and characterization of genes required for post-translational modification of *Campylobacter coli* VC167 flagellin. *Molecular microbiology*, 19(2), 369-378.

Guibourdenche, M., Roggentin, P., Mikoleit, M., Fields, P. I., Bockemühl, J., Grimont, P. A., & Weill, F.-X. (2010). Supplement 2003–2007 (No. 47) to the white-Kauffmann-Le minor scheme. *Research in microbiology*, 161(1), 26-29.

Guran, H. S., Mann, D., & Alali, W. Q. (2017). *Salmonella* prevalence associated with chicken parts with and without skin from retail establishments in Atlanta metropolitan area, Georgia. *Food Control*, 73, 462-467.

Hafez, H., Stadler, A., & Kösters, J. (1997). Surveillance on *Salmonella* in turkey flocks and processing plants. *Vet. Res*, 46, 1372-1374.

Hazeleger, W. C., Wouters, J. A., Rombouts, F. M., & Abee, T. (1998). Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. *Applied and environmental microbiology*, 64(10), 3917-3922.

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., Rasschaert, G., Heyndrickx, M., & Pasmans, F. (2011). *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Veterinary microbiology*, 152(3-4), 219-228.

Hofreuter, D. (2014). Defining the metabolic requirements for the growth and colonization capacity of *Campylobacter jejuni*. *Frontiers in cellular and infection microbiology*, 4, 137.

Hofreuter, D., Tsai, J., Watson, R. O., Novik, V., Altman, B., Benitez, M., Clark, C., Perbost, C., Jarvie, T., & Du, L. (2006). Unique features of a highly pathogenic *Campylobacter jejuni* strain. *Infection and Immunity*, 74(8), 4694-4707.

- Ibarra, J. A., & Steele-Mortimer, O. (2009). *Salmonella*—the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cellular microbiology*, 11(11), 1579-1586.
- James, W. O., Williams, W. O., Prucha, J. C., Johnston, R., & Christensen, W. (1992). Profile of selected bacterial counts and *Salmonella* prevalence on raw poultry in a poultry slaughter establishment. *Journal of the American Veterinary Medical Association*, 200(1), 57-59.
- Je, H. J., Singh, S., Kim, D. W., Hur, H. S., Kim, A. L., Seo, E. J., & Koo, O. K. (2023). Systematic Review and Meta-Analysis of *Campylobacter* Species Contamination in Poultry, Meat, and Processing Environments in South Korea. *Microorganisms*, 11(11), 2722.
- Jiménez, S., Tiburzi, M., Salsi, M., Moguilevsky, M., & Pirovani, M. (2009). Survival of *Salmonella* on refrigerated chicken carcasses and subsequent transfer to cutting board. *Letters in Applied Microbiology*, 48(6), 687-691.
- Jung, Y., Porto-Fett, A. C., Shoyer, B. A., Henry, E., Shane, L. E., Osoria, M., & Luchansky, J. B. (2019). Prevalence, levels, and viability of *Salmonella* in and on raw chicken livers. *Journal of Food Protection*, 82(5), 834-843.
- Kiggins, E., & Plastringe, W. (1958). Some metabolic activities of *Vibrio fetus* of bovine origin. *Journal of Bacteriology*, 75(2), 205-208.

Kist, M. (1986). Who discovered *Campylobacter jejuni/coli*? A review of hitherto disregarded literature. *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene. Series A, Medical microbiology, infectious diseases, virology, parasitology*, 261(2), 177-186.

LaRock, D. L., Chaudhary, A., & Miller, S. I. (2015). *Salmonellae* interactions with host processes. *Nature Reviews Microbiology*, 13(4), 191-205.

Lázaro, B., Cárcamo, J., Audicana, A., Perales, I., & Fernández-Astorga, A. (1999). Viability and DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. *Applied and environmental microbiology*, 65(10), 4677-4681.

Lewis, A. M. M., M.C.; Fink, R.C. (2019). *Salmonella*. In M. P. B. Doyle, R.L. (Ed.), *Food Microbiology: Fundamentals and Frontiers* (5th Ed. ed., pp. 225-273). ASM Press

Lillard, H. (1980). Effect on broiler carcasses and water of treating chiller water with chlorine or chlorine dioxide. *Poultry Science*, 59(8), 1761-1766.

Line, J., & Bailey, J. (2006). Effect of on-farm litter acidification treatments on *Campylobacter* and *Salmonella* populations in commercial broiler houses in northeast Georgia. *Poultry Science*, 85(9), 1529-1534.

Line, J. E. (2001). Development of a selective differential agar for isolation and enumeration of *Campylobacter* spp. *Journal of Food Protection*, 64(11), 1711-1715.

Linton, D., Lawson, A., Owen, R., & Stanley, J. (1997). PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *Journal of clinical microbiology*, 35(10), 2568-2572.

Löfström, C., Josefsen, M. H., Hansen, T., Søndergaard, M. S. R., & Hoorfar, J. (2015). Fluorescence-based real-time quantitative polymerase chain reaction (qPCR) technologies for high throughput screening of pathogens. In High throughput screening for food safety assessment (pp. 219-248). Elsevier.

Louwen, R., Heikema, A., van Belkum, A., Ott, A., Gilbert, M., Ang, W., Endtz, H. P., Bergman, M. P., & Nieuwenhuis, E. E. (2008). The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. *Infection and Immunity*, 76(10), 4431-4438.

Malorny, B., Löfström, C., Wagner, M., Krämer, N., & Hoorfar, J. (2008). Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. *Applied and environmental microbiology*, 74(5), 1299-1304.

Malorny, B., Hoorfar, J., Bunge, C., & Helmuth, R. (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and environmental microbiology*, 69(1), 290-296.

Mazengia, E., Samadpour, M., Hill, H., Greeson, K., Tenney, K., Liao, G., Huang, X., & Meschke, J. (2014). Prevalence, concentrations, and antibiotic sensitivities of *Salmonella* serovars in poultry from retail establishments in Seattle, Washington. *Journal of Food Protection*, 77(6), 885-893.

McEntire, J., Acheson, D., Siemens, A., Eilert, S., & Robach, M. (2014). The Public Health Value of Reducing *Salmonella* Levels in Raw Meat and Poultry. *Food Protection Trends*, 34(6).

Meade, K. G., Narciandi, F., Cahalane, S., Reiman, C., Allan, B., & O'Farrelly, C. (2009). Comparative in vivo infection models yield insights into early host immune response to *Campylobacter* in chickens. *Immunogenetics*, 61, 101-110.

Millr, R. G., Tate, C., Mallinson, E., & Scherrer, J. (1991). Xylose-lysine-tergitol 4: an improved selective agar medium for the isolation of *Salmonella*. *Poultry Science*, 70(12), 2429-2432.

Miller, S. (2000). *Salmonella* species, including *Salmonella Typhi*. *Principles and practice of infectious diseases*, 2344-2363.

Mion, L., Parizotto, L., dos Santos, L. A., Webber, B., Cisco, I. C., Pilotto, F., Rodrigues, L. B., do Nascimento, V. P., & dos Santos, L. R. (2016). *Salmonella* spp. isolated by miniaturized most probable number and conventional microbiology in poultry slaughterhouses. *Acta Scientiae Veterinariae*, 44, 5-5.

Molnar, G., Mertsola, J., & Erkkö, M. (1982). Guillain-Barré syndrome associated with *Campylobacter* infection. *British Medical Journal (Clinical research ed.)*, 285(6342), 652.

Müller, S. I., Valdebenito, M., & Hantke, K. (2009). Salmochelin, the long-overlooked catecholate siderophore of *Salmonella*. *Biometals*, 22, 691-695.

O'Bryan, C. A., Ricke, S. C., & Marcy, J. A. (2022). Public health impact of *Salmonella* spp. on raw poultry: Current concepts and future prospects in the United States. *Food Control*, 132, 108539.

Oscar, T. (2021). *Salmonella* prevalence alone is not a good indicator of poultry food safety. *Risk analysis*, 41(1), 110-130.

Owen, M., Willis, C., & Lamph, D. (2010). Evaluation of the TEMPO® most probable number technique for the enumeration of *Enterobacteriaceae* in food and dairy products. *Journal of applied microbiology*, 109(5), 1810-1816.

Oyarzabal, O. A. (2005). Reduction of *Campylobacter* spp. by commercial antimicrobials applied during the processing of broiler chickens: a review from the United States perspective. *Journal of Food Protection*, 68(8), 1752-1760.

Park, S. H., Aydin, M., Khatiwara, A., Dolan, M. C., Gilmore, D. F., Bouldin, J. L., Ahn, S., & Ricke, S. C. (2014). Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry products. *Food microbiology*, 38, 250-262.

Poudel, S., Li, T., Chen, S., Zhang, X., Cheng, W.-H., Sukumaran, A. T., Kiess, A. S., & Zhang, L. (2022). Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter* isolated from broilers and broiler meat raised without antibiotics. *Microbiology Spectrum*, 10(3), e00251-00222.

Rahn, K., De Grandis, S., Clarke, R., McEwen, S., Galan, J., Ginocchio, C., Curtiss Iii, R., & Gyles, C. (1992). Amplification of an *invA* gene sequence of *Salmonella Typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and cellular probes*, 6(4), 271-279.

Robert Koch Institute (RKI,2018). *Epidemiologisches Bulletin 23/2018: Campylobacter-Enteritis*. Robert Koch Institute. Available at https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2018/Ausgaben/23_18.pdf?__blob=publicationFile. Accessed on August 28,2024

Rollins, D., & Colwell, R. (1986). Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and environmental microbiology*, 52(3), 531-538.

Rothrock, M. J., Hiett, K. L., Kiepper, B. H., Ingram, K., & Hinton, A. (2013). Quantification of zoonotic bacterial pathogens within commercial poultry processing water samples using droplet digital PCR. *Advances in Microbiology*, 3(05), 403.

Sanderson, K. E., & Nair, S. (2013). Taxonomy and species concepts in the genus *Salmonella*. In *Salmonella in domestic animals* (pp. 1-19). Cabi Wallingford UK.

Sarlin, L., Barnhart, E., Caldwell, D., Moore, R., Byrd, J., Caldwell, D., Corrier, D., Deloach, J., & Hargis, B. (1998). Evaluation of alternative sampling methods for *Salmonella* critical control point determination at broiler processing. *Poultry Science*, 77(8), 1253-1257.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors—occurrence, properties, and removal. *Journal of applied microbiology*, 113(5), 1014-1026.

Sebald, M., & Veron, M. (1963). Base DNA content and classification of *vibrios*.

Sellars, M. J., Hall, S. J., & Kelly, D. J. (2002). Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen. *Journal of bacteriology*, 184(15), 4187-4196.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., & Teixeira, P. (2011). *Campylobacter* spp. as a foodborne pathogen: a review. *Frontiers in microbiology*, 2, 200.

Simm, R., Ahmad, I., Rhen, M., Le Guyon, S., & Römling, U. (2014). Regulation of biofilm formation in *Salmonella enterica* serovar Typhimurium. *Future microbiology*, 9(11), 1261-1282.

Smith, J. (2013). *Campylobacter*, chicken, and the regulatory performance standard.

Stern, N. J., Wojton, B., & Kwiatek, K. (1992). A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. *Journal of Food Protection*, 55(7), 514-517.

Sun, Y.-H., Rolán, H. G., & Tsolis, R. M. (2007). Injection of flagellin into the host cell cytosol by *Salmonella enterica* serotype Typhimurium. *Journal of Biological Chemistry*, 282(47), 33897-33901.

Sylte, M., Inbody, M., Johnson, T., Looft, T., & Line, J. (2018). Evaluation of different *Campylobacter jejuni* isolates to colonize the intestinal tract of commercial turkey poults and selective media for enumeration. *Poultry Science*, 97(5), 1689-1698.

Taylor, N., Mills, J., Johnson, R., & Bailey, J. S. (2020). Evaluation of the TEMPO® CAM (*Campylobacter*) Assay for the Detection of *Campylobacter* from Poultry Samples. *IAFP 2020*.

Thames, H. T., Fancher, C. A., Colvin, M. G., McAnally, M., Tucker, E., Zhang, L., Kiess, A. S., Dinh, T. T., & Sukumaran, A. T. (2022). The prevalence of *Salmonella* and *Campylobacter* on broiler meat at different stages of commercial poultry processing. *Animals*, 12(18), 2460.

Van Der Heijden, J., & Finlay, B. B. (2012). Type III effector-mediated processes in *Salmonella* infection. *Future microbiology*, 7(6), 685-703.

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., Woodward, D. L., & Rodgers, F. G. (2002). Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. *Journal of clinical microbiology*, 40(12), 4744-4747.

Wang, J., Vaddu, S., Bhumanapalli, S., Mishra, A., Applegate, T., Singh, M., & Thippareddi, H. (2023). A systematic review and meta-analysis of the sources of *Salmonella* in poultry production (pre-harvest) and their relative contributions to the microbial risk of poultry meat. *Poultry Science*, 102(5), 102566.

Wang, Y., Chen, Q., Cui, S., Xu, X., Zhu, J., Luo, H., Wang, D., & Li, F. (2014). Enumeration and characterization of *Salmonella* isolates from retail chicken carcasses in Beijing, China.

Foodborne pathogens and disease, 11(2), 126-132.

Wassenaar, T. M., Bleumink-Pluym, N., & Van Der Zeijst, B. (1991). Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. *The EMBO journal*, 10(8), 2055-2061.

Williams, A., & Oyarzabal, O. A. (2012). Prevalence of *Campylobacter* spp. in skinless, boneless retail broiler meat from 2005 through 2011 in Alabama, USA. *BMC microbiology*, 12, 1-7.

Young, K. T., Davis, L. M., & DiRita, V. J. (2007). *Campylobacter jejuni*: molecular biology and pathogenesis. *Nature Reviews Microbiology*, 5(9), 665-679.

Ziprin, R. L., Droleskey, R. E., Hume, M. E., & Harvey, R. B. (2003). Failure of viable nonculturable *Campylobacter jejuni* to colonize the cecum of newly hatched leghorn chicks.

Avian diseases, 47(3), 753-758.

CHAPTER 3

Evaluation of *Campylobacter* and *Salmonella* enumeration methods in various poultry matrices

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ABSTRACT

This study was conducted with the aim to compare enumeration methods for *Campylobacter* and *Salmonella* populations in poultry products, specifically ground chicken, ground turkey, and chicken wing rinses. Samples were inoculated with a cocktail of ciprofloxacin-resistant *Campylobacter coli* and *C. jejuni* and nalidixic acid-resistant *Salmonella Typhimurium*. For *Campylobacter*, conventional plating on campy-cefex agar was compared with automated MPN for all matrices, and an additional PCR-based assay (BAX® Campyquant™) was employed for chicken wing rinses. For *Salmonella*, conventional plating on Tryptic Soy Agar (TSA) was compared with miniaturized MPN and two PCR-based quantification assays 1 and 2 (GENE-UP® QUANT *Salmonella* and BAX® Salquant™, respectively). Two levels of inoculation, high (~8 log CFU/ mL) and low (~5 log CFU/ mL), were used across all the enumeration methods for both pathogens. For *Salmonella*, at the high level of inoculation, miniaturized most probable number (mMPN) was the most effective, followed by PCR assay 1 and plating, while PCR assay 2 had the least recovery ($p \leq 0.05$). At lower levels mMPN, PCR assay 1 & 2 performed similarly ($p > 0.05$), while plating had significantly lower bacterial populations ($p \leq 0.05$). For *Campylobacter*, the plating method was significantly higher than automated MPN ($p = 0.0353$) at a high inoculation level, but there was no statistical difference ($p > 0.05$) at a lower level of inoculation. In the case of chicken wing rinses, both plating and automated MPN yielded significantly higher ($p \leq 0.05$)

results as compared to PCR-based assay, whereas, at the low levels, PCR-based quantification assay was significantly higher than both plating and automated MPN ($p<.0001$). This study underscores the need to select enumeration methods based on contamination level and operational requirements. While conventional methods are reliable for bacterial quantification at higher contamination levels, PCR-based assays are more accurate, robust, and effective at lower detection levels.

INTRODUCTION

Foodborne pathogens have been a significant public health challenge, causing over 48 million illnesses every year, with *Salmonella* and *Campylobacter* being among the most common foodborne pathogens. In recent years, there has been a rising trend in the number of illnesses caused by foodborne pathogens, accounting for up to 48 million illnesses every year (CDC, 2024). The majority of these infections are of animal origin, poultry being one of the leading sources (Hansson et al., 2015). Poultry products are the most economical and readily available source of protein, the improper handling of raw meat, consumption of undercooked poultry meat as well and cross-contamination increase the prospect of causing infections in humans via direct or indirect contact. As evidenced in *Campylobacter*, improper handling and preparation and consumption of broiler meat accounts for 20-30% of human cases of campylobacteriosis, while the chicken reservoir may be responsible for 50 to 80% of human campylobacteriosis (EFSA, 2013).

Campylobacter colonizes the gastrointestinal tract of live chicks, and due to leakage of contents while processing, it causes further contamination in other carcasses. In humans, the infection can

be transmitted via various routes, such as handling, processing, preparing, and consumption of raw and or undercooked poultry (Umaraw et al., 2017). The infection has an incubation period of 2-5 days and exhibits symptoms like fever, headache, muscle pain, sometimes bloody diarrhea, cramp-like abdominal pain, and general malaise; severe cases can occur in YOPI (children, older adults, and people with weakened immune systems (CDC, 2024). In some instances, *Campylobacter* infections can result in post-infectious complications such as irritable bowel syndrome, reactive arthritis, and autoimmune diseases like Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome (MFS). GBS is a severe neurological condition that can cause paralysis, with a mortality rate of 2 to 3%, primarily due to respiratory failure (Molnar et al., 1982).

In the case of *Salmonella*, the infection can be transmitted vertically and horizontally. Vertically transmitted *Salmonella* in the reproductive organs of breeder hens may lead to contamination of egg contents during ovogenesis (Gantois et al., 2009). Development of infection can start with simply consuming contaminated food or water. The bacteria then further move and attach to the intestinal epithelium, causing inflammation to spread throughout the body (LaRock et al., 2015). The predominant sources of *Salmonella* contamination within the poultry production continuum are the hatchery (48.5%), litter (25.4%), feces (16.3%), internal poultry house environment (7.9%), external poultry house environment (4.7%), feed (4.8%), chicks (4.7%), and drinker water, listed in order of prevalence (Wang et al., 2023).

There are many ongoing initiatives for the adoption of different physical and chemical interventions, such as Good Manufacturing Practices (GMP), Hazard Analysis, and Critical Control Points (HACCP) during poultry processing, which help to assess and control the incidence of pathogens like *Salmonella* and *Campylobacter* in poultry processing facilities

(USDA, 2021a). According to the Food Safety and Inspection Service (FSIS) performance standards for *Campylobacter* in raw poultry products, the maximum acceptable percentage of positive tests for young chicken carcasses is 10.4% and 5.4% for turkey carcasses. Additionally, new policies have been proposed by the United States Department of Agriculture (USDA) to reduce *Salmonella* in raw poultry products and focus on preventing contaminated products from entering the market. The proposed guidelines set a limit of 10 colony-forming units (CFU) per g/mL for *Salmonella* in poultry products. Targets specific serotypes of public health significance for chicken are Enteritidis and Typhimurium I,4,[5],12:I:- and for turkey are Hadar, Typhimurium, and Muenchen. The proposed regulations also require poultry establishments to develop microbial monitoring programs to prevent contamination throughout the production chain (USDA, 2021).

Despite efforts by the regulatory agencies and stringent guidelines in the poultry processing facilities, there still has been a continuous incidence of foodborne illnesses associated with *Campylobacter* and *Salmonella* in poultry (Ricke et al., 2019). One of the primary reasons behind this can be attributed to reliance on prevalence data and limited efforts to estimate the concentration of *Salmonella* and *Campylobacter* in poultry products (O'Bryan et al., 2022). The prevalence of these pathogens can be low, but they can still multiply and proliferate if provided optimum conditions. Thus, there is a significant knowledge gap between the prevalence of *Salmonella* and *Campylobacter* in poultry and the actual foodborne illnesses caused by these pathogens, which needs to be addressed. To date, there has been significant reliance on techniques like most probable number (MPN), conventional plating using selective and differential media, and broths for enumerating pathogens. Despite these methods advantages,

simplicity, lower capital cost requirements, and reliable detection, they are labor-intensive and time-consuming. A series of selective plating or enrichment in selective broths can easily take up to 4-6 days until the determination of the final bacterial load in the sample. Until then, the product is already in commerce and can pose a substantial public health risk.

Various methods like automated MPN and rapid-PCR-based quantification assays have helped in reducing the time needed to determine the bacterial loads to a few hours with high accuracy.

Traditional methods, which are known for high precision and capability to identify a wide range of pathogens, cannot be eliminated. However, quantification methods have multiple advantages, such as lowering the limit of detection and faster results by decreasing the time from sample evaluation to result, which enables faster data-driven decision-making. These methods have shortened the enrichment times and aid in quick detections, which significantly helps in the identification and detection of pathogens in case of low-level contamination, which can pose a significant food safety risk if it goes undetected. Although the initial investment in setting up these advanced techniques is higher, it has numerous benefits like: reduced labor and material costs, fewer product recalls, and reduced production downtime. Given the primary objective of the poultry industry in minimizing contamination and maximizing the safety of poultry products, this research aims to investigate and compare different conventional and automated rapid quantification methods for the detection of *Salmonella* and *Campylobacter*. The focus of this study was to compare conventional plating, MPN techniques, and PCR-based quantification assays.

MATERIALS AND METHODS

Sample Collection and Inoculum Preparation

Fresh ground chicken, ground turkey, and chicken wings were procured from a local grocery store on three different processing days, corresponding to three independent replications. Control samples were analyzed separately for each batch and were negative for *Salmonella* and *Campylobacter*.

For *Campylobacter* inoculum, ciprofloxacin-resistant strains of wild-type poultry *Campylobacter jejuni* and laboratory-induced ciprofloxacin-resistant strains of *Campylobacter coli* were recovered from frozen glycerol stock and streaked onto Campy-Cefex agar (HiMedia Laboratories Pvt. Limited, Mumbai, India) supplemented with 5 ppm ciprofloxacin (Sigma-Aldrich, St. Louis, MO) using a 1 µl inoculation loop, and incubated at 42°C for 48 h under microaerophilic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen). After 48 h, a single isolated colony was streaked for isolation onto Campy-line selective agar prepared using the method described in (Oyarzabal et al., 2005) supplemented with 5 ppm ciprofloxacin and incubated at 42°C for 48 h microaerophilic conditions. For enhanced selectivity and to obtain pure cultures, further sub-culturing was done by taking an isolated colony and streaking it onto fresh Campy-line selective agar and incubating it at 42°C for 48 h under microaerophilic conditions. Following this step, 3-4 colonies from the Campy-line plates were isolated and streaked onto Campy-cefex + 5 ppm ciprofloxacin agar. Four plates were used per stain and incubated at 42°C for 48 h under microaerophilic conditions. *Campylobacter* lawns were

harvested using 1 mL of sterile phosphate buffer saline (PBS; Fisher Scientific, Fair Lawn, NJ) onto each plate and swabbed with a sterile cotton-tipped swab (15 cm; Puritan Medical Products Co LLC, Guilford, ME) and transferred into a tube containing 9 mL of PBS. After harvesting lawns, the suspension was transferred into a 15 mL conical Falcon tube and centrifuged at 5,000 x g for 10 min. Each pellet was washed by re-suspending in 10 mL of PBS and centrifuging at 5,000 x g for 10 min. The pellet was then re-suspended in 10 mL of PBS, and 5 mL from each strain was collected to form a cocktail.

For *Salmonella*, a loopful of nalidixic acid-resistant *Salmonella Typhimurium* was obtained from freezer glycerol stock using 1 µl inoculation loop and streaked for isolation onto a Tryptic Soy Agar (TSA; BD, Franklin Lakes, NJ) supplemented with 200 ppm nalidixic acid (Sigma-Aldrich, St. Louis, MO). Plates were then incubated at 37°C for 24 h. An isolated colony from each TSA agar plate was then used to inoculate 10 mL of Tryptic Soy Broth (TSB; Remel, Lenexa, KS) supplemented with 200 ppm nalidixic acid. Tubes were then incubated with shaking at 100 rpm at 37°C for 18 to 24 h. Following incubation, the cultures were transferred into two 15 mL conical tubes and centrifuged at 5,000 X g for 20 min. The pellet was then re-suspended in 5 mL of phosphate buffer saline (PBS) and rewashed by centrifuging at 5,000 x g for 10 min. Once the washing was completed, pellets were re-suspended in 0.5 mL PBS and added to a 10 mL tube containing PBS for further inoculation. *Salmonella* and *Campylobacter* inoculum were separately prepared and plated for each replication to verify the inoculation levels for each replication. Fresh inoculum was prepared on each experiment day, and uninoculated samples served as a negative control for the experiment.

Sample Inoculation

For ground chicken and ground turkey, six separate samples, each of 25 g, were aseptically weighed for testing *Salmonella* and *Campylobacter*. Each 25 g sample was then individually placed in a Whirl-pak Filter bag (Nasco, Fort Atkinson, WI). Out of six samples in each matrix for both pathogens, three corresponded to high-level inoculation ($\sim 8 \log \text{CFU/g}$), and the other three corresponded to low-level inoculation ($\sim 5 \log \text{CFU/g}$). The inoculation procedure was standardized for *Salmonella* and *Campylobacter*. Each sample was inoculated with 1 mL of respective inoculum and hand massaged for 2 min, followed by a 15-min period of bacterial attachment. Following the attachment period, 125 mL of Buffered peptone water (BPW; Difco, Sparks, MD) was added to samples inoculated with *Salmonella*, and 25 mL of BPW was added to samples inoculated with *Campylobacter*. Samples were homogenized (Seward, Worthing, West Sussex, UK) at 230 rpm for 2 min.

For chicken wing rinses, a total of twelve samples of 0.45kg of skin-on chicken wings were aseptically weighed and placed in a Whirl Pak filter bag prior to inoculation. Out of these twelve samples, six samples were used for *Salmonella* and the remaining six for *Campylobacter* testing. Each set of six samples was further divided, where three samples corresponded to sample inoculation at high-level ($\sim 8 \log \text{CFU/g}$) and the remaining three for low-level ($\sim 5 \log \text{CFU/g}$) inoculation. The samples were dip-inoculated in 10 mL of *Salmonella* and *Campylobacter* inoculum separately, followed by hand-massaging for 1 min. The inoculated samples were then placed on a sterile aluminum tray, separately, for each level of inoculation and replication for 15 min, allowing bacterial attachment in the biosafety cabinet (Thermo Fisher Scientific, Ward Hill, MA). Following this, 450 mL of BPW was added to sample bags and vigorously shaken for 1 min. Separate dilutions were prepared for each matrix at each inoculation level for further processing

and quantification.

Microbiological Analysis

Quantification of Salmonella spp.

Conventional Plating: *Salmonella* quantification was performed using the traditional plating method. Samples were inoculated at two levels for *Salmonella* spp. From each sample, 10 mL aliquot from sample rinses was transferred to sterile test tubes and was serially diluted in PBS. Subsequently, 100 µL from each dilution was spread plated onto Tryptic Soy Agar (TSA) supplemented with 200ppm nalidixic acid. Plates were incubated at 37°C for 24 h. After the incubation, presumptive *Salmonella* spp. colonies were reported as CFU/g or CFU/mL, depending on each sample type and inoculation level.

Miniaturized Most Probable Number (mMPN): Quantification of *Salmonella* using mMPN was performed using the method described by Chenu et al., 2013. 1 mL of the original sample was added to the first three tubes of a 96-well microtube plate. The samples were added in triplicates, and serial dilutions (100:900 µL) were performed in BPW using a multichannel pipette until 10^{-8} in the final microtube for each sample. All tubes were repeatedly mixed by pipetting and incubated overnight at 37°C. Following incubation, 100 µL of the BPW solution from each well was transferred into the corresponding microtube of a new microtube plate containing 900 µL Modified Semi-Solid Rappaport-Vassiliadis (MSRV) (BD, Franklin Lakes, NJ). The samples were again further diluted and mixed using a multichannel pipette and incubated at 42°C for 24 h. A change in the color of tubes and the formation of white-hallow tubes was signified as presumptive positive for *Salmonella*. This was further confirmed by using a 10 µL multichannel pipette and dipping into each replicate of cluster tubes and poking into RF Chromogenic Agar

for *Salmonella* (R&F Products, Downers Grove, IL). The number of wells with reddish-pink raised colonies was presumed positive for *Salmonella*. Populations of *Salmonella* for each sample were determined using the BAM-MPN calculator (Garthright & Blodgett, 2003).

PCR-based quantification assay: *Salmonella* was quantified using two different PCR-based assays, PCR assay 1 (GENE-UP® QUANT *Salmonella*) and PCR assay 2 (BAX® Salquant™).

For PCR-based assay 1, 40 mL of sample was transferred into the conical tube, and further sample preparation was completed according to the GENE-UP®QUANT *Salmonella* (bioMérieux, Marcy-l'Étoile, France) assay in accordance with the manufacturer's protocol. The PCR tube was then analyzed in the GENE-UP® PCR instrument, as per the GENE-UP® PCR instrument user guide, following AOAC® approved TRUE non-enrichment assay. Once the PCR run is complete, all assay Cp data are transferred to the appropriate SLM Quant Enumeration Calculator for the final enumeration as per the manufacturer's guidelines.

For PCR-based assay 2, 30 mL of rinsate from each sample (primary enrichment) was combined with 30 mL of pre-warmed BAX-MP (Hygiena, Camarillo, CA) media supplemented with 40 mg/L novobiocin pre-warmed at 42°C in a 24 oz filtered Whirl-Pak bag, homogenized for 1 min at 230 rpm, and incubated at 42°C for 8 h for ground chicken and ground turkey and 6 h for chicken wing rinses. Following incubation, each sample was processed utilizing BAX® System (SalQuant™ ;Hygiena BAX® System; Hygiena, Camarillo, CA) RT *Salmonella* assay following the AOAC 081201 protocol (AOACInternational, 2023) and the CT value obtained was inserted into the Salquant™ curve to determine the estimated log-pre-enrichment levels of *Salmonella* in each sample.

Quantification of Campylobacter spp.

Conventional Plating: *Campylobacter* quantification was performed using the traditional plating method. Samples were inoculated at two levels for *Campylobacter*. From each sample, 10 mL aliquot from sample rinses was transferred to sterile test tubes and was serially diluted in PBS. Subsequently, 100 µL from each dilution was spread plated Campy-Cefex agar (HiMedia Laboratories Pvt. Limited, Mumbai, India) supplemented with 5 ppm ciprofloxacin. Plates were incubated at 42°C for 44-48 h under microaerophilic conditions (85% N₂, 10% CO₂, and 5% O₂). After the incubation, typical *Campylobacter* spp. colonies were reported as log CFU/g for ground meat samples and log CFU/mL for chicken wing rinse samples.

Automated MPN Method: Quantification of *Campylobacter* was done using the automated MPN technique (ISO 16140/AFNOR) with the TEMPO® System (BioMérieux, Paris, France). Each vial containing Tempo® CAM culture medium was reconstituted by adding 3 mL of sterile DI water and mixed. Subsequently, samples were prepared using manufacturers guidelines and mixed gently to prevent oxygenation of the media (Line et al., 2011). The sample was then loaded into micro channeled Tempo® cards, which were vacuum sealed (TEMPO®, BioMérieux, Marcy l'Étoile, France) and incubated for 44-48 h at 42°C under microaerophilic conditions. Following incubation, the cards were examined for *Campylobacter* populations (Tempo® Reader), and the results were reported as log CFU/g for ground meat samples and log CFU/mL for chicken wing rinse samples.

PCR-based Quantification Assay: PCR quantification of *Campylobacter* in chicken wing rinses was performed using CampyQuant™ (Hygiena BAX System; Hygiena, Camarillo, CA). From each sample 30 mL of primary enrichment rinse were transferred in a 24 oz filtered Whirl-Pak bag (Nasco, Fort Atkinson, WI). An equal volume of 30 mL of pre-warmed 2X Bolton's Broth +

2X Bolton's Broth Supplement at 42°C (HiMedia Laboratories, Mumbai, India) + additional 5 ppm ciprofloxacin due to antibiotic resistant strains used into a filtered Whirl-Pak bag. The combined sixty milliliters mixture was then incubated for 20 h at 42°C in microaerophilic conditions. Following the incubation, each sample was processed utilizing BAX® System RT *Campylobacter* assay, and the CT value obtained was inserted into the Campyquant™ curve to determine the estimated log-pre-enrichment levels of *Campylobacter* in each sample.

Statistical analysis

The data were analyzed using both one-way and two-way analysis of variance (ANOVA) where appropriate. Differences between group means were further evaluated using Tukey's post hoc test for pairwise comparisons. All statistical analyses were performed using JMP PRO 17, and significance was defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

Comparative Analysis of Enumeration Methods for *Salmonella*:

Salmonella populations recovered from the ground turkey, ground chicken, and chicken wing rinses at high- and low-level inoculation were enumerated using four different methods, i.e., conventional plating, mMPN, PCR-based quantification assays (PCR Assay 1, PCR Assay 2). The performance of these methods at the high inoculation level (H) was significantly different (figure 2.1). The mMPN method yielded the highest mean log CFU recovery CA~7.5 log CFU/mL, significantly higher than all other methods ($p \leq 0.05$). In contrast, PCR assay 1 and plating methods showed comparable log CFU values, averaging CA~ 6.5 log CFU/mL, and not significantly different from each other ($p > 0.05$). The PCR assay 2 demonstrated the lowest mean log CFU recovery ~4.5 log CFU/mL, significantly lower than mMPN, PCR assay 1, and

plating methods ($p \leq 0.05$). These results indicate that at a high inoculation level, mMPN is the most effective method for detecting high bacterial concentrations, closely followed by PCR assay 1 and plating.

At low inoculation level (L), the mMPN method showed higher mean log CFU (CA ~ 4.5 log CFU/mL) than other methods. However, these differences were not statistically significant ($p > 0.05$) from the PCR assay 1 and PCR assay 2. The plating method showed the lowest log CFU recovery (CA ~ 3.5 log CFU/mL), which was significantly different from mMPN ($p \leq 0.05$) but not from PCR assay 1 and PCR assay 2 ($p > 0.05$). This suggests that at lower bacterial loads, while mMPN remains an efficient method, PCR assay 1 and PCR assay 2 provide comparable results. Based on the results obtained, differences in bacterial recovery were subjective to the enumeration method employed rather than the matrix.

In ground turkey, at the high inoculation level (H), significant differences ($p \leq 0.05$) are observed among the enumeration methods (figure 2.2). The mMPN method had the highest log CFU/g (CA $\sim \log 7.5$ CFU/g), significantly greater than all other methods ($p \leq 0.05$), which was followed by PCR assay 1 and plating with similar recovery of CA $\sim \log 6.5$ CFU/g, while PCR assay 2 had the lowest recovery (CA $\sim \log 4.5$ CFU/g), which was significantly lower than mMPN, PCR assay 1, and plating ($p \leq 0.05$). At the low inoculation level (L), mMPN had the highest recovery (CA ~ 4.5 log CFU/g), but no significant differences ($p > 0.05$) were observed between PCR assay 1, plating, and PCR assay 2 (CA $\sim \log 3.5$ – 4.0 CFU/g). This indicates that all methods were similar in quantifying pathogens at lower concentrations.

In ground chicken, at high inoculation level (H), significant differences ($p \leq 0.05$) were observed among the four enumeration methods (figure 2.3). The mMPN method recorded the highest

mean recovery of *Salmonella* (CA ~ 7.38 log CFU/g). PCR assay 1 and plating methods showed similar ($p > 0.05$) recovery of *Salmonella* population (CA ~ 6.0 log CFU/g). However, both the PCR assay 1 and traditional plating methods significantly differed from PCR assay 2 ($p \leq 0.05$). At low inoculation level (L), all four methods—mMPN, PCR assay 1, traditional plating, and PCR assay 2 recovered similar log CFU/mL of *Salmonella* (CA ~ 3.5 to 4.0 log CFU/g), with no significant differences ($p > 0.05$) observed among them. These results suggest that, while there are differences in method performance at higher inoculation levels, all methods are comparable for the detection of *Salmonella* populations at low inoculation levels.

For chicken wings inoculated at high level (H), significant differences ($p \leq 0.05$) were observed among the four enumeration methods (figure 2.4). The recovery of *Salmonella* from the mMPN method was 7.5 log CFU/mL, indicating it is the most sensitive method for detecting high bacterial concentrations. This was significantly higher ($p \leq 0.05$) than the other three methods, indicating its superior performance at high bacterial concentrations. PCR assay 1 and conventional plating showed similar performance, with mean recovery of approximately 6.0 log CFU/mL and 5.5 log CFU/mL, respectively. The difference between these two methods was not statistically significant ($p > 0.05$) at high inoculation levels. However, in the case of PCR assay 2, the lowest population of *Salmonella* (CA ~ 4.5 log CFU/mL) was recovered, which was significantly lower than mMPN, PCR assay 1, and plating ($p \leq 0.05$). This pattern of underestimation of bacterial count by PCR assay 2 was consistent in different matrices, including ground turkey and ground chicken. The results suggest that, at higher bacterial concentrations, this enumeration method can have limitations in performance due to assay constraints. While at lower levels of inoculation, the uniformity across all methods demonstrates that all methods are

equally capable of enumerating *Salmonella* concentrations, with the choice of the method further depending on laboratory preferences, operational requirements, and time to obtain results.

The results from this study suggest that mMPN is the most reliable method for quantifying *Salmonella* at higher loads, followed by PCR assay 1 and conventional plating. However, there can also be a trend observed for PCR assay 2, that the limitation in quantifying the level of pathogen accurately at higher bacterial loads can be attributed to populations exceeding the upper limit of quantification (ULQ) for this assay, thus restricting the ability to provide accurate estimates and underestimate the actual bacterial loads (Vashist & Luong, 2018). A potential issue with such a limitation can potentially arise when used in situations with a high likelihood of contamination with high concentrations of *Salmonella*. Enumeration methods employed in this study, like mMPN and conventional plating, are more reliable in these scenarios as they can quantify pathogens at higher bacterial loads. However, in real-world scenarios, the need for detection arises mostly at lower bacterial concentrations, posing a food safety concern as pathogens like *Salmonella* multiply quickly in optimum temperature and time and pose the risk of potential foodborne illnesses. The results from this experiment signify the robustness of all four methods to quantify levels of *Salmonella* at lower inoculation levels, indicating their suitability for routine food testing and monitoring. Thus, the choice of the enumeration method can be flexibly made depending on the specific needs, time constraints, and budget. This adaptability is essential for small to medium-sized businesses with financial limitations. Our study indicates that the mMPN method demonstrates high effectiveness across all matrices and both levels of contamination, indicating it is the most effective enumeration method for expected contamination levels amongst the other enumeration methods tested. The mMPN

methods offer quick, accurate, and cost-effective detection of target pathogens (Fung & Kraft, 1969; Oscar, 2004). This method entails two enrichment stages - primary enrichment in BPW which allows the recovery and quantification of injured and stressed cells. This is followed by secondary enrichment in MSRV broth supplemented with novobiocin for selective enrichment. In comparison to traditional MPN, all the steps are conducted on a single microtiter plate, streamlining the process and making it efficient and practical (Chenu et al., 2013).

However, conventional plating provides rapid results without necessitating an enrichment step and at a lower cost than the mMPN method (Brichta-Harhay et al., 2007). It also has certain limitations, like the variability in the choice of media, potential human error, and contamination with non-target bacteria, which can affect the accuracy of results and potentially lead to underestimation of the actual pathogen load in the sample.

In contrast, enrichment-based enumeration methods, such as the mMPN method and PCR-based quantification assays (PCR assay 1 and PCR assay 2 in this study), consistently deliver more dependable results due to enhanced selectivity for the target pathogen (Brichta-Harhay et al., 2008). In addition, the time required to quantify pathogens is a critical factor in determining the enumeration method. In such cases, PCR-based quantification methods are advantageous, even though a high initial cost is required to set up, but can rapidly analyze the bacterial load in samples before they are released into commerce. In this study, two PCR-based quantification methods were employed, quantifying the bacterial load in less than 24 h. PCR-based assay 1 required no enrichment time, and *Salmonella* counts were obtained in less than 4 h (Schmidt et al., 2024). In the case of PCR-based assay 2, the samples required a shortened enrichment time as compared to the conventional mMPN method, and the *Salmonella* counts were determined in

less than 24 h. In such cases, where time is a critical factor, PCR-based assays outperform mMPN and plating, which are widely employed methods but also require extended periods, increased labor, and large enrichment periods (Kim et al., 2017). This rapid turnaround time can be an advantage, especially needed in the poultry industry, where strict adherence to regulatory guidelines and compliance with USDA-FSIS standards is mandated. The PCR-based quantification methods can allow quick surveillance and routine testing for foodborne pathogens to ensure a safer food supply and employ timely corrective actions.

Comparative Analysis of Enumeration Methods for *Campylobacter*

Campylobacter populations recovered from the ground turkey, ground chicken, and chicken wing rinses at high-level and low-level inoculation were enumerated using two different methods, i.e., conventional plating and automated MPN. Chicken wing rinses were analyzed using the third additional method as well i.e., validated PCR-based quantification method. The performance of these methods at high inoculation levels (H) was significantly different. (figure 2.5). The plating method yielded the highest mean log CFU recovery (CA ~6.09 log CFU/mL), significantly higher than automated MPN (CA~5.88 log CFU/ mL) ($p \leq 0.05$). At low-level inoculation, both automated MPN (CA ~ 2.836 log CFU/mL) and conventional plating (CA~ 3.015 log CFU/ mL) reported similar log CFU recovery, with no significant difference ($p > 0.05$) from each other. The results from the quantification levels at both levels of inoculation indicate that variation in bacterial counts is not influenced by the matrix type employed in the study but rather influenced by the choice of enumeration method.

In the ground turkey, at both high inoculation levels (H) and low inoculation levels (L), significant differences ($p \leq 0.05$) were observed among the enumeration methods (figure 2.6). At

high inoculation levels, plating had the highest recovery (CA ~ 6.11 log CFU/ g), which was significantly greater than the automated MPN method (CA~ 5.7 log CFU/g) ($p \leq 0.05$). At a low level of inoculation, the plating method reported higher recovery (CA~ 3.166 log CFU/g), which was significantly different from the automated MPN method (CA~ 2.756 log CFU/g) ($p \leq 0.05$). The results highlight the importance of choosing the method of enumeration based on expected contamination levels, as the enumeration methods may perform differently at high and low bacterial concentrations.

In ground chicken, at high level of inoculation (H), significant differences ($p \leq 0.05$) were observed between the enumeration methods (figure 2.7). Conventional plating reported the highest recovery (CA~ 6.00 log CFU/ g), which was significantly greater than automated MPN (CA~ 5.66 log CFU/ g) ($p \leq 0.05$). At a low level of inoculation (L), no significant differences ($p > 0.05$) were observed between plating and automated MPN with recoveries of CA~ 2.8 log CFU/ g and CA~2.6 log CFU/ g, respectively. The comparable performance at lower contamination levels suggests that the method selection is more influenced by factors such as cost, user-friendliness, and resource availability.

In chicken wings, at high inoculation level (H), significant differences ($p \leq 0.05$) were observed in three enumeration methods (figure 2.8). Plating (CA~6.17 log CFU/ mL) and automated MPN (CA~6.2 log CFU/mL) had similar recovery with no significant difference between them. The bacterial counts obtained from plating and automated MPN were significantly higher than the PCR-based quantification (CA~4.6 log CFU/ mL) ($p \leq 0.05$). At the low levels of inoculation (L), the PCR-based quantification assay showed the highest recovery (CA~ 4.45 log CFU/ mL), which was significantly greater than the plating (CA~ 3.13 CFU/ mL) and automated MPN

(CA~3.07 CFU/ mL) method ($p \leq 0.05$). The experimental results indicate that while plating and automated MPN perform well at high bacterial concentrations, the PCR-based assay can play an instrumental role in quantifying bacteria at lower levels, which plays a crucial role in the early detection of pathogens in industrial settings.

The variations in method performance in different matrices and inoculation levels emphasize the necessity for need-based approaches. At high -inoculation levels, *Campylobacter* recovery was significantly higher in conventional plating as compared to automated MPN. Plating requires extended incubation periods, specialized growth media, and considerable manual effort to accurately identify and enumerate *Campylobacter* colonies, which can take several days (Hansson et al., 2015). While this traditional culture-based method remains the benchmark for its specificity and ability to detect viable organisms, the laborious nature and prolonged time-to-result make it less practical for high-throughput settings or situations requiring rapid decision-making (Humphrey et al., 2007). In addition, certain types of growth media can selectively promote the growth of specific strains of *Campylobacter*, which can result in underestimating or inaccurately estimating the total *Campylobacter* level in a particular sample. Automated MPN, on the other hand, provides a semi-quantitative approach due to its estimation based on statistical probability rather than direct counting. It estimates the growth patterns based on pre-established references to estimate microbial concentrations. This approach balances sensitivity with operational efficiency. It reduces the manual workload and minimizes human error by automating the enumeration process (Owen et al., 2010).

Automated MPN offers certain advantages, such as the processing of multiple samples in comparatively half the amount of time as compared to conventional plating. The efficiency of

this method plays an instrumental role in industrial settings where numerous samples are assessed for bacterial load across processing lines on a day-to-day basis (Katase & Tsumura, 2011). In addition to that, data tracking can be easily maintained and traced back in case of any recall while assessing for CCPs (Critical control points). Automated MPN outpaces plating in speed; the time required for result generation is still equivalent to plating and is comparatively time-consuming when compared to molecular methods like PCR-based quantification assays (Hansson et al., 2015). In the case of automated MPN, it is crucial to emphasize the precise preparation of the dilution series. Any deviations in this process between operators or laboratories can impact the dependability of the results acquired (Owen et al., 2010). For the analysis of chicken wing samples, three methods were employed to quantify *Campylobacter* levels: PCR based assay, plating, and automated MPN. The PCR-based assay was included specifically for this matrix due to the availability of a validated method. The PCR-based method offers the advantage of exhibiting high sensitivity, detecting low contamination levels, and replacing extensive culturing. It has a rapid detection mechanism with a 20h enrichment period, which is shorter than the incubation times required for automated MPN and conventional plating methods (Bodie et al., 2024). Time efficiency proves especially advantageous in those food safety testing environments, where rapid responses to potential contamination are essential. At higher levels of contamination, similar limitations were evident, as observed with PCR Assay 2 in *Salmonella* detection due to its upper limit of quantification. The assay has a defined limit of quantification with an enumerable range from 10-10,000 CFU/mL, beyond which its accuracy diminishes due to saturation effects and the presence of amplification inhibitors when bacterial loads are high (Ricke et al., 2019). Apart from the initial

high set-up cost, these PCR-based assays can empower poultry processors to identify points with higher *Campylobacter* loads and thus take necessary corrective actions to reduce the risk of foodborne illnesses.

From the comparison of enumeration methods conducted in the above experiment, the selection of the enumeration method should be based on factors such as contamination levels, available resources, and testing throughput requirements. Our study demonstrated that the choice depends on the specific needs of the testing environment. While plating is accurate and capable of enumerating viable cells, it is unsuitable for rapid or large-scale testing due to its labor-intensive nature and reduced time efficiency. Automated most probable number (MPN) could quantify *Campylobacter* levels in close competence to conventional plating, making it suitable for routine surveillance where moderate throughput and cost considerations are important.

PCR-based quantification assays are ideal for screening at levels where a quantifiable range of *Campylobacter* is low. Still, limited validation in matrices poses a limitation in the scope of application across various food matrices.

Future research should concentrate on refining these methods by further evaluating the performance of these assays at lower contamination levels than tested in this study. In addition to that, this study further suggests that there should be impetus to reducing the incubation periods, which poses a significant limitation in the quantification of *Campylobacter* as a prolonged 48h incubation time under microaerophilic conditions puts a substantial drawback in timely results.

CONCLUSION

This study underscores the importance of choosing appropriate enumeration methods based on the contamination levels and operational requirements for detecting *Salmonella* and

Campylobacter. The results from this study revealed that the mMPN method was consistently the most reliable method for detecting *Salmonella* at higher contamination levels. In the case of *Campylobacter*, conventional plating, and automated MPN performed comparably similar. Automated MPN can be a preferred method in industrial settings where high-throughput testing is performed. At lower contamination levels, PCR-based quantification assays demonstrated high efficiency in detecting pathogen loads due to lower detection limits, enhanced sensitivity, and no or minimal requirement of enrichment period. The expedited turnaround times enable poultry processors to make data-driven, timely decisions and accelerate pathogen detection. PCR-based quantification assays also address the critical need for early pathogen detection at lower levels, minimizing the risk of preventing potential outbreaks and ensuring food safety compliance. Future research should further evaluate the performance of these quantification assays at lower contamination levels to understand each assay's relative strengths and limitations. Additionally, the data generated from quantification methods could play an instrumental role in developing predictive models and performing risk assessment studies. Presently, the limited availability of validated PCR-based quantification assays across different matrices restricts its scope of application, especially for *Campylobacter*. Future research should focus on its broad applicability across various poultry matrices. Additionally, prolonged incubation times, especially for *Campylobacter*, limit the availability of timely results, thus highlighting the need for quick detection methodologies. Ultimately, this research experiment's findings will enhance poultry products overall safety and quality, benefiting consumers and industries alike.

REFERENCES

AOAC International (2023). *AOAC performance tested method 081201:BAX System Real-Time PCR Assay for Salmonella*. Available at

https://members.aoac.org/aoac/PTM_Validated_Methods. Accessed on September 2,2024

Brichta-Harhay, D., Arthur, T., Bosilevac, J., Guerini, M., Kalchayanand, N., & Koohmaraie, M. (2007). Enumeration of *Salmonella* and *Escherichia coli* O157: H7 in ground beef, cattle carcass, hide, and fecal samples using direct plating methods. *Journal of applied microbiology*, 103(5), 1657-1668.

Brichta-Harhay, D., Arthur, T., & Koohmaraie, M. (2008). Enumeration of *Salmonella* from poultry carcass rinses via direct plating methods. *Letters in Applied Microbiology*, 46(2), 186-191.

Bodie, A. R., Dittoe, D. K., Applegate, S. F., Stephens, T. P., & Ricke, S. C. (2024). Adaptation of a Commercial Qualitative BAX® Real-Time PCR Assay to Quantify *Campylobacter* spp. in Whole Bird Carcass Rinses. *Foods*, 13(1), 56. <https://www.mdpi.com/2304-8158/13/1/56>

Centers for Disease Control and Prevention (CDC; 2024a). *Campylobacter Infections*. Available

at <https://www.cdc.gov/campylobacter/about/index.html>. Accessed on August 26, 2024

Centers for Disease Control and Prevention, (CDC; 2024b) *Foodborne Illnesses and Germs*.

Available at <https://www.cdc.gov/foodborneburden/index.html>. Accessed on August 26, 2024

Chenu, J. W., Pavic, A., & Cox, J. M. (2013). A novel miniaturized most probable number method for the enumeration of *Campylobacter* spp. from poultry-associated matrices. *Journal of microbiological methods*, 93(1), 12-19.

European Food Safety Authority. (EFSA; 2013). Scientific Opinion on the public health hazards to be covered by inspection of meat (bovine animals). *EFSA Journal*, 11(6).

<https://doi.org/10.2903/j.efsa.2013.3266>

Fung, D. Y., & Kraft, A. A. (1969). Rapid evaluation of viable cell counts by using the microtiter system and MPN technique. *Journal of Food Protection*, 32(10), 408-409.

Garthright, W., & Blodgett, R. (2003). FDA's preferred MPN methods for standard, large, or unusual tests, with a spreadsheet. *Food microbiology*, 20(4), 439-445.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T. J., & Van Immerseel, F. (2009). Mechanisms of egg contamination by *Salmonella* Enteritidis. *FEMS microbiology reviews*, 33(4), 718-738.

Hansson, I., Nyman, A., Lahti, E., Gustafsson, P., & Engvall, E. O. (2015). Associations between *Campylobacter* levels on chicken skin, underlying muscle, caecum, and packaged fillets. *Food microbiology*, 48, 178-181.

Humphrey, T., O'Brien, S., & Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: a food production perspective. *International journal of food microbiology*, 117(3), 237-257.

Hygiena. (2019). *Quantification and Limits Testing Product Instructions Using BAX Assays*.

<https://www.hygiena.com/documents/81111/quantification-and-limits-testing-product-instructions-using-bax-assays.pdf>

Katase, M., & Tsumura, K. (2011). Enumeration of micro-organisms in processed soy products with an automated most probable number method compared with the standard plate method.

Letters in Applied Microbiology, 53(5), 539-545. <https://doi.org/10.1111/j.1472-765X.2011.03143.x>

Kim, S. A., Park, S. H., Lee, S. I., & Ricke, S. C. (2017). Development of a rapid method to quantify *Salmonella Typhimurium* using a combination of MPN with qPCR and a shortened time incubation. *Food microbiology*, 65, 7-18.

LaRock, D. L., Chaudhary, A., & Miller, S. I. (2015). *Salmonellae* interactions with host processes. *Nature Reviews Microbiology*, 13(4), 191-205.

Molnar, G., Mertsola, J., & Erkkö, M. (1982). Guillain-Barré syndrome associated with *Campylobacter* infection. *British Medical Journal (Clinical research ed.)*, 285(6342), 652.

Line, J., Stern, N., Oakley, B., & Seal, B. (2011). Comparison of an automated most-probable-number technique with traditional plating methods for estimating populations of total aerobes, coliforms, and *Escherichia coli* associated with freshly processed broiler chickens. *Journal of Food Protection*, 74(9), 1558-1563.

Oyarzabal, O. A., Macklin, K. S., Barbaree, J. M., & Miller, R. S. (2005). Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. *Applied and environmental microbiology*, 71(6), 3351-3354.

O'Bryan, C. A., Ricke, S. C., & Marcy, J. A. (2022). Public health impact of *Salmonella* spp. on raw poultry: Current concepts and future prospects in the United States. *Food Control*, 132, 108539.

Oscar, T. (2004). Simulation model for enumeration of *Salmonella* on chicken as a function of PCR detection time score and sample size: implications for risk assessment. *Journal of Food Protection*, 67(6), 1201-1208.

Owen, M., Willis, C., & Lamph, D. (2010). Evaluation of the TEMPO® most probable number

technique for the enumeration of *Enterobacteriaceae* in food and dairy products. *Journal of applied microbiology*, 109(5), 1810-1816.

Ricke, S. C., Feye, K. M., Chaney, W. E., Shi, Z., Pavlidis, H., & Yang, Y. (2019).

Developments in rapid detection methods for the detection of foodborne *Campylobacter* in the United States. *Frontiers in microbiology*, 9, 3280.

Schmidt, J. W., Carlson, A., Bosilevac, J. M., Harhay, D., Arthur, T. M., Brown, T., Wheeler, T. L., & Vipham, J. L. (2024). Evaluation of methods for identifying poultry wing rinses with *Salmonella* concentrations greater than or equal to 10 CFU/mL. *Journal of Food Protection*, 100362.

U.S. Department of Agriculture, Food Safety and Inspection Service, (USDA-FSIS, 2021). FSIS Guidance Document for Controlling *Salmonella* and *Campylobacter* in Poultry (FSIS-GD-2021-00060). U.S. Department of Agriculture. Available at:
https://www.fsis.usda.gov/sites/default/files/media_file/2021-07/FSIS-GD-2021-0006.pdf.

Accessed on August 26,2024

Umaraw, P., Prajapati, A., Verma, A. K., Pathak, V., & Singh, V. (2017). Control of *Campylobacter* in poultry industry from farm to poultry processing unit: A review. *Critical reviews in food science and nutrition*, 57(4), 659-665.

Vashist, S. K., & Luong, J. H. (2018). Bioanalytical requirements and regulatory guidelines for immunoassays. In *Handbook of immunoassay technologies* (pp. 81-95). Elsevier.

Wang, J., Vaddu, S., Bhumanapalli, S., Mishra, A., Applegate, T., Singh, M., & Thippareddi, H. (2023). A systematic review and meta-analysis of the sources of *Salmonella* in poultry production (pre-harvest) and their relative contributions to the microbial risk of poultry meat. *Poultry Science*, 102(5), 10256

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Figure 2.1 - Graphic representation of *Salmonella* counts (log CFU/ mL) recovered from poultry meat samples. In the graph, black bars represent the high inoculation level. In contrast, hatched bars denote the low inoculation level. Each bar includes standard error bars to show variability in the measurements. mMPN, PCR-based quantification assays, and conventional plating determined *Salmonella* inoculum. ^(a-c ; x-y) Denote means with different letters indicate significant differences ($p \leq 0.05$) between samples analyzed using various methods of enumeration and different inoculation stages

Figure 2.2 - *Salmonella* counts (log CFU/ g $\pm$ S.D.) recovered from ground turkey samples subjected to high (H) and low (L) inoculation levels, enumerated by mMPN, PCR Assay 1, PCR Assay 2, and conventional plating. The height represents the mean log CFU/g in each bar, and the error bars represent the standard deviation. Different letters (^{a-c} for high inoculation; ^{x-y} for low inoculation) indicate statistically significant differences ($p < 0.0001$) between counts obtained from different enumeration methods at each inoculation level. Bars sharing the same letter are not significantly different.

Figure 2.3 - *Salmonella* counts (log CFU/ g $\pm$ S.D.) recovered from ground chicken samples at high (H) and low (L) inoculation levels, enumerated by mMPN, PCR Assay 1, PCR Assay 2, and conventional plating. The height represents each bar's mean log CFU/ g, and the error bars represent the standard deviation. Different letters (^{a-c} for high inoculation and ^x for low inoculation)

indicate statistically significant differences ($p \leq 0.05$) between counts obtained from different enumeration methods at each inoculation level.

Figure 2.4 - *Salmonella* counts (log CFU/ mL $\pm$ S.D.) recovered from chicken wing rinse samples subjected to high (H) and low (L) inoculation levels, enumerated by mMPN, PCR Assay 1, PCR Assay 2, and conventional plating. The height represents each bar's mean log CFU/ mL, and the error bars represent the standard deviation. Different letters (^{a-c} for high inoculation; ^{x-y} for low inoculation) indicate statistically significant differences ($p < 0.0001$) between counts obtained from different enumeration methods at high inoculation levels and ($p \leq 0.05$) for low-level inoculation. Bars sharing the same letter are not significantly different

Figure 2.5 - Graphic representation of *Campylobacter* counts (log CFU/ mL) recovered from meat samples. In the graph, black bars represent the high inoculation level, while hatched bars denote the low. Each bar includes standard error bars to show variability in the measurements (^{a-c; x}), denoting means with different letters indicate significant differences ($p \leq 0.05$) between samples analyzed using different methods of enumeration and different inoculation stage

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Figure 2.8 - *Campylobacter* counts (log CFU/ mL $\pm$ S.D.) recovered from chicken wing rinse samples at high (H) and low (L) inoculation levels, enumerated by automated MPN, PCR Assay, and conventional plating. The height represents the mean log CFU/ mL in each bar, and the error bars represent the standard deviation. Different letters (^{a, b} for high inoculation; ^{x, y} for low inoculation) indicate statistically significant differences ($p < 0.0001$) between counts obtained from different enumeration methods at each inoculation level

Figure 2.1

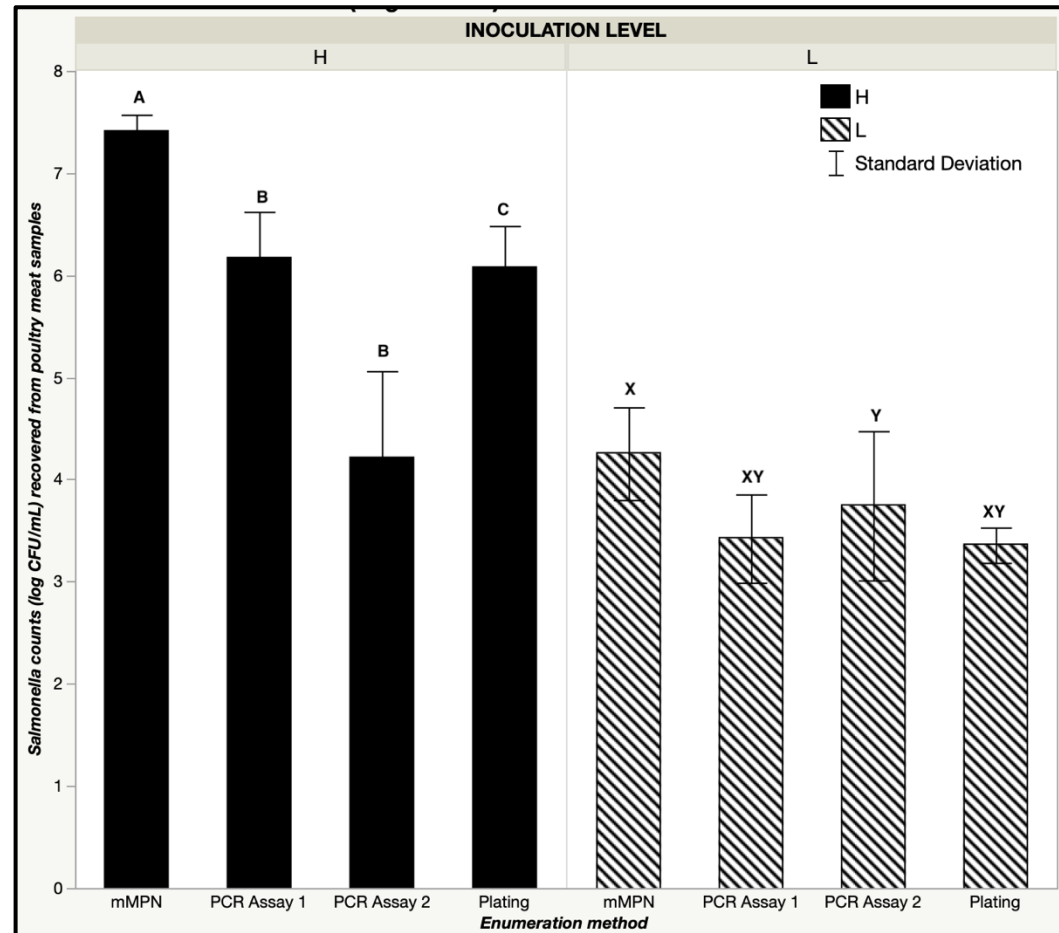


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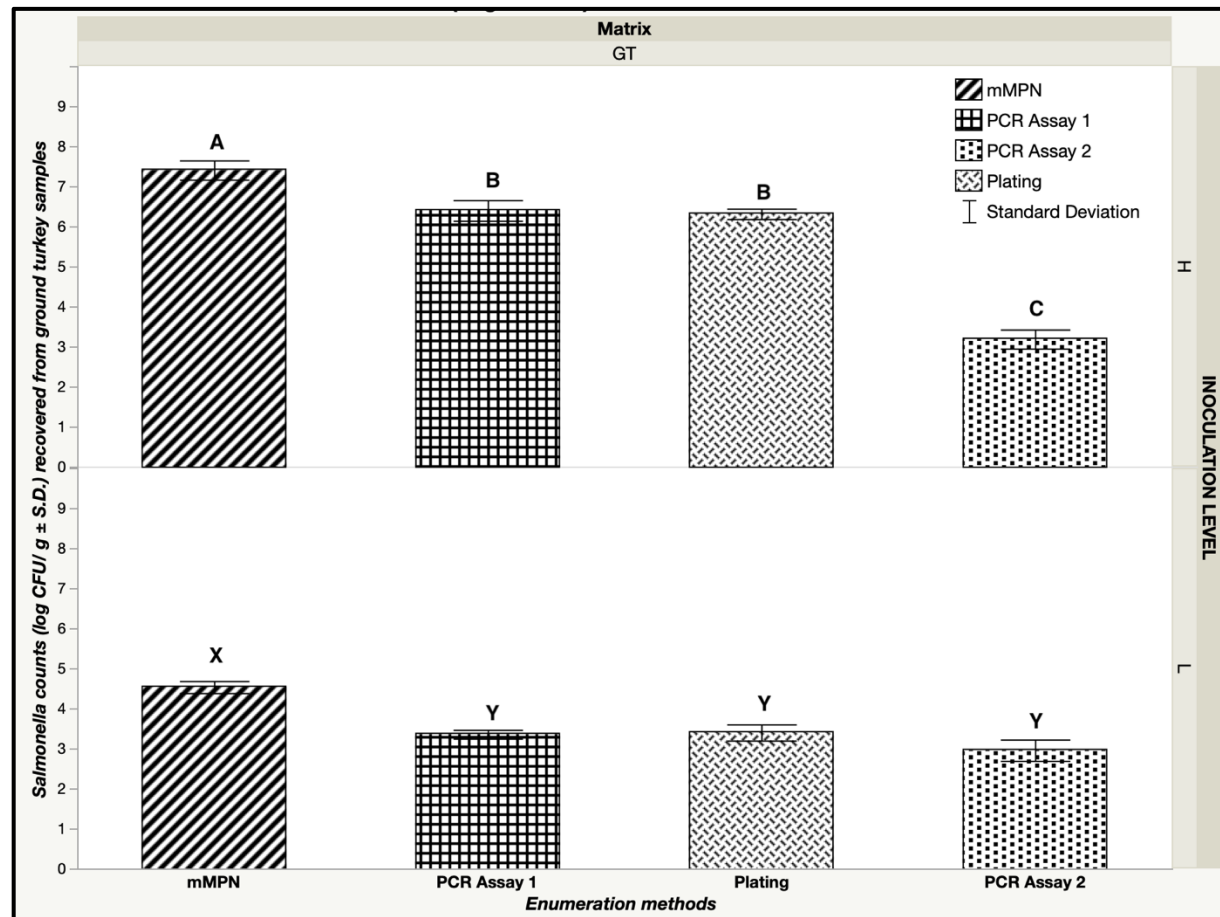


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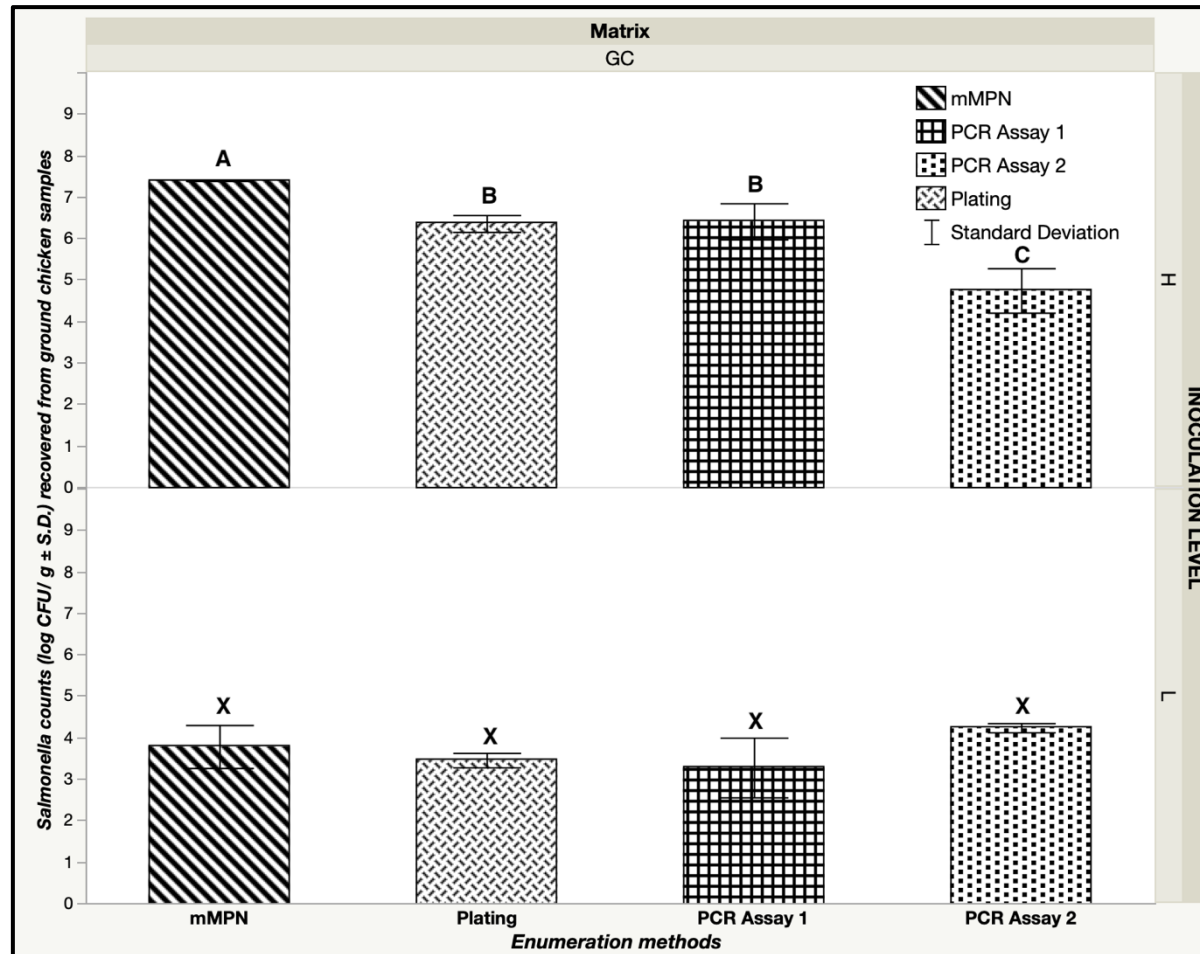


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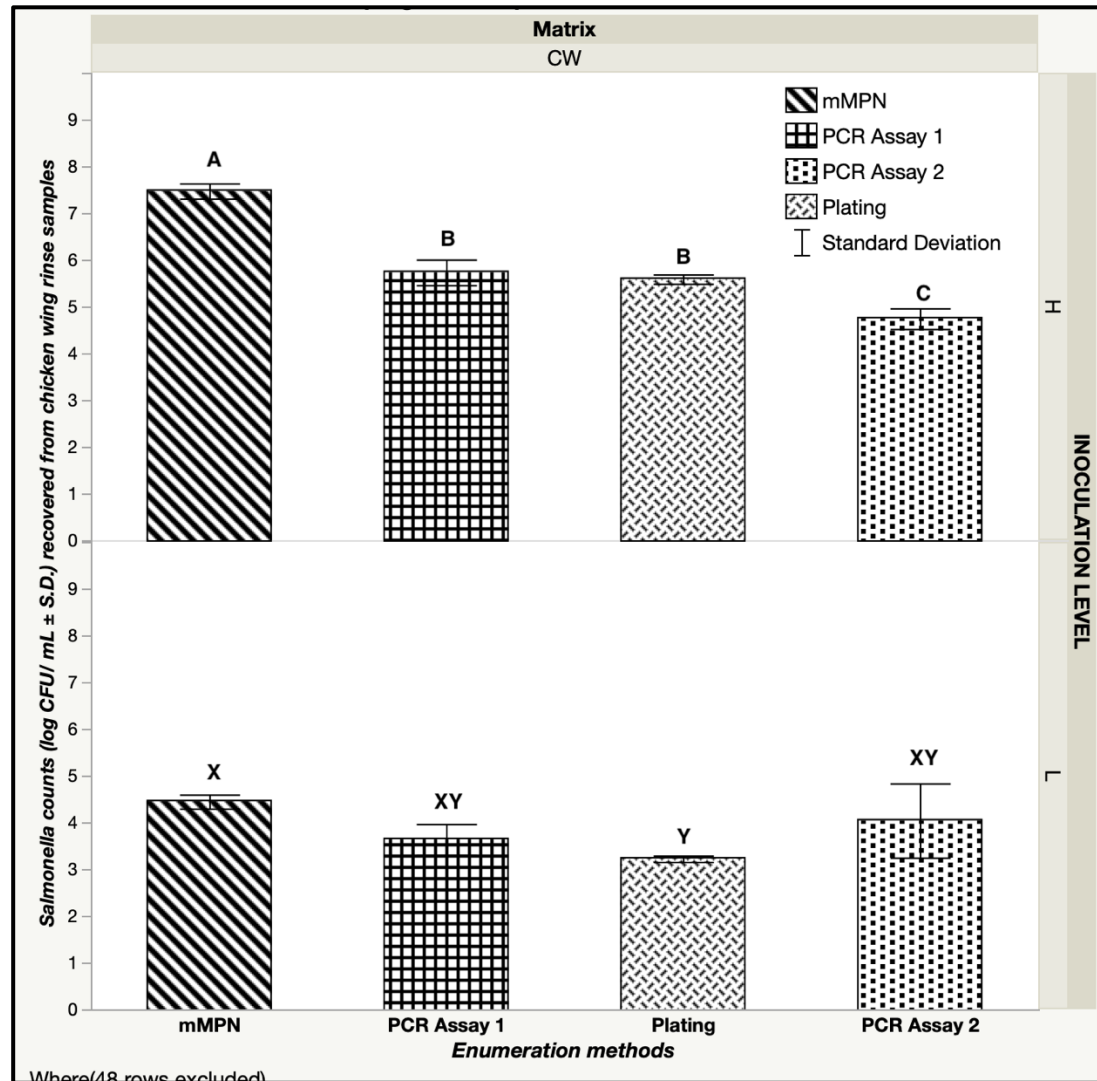


Figure 2.5

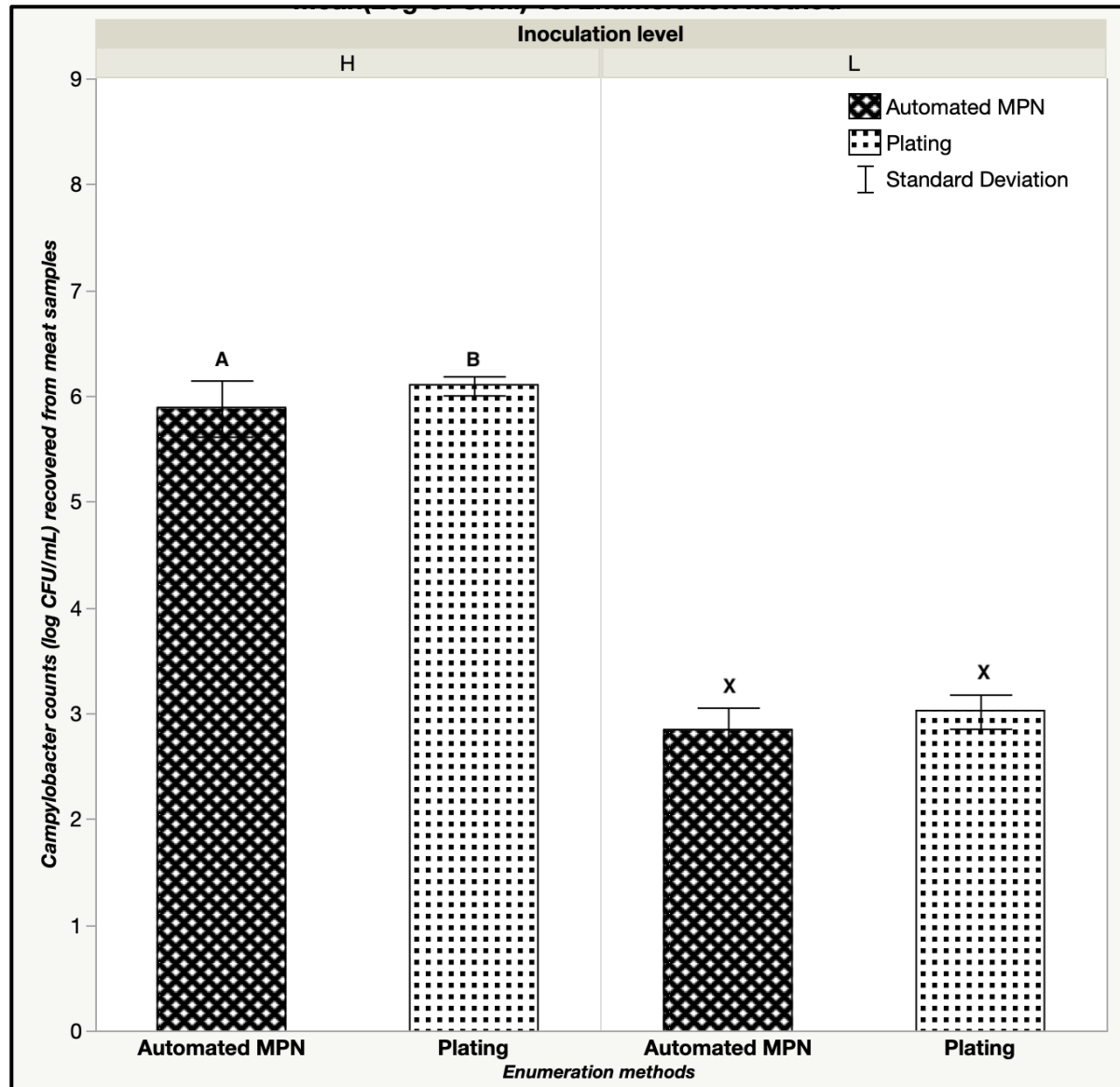


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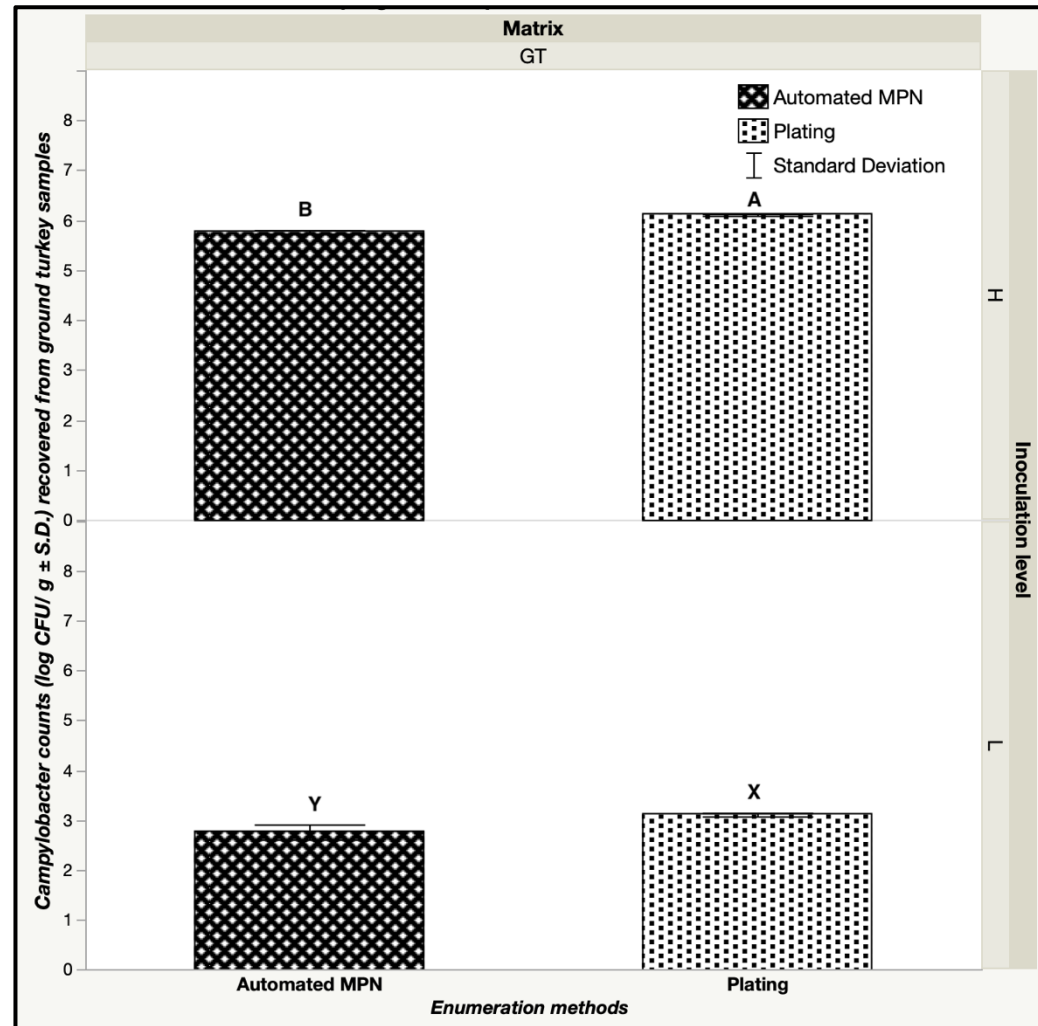


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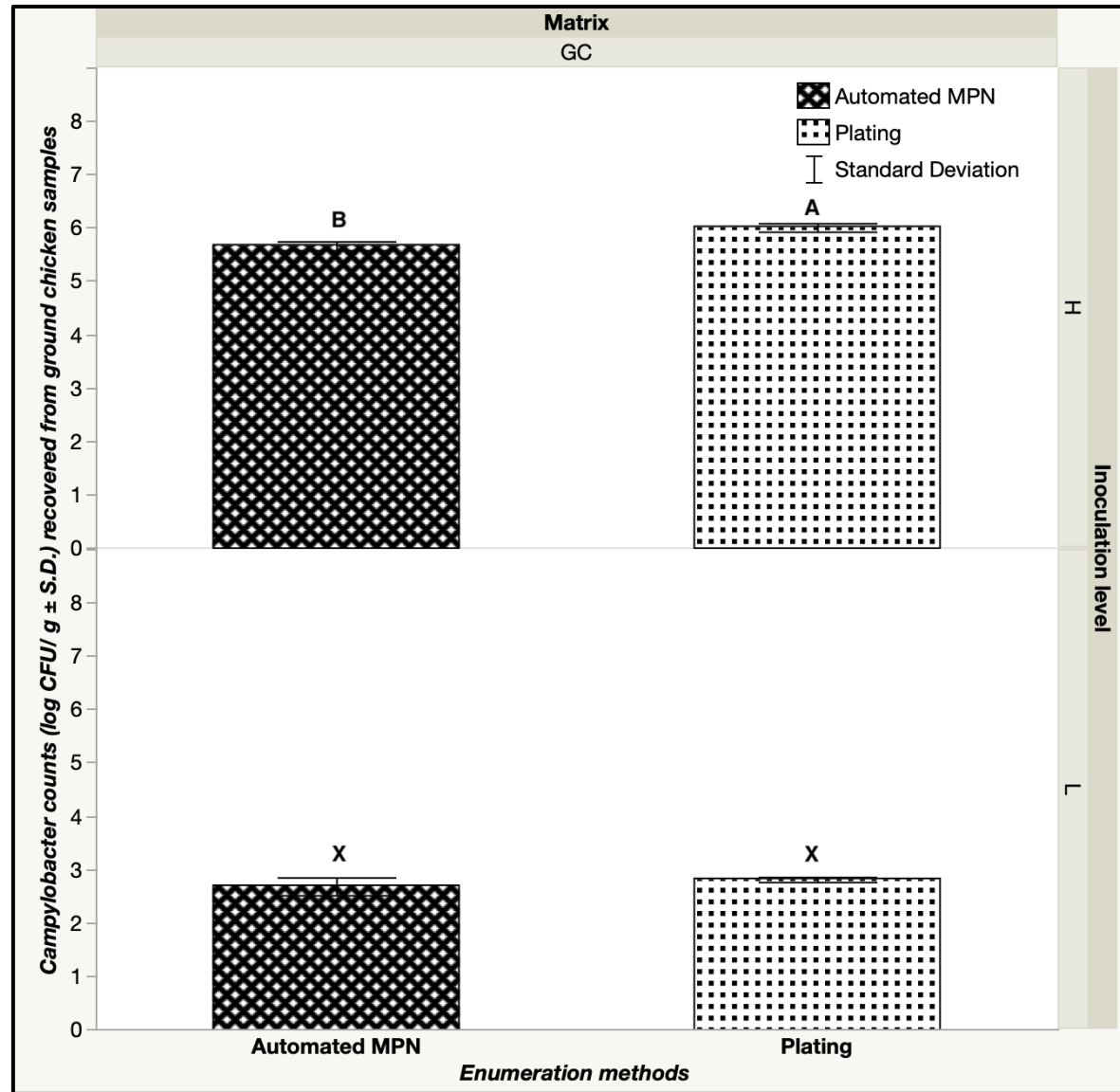
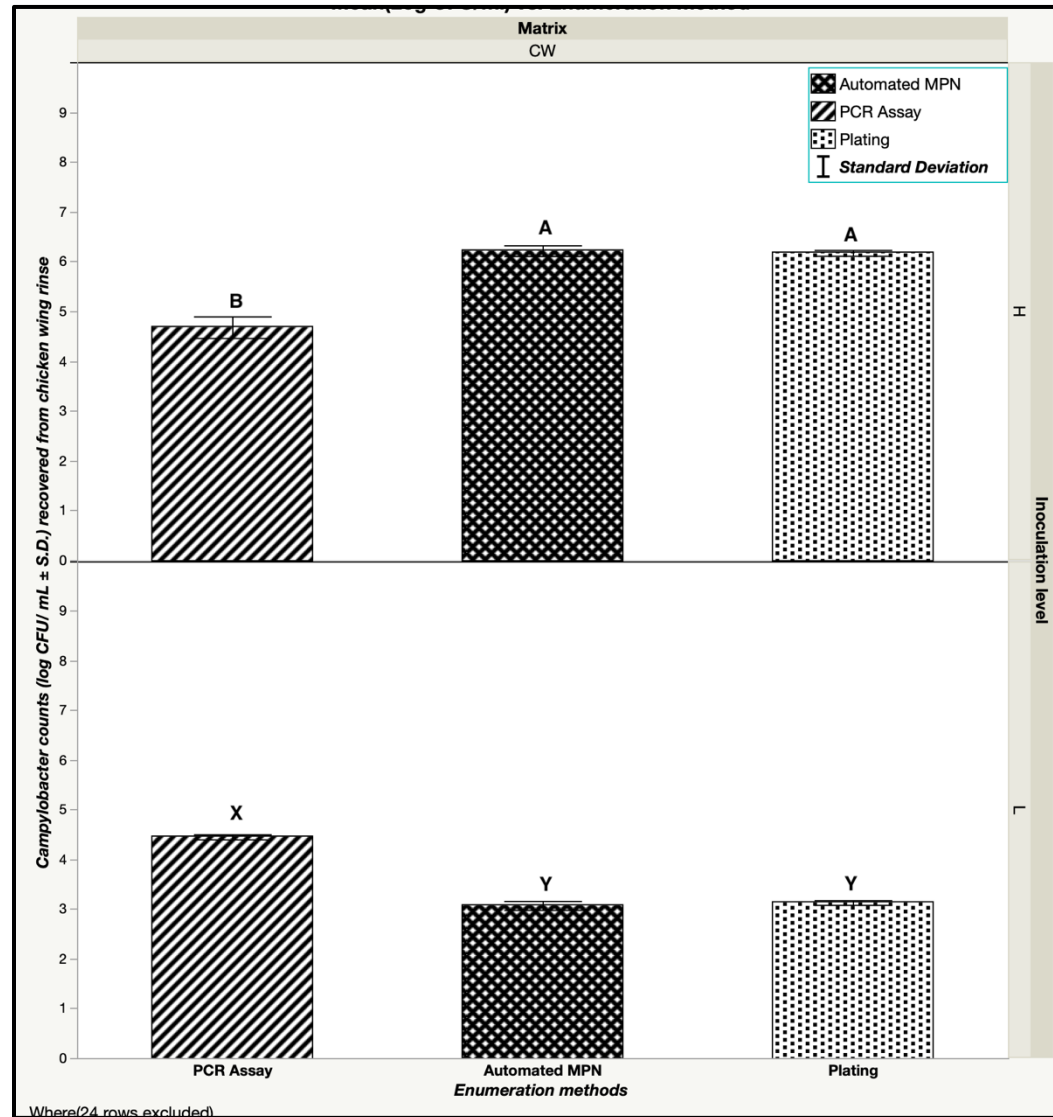


Figure 2.8



CHAPTER 4

OVERALL CONCLUSION AND FUTURE RESEARCH

This study provided a comprehensive comparison of various enumeration methods for the quantification of *Salmonella* and *Campylobacter* across varying degrees of contamination in different poultry matrices. Our results suggest that conventional and rapid automated methods demonstrated varying degrees of accuracy depending on contamination load. Conventional methods such as plating and MPN are considered reliable methods, but the prolonged time for enumeration limits their efficacy and adoption at the industrial scale as it limits the ability to make timely decisions for releasing poultry and poultry products to commerce, thus reducing the chances of foodborne illnesses. In contrast to the traditional methods, rapid PCR-based quantification assays enable the processor to detect lower contamination levels of pathogens due to the increased limit of detection and obtain information in a shorter time frame. This helps in making timely data-driven decisions and reducing the risks associated with the pathogens. Due to their limited adoption, there has been a significant gap in published data comparing the efficacy of culture-based enumeration methods with rapid pathogen quantification methods. This study provides a thorough understanding and robust comparison of widely employed methods—conventional plating, most probable number (MPN), automated MPN, and PCR-based quantification methods for *Salmonella* and *Campylobacter* in poultry.

For *Campylobacter*, conventional plating is accurate and capable of enumerating viable cells;

however, it is not suitable for rapid or large-scale testing due to its labor-intensive nature and reduced time efficiency. Automated Most Probable Number (MPN) could quantify *Campylobacter* levels similarly to conventional plating, making it suitable for routine surveillance where moderate throughput and cost considerations are important. PCR-based quantification assays are ideal for screening at levels where a quantifiable range of *Campylobacter* is low. From this study, it was observed that for *Salmonella*, mMPN was the most effective quantification method irrespective of contamination levels, offering accurate and cost-effective pathogen detection, but it has limitations such as media variability, potential human error, and prolonged time for result delivery. PCR-based rapid quantification assays are performed like mMPN, especially at lower contamination levels, where there is the most need for quantification and detection. These methods can allow for quick detection and enable processors to make timely decisions and faster pathogen monitoring.

Future research should focus on refining the limitations observed in this study. The upper limit of quantification for PCR-based quantification assay can pose a significant risk if the contamination levels are high, causing an underestimation of pathogen load. Investigating ways to improve the range of the assay and expand the quantifiable range can help in increasing their utility.

Additionally, limited validation in matrices poses a limitation in the scope of application across various food matrices. Future research should focus on refining these methods. This study further suggests that reducing the incubation periods should be focused on, which poses a significant limitation in the quantification, especially in the case of *Campylobacter*, as a prolonged 48h incubation time under microaerophilic conditions presents a substantial drawback in timely results. In summary, continuous research and progress in pathogen quantification methods are

essential to address the changing requirements of the poultry industry and guarantee efficient and prompt food safety.