

SELECTIVE PRE-ENRICHMENT METHOD TO LESSEN TIME NEEDED TO RECOVER
SALMONELLA FROM COMMERCIAL POULTRY PROCESSING SAMPLES

by

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(Under the Direction of Nikki Shariat)

ABSTRACT

Conventional *Salmonella* surveillance requires a week for isolation, confirmation, and subsequent serotyping. To reduce this time, we sought to optimize pre-enrichment step for *Salmonella* recovery by addition of selective chemicals from enrichment broths to buffered peptone water (**BPW**). In the first study, duplicate carcass drips samples were collected immediately after feather removal in each of nine commercial processing plants (n=10). In second study, drip samples were collected at post-pick, post-inside-outside bird washer (**IOBW**), and post-chill; duplicate wing rinses were performed pre- and post-antimicrobial wing rinsates at three different processing plants (n=180). All the samples were cultured using traditional methods. The recovery of *Salmonella* using selective pre-enrichment culture conditions were similar to selective enrichment conditions (Fisher's exact test, $P_1 = 1$; $P_2 = 0.72$). These results suggest that a selective pre-enrichment step reduces a day in *Salmonella* isolation and serovar identities determined using CRISPR-SeroSeq also matched between selective pre-enrichment and enrichment methods.

INDEX WORDS: *Salmonella*, broiler processing, pre-enrichment, selective enrichment, serotyping, CRISPR-SeroSeq

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DEDICATION

Dedicated to my family and friends for their unconditional love and support throughout my life.

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NOMENCLATURE

BGS	Brilliant Green Sulfa Agar
BPW	Buffered Peptone Water
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPR-SeroSeq	Serotyping by CRISPR Sequences
CPC	Cetylpyridinium Chloride
H ₂ S	Hydrogen sulfide
LIA	Lysine Iron Agar
RV	Rappaport-Vassiliadis broth, modified
PAA	Peroxyacetic Acid
PBS	Phosphate Buffered Saline
TSI	Triple Sugar Iron Agar
TT	Tetrathionate broth
USDA-FSIS	United States Department of Agriculture-Food Safety Inspection Service
XLT-4	Xylose Lysine Tergitol-4 Agar
BGS	Brilliant Green Sulfa Agar

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CHAPTER 1

INTRODUCTION

Contaminated broiler meat contributes to 17% of human salmonellosis cases in the United States (IFSAC, 2019). To reduce these chicken-borne illnesses, the United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) agency and poultry companies perform *Salmonella* surveillance after the antimicrobial interventions that are used during slaughter and processing (USDA-FSIS, 2016).

Salmonella is a diverse species with more than 2600 serovars (Grimont and Weill, 2007; Andino and Hanning, 2015). Conventional *Salmonella* isolation requires multiple days to confirm and characterize the *Salmonella* (USDA-MLG, 2014). During routine *Salmonella* surveillance using conventional protocol, selective enrichment media such as Rappaport Vassiliadis (RV) and Tetrathionate (TT) broths may impact the serovars being identified after plating as some serovars grow better than others depending upon the media (Harvey and Price, 1967; Singer et al., 2009; Temelli et al., 2010; Gorski, 2012; Cox et al., 2019, 2020; Larsen 2021). Past studies reported that *Salmonella* often exists as multi-serovar populations within the pre- and post-harvest poultry samples (Berghaus et al., 2013; Bourassa et al., 2015; Cox et al., 2019; Ramirez-Hernandez et al., 2019; Obe et al., 2021). Due to this, picking 1-2 colonies from the agar plate would impact the serovar detected. Typical colonies picked would be the serovar that are present in abundance and the remaining background serovars present in the sample would remain undetected. These background or undetected serovars could be clinically important and exhibit serious limitations

with traditional surveillance methods. There are two major limitations with conventional protocol: 1) The use of selective enrichment broths would take longer time and introduces serovar bias depending upon the media used, 2) typical picking of colonies from the selective agar plate would identify only abundant serovar present in the samples. These limitations can be addressed by optimizing the *Salmonella* culturing protocol and with the use of any high throughput sequencing technology for further characterization. CRISPR-SeroSeq is a novel technology, which can identify all *Salmonella* serovars and their relative frequency in an individual sample (Thompson et al., 2018; Cox et al., 2019).

The goal in this work was to improve culture detection of *Salmonella* in conjunction with CRISPR-SeroSeq. The first goal was to improve the process of culture detection of *Salmonella* by reducing the time required for isolation protocol while limiting serovar bias. The second goal was to analyze the *Salmonella* serovar populations through different stages of processing using a newly developed selective pre-enrichment broth to determine if some serovars can survive processing interventions better than others.

I hypothesized that increasing the selectivity of pre-enrichment broth would reduce the background flora and enable *Salmonella* detection by direct plating within 24 hours. This alteration would show similar *Salmonella* recovery rate to traditional culture methods, thus avoiding the additional selective enrichment step and shortening the protocol by 24 hours. The use of CRISPR-SeroSeq would enable to discern the serovar populations present within poultry samples, which helps to identify serovars of concern in processing samples to mitigate *Salmonella* effectively. Further, I hypothesized that this novel alternative protocol would help the poultry industry to successful biomap any changes in *Salmonella* serovars through processing.

The overall aim of this research was to improve the *Salmonella* isolation protocol while reducing the time needed but increasing the resolution of *Salmonella* identified. In the first study, I added one selective agent from RV broth (malachite green), one from TT broth (bile salts), and novobiocin after four hours to the pre-enrichment broth to identify *Salmonella* within 24 hours from post-pick processing samples. Due to addition of selective agents to the non-selective pre-enrichment broth, I referred it as selective pre-enrichment broth. In the second study, I investigated the efficacy of selective pre-enrichment tested in the first study, to isolate *Salmonella* at five different points during processing from high to low *Salmonella* incidence from three different processing facilities. In both studies, I cultured the samples using selective pre-enrichment broths and compared the outcomes to conventional culture methods to determine their efficiency in detecting *Salmonella* at different stages of processing. I also used CRISPR-SeroSeq on all the culture positive samples to investigate serovar profiles detected using selective pre-enrichments were similar when compared to isolation after standard selective enrichment in RV and TT broths.

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CHAPTER 2

LITERATURE REVIEW

Approximately 48 million people become ill from foodborne illnesses in the United States every year (CDC, 2011). Among these cases, 9.4 million illnesses were attributed to known pathogens referred to as “31 major pathogens” for which the Centers for Disease Control and Prevention (CDC) can identify the causative pathogen and the remaining 38.4 million illnesses have been attributed to unidentified pathogens (Scallan et al., 2011). The CDC estimates that one in six Americans get sick every year, 128,000 are hospitalized, and 3,000 die due to foodborne illnesses (Scallan et al., 2011). Among the 31 major pathogens, fifteen pathogens accounted for more than 95% of illnesses and deaths, with an economic burden of \$17.6 billion (USDA-ERS, 2019).

The Foodborne Diseases Active Surveillance Network (FoodNet) operates in collaboration with CDC, ten state health departments, U.S. Department of Agriculture’s Food Safety and Inspection service (USDA-FSIS) and the Food and Drug Administration (FDA) which conducts active population-based surveillance of foodborne infections (CDC, 2020). FoodNet monitors trends in foodborne infections in residents of these ten states and recorded the most significant rate of infection (per 100,000 population) for *Campylobacter* (19.5) and *Salmonella* (17.1) (Tack et al., 2019). *Campylobacter* has been the most common pathogen diagnosed in foodborne bacterial infections, causing approximately 37.6%, i.e., 9,731 cases out of 25,866 foodborne bacterial infections. *Salmonella* is the second most frequent cause of foodborne bacterial disease, causing

around 33%, i.e., 8,556 cases out of 25,866 foodborne bacterial infections (Tack et al., 2019), whereas the CDC estimated the incidence of foodborne *Salmonella* illnesses ranges from 645,000 cases to 1.7 million cases considering the unreported cases as well (Scallan et al., 2011).

In terms of economic burden, *Salmonella* imposes an estimated \$3.7 billion economic burden in a typical year (Hoffmann et al., 2015). Around 90 percent of this burden, \$3.3 billion, is due to deaths; 8% is due to hospitalizations; and the remaining 2% is due to non-hospitalized cases (Hoffmann et al., 2015). The USDA-FSIS and CDC recently launched a new effort to reduce foodborne *Salmonella* infections further due to its significant economic burden and number of illnesses have not dropped in recent years (CDC, 2019; USDA-FSIS, 2021). As *Salmonella* is a concern for public health, detailed knowledge and understanding of its presence in the environment is required to effectively control this pathogen to mitigate the salmonellosis cases in the humans.

SALMONELLA AS A MAJOR FOODBORNE PATHOGEN

Salmonella was first discovered and isolated in 1855 by Theobald Smith from a pig's intestine infected with classical swine fever. The bacteria were named after Dr. Daniel Elmer Salmon, who worked with Theobald Smith (Steele, 1969). *Salmonella* is rod-shaped, facultative anaerobic, flagellated, and gram-negative bacteria in *Enterobacteriaceae* family. Salmonellae are non-fastidious species that can survive outside living hosts in the environment, and capable of infecting many hosts. The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori* (Tindall et al., 2005; Grimont, and Weill, 2007). *S. enterica* is divided into six subspecies *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) based on their phylogeny (Grimont, and Weill, 2007).

Among these subspecies, *Salmonella enterica* subsp. *enterica* is the major cause of infections in both humans and animals with more than 2500 serovars (Brenner et al., 2000). The

antigenic formulae of *Salmonella* serovars are defined and maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (WHO Collaborating Centre) and new serovars are listed in annual updates of another system of classification called Kauffmann-White classification system (Grimont and Weill, 2007).

Kauffman and White introduced a scheme to classify *Salmonella* serovars based on the major antigenic determinants such as somatic (O), flagellar (H) antigens and capsular (Vi) antigens (Brenner et al., 2000; Grimont and Weill, 2007; Guibourdenche et al., 2010). The somatic (O) antigen is heat-stable and found in the outer bacterial membrane, which forms the oligosaccharide component of the lipopolysaccharide (LPS) of the bacterial cells (Grimont and Weill, 2007). A serovar can express more than one somatic antigen on its surface (Schnaitman and Klena, 1993; Reeves et al., 1996). *Salmonella* have biphasic flagella that possess heat-labile H antigens which are involved in activation of host-immune responses. These bacteria can be diphasic (phase I H antigen (H1) and phase II H antigen (H2), capable of expressing one protein at a time coordinated via phase variation mechanism (Silverman et al., 1979; Bonifield and Hughes, 2003; Yamamoto and Kutsukake, 2006, McQuiston et al., 2011). The genes encoding the H1 antigen are present in all salmonellae responsible for immunological identity (Macnab, 1992), while some serovars lack non-specific H2 antigen and are thus, designated as monophasic serovars (McQuiston et al., 2011). The heat sensitive virulence (Vi) antigen is the subtype of K antigen located on the surface of bacteria capsule and is rarely found in majority of the serovars (Brenner et al., 2000). The various combinations of these somatic antigens (n=46) and flagellar (n=114) antigens helps in identification of different *Salmonella* serovars (~2500 serovars) (McQuiston et al., 2004; Grimont and Weill, 2007; Guibourdenche et al., 2010). However, the formal identification of any specific

serovar can be achieved only by extensive serotyping of all the surface antigens manually (Chiu et al., 2004). Since this relies on having a vast repertoire of antisera, many isolates are sent to specific reference laboratories for serotyping (McQuiston et al., 2004; Fitzgerald et al., 2006). Usually, clinical laboratories prefer to conduct simple agglutination reactions to antibodies or antisera specific to the somatic O antigens, which groups *Salmonella* into several serogroups such as O:2 (A), O:4 (B), O:7 (C1), O:8 (C2, C3) and O:9 (D1) (Chiu et al., 2004). This serogrouping provides valuable information for epidemiological studies and genus identifications of *Salmonella* infections (Wattiau et al., 2011). While there are many hundred serovars in *Salmonella enterica* subspecies grouped under several serogroups, there are a limited number of these that are frequently responsible for human infections (Hendriksen et al., 2011). *Salmonella* serovars show different phenotypes and inhabit different animal hosts.

***SALMONELLA* SEROVARS**

For *S. enterica* subsp. *enterica*, many serovars were named after the geographical region where they were first isolated (e.g., serotype London was isolated from a patient with last name “L” in United Kingdom) and some were named after the illness itself (e.g., *Salmonella typhi* because it causes typhoid fever) (Brenner et al., 2000; Gossner et al., 2016). The two most common *Salmonella* serovars related to human gastrointestinal illnesses are *Salmonella* ser. Enteritidis and *Salmonella* ser. Typhimurium. The typhoidal serovars (i.e., *S. Typhi* and *S. Paratyphi* A, B, C or Sendai) of *Salmonella* causes enteric fever, whereas the nontyphoidal *Salmonella* (NTS) serovars are responsible for causing self-limiting gastroenteritis in the human host (Gal-Mor et al., 2014). Serovars belonging to the other subspecies were classified according to the name of the subspecies by their antigenic formulae. Over 2500 serovars have been identified so far with over 50% belonging to *Salmonella enterica* subsp. *enterica*, which contains the most serovar diversity

(~1500 serovars) (Grimont and Weill, 2007). These serovars can be specialist serovars (adapted to narrow range of hosts) or generalist serovars such as *S. Enteritidis* and *Typhimurium* infecting both humans and animals (Gal-Mor et al., 2014). It is necessary to understand the nature of transmission from primary host or primary reservoir to secondary host.

Host restricted serotypes

The *Salmonella* serovars associated with one particular species exclusively, such as Typhi, Abortusequi, Pullorum, Gallinarum and Abortusovis are referred as host-restricted serovars (Uzzau et al., 2000). *Salmonella* serovars Pullorum and Gallinarum are non-motile, host-specific which causes Pullorum disease (PD) and Fowl typhoid (FT), highly septicemic diseases in poultry (Shivaprasad, 2000). In 1935, the US Federal Government started National Poultry Improvement Plan (NPIP) to reduce the mortality of chickens from these serovars (Andino and Hanning, 2015). In the 1950s, breeders and hatcheries of the poultry industry in the United States carried out blood analysis, tube agglutination, and rapid serum test extensively to reduce the incidence of *S. Pullorum* and *S. Gallinarum* (Shivaprasad, 2000) under the uniform national management standards (Uzzau et al., 2000; Andino and Hanning, 2015). Control programs such as good hygiene management, biosecurity enforcement, serological tests, and slaughter policies helped to eradicate these salmonellae (Bullis, 1977; Uzzau et al., 2000; Lutful Kabir, 2010). Furthermore, vaccination was implemented to control pullorum and fowl typhoid diseases. These serovars have been eradicated in developed countries, including the United States, Canada, and Western Europe, but other areas of the world still face challenges (Barrow and Freitas Neto, 2011). Two decades later, both conditions were eradicated, and by 1975, there was no evidence of infection in commercial poultry in the United States (Bullis, 1977; Uzzau et al., 2000; Lutful Kabir, 2010). It has been hypothesized that clearing poultry flocks of ser. Gallinarum and ser. Pullorum opened a favorable

niche for *Salmonella* ser. Enteritidis (Baumler, 2000; Uzzau et al., 2000; Cogan and Humphrey, 2003; Kumar et al., 2009).

Salmonella serovar Typhi, the etiologic agent of typhoid fevers, primarily infects humans and primates (Pascopella et al., 1995). Serovar Typhi is a motile, non-lactose fermenting bacillus that causes most cases of typhoid or enteric fever in humans worldwide. *S.* Typhi is considered as host-restricted because it is confined to humans and primates. *Salmonella* ser. Paratyphi A, B, C and ser. Sendai are also restricted to humans causes enteric fever. *Salmonella* ser. Miami, which is serologically related to ser. Sendai is mostly confined to humans, which triggers gastroenteritis rather than enteric fever (Selander et al., 1990; Feng et al., 2019). *Salmonella* ser. Abortusovis is a bacterial pathogen adapted to sheep without causing any infections in humans and considered host-specific, resulting in abortions in pregnant ewes, stillbirths, neonatal mortality, and abortions only in sheep (Uzzau et al., 2000).

Host adapted serovars

A serovar is capable of causing infection in a specific host population provided appropriate conditions for its replication are present in the defined host. This phenomenon is known as host specificity or host adaptation (Steinbach et al., 2000; Kingsley and Bäumler, 2000). *Salmonella* serovars Dublin and Choleraesuis are host-adapted in bovines and swine, respectively (Uzzau et al., 2000). Contact with infected animals or the ingestion of contaminated products might result in the transmission of such serovars to humans with potential to cause severe illnesses. *Salmonella* serovar Choleraesuis is an intracellular, facultative, host-adapted, highly invasive pathogen causing swine paratyphoid. *Salmonella* serovar Choleraesuis var. Kunzendorf is the most common serovar associated with swine disease called swine parathyroid in Europe, and USA (Allison et al., 1969) but rarely involved in human infections. However, when it infects humans, it typically

causes systemic infection, acute gastroenteritis and metastatic infections with bacteremia, which often requires parenteral antimicrobial therapy. Pneumonia, arthritis, aortitis, and endocarditis may also complicate the infection further in humans (Chiu et al., 2002).

Salmonella serovar Dublin is a bovine-adapted serovar and affects both young and adult cattle that causing enteritis and systemic disease in affected cattle. The persistent capacity of this serovar makes it hard to diagnose in affected cattle, because of its capability to remain in chronic carrier state along while being intermittently shed in feces (McDonough et al., 1999; Vignaud et al., 2017). *Salmonella* Dublin is capable of zoonotic infection in humans after contact with carrier animals but can also be transmitted via contaminated food or water and causes blood stream infection, multi-drug resistance association and gastroenteritis. Sometimes it can cause systemic infection with metastatic abscesses (Uzzau et al., 2000). Serovar Dublin rarely causes human illness, though in December 2019, an outbreak from contaminated ground beef was reported that resulted in nine hospitalizations and one death (CDC, 2017a). Consumption of contaminated poultry meat is considered a primary vehicle for *Salmonella* transmission among all the food animals and overlapping of serovars was reported between humans and poultry meat globally (Antunes et al., 2016).

SALMONELLA IN BROILERS

Chicken meat is an affordable protein source and the most consumed meat in the United States with 96.4 pounds per capita consumption (USDA-Economic Research Service, 2020). A survey in 2018 reported that nearly half of Americans eat chicken twice in a week (Technomic survey, 2018). The United States has world's largest broiler industry producing 51.95 million pounds of meat with a worth of \$21.7 billion (USDA, 2020). The top five broiler producing states are Georgia (8.08 billion pounds), North Carolina (7.88 billion pounds), Arkansas (7.34 billion

pounds), Alabama (6.6 billion pounds), and Mississippi (4.61 billion pounds) (USDA-NASS, 2021). In 2021, 1.1 million pounds of food products were recalled due to *Salmonella* contamination (USDA-FSIS, Recalls, 2021). The USDA ERS estimated the economic burden of *Salmonella* (non-typhoidal) as \$3.66 billion for 2014 considering all the hospitalizations, medical costs, number of cases, productivity losses and deaths (USDA-ERS, 2019).

With the rate of consumption of poultry meat and poultry products in American populations, there is a major concern associated with foodborne illnesses caused by *Salmonella* (Zeng et al., 2019). *Salmonella enterica* species are the primary concern for poultry processors, because raw chickens were attributed to 17% of one million foodborne illnesses in the United States (IFSAC, 2021). Despite various preventative and control measures implemented to mitigate *Salmonella* on poultry products, *Salmonella* remained a major concern in poultry industry due to resistant strains that have rapidly emerged and been associated with outbreaks (Williams et al., 2014; Antunes et al., 2016). The most common foodborne disease caused by consumption of contaminated poultry meat is salmonellosis. Chai et al. (2017) reported that 279 of the total 1,114 foodborne outbreaks (25%) were linked to poultry in the United States from 1998-2012. The CDC also reported 2,485 illnesses and one death from 64 individual outbreaks in the years 2011-2017 were attributed to *Salmonella* from contaminated chicken (Table.1) (Source: *Salmonella* outbreaks in CDC website from 2011-2017). Furthermore, chicken meat was responsible for 987 illnesses from 2013 to 2021 in the United States (Table. 2). The top three serovars known for causing significant human illnesses associated with poultry were Heidelberg, Enteritidis and Typhimurium (Foley et al., 2011; Shah et al., 2017; O'Bryan et al., 2022), followed by Infantis in the recent years (Mcmillan et al., 2020).

Because *Salmonella* in poultry is a concern, the USDA-FSIS sets performance standards for *Salmonella* in poultry products and performs monitoring by taking verification samples of post-chill broiler carcasses, parts and comminuted chicken on a continuous 52-week moving window basis. The sequential results from a single establishment within a 52-week time should meet with the performance standards set by the USDA-FSIS. The current performance standards for *Salmonella* on post-chill whole chicken carcass, comminuted chicken and portions are 9.8%, 25%, and 15.4% respectively (USDA-FSIS, 2016).

In the United States, serovar Infantis is one of the epidemiologically important NTS serovars within poultry and has been commonly associated with human salmonellosis in the last five years (McMillan et al., 2020). In 2017-18, serovar Infantis was seen more frequently in the samples collected by USDA-FSIS as a part of their *Salmonella* surveillance (McMillan et al., 2020). Serovar Kentucky has evolved as the most predominant serovar associated with U.S poultry with less association to human illness (CDC, 2016), but known to harbor antibiotic resistance genes (Shah et al., 2017; Hawkey et al., 2019). Across the United States from 2016 to 2020, the *Salmonella* prevalence in broiler carcass rinses collected at processing plants increased from 3.64% to 4.49% and the relative amount of *Salmonella* Infantis identified within the raw chicken parts were on the rise (USDA-FSIS, 2020). This risk is increased by the complexity of *Salmonella* serovars, as different serovars were responsible for multi-state outbreaks in the United States attributed to chicken. The change in these serovars (e.g., emerging serovars such as *S. Infantis*) responsible for outbreaks from time to time shows the importance of routine *Salmonella* surveillance to evaluate the prevalence of serovars in poultry environments and identify any next potential outbreaks from a particular serovar.

In conclusion, *Salmonella* outbreaks associated with broiler meat provide a significant impact on public health and shows the necessity of improved detection protocols to identify all the *Salmonella* serovars present in poultry samples. As poultry meat consumption is increasing each year, recognizing the differences between prevalent *Salmonella* serovars and their phenotypes helps in better understanding of *Salmonella* contamination sources and help to develop effective mitigation strategies from pre-harvest to post-harvest, possible only with accurate *Salmonella* surveillance serovar data.

Pre-harvest *Salmonella* contamination – broiler production

The increase in *Salmonella* prevalence in broiler processing plants raises a concern for control measures during live production (Bailey, 1993; Russell, 2012). The poultry industry is vertically integrated, where a single company may operate all aspects of different stages in chicken life such as maintenance of parent flocks (breeders), hatcheries, broilers, feed mills and processing plants. Primary breeding companies maintains the grandparent stock and supplies parent stock which will be grown in pullet and breeder grow-out farms (Taube-Netto, 1996). Eggs from breeder farms are hatched in hatchery facilities within the complex (Ollinger et al., 2005) and broilers are grown in multiple grow-out operations (De Vylder et al., 2011; Volkova et al., 2011).

Vertical integration allows the production process to run efficiently and economically and allows implementation of stricter biosecurity measures to control foodborne pathogens (Bailey et al., 2001; Liljebjelke et al., 2005; Dórea et al., 2010). *Salmonella* can enter the production chain through various sources such as hatcheries, vertical transmission via infected breeder or horizontal transfer within subpopulation of birds, workers, rodents, and feed mills (Henzler and Opitz, 1992; Davies and Wray, 1997; Bailey et al., 2001; Liljebjelke et al., 2005; Park et al., 2008; Roche et al.,

2009; Dórea et al., 2010; Dale et al., 2015; Magossi et al., 2019; Sargeant et al., 2021; Pal et al., 2021).

Day-old chicks in hatcheries are prone to *Salmonella* colonization through various exposure routes (Cox et al., 1990, 1996; Blankenship et al., 1993; Bailey et al., 2001; Kallapura et al., 2014 a, b). Intervention strategies such as vaccination and feeding of probiotics or prebiotics were shown to reduce broiler gut colonization with *Salmonella* thereby controlling vertical transmission from infected breeder to progeny (Bailey et al., 2007; Totton et al., 2012; Kerr et al., 2013). Horizontal transmission of *Salmonella* within flocks can contribute to increased levels of *Salmonella* in live production (Park et al., 2008; Vandeplas et al., 2010; Berghaus et al., 2012, 2013). *Salmonella* can also survive in poultry feeds and litter even under low water activity conditions (Williams and Benson, 1978; Davies and Wray, 1997; Davies and Hinton, 2000). The major contributing factor for *Salmonella* dissemination in broiler houses is due to fecal shedding in confined environment surrounding the birds (Rodriguez et al., 2006; Park et al., 2008; Volkova et al., 2010; Doyle and Erickson, 2012). Shedding of watery feces is due to stress induced by catching, shipping, and transportation and can result in *Salmonella* cross-contamination within the flock before reaching the processing plant (Handley et al., 2015). Previous studies observed a positive correlation between prevalence of *Salmonella* at farm and processing plants (Franz et al., 2012; Berghaus et al., 2013b).

Commercial broilers undergo feed withdrawal for 8-12hrs before transportation to reduce fecal shedding during processing (Mainali et al., 2009; Cox and Pavic, 2010). However, this practice can lead to ingestion of contaminated bedding with *Salmonella* (Ramirez et al., 1997; Byrd et al., 1998; Corrier et al., 1999). Transit and wait times before processing, can increase the risk of intra-flock cross-contamination of *Salmonella* by fecal shedding in the transport crates

(Mead et al., 1994; Slader et al., 2002; Corry et al., 2002; Mainali et al., 2009). *Salmonella* spread in the broiler farms could be controlled by aeration of litter during rearing (Bodí et al., 2013). Previous studies reported effective cleaning, sanitation and drying of crates could reduce the microbes such as *Campylobacter* and *Salmonella* during transportation of birds (Corry et al., 2002; Atterbury et al., 2020; Dzieciolowski et al., 2022). Preventative measures such as administration of vaccines at the breeder level, pelleting feed and addition of antimicrobial products (like bacteriocins, bacteriophages, synbiotics, prebiotics, and probiotics) in the feed can be helpful in reducing *Salmonella* colonization in broilers (Joerger, 2003; Patterson and Burkholder, 2003; Ricke, 2003; Hume, 2011; Handley et al., 2015). While these techniques can be effective broilers may be colonized with *Salmonella*.

Salmonella transmission within integrated broiler companies is complex due to various sources of contamination and phenotypes (Davies and Wray, 1997; Murray, 2000; Bailey et al., 2001; Park et al., 2008; Dórea et al., 2010; Foley et al., 2013). Apart from pre-harvest intervention strategies to reduce *Salmonella* in incoming birds to processing plant, interventions at the processing plant would eliminate *Salmonella* from poultry meat before retail display (Handley et al., 2015).

Post-harvest *Salmonella* contamination – processing plant

Processing of broiler carcasses consists of various steps from first processing to second processing. First processing includes hanging, stunning, bleeding, scalding, feather picking, removal of feet and oil gland, vent cutting, opening of vent, evisceration, crop removal, neck breaking, lung removal, Inside and outside bird washer (IOBW) (Figure. 1). Second processing includes pre-chilling, main chilling and post chilling followed by deboning and parts cut up (Figure

1). *Salmonella* cross-contamination between carcasses can occur during all the stages of processing (Bailey et al., 2001; Fries, 2002).

During scalding, carcasses are immersed in tanks containing water at 50°C-60°C with countercurrent flow of water relative to carcasses (Humphrey and Lanning, 1987; James et al., 1992; Slavik et al., 1995; Buhr et al., 2014; Pacholewicz et al., 2016). Scalding helps to loosen feather follicles and is a concern for possible cross-contamination, as the opened follicles may retain *Salmonella* for longer periods (Bailey et al., 2001; Fries, 2002; Carrasco et al., 2012; Handley et al., 2015; Antunes et al., 2016; Blevins et al., 2018; Akil and Ahmad, 2019). However, the high temperatures in the scald water can be effective in killing, removing, or causing injury to bacteria (Berrang et al., 2000). Defeathering, sometimes called picking, is done with a picker machine that contains several metal rotating plates equipped with rubber fingers to remove feathers from the post-scald carcasses (Allen et al., 2003). These rubber fingers can contribute to cross-contamination of *Salmonella* during defeathering (Nde et al., 2007). A New York rinse is often used after defeathering and before evisceration to reduce the bacterial load via a quick antimicrobial spray on carcasses (Stopforth et al., 2007).

Evisceration is a multistep process to remove the viscera from the carcass. The rupture of any intestinal and crop contents during this process is a significant source of bacterial contamination as pathogens can spread between carcasses (Hargis et al., 1995; Sarlin et al., 1998; Smith et al., 2007; Finstad et al., 2012; Pérez et al., 2014; Rouger and Zagorec, 2017; Gu et al., 2020). Use of high-pressure sprayers has been proven to be effective at reducing such contamination (Finstad et al., 2012). Next, carcasses will pass through series of washers that spray water inside and outside each carcass (inside and outside bird washer; IOBW) to remove contaminants prior to chilling. Some processing plants utilize chlorine in the water that is sprayed

with high pressure nozzles (Barbut, 2002), but other antimicrobial such as peracetic acid (PAA) may be more effective at reducing the *Salmonella* contamination during this step (González et al., 2019). Persistence of *Salmonella* entrapped in the skin might contribute to cross-contamination (Lillard, 1989; Rimet et al., 2019).

USDA-FSIS adopted a new means of self-monitoring for integrators called Hazard Analysis Critical Control Point (HACCP). The Code of Federal Regulation, 9 CFR 417.2 (b)(1), required that every USDA-inspected meat processing establishment develop and implement a written HACCP plan covering each product generated and how they would deal with food safety hazards, to identify and control possible contamination at specific points during production along with the use of Good Manufacturing Practices (GMP). Contaminated carcasses observed after evisceration and before chilling will be transferred to salvage or reprocessing line [On-Line Reprocessing (OLR) or Off-Line Reprocessing (OFLR)] (Rasekh et al., 2005; Codex Guidelines, 2011). Transferred carcasses are processed on separate salvage line to remove any visible fecal contamination or trim the damaged carcass followed by additional antimicrobial wash processes before pre-chiller, thus reducing the chance of cross-contamination during chilling (Rasekh et al., 2005).

Chilling is the final step in the first processing series (Obe et al., 2020) and an important step to effectively reduce *Salmonella* on the broiler carcasses (Cox et al., 1974; Brant, 1975). There are two types of chilling systems, immersion and air; the former is most commonly used in the United States while the latter is more common in Europe (Sams, 2000). After the IOBW, carcasses may be immersed in a pre-chill tank for 10-15 minutes to reduce the temperature of carcasses from 38°C to 30-35°C. Following pre-chill, carcasses are transferred to the main chill tank for 45 to 60

min submerged in cold water to achieve a deep muscle temperature of 4°C (Thomson et al., 1974). Immersion chilling may include the use of approved antimicrobials listed by USDA as safe in the production of poultry meat, such as chlorine, per acetic acid (PAA), acidified sodium chlorite (ASC), cetylpyridinium chloride (CPC), trisodium phosphate (TSP), or electrolyzed sodium chloride (Stopforth et al., 2007). FSIS-approved chemicals list for use in meat, poultry, and processed egg products are available in the USDA website (USDA, 2021). Earlier studies observed immersion chilling with chlorine could be a source of cross contamination (Lillard, 1989; James et al., 2006), contradicted by major studies observed reduction in *Salmonella* prevalence after immersion chilling (Knivett, 1971; Thomson et al., 1981; Dickens and Cox, 1992; Stopforth et al., 2007; Bauermeister et al., 2008; Northcutt et al., 2008). However, in the recent past, inclusion of PAA in immersion chilling has been proven to be more effective than chlorine in reducing microbial load compared to chlorine (Nagel et al., 2013; Wideman et al., 2016). Bauermeister et al., (2008) and Thames and Sukumaran (2020) reviewed the efficiency of different antimicrobials from various studies and reported that PAA is the most effective antimicrobial to use in the processing of chicken with improved reduction of *Salmonella* levels compared to chlorine.

Some studies reported that flocks entering the chiller with a lower prevalence of *Salmonella* had fewer positive carcasses post-chill when compared to flocks with higher initial levels (Thomson et al., 1981; Dickens and Cox., 1992). These findings are supported by a study conducted by Berghaus and their colleagues, also reported a reduction in *Salmonella* level from 3.44 MPN (most probable number) per carcass rinses performed at outside of the plant to 2.32 MPN after post-chill (Berghaus et al., 2013). The reduction in *Salmonella* prevalence through processing was also observed due to multi-hurdle approach followed by the processing plants with antimicrobials at several locations (Northcutt et al., 2008). At the end of primary processing, chilled carcasses

may be cut to required specifications using automated cutting machines which could acts as a source of *Salmonella* contamination (Guo et al., 2011); synovial fluid could be reservoir of *Salmonella* (Sexton et al., 2018).

In second processing, chilled poultry carcasses are separated into different parts and meat is separated from bone (Baker and Bruce, 1996). A dressed poultry carcass can be divided into major parts such as wings, thighs, drumsticks, breasts and back (Romans et al 1994). These products are sold to restaurants (Fletcher, 2004). During the 1980's, there was a shift to more use of boneless and skinless poultry products (Fletcher, 2004), which were wrapped in tray packs. With the increase in second processing, consumption of poultry meat increased especially as boneless-skinless meat. Second processing involves operations such as cutting, deboning, and portioning either by manual or mechanical cutting. Hand deboning remained as a popular method in the United States processing plants requiring many employees (Froning, 1979); Stadelman et al 1988). A higher prevalence of *Salmonella* was seen after second processing compared to first processing and this might be due to frequent contamination of equipment used in portioning of parts (Hardie et al., 2019; Ramirez-Hernandez et al., 2019; Thames et al., 2021). The ability of *Salmonella* to form biofilms, thus surviving sanitation procedures can contribute to cross-contamination from the machinery (Obe et al., 2020). Previous studies reported the reduction of serovar Kentucky prevalence between carcasses and parts replaced by an increase in other serovars such as serovars Enteritidis and Infantis (CDC, 2013; Tack et al., 2019). The separation of a carcass into parts could release internalized *Salmonella* such as *S. Enteritidis* (Barrow, 1991; Suzuki, 1994) and some serovars might tolerate the antimicrobial interventions applied during processing (Abdullah et al., 2017). Despite cleaning and sanitation procedures, the ability of biofilm formation by *Salmonella* serovars may contribute to the persistence of tolerant strains in processing plants

(Arnold and Silvers, 2000; Chia et al., 2009; Dantas et al., 2020; Obe et al., 2021b; Zeng 2021). Antimicrobial interventions such as PAA dip during processing of parts can be beneficial to reduce *Salmonella* prevalence on the parts (Modzelewska-Kapitula and Maj-Sobotka, 2014; Thames et al., 2021). Third processing involves additional operations involving marination, cooking, coating, and making some value-added products (nuggets, tenders, patties, and fillets) (Baker and Bruce, 1996; Fletcher, 2004). *Salmonella* persisting through the earlier processing steps could increase the risk of infection due to improper cooking or baking by the consumers (Roccato et al., 2015).

Despite the efforts of the poultry industry to reduce *Salmonella* levels, there is no correlation between *Salmonella* incidence and human illnesses reported (O'Bryan et al., 2022). *Salmonella* contamination of poultry products remains a complex issue throughout live production and various processing steps (Foley et al., 2011; Antunes et al., 2016). Given the various sources of *Salmonella* contamination from hatcheries to processing plant and various phenotypes shown by *Salmonella* strains, it is important to optimize culture conditions used in conventional *Salmonella* surveillance protocol. The culture-detection protocol should be effective and able to identify all *Salmonella* serovars present in poultry samples without missing any pathogenic serovar.

SALMONELLA ISOLATION

Culturing of *Salmonella*

Traditional *Salmonella* isolation protocol followed by the USDA-FSIS takes atleast a week to confirm the presence of *Salmonella* (USDA-FSIS, 2014). A single carcass is removed from the processing line after chilling and rinsed in 400ml of neutralized buffered peptone water (nBPW) for one minute. A 30 mL aliquot of the rinsate is removed and used for *Salmonella* analysis. In the laboratory, a further 30 mL of nBPW is added to the 30 mL rinsate and incubated for 24 hours at

37°C. This incubation is a non-selective pre-enrichment step which helps to recover the injured *Salmonella* present in the sample (D'Aoust, 1981; Busse, 1995). The presence of antimicrobial residues on carcasses being tested, especially PAA being the most used antimicrobial in the chiller could result in false negatives (Gamble et al., 2017). This has led FSIS to incorporate neutralized buffered peptone water (nBPW), which contains sodium thiosulphate that neutralizes PAA in the *Salmonella* detection protocols (Gamble et al., 2016; Bourassa et al., 2017). Subsequent to incubation for 24 hrs, two aliquots from the pre-enrichment (0.1 mL and 1mL) step are transferred in parallel to two selective enrichment broths Rappaport-Vassiliadis (RV) and Tetrathionate (TT), respectively, and incubated for further 24 hours at 37°C (Oscar Teague and A. W. Clurman, 1916; Knox, 1945; Vassiliadis, 1983; Peterz et al., 1989). RV broth has a low pH that is well tolerated by *Salmonella* and contains magnesium chloride and malachite green. The former creates hypertonic conditions that inhibit growth of *Proteus* and *E. coli* by osmotic pressure, whereas the presence of malachite green inhibits coliforms (Vassiliadis et al., 1956; Vassiliadis et al., 1981; Vassiliadis, 1983; Peterz et al., 1989). TT broth contains bile salts, which inhibits Gram-positive bacteria, and an iodine-iodide (I₂-KI) solution that induces TT production to provide a metabolic advantage to *Salmonella* as it produces tetrathionate reductase (Knox, 1945; D'Aoust, 1981; Moats, 1981; Winter et al., 2010). Calcium carbonate present in TT broth neutralizes sulfuric acid produced while *Salmonella* reduces the tetrathionate. Thus, pH balance is maintained within the broth, and this helps to promote competitive exclusion of other bacteria while enabling selectivity of *Salmonella* species (Knox, 1945; D'Aoust, 1981; Moats, 1981).

After selective enrichment, samples are subsequently streaked onto selective agar plates and incubated for another 24 hours. Most of the *Salmonella* serovars do not ferment lactose and produce hydrogen sulfide except for a few serovars such as *S. Paratyphi A* and *S. Choleraesuis*.

This unique property of *Salmonella* in *Enterobacteriaceae* family has been used for the development of selective and differential media for the presumptive identification of *Salmonella* (Rambach, 1990). Xylose lysine Tergitol-4 (XLT-4) is the most common selective agar used as *Salmonella* grows as distinct black H₂S-positive colonies. Other selective and differential media such as xylose lysine deoxycholate agar (XLD), brilliant green sulphate (BGS), and Hektoen enteric (HE) agar can also be used to isolate *Salmonella* colonies. These often have the antibiotic novobiocin added (Komastu and Restaino, 1981). Novobiocin is commonly used to inhibit Gram-positive bacteria, but *Proteus* spp. are also susceptible. Among the Gram-negative bacilli, *Salmonella* is strongly resistant to novobiocin (Jeffries, 1959; Gast and Porter, 2020). After incubation, one to three presumptive *Salmonella* colonies are picked and restreaked before confirmation. Suspected *Salmonella* colonies are inoculated in triple sugar iron (TSI) and lysine iron agar (LIA) slants and incubated for phenotypic confirmation. Serological tests, including somatic (O) antigen agglutination tests and flagellar (H) antigen agglutination tests are useful for confirmation (Andrews et al., 2013). Thus, traditional *Salmonella* isolation at a minimum takes a week; reducing this time would be beneficial.

One way to decrease the time required for *Salmonella* isolation is to optimize the pre-enrichment step by increasing the selectivity for *Salmonella*, without sacrificing the ability to allow damaged salmonellae to be resuscitated. This would be a delicate balance that requires combining non-selective and selective enrichment steps or removing either pre-enrichment or selective enrichment steps. With the pre-enrichment of dry poultry feed samples in minimal agar medium or BPW showed better *Salmonella* recovery rates compared to directly enrichment in TT broth (Juven et al., 1984). Chen et al., (1993) showed the efficiency of pre-enrichment step in *Salmonella* recovery using five different serovars. Likewise, pre-enrichment of poultry litter samples with

Salmonella showed higher recovery in BPW compared to direct enrichment in Tetrathionate Brilliant Green (TBG) broth (Read et al., 1994). Vanderzee (1994) showed that when novobiocin was added to BPW pre-enrichment broth along with ammonium-iron (III) - citrate and ferrioxamine E and G favored ser. Enteritidis isolation in egg products (van der Zee, 1994). Hoorfar and Baggesen (1998) reported better recovery rate of *Salmonella* in naturally contaminated poultry skin samples when pre-enriched in Universal Pre-enrichment (UP) broth compared to BPW. Another group investigated the addition of malachite green (MG) (0.1g/L) to BPW and reported that the addition of MG to BPW improved *Salmonella* isolation from heavily contaminated samples (Schothorst and Renaud, 1985). The addition of novobiocin (22 µg/ml) to the BPW, caused a reduction in number of competitive organisms and this helped in *Salmonella* isolation (Jensen et al., 2003). Myint et al., (2006). reported *Salmonella* was detected by Polymerase Chain Reaction (PCR) in chicken meat only when samples were pre-enriched in BPW with a minimum incubation for 12 hrs followed by eight hours in TT-H (Tetrathionate – Hajna) broth. Recently, a research team tried to test the Food and Drug Administration – Bacteriological Analytical Manual (FDA BAM) TT formulation by reducing selection in TT broth by use of 1% I₂-KI solution and no brilliant green (Daquigan et al., 2016). They were able to improve the growth of *Salmonella enterica* species compared to all other competitive bacteria a day earlier on raw chicken samples and reported that the reduced concentration of I₂-KI improved recovery of *Salmonella*. Collectively, this body of work shows the necessity and improvement through pre-enrichment step compared to direct enrichment in TT broth. Also, reduction in the selective pressure in TT broth or addition of selective ingredients such as malachite green from RV broth has helped in efficient recovery of *Salmonella*. Despite the culture-conditions used for *Salmonella*

isolation, another challenge is to identify all the *Salmonella* populations in each sample, because *Salmonella* often exists as multi-serovar populations.

Multi-serovar populations

Previous studies have shown that multiple serovars may be present in individual poultry samples. In one preharvest study, there was evidence that 6.3% of samples had more than one serovar (Berghaus et al., 2013). Thompson et al., (2018) reported 91% of samples analyzed possessed more than one serovar and one sample harbored four different serovars. To add to the complexity of *Salmonella* isolation, a simple carcass rinse may not be sufficient to detect all the serovars present on the carcass (Bourassa et al., 2015; Cox et al., 2019); this might be because certain serovars are more strongly attached to the chicken skin than others (Lillard, 1989; Rimet et al., 2019; Obe et al., 2021b). Cox et al., (2019) study detected an average of five serovars pre-chill per broiler carcass with up to nine unique serovars on a single carcass, more serovar diversity was witnessed with a whole carcass overnight enrichment method compared to one-min carcass rinse. Ramirez-Hernandez et al., (2019) identified more than one serovar on processing samples collected at four different locations within a plant. A recent study detected the presence of two distinct serovars in carcass rinses by just picking maximum of two colonies from the agar plate (Boubendir et al., 2021). Obe et al., (2021a) observed the presence of multiple *Salmonella* serovars in all broiler carcass rinse samples they evaluated. As Cason et al., (2010) calculated, if a sample contains more than two serovars, a microbiologist needs to pick up 32 colonies from a plate to have 95% probability to identify the background serovar present in the given sample, which is a laborious process. This body of work demonstrates that *Salmonella* often occurs in mixed populations of multiple serovars.

Serovar bias based on media used for isolation

Because *Salmonella* is often found as a multi-serovar population, the choice of methodology and growth media could influence isolation and identification, where fastest growing serovar may be the only one detected. Background serovars could either remain undetected due to outgrowth of dominant serovar or suppressed due to higher sensitivity to selective pressure present in the media. Studies have reported significant limitations in *Salmonella* isolation related to the finding that some serovars grow better than others under different culture conditions. *Salmonella* serovars can exhibit different phenotypes, and thus their growth characteristics can also differ based upon the method and type of media used. Harvey and Price (1967) reported better growth of ser. Choleraesuis in brilliant green broth and ser. Pullorum in selenite F broth. Singer et al., (2009) observed *S. Enteritidis* survived better in R-10 broth (similar to RV broth) and observed the dominance of *S. Newport* over *Enteritidis* when grown together. While Cox et al., (2019) observed the opposite, preferential survival of ser. *Enteritidis* in TT broth and ser. *Schwarzengrund* in RV broth. Cox et al., (2020) observed *S. Kentucky* was recovered significantly better in Gram Negative broth and *S. Heidelberg*, *Seftenberg* in TT broth. Another study showed better survival of ser. *Montevideo* in RV broth compared to TT broth and ser. *Typhimurium* in TT broth compared to RV broth, when grown together in 1:1 ratio (Larsen et al., 2021). Other studies have reported ser. *Typhimurium* survived better in TT broth compared to RV broth (Singer et al., 2009; Gorski, 2012). Gorski observed reduced growth of serovars *Typhimurium*, *Saintpaul*, and *Schwarzengrund* in standard RV broth (Gorski, 2012). Plating media such as XLT-4 agar more frequently isolated serovar *Kentucky* compared to BGS agar (Cox et al., 2020). The serovar *S. Typhi* cannot be detected on XLT-4 media due to lack of H₂S forming ability (Miller et al., 1991; Mallinson et al., 2000; Miller and Mallinson, 2000; Wang and Hammack, 2014). *Salmonella* exists as multi-serovar

populations therefore, choice of media used especially either RV or TT broth can influence which serovar is detected in a sample.

An efficient culture detection protocol is required to reduce the serovar bias introduced during selective enrichment step. In most instances, colonies of the dominant serovar would be picked from selective agar for *Salmonella* serotyping which could mask true background serovar diversity serovars. This is a concern as pathogenic serovars may be overlooked. It is also not possible to pick all the colonies present on a plate for further analysis. Therefore, an effective and accurate molecular technology such as CRISPR-SeroSeq is useful to identify all serovars present in a sample and address the limitations of conventional protocols. After culture detection of *Salmonella*, the characterization of *Salmonella* isolates is helpful to both poultry industry and regulatory agencies in two ways. First, the identification of serovars associated with foodborne illnesses at live production or at processing plant will help in developing and selecting autogenous vaccines to reduce the *Salmonella* in broilers. Second, determine serotyping and subtyping of isolates helps to identify trends in *Salmonella* illnesses and observe if there is overlapping of serovars between poultry and humans. It also facilitates tracking the source of contamination of a foodborne outbreak of salmonellosis.

CHARACTERIZATION OF *SALMONELLA* ISOLATES

With the phenotypic diversity of *Salmonella* attributed to different food matrices, there is a focus on shortening the time while increasing in-depth resolution. This diversity by species, serovar and strain, explains the need for effective resolution in routine surveillance relative to foodborne outbreaks. Advancements in molecular detection techniques addressed the need for efficient serotyping and subtyping. Molecular subtyping is useful to compare genetic makeup of the *Salmonella* isolates and determine if they have the same cluster or source of contamination.

Manual Serotyping

Traditional serotyping is based on immunologic reactivity between antisera and *Salmonella* surface antigens. *Salmonella* phenotypic serotyping depends on ‘O’ antigen which is encoded by the *rfb* gene cluster (Liu et al., 2014) and flagellar H antigens (H1 and H2 antigens) encoded by *fliC* and *fliB* respectively (McQuiston et al., 2004). There are 46 types of “O antigens” and 114 types of “H antigens” under KWM (Kauffmann-White-Le minor) scheme for *Salmonella* serotyping (Grimont and Weill, 2007). The traditional method is labor intensive and difficult to perform for large number of *Salmonella* isolates.

Molecular Subtyping Methods

Pulsed Field Gel Electrophoresis (PFGE)

PFGE works by slicing the DNA at specific locations with restriction enzymes in agarose (Schwartz et al., 1983; Schwartz and Cantor, 1984). DNA fragments are separated using periodic application of electricity in different directions. By using software, PFGE patterns are compared to find any potential matches of isolates across different states and countries with the outbreak strain (Ricke, 2014). PFGE is considered as gold standard for molecular subtyping, but not serotyping in *Salmonella* foodborne outbreak investigations (Foley et al., 2009). Deng et al., (2015) reported PFGE subtyping as less discriminatory approach compared to advanced subtyping methods to discern isolates at strain level.

Multi-Locus Sequence Typing (MLST)

MLST is another subtyping approach which differentiates isolates based on genome fragments (450-500 bp in length) of five to seven housekeeping genes affected, either by mutation or recombination (Maiden et al., 1998; Urwin and Maiden, 2003). Sequences are given different allele numbers at single or multiple sites. Unique MLST schemes are utilized to investigate

outbreaks (Harbottle et al., 2006). Previous studies reported MLST was less discriminatory than whole genome sequencing (WGS) based subtyping and time consuming (Larsen et al., 2012).

Multiple Locus Variable number tandem repeat Analysis (MLVA)

Bacterial genomes contain repeated DNA sequences at different sites or loci on chromosomal regions which can be duplicated or deleted as a process of natural evolution. The total length of the locus and number of repeats at specific loci is variable between different strains. This allows strain comparison by amplifying the target loci by PCR and provides results with series of numbers for each strain. The analysis from MLVA can be used in conjunction with PFGE to differentiate between closely related *Salmonella* isolates (Weigel et al., 2004; Van Belkum, 2007). MLVA is reported as more discriminatory than PFGE (Boxrud et al., 2007; Cho et al., 2007).

Whole genome sequencing (WGS)-based subtyping

The emergence of high throughput DNA sequencing makes WGS an affordable, and precise procedure (Brunham and Hayden, 2012; Köser et al., 2012; Allard et al., 2013). WGS-based subtyping consists of single nucleotide polymorphism (SNP)-based analysis, Multilocus sequence typing (MLST), ribosomal MLST (rMLST), whole genome MLST (wgMLST), and core genome MLST (cgMLST) (Mohammed and Thapa, 2020). MLST works by comparing gene-to-gene variation in the test genome to a reference genome. SNP analysis is more discriminatory compared to above mentioned methods and works by base-to-base analysis (Brown et al., 2019). In 2010, WGS was used in a foodborne outbreak investigation and later studies also reported WGS based subtyping as highly discriminatory approach compared to other approaches discussed above (Deng et al., 2014, 2015). However, the caveat for WGS approach is the requirement of an appropriate reference genome which can be difficult to obtain in all the scenarios.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - Sequencing by Serotyping (SeroSeq)

CRISPR-based subtyping approaches have been used successfully to subtype *Salmonella*. CRISPRs are present in nearly 45% of the sequenced bacterial genomes, including *Salmonella* (Grissa et al., 2007; Touchon and Rocha, 2010). CRISPR arrays consist of highly variable spacer sequences interspaced with conserved direct repeat sequences acquired from plasmids or bacteriophages (foreign DNA) early in the evolution process (Barrangou et al., 2007; Garneau et al., 2010). The CRISPR spacer content has been a useful tool which that can be used to identify different serovars and strains of bacteria (Shariat and Dudley, 2014).

The genome of *Salmonella* includes two CRISPR loci; CRISPR 1 and CRISPR 2, located <10kb apart from each other (Touchon and Rocha, 2010; Fabre et al., 2012). The direct repeats are 29 nucleotides long and spacers are 32 nucleotides long (Touchon and Rocha, 2010). CRISPR spacer content is highly conserved within a serovar, and this property can be used to effectively discern *Salmonella* serovars (Liu et al., 2011b; Fabre et al., 2012; Shariat et al., 2013b; Bachmann et al., 2014; Rauch et al., 2018; Richards et al., 2020). CRISPR-SeroSeq is a novel molecular tool that utilizes next-generation sequencing of *Salmonella* CRISPR amplicons to identify multiple serovars in a single sample using serovar-specific CRISPR signatures (Thompson et al., 2018; Cox et al., 2019a; Rasamsetti et al., 2021; Siceloff et al., 2021). This molecular tool can be used to map the relative frequencies of serovars present in a single food sample and can detect serovars comprising as low as 0.003% of the population (Thompson et al., 2018a). As CRISPR-SeroSeq evaluates all the genomic DNA present in an enrichment broth, it can identify both dominant and background serovars in a particular sample, addressing the limitations of culturing.

CRISPR-SeroSeq analysis has demonstrated that *Salmonella* often exists as multi-serovar populations in samples from broiler houses and processing plants (Thompson et al., 2018; Cox et al., 2019a; Rasamsetti et al., 2021). Thereby, CRISPR-SeroSeq highlights the challenge of traditional culture-based *Salmonella* isolation where characterization is limited to analyzing the most abundant serovars in mixed populations. This has significant implications for food safety and control of *Salmonella* in poultry production. With the availability of CRISPR-SeroSeq, the identification of *Salmonella* serovars and the existence of culture bias can be compared more accurately studied.

SUMMARY

Salmonella is a leading cause of foodborne bacterial illnesses in the United States. To effectively mitigate *Salmonella* at multiple steps in the chain of poultry meat production from farm to fork, an appropriate and stringent surveillance system is required. As *Salmonella* often exists as multi-serovar populations, an ideal culture method should allow equal survival of all serovars in enrichment media without any bias. A reduction in time needed to isolate *Salmonella* would be beneficial to the industry considering the shelf-life of food products. Characterization and serotyping of *Salmonella* isolates are the basis of current surveillance system to effectively mitigate *Salmonella*. Advanced molecular approaches such as CRISPR-SeroSeq can be used to analyze multi-serovar populations of *Salmonella* in poultry samples. This information is useful to choose vaccines for application at breeder level, developing serovar driven intervention strategies at processing plants and prevent future outbreaks. Though, WGS subtyping can provide in-depth resolution of *Salmonella* populations, WGS can be done only on isolates recovered from a selective plate where there is a greater amount of chance of picking up only the most abundant serovar, but not background serovar which could be pathogenic. CRISPR-SeroSeq addresses this concern by

analyzing DNA of all the *Salmonella* serovars (both abundant and background) present in an individual sample, thereby providing the relative serovar frequencies based on serovar-specific spacer content present in a given sample. An ideal culture medium for *Salmonella* culture detection would be beneficial, where the selective pressure is reduced to support survival of all salmonellae present in the sample, removing serovar bias introduced due to selective enrichment while reducing time in detection protocol. An improved culture detection protocol along with advanced molecular serotyping technique such as CRISPR-SeroSeq would be a huge improvement for accurate *Salmonella* surveillance in the poultry industry. This could be helpful in bio mapping all the *Salmonella* serovars found in the processing samples and developing serovar driven strategies to successfully mitigate *Salmonella* in the poultry industry.

Table 1.1. Foodborne illnesses (multi-state outbreaks) caused by *Salmonella* serovars in chicken meat

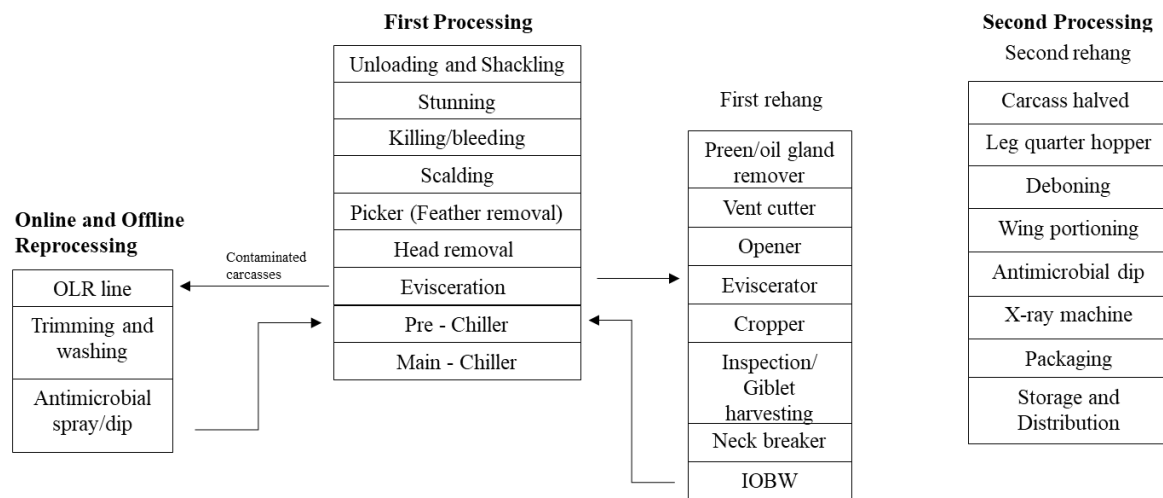
Source	Serovar associated with outbreak	Year	Number of illnesses
Raw chicken	Ser. Heidelberg	2013	634
Raw chicken	Ser. Heidelberg	2013	134
Raw chicken	Ser. Heidelberg	2014	9
Breaded chicken	Ser. Enteritidis	2015	5
Breaded chicken	Ser. Enteritidis	2015	15
Raw chicken	Ser. Infantis	2018	129
Raw chicken	Ser. 1,4,[5],12: i:-	2018	25
Breaded Chicken	Ser. Enteritidis	2021	36
Total illnesses			987

Source: CDC website, Outbreaks involving *Salmonella* (2013-2021).

Table 1.2. CDC *Salmonella* surveillance reports - Annual (2011-17)

Food category	Year	No. of Outbreaks	No. of Illnesses	No. of hospitalizations	Deaths
Chicken	2017	11	299	16	0
	2016	8	307	42	1
	2015	9	62	9	0
	2014	11	227	23	0
	2013	9	700	213	0
	2012	9	345	109	0
	2011	7	545	36	0
Total		64	2485	448	1

Source: CDC website, Outbreaks involving *Salmonella* (2011-2017).



Adapted from Obe et al., 2020

Figure 1.1: Layout of poultry processing steps

Abbreviation: IOBW, Inside and Outside Bird Washer

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CHAPTER 3

SELECTIVE PRE-ENRICHMENT METHOD TO LESSEN TIME NEEDED TO RECOVER *SALMONELLA* FROM COMMERCIAL POULTRY PROCESSING SAMPLES¹

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Key Words: *Salmonella*, broiler processing, novobiocin, bile salts, pre-enrichment

ABSTRACT

Conventional *Salmonella* detection is time consuming, often employing a 24-hour pre-enrichment step in buffered peptone water (BPW), followed by a 24-hour selective enrichment in either Rappaport Vassiliadis (RV) or tetrathionate (TT) broths before streaking onto selective indicator agar. To reduce this time, we sought to optimize pre-enrichment for *Salmonella* recovery by evaluating the addition of selective chemicals to BPW. Duplicate samples each representative of 500 carcasses were collected by catching processing water drip under moving carcass shackle lines immediately after feather removal in each of nine commercial processing plants. Carcass drip samples were cultured under selective pre-enrichment conditions in parallel with BPW pre-enrichment followed by RV and TT selective enrichment. Addition of bile salts (1 g/L) and novobiocin (0.015 g/L) resulted in *Salmonella* recovery from 89% samples when plated directly after pre-enrichment compared to 67% recovery in non-selective BPW alone. *Salmonella* serovar identities were determined using CRISPR-SeroSeq. Overall, serovars matched between selective pre-enrichment and traditional enrichment methods. These data suggest that increasing the selectivity of *Salmonella* pre-enrichment step may lessen the need for a separate selective enrichment step thereby reducing time required for *Salmonella* isolation by 24 hours.

INTRODUCTION

Salmonella is a leading bacterial cause of foodborne illnesses in the United States, causing an estimated one million illnesses each year (Scallan et al., 2011c; Tack et al., 2019b) and imposing an annual economic burden of approximately \$3.7 billion (Hoffmann et al., 2015). Despite improvements to mitigate *Salmonella* during processing, chicken and chicken-related products remain a source of *Salmonella* outbreaks (IFSAC, 2021). In addition to regulatory surveillance by the United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS), many poultry companies perform additional *Salmonella* surveillance. Accurate and rapid surveillance for *Salmonella* is required as a key step in assessing whether mitigation strategies are effective.

The gold standard for *Salmonella* detection on broiler carcasses is a culture-based protocol that includes several steps and takes multiple days to complete (USDA - FSIS, 2014). In the first non-selective pre-enrichment step, a single carcass is rinsed in 400 mL of buffered peptone water (BPW) for one minute and then 30 mL of the rinsate is removed and added to the same volume of fresh BPW before being incubated for 24 hours (USDA, 2019). In the second selective-enrichment step, the pre-enriched BPW is inoculated into two selective enrichment broths such as Rappaport-Vassiliadis (RV) and Tetrathionate (TT) broths in parallel and incubated for an additional 24 hours to reduce the other competitive bacteria present in the samples and allow only *Salmonella* to proliferate within these broths (Oscar Teague and A. W. Clurman, 1916; Knox, 1945; Vassiliadis, 1983; Peterz et al., 1989). Followed by the selectively enriched samples were streaked onto selective agar plates such as Xylose lysine Tergitol-4 (XLT-4) agar and incubated for another 24 hours. After incubation, one to three presumptive *Salmonella* colonies are picked and restreaked before confirmation by various molecular, biochemical, or serological tests, including somatic (O)

antigen agglutination tests and flagellar (H) antigen agglutination tests (H. Andrews et al., 2021). Thus, traditional *Salmonella* isolation at a minimum takes 4-5 days, and reducing this time may be beneficial.

BPW is a non-selective broth, and this incubation is considered a non-selective pre-enrichment step that is required to resuscitate *Salmonellae* from injury imparted during processing (D'Aoust, 1981; Busse, 1995). The selective chemicals like bile salts, brilliant green dye and malachite green dyes are added to the selective enrichment broths to reduce the other competitive bacteria in *Salmonella* isolation (Oscar Teague and A. W. Clurman, 1916; Rappaport et al., 1956; Peterz et al., 1989; Daquigan et al., 2016). In enrichment broths such as RV, selectivity depends on low pH (5.2), high ionic strength (due to $MgCl_2$) and malachite green. The magnesium chloride creates hypertonic conditions that inhibit growth of *Proteus* spp. and *E. coli* by osmotic pressure, whereas the presence of malachite green inhibits coliforms (Rappaport et al., 1956; Vassiliadis et al., 1981; 1983; Peterz et al., 1989). The conditions such as high osmotic pressures, low pH levels, resistance to malachite green and survive at less demanding nutritional requirements were well tolerated by *Salmonella* and thus helps in its proliferation (Dwivedi et al., 2014). TT broth contains an iodine-iodide (I_2 -KI) solution that induces tetrathionate production which suppresses coliforms and other microflora, providing a metabolic advantage to microorganisms such as *Salmonella* that produce tetrathionate reductase (Knox, 1945; D'Aoust, 1981; Moats, 1981; Winter et al., 2010). TT broth also contains bile salts that cause oxidative DNA damage and some TT broth formulations also contain brilliant green, which blocks sucrose or lactose fermentation (Bakker et al., 1992; Bernstein et al., 1999; Merritt and Donaldson, 2009). The lipopolysaccharide (LPS) in Gram negative bacteria limits permeability of these agents to the cytosol, thus protects them from the action of bile salts and brilliant green (Provenzano et al., 2000; Cremers et al., 2014). TT Hajna

broth contains the bile salt sodium deoxycholate and also contains brilliant green (Hajna and Damon, 1956; Dwivedi et al., 2014).

The most common selective and differential agar used for *Salmonella* isolation is XLT-4, which has largely replaced xylose lysine deoxycholate (XLD) agar (USDA - FSIS, 2014). *Salmonella* colonies present as distinct black colonies on both XLT-4 and XLD agars due to H₂S production. The presence of H₂S indicators such as sodium thiosulfate and ferric ammonium citrate act as differential agents from other Enterobacteriaceae (Miller et al., 1991; Mallinson et al., 2000). H₂S-negative strains appear as pinkish-yellow colonies on XLT-4 (Miller et al., 1991) and thus second selective media such as brilliant green sulphate (BGS) and Hektoen enteric (HE) are suggested (Komastu and Restaino, 1981; Mallinson et al., 2000). The inclusion of tergitol 4 in XLT-4 inhibits all gram-positive bacteria and also inhibits the growth of *Proteus* spp. and *Pseudomonas* spp. (Tate et al., 1990; Miller et al., 1991; Mallinson et al., 2000).

Novobiocin is a bacteriostatic antibiotic that inhibits bacterial DNA synthesis. The LPS present on Gram negatives limits permeability of novobiocin, so this antibiotic typically targets Gram positive bacteria (Bisacchi and Manchester, 2015; May et al., 2018). Further LPS modifications in *Salmonella* render it more resistant to novobiocin than other gram negatives such as *Klebsiella* spp., *E. coli*, and *Proteus* spp. (Sabath et al., 1968; Nobre et al., 2015; Mouton, 2017). Novobiocin is often added to BGS and HE agars and XLD to increase *Salmonella* selection (Jeffries, 1959; Gast and Porter, 2020).

Earlier studies have sought to increase selection at different stages in *Salmonella* isolation, chiefly to reduce the background microflora in highly contaminated samples. For example, by adding novobiocin to BPW or TT broths (Jeffries, 1959; Jensen et al., 2003; Wang and Hammack, 2014) or by adding malachite green to BPW broth (Schothorst and Renaud, 1985). *Salmonella*

enterica is a hugely diverse species that is represented by over 2500 distinct serovars that are defined by their cell-surface O and H antigens (Grimont, P.A.D. and Weill, 2007). Past studies indicated these serovars exhibit different growth characteristics even when grown in same enrichment, thus introducing biases when enriched (Harvey and Price, 1967; Singer et al., 2009; Temelli et al., 2010; Gorski, 2012; Cox et al., 2019a; b, 2020). For example, serovars Heidelberg and Senftenberg were preferentially recovered from TT broth compared to Gram negative broth (Cox et al., 2020a), serovar Newport showed dominance over serovar Typhimurium and serovar Enteritidis when grown together (Singer et al., 2009), serovar Enteritidis grew better in the RV enrichment broth variant, R-10 (Singer et al., 2009), and serovars Typhimurium and Saintpaul exhibit reduced growth in RV broth (Gorski, 2012). On plating media, serovar Kentucky appears to be more frequently isolated from XLT-4 than BGS (Cox et al., 2020b). Some serovars cannot be isolated on XLT-4 media, including serovar Typhi and many strains of serovar Cholerasuis (Mallinson et al., 2000); (Miller et al., 1991; Miller and Mallinson, 2000). Serovar Typhi cannot be propagated in RV broth, as it is sensitive to malachite green, so TT broth serves as the best enrichment when this serovar is present (Wang and Hammack, 2014).

Salmonella contains two CRISPR loci; CRISPR 1 and CRISPR 2, which are located <10kb apart (Touchon and Rocha, 2010; Fabre et al., 2012). The direct repeats are 29 nucleotides long and spacers are 32 nucleotides long (Touchon and Rocha, 2010). CRISPR spacer content is highly conserved within a specific serovar, and this property can be used to effectively discern *Salmonella* serovars (Liu et al., 2011a; Fabre et al., 2012; Shariat et al., 2013a; Bachmann et al., 2014; Vosik et al., 2018; Rauch et al., 2018; Richards et al., 2020). CRISPR-SeroSeq is a molecular tool that utilizes next generation amplicon sequencing of *Salmonella* CRISPR arrays to identify multiple serovars in a single sample (Thompson et al., 2018; Cox et al., 2019a). Using

this approach, we recently reported that serovars Enteritidis and Schwarzengrund were preferentially enriched in TT and RV broths respectively (Cox et al., 2019a).

We postulated that increasing the selectivity of the pre-enrichment step would not only reduce the background flora, as shown by others, but would enable direct plating from a selective pre-enrichment culture. This would avoid the need for an additional 24-hour selective enrichment culture and reduce the overall time required for *Salmonella* isolation by a day. Our rationale in the current study was to provide sufficient suppression of background flora using these agents, such that *Salmonella* would recover and proliferate better with reduced competition in the pre-enrichment broth. Since the purpose of pre-enrichment is to allow resuscitation of damaged *Salmonellae*, the timing of adding these components is an important consideration, and this would be a delicate balance that requires combining non-selective and selective enrichment steps. One selective agent from RV broth (malachite green), one from TT broth (bile salts), and also novobiocin were selected for this study. Bile salts were chosen instead of tetrathionate as they have been broadly used to limit the growth of Gram-positive bacteria and because tetrathionate may be too selective. Our objective was to test whether the delayed addition of these selective agents during pre-enrichment step could reduce the time required to isolate *Salmonella*. Given the differences in *Salmonella* serovar isolation under different media conditions, we also used CRISPR-SeroSeq to investigate whether the selective pre-enrichments used here resulted in isolation of different serovars when compared to isolation after standard selective enrichment in RV and TT broths.

MATERIALS AND METHODS

Sample collection

Carcass drip samples were collected as described by Line et al. (2013). Briefly, a sanitized plastic tub was placed beneath the moving shackle line following feather removal in commercial poultry processing plants (Figure 2.1). The tub remained in place collecting all dripping processing water for the amount of time needed to allow approximately 500 carcasses to pass as determined by counting carcasses with a timer. The time required varied depending on processing parameters and line speed; ranged from 2 minutes and 50 seconds to 4 minutes and 4 seconds. Carcass drip samples were transferred to sterile Nalgene bottles, which were sealed, placed on ice and shipped overnight in cold insulated packaging to the laboratory for bacterial culture. In Experiment 1, samples were collected in duplicate from four different processing plants for eight total samples and in Experiment 2, in duplicate from five different plants for ten total samples. For plants 2-4, and 5-9 dual processing lines were running, and a single drip line sample was collected from each line. From plant 1, duplicate samples were collected on the same line. In total 18 samples, representing nine different processing facilities, were examined in this whole study.

***Salmonella* isolation and confirmation**

Samples were diluted 1:1 with double strength BPW (2XBPW) and incubated for four hours at 37°C in a shaking incubator (Figure 2.1). Subsequently, four aliquots of 30ml were placed into separate sterile culture flasks and selective ingredients were added in different combinations to the following final concentrations: novobiocin 0.015 g/L; bile Salts 1 g/L; malachite green 0.1 g/L (Hardy diagnostics, Santa Maria, CA). In Experiment 1, the following three combinations were used: i) bile salts and novobiocin, ii) malachite green and novobiocin, and iii) bile salts, malachite green, and novobiocin. In Experiment 2, the following three conditions were used: i) bile salts, ii)

novobiocin, iii) bile salts and novobiocin. For all samples a non-selective BPW control was included. When BPW is used alone, the condition was referred to as non-selective pre-enrichment condition in the manuscript. The addition of malachite green, bile salts and novobiocin individually or in different combinations to the BPW were referred to as selective pre-enrichment condition in the manuscript. All culture flasks continued to incubate for an additional 20 hours at 37°C in a shaking incubator. After incubation, all broths were streaked for isolation onto XLT-4 agar (Hardy Diagnostics, Santa Maria, CA) and plates were incubated for 24 hours at 37°C (Figure 2.1). The streaking of aliquots from either pre-enrichment or enriched cultures on XLT-4 agar was referred to as selective plating in the manuscript. As a positive control, 0.1 ml and 1 ml of the BPW-only non-selective pre-enrichment cultures were also sub-inoculated into RV and TT selective enrichment broths (Hardy Diagnostics, Santa Maria, CA) respectively (Figure 2.1), performed according to conventional protocol followed by USDA-FSIS. The samples from non-selective BPW, when inoculated in to either RV or TT broth were referred to as selective enrichment condition in the manuscript. These selective enrichment broths were incubated for 24 hours at 37°C in a shaking incubator before being streaked onto XLT-4 agar (Figure 2.1) which was incubated as described above. XLT-4 plates were examined for development of typical black H₂S colonies and up to six characteristic *Salmonella* colonies were picked and streaked for isolation onto new XLT-4 plates, which were incubated as described above. Isolated *Salmonella* colonies were serogrouped using *Salmonella* polyvalent and single factor antisera until mixture results in marked agglutination (Difco, Madison, WI) according to the manufacturer's instructions. The samples from Plant-9 were serogrouped by CRISR-SeroSeq, based on its relative frequency as major serovar within the sample.

DNA isolation and CRISPR-SeroSeq

One ml of each pre-enriched and enriched broth was centrifuged at 15,000 RPM for two minutes to pellet the bacteria, and the supernatant was removed. Pellets were stored at -20°C until analysis. Total genomic DNA was isolated from samples found to be *Salmonella* positive by culture; isolation was done using the Genome Wizard Kit (Promega, Madison, WI) according to the manufacturer's instructions. DNA pellets were resuspended in 200 µL of molecular grade water and stored at -20°C until use. DNA samples were diluted 1:20 in water and 2 µL was used as a template in the first step CRISPR-SeroSeq PCR to amplify *Salmonella* CRISPR spacer sequences with the following primers: forward primer, 5'-tcgtcggcagcgtcagatgtgtataagagacagcgcgccagcggggataaac-3' and reverse primer 5'-gtctcgtgggctcggagatgtgtataagagacaggctggcgcgggggaacac-3' (Thompson et al., 2018a). The underlined portion of the primers binds to the conserved direct repeat sequences found in *Salmonella* CRISPR arrays, and result in a heterogeneous mix of PCR amplicons containing different spacers, typically 1-3 spacers per amplicon. A 5 µL aliquot of the PCR was analyzed by gel electrophoresis to confirm CRISPR amplification and the remaining 25 µL was purified using Ampure Beads (Beckman Coulter, Indianapolis, IN) according to manufacturer's protocol and resuspended in 40 µL of water. From this, 5µL was used as a template in the second PCR with primers containing sequences for the addition of Illumina adaptors and dual barcodes as per the Illumina Nextera protocol (Illumina, San Diego, CA). Again, the PCR products were analyzed by gel electrophoresis and then were pooled in approximate equimolar proportions and sequenced using an Illumina NextSeq (Wright Labs, Huntingdon, PA). The pooled sequencing sample also contained two negative controls (water used as template in PCR1 and PCR2, and water used as a template for PCR2) and a single positive control (serovar Enteritidis genomic DNA with known

CRISPR content). Sequencing reads without a 100% dual-indexed barcode sequence match were removed. CRISPR-SeroSeq analyses were performed using a Python script that scans sequence reads and uses BLAST to match sequence reads to a database of over 130 serovars, before writing the output directly to Microsoft Excel (Thompson et al., 2018; Cox et al., 2019a). For each plant sample, the reads for each sample were normalized to the reads from the culture condition with the lowest number of sequencing reads (Supplemental Table 2.3). From this normalization, individual spacer sequences with fewer than five reads were not included in the analysis. Serovars were confirmed only if they contained multiple CRISPR spacers that were unique to that serovar and if the cumulative number of reads for all spacers in that serovar constituted a relative frequency of at least 0.001% of the sample's total serovar population.

Statistical analysis

Fisher's exact test was used to compare the number of samples positive for *Salmonella* after enrichment in selective pre-enrichment media compared to non-selective media in all 18 samples and the *p* value was reported. A two-tailed *t* test was used to compare mean serovar diversity between conventional enrichments and the selective pre-enrichments, with serovar diversity being determined by the number of different serovars detected in each sample under each condition.

RESULTS

Experiment 1: *Salmonella* recovery in BPW with combinations of selective ingredients

Duplicate carcass drip samples were collected from four processing plants and the data are presented in Table 2.1. All eight samples were positive for *Salmonella* when pre-enriched in standard non-selective BPW and then selectively enriched in either RV (37.5%; 3/8 samples) or TT broth (87.5%; 7/8 samples). This includes both samples from Plant 2 that were positive after

both RV and TT enrichment. All isolates from Plants 2-4 serogrouped as O:8, and most isolates from Plant 1 serogrouped as O:4 with one sample belongs to O:7 (Table 2.1). All eight samples were also positive after direct plating from at least one of the selective pre-enrichment conditions. Conversely, only 62.5% (5/8) of samples plated directly after non-selective pre-enrichment in BPW were positive for *Salmonella*. The addition of bile salts and novobiocin to BPW resulted in a more consistent recovery of *Salmonella* (75%; 6/8 samples) than in BPW alone and is concordant with *Salmonella* recovery from TT broth (Table 2.1). The addition of malachite green and novobiocin to BPW enabled early recovery of *Salmonella* from both Plant 1 samples and from a single sample from Plant 4. Notably, *Salmonella* from Plant 1 was not recovered using BPW with bile salts and novobiocin. Further, the addition of bile salts, malachite green, and novobiocin seemed to be over-selective, resulting in only four samples that were positive for *Salmonella* compared to BPW. In Experiment 1, we combined novobiocin with either bile salts or malachite green, rather than adding the components individually. Our rationale here was two-fold: i) to reduce the overall culture conditions to be tested, and ii) since RV and TT selection broths are used in parallel and each is often plated onto media containing novobiocin, combining them made sense. Since BPW containing bile salts and novobiocin yielded the greatest number *Salmonella* positive samples and had better recovery than any of the other selective pre-enrichment conditions, we sought to determine whether this required the individual action of the selective components, or the combination of both.

Experiment 2: *Salmonella* recovery in BPW with individual selective ingredients

In Experiment 2, carcass drip samples were collected in duplicate from an additional five different broiler processing plants (plants 5-9) and the resulting 10 samples were pre-enriched with either bile salts or novobiocin, or both together. As above, all samples were positive after pre-

enriched in non-selective BPW, followed by selective enrichment in at least one of the broths either RV or TT. RV enrichment yielded 70% positive samples (7/10 samples) and TT enrichment resulted in 100% *Salmonella* positive samples (Table 2.2). Similar to Experiment 1, most isolates were grouped as serogroup O:8 (Table 2.2). Only 70% of samples yielded *Salmonella* colonies when plated directly after non-selective pre-enrichment in BPW (Table 2.2), and these were different from those found to be positive post RV enrichment. Selective pre-enrichment with bile salts alone or novobiocin alone yielded 90% and 80% samples that were *Salmonella* positive, respectively, whereas the combination of bile salts and novobiocin resulted in 100% *Salmonella* recovery (Table 2.2). Collectively, for plants 1-9, increasing the selectivity of the pre-enrichment step (with either bile salts and novobiocin or malachite green and novobiocin) enabled recovery of *Salmonella* from all samples compared to direct plating from a non-selective pre-enrichment with BPW-alone (Fisher's exact test, $p = 0.0191$).

***Salmonella* serovar profiles under different pre-enrichment and enrichment conditions**

Since media choice can influence the growth of different serovars, we employed CRISPR-SeroSeq to assess serovar populations within individual samples. CRISPR-SeroSeq was performed on positive samples from Experiments 1 and 2 that were selectively pre-enriched with bile salts and novobiocin combined, and from samples selectively enriched in RV or TT broth, whichever yielded *Salmonella* colonies. We also examined samples from plant 1, where the selective pre-enrichment condition with the addition of malachite green and novobiocin yielded *Salmonella* colonies while other selective pre-enrichment conditions failed to recover *Salmonella*. We could not accurately analyze the samples shown positive from non-selective BPW due to low reads obtained, attributed to presence of other Enterobacteriaceae whose DNA can inhibit the PCR. We did not attempt performing CRISPR-SeroSeq on positives recovered with bile salts, malachite

green and novobiocin combination in BPW due to its over selective nature, inconsistent recovery and to maintain a comparison between both Experiments 1 and 2.

When considering duplicates and different culture conditions as different individual samples, a total of 44 CRISPR-SeroSeq samples were present and there were about four to six samples per plant (plants 1-9). We identified nine different serovars and one untypeable serovar (Figure 2.2). The average number of serovars detected per culture sample was two, and the most diverse samples contained four different serovars. Regarding serovar presence and their relative frequency data (Figure 2.2), fourteen samples harbored a single serovar, and in all cases, that was serovar Kentucky. It was the most frequently (40/44 samples) identified and predominant serovar (37/40) found in all plants with only one exception, not detected from plant 1, replicate 2 (Figure 2.2). This is concordant with the serogrouping data presented in Tables 2.1 and 2.2, as serovar Kentucky expresses the O:8 antigen. Serovar Typhimurium was detected in samples from three different plants (plants 1, 8, and 9), and interestingly for plants 8 and 9, it was a minority serovar (i.e., not the most abundant serovar) in all, but one sample (Figure 2.2). As in previous work, we also saw a propensity for serovar Enteritidis enrichment in TT compared to RV (plant 5, replicate 2 and plant 9, replicate 1). Serovar Berta was seen only in samples from plant-7 in both selectively pre-enriched and enriched culture conditions. Serovar Braenderup was found in five samples, and four of these were selectively enriched cultures, which suggests that this serovar is not as competitive in the selective pre-enrichment environment as in the enrichments. Serovars Mbandaka, Infantis and Montevideo were each only identified in single culture samples, when culture conditions consist of bile salts in either selective pre-enriched or enriched cultures.

Occasionally, the relative abundance between the serovars was different, depending on the culture condition used, such as the switch between serovars Typhimurium and Schwarzengrund in

replicate 2 from plant 1, or between serovars Typhimurium and Kentucky in replicate 2 from plant 9 (Figure 2.2). There were 4/18 instances where the predominant serovar found after selective enrichment in at least one of RV or TT was not the predominant serovar after selective pre-enrichment. In these cases, the serovar was also identified in the pre-enriched sample, albeit at a lower relative frequency. The mean number of serovars detected in selectively enriched samples (RV or TT broth) compared to selective pre-enriched samples was not altered much with any significant statistical difference (two-tailed t test, $p = 0.62$), suggesting no change in serovar diversity. Overall, the profile of serovars found in selectively enriched cultures was comparable to those found in selective pre-enriched cultures (Figure 2.2).

DISCUSSION

Salmonella outbreaks are often linked to poultry and poultry-related products; between 2010 to 2018, 87 multistate outbreaks were associated with poultry, resulting in more than 6200 cases of salmonellosis (Centers for Disease Control and Prevention, 2013). Conventional culture-based methods used by USDA-FSIS and the US Food and Drug Administration to isolate *Salmonella* involve protocols that take multiple days to complete (USDA, 2014; Andrews et al., 2018). While there are commercial products available that enable rapid determination of whether a sample is *Salmonella* positive (e.g., *Salmonella* BAX system, SureTect from ThermoFisher, and DNable *Salmonella* detection from EnviroLogix), there is still a need to be able to isolate and characterize individual *Salmonella* colonies, especially within the context of an outbreak. Our findings here demonstrate that addition of ingredients that confer *Salmonella* selection in RV or TT enrichment broths can be added to the pre-enrichment step and allow for *Salmonella* isolation recovery a day earlier.

Early studies showed that a non-selective pre-enrichment step is required before selection in RV or TT broths for the successful recovery of *Salmonella* isolates in both artificial and naturally contaminated samples (Edel and Kampelmacher, 1973; D'Aoust, 1981; Hoorfar and Visby Mortensen, 2000). Many studies have addressed reducing the time required for *Salmonella* isolation, either by shortening the pre-enrichment step or by removing it entirely. There have been discrepancies in the time required for pre-enrichment, with one paper reporting that five to six hours is sufficient, while another showed that 24 hours was required (D'Aoust et al., 1990; Chen et al., 1993; Daquigan et al., 2016). Shortening the pre-enrichment time often resulted in false negatives (Moats, 1981; D'Aoust et al., 1990). Two studies showed higher *Salmonella* recovery rates from poultry farm samples when directly enriched in either selenite cystine or TT broth without pre-enrichment (Pangloli et al., 2003; Schultz et al., 2012). In the Schultz study, TT broth served as a pre-enrichment for enrichment in modified semi-solid RV agar (Schultz et al., 2012), and this approach has been approved by the National Poultry Improvement Plan for environmental samples collected during live production.

Other groups have investigated the addition of selective ingredients to BPW, including novobiocin and malachite green (individually), and reported increase in *Salmonella* recovery (Schothorst and Renaud, 1985; Jensen et al., 2003) and also the addition of novobiocin or sodium deoxycholate (the selective component of bile salts) in improving Shiga toxin-producing *E. coli* isolation (Margot et al., 2015; Amagliani et al., 2018). However, we know of no previously published work on investigating selective pre-enrichment for *Salmonella* recovery in poultry processing samples by comparing combinations of bile salts, malachite green and novobiocin being added to BPW.

To allow the resuscitation of potentially injured *Salmonella* and enable multiplication during the lag phase, we pre-enriched the samples in non-selective BPW for four hours before introducing any selection. We did not find any false negatives as all samples were positive after addition of bile salts and novobiocin or malachite green and novobiocin to the pre-enrichment media, with the former combination resulting in recovery from 89% (16/18) samples. While separate addition of bile salts or novobiocin resulted in high *Salmonella* recovery, the combination of both showed slightly better recovery, which is likely due to the combinatorial action of novobiocin and bile salts in inhibiting gram positives, and also *Proteus* spp. It is necessary to maintain a balance between a selective pressure that limits growth of competitive bacteria and an ability for injured *Salmonella* to recover (Carrique-Mas and Davies, 2008). Our data supports this, where addition of all three components together (bile salts, malachite green, and novobiocin) to BPW resulted in lower *Salmonella* recovery rates among all selective pre-enrichment conditions investigated, likely due to excessive selective pressure. In this work, we used the selective ingredients at the same concentration that they are used at in selective enrichment broths (bile salts, 1 g/L, and malachite green, 0.1 g/L). The culture-fitness characteristics can change based upon the concentration of ingredients used and pH of the pre-enrichment media (Gorski, 2012), therefore future work will include titrating the concentrations of each component to determine whether more optimal concentrations can be used to improve *Salmonella* isolation.

Salmonella isolation from plant 1 samples were unique across the nine plants we investigated. Here, addition of malachite green and novobiocin to the pre-enrichment allowed early recovery, whereas no *Salmonella* was recovered from the bile salts and novobiocin culture. This sample contained high relative amounts of serovar Schwarzengrund. Previous work from our group has demonstrated that this serovar is isolated at higher frequency after enrichment in RV

compared to TT (Cox et al., 2019a), therefore it is not surprising that we were able to recover it in malachite green, a component of RV.

Previous studies have shown that different *Salmonella* serovars are recovered from the same samples when enriched in different selective enrichment broths (Gorski, 2012; Cox et al., 2019). Further, it is becoming more evident that *Salmonella* exists in multi-serovar populations in broiler houses, and also on individual carcasses at processing (Berghaus et al., 2013; Thompson et al., 2018; Cox et al., 2019) and that picking and characterizing only a small number of colonies from indicator agar only represents the most abundant serovars present in a sample (Cox et al., 2019). Therefore, using CRISPR-SeroSeq, we performed an in-depth analysis on the culture-positive *Salmonella* samples to investigate the profiles of serovars found between selective pre-enrichment broth with bile salts and novobiocin and selective enrichment broth.

Across the dataset, the identity of serovars found within samples from the same plant matched, with few exceptions. With the exception of Braenderup in plant 5-replicate 2, the predominant serovar in all enriched samples, that with the highest relative frequency within a sample, was also detected in the selective pre-enriched samples. While there was no statistical difference in the level of serovar diversity between enriched and selectively pre-enriched samples, we noted that in 39% (7/18) cases, the number of serovars was higher in enriched samples, compared to 28% cases where that number was higher in selectively pre-enriched samples, and the remaining 33% samples where the number of serovars matched between the two treatments.

There are caveats to the work presented here, namely that we collected our samples post-pick, but before the carcasses were exposed to many of the antimicrobial processing aids which may be used during broiler processing. This was done deliberately as we anticipated that these samples would be more likely to be *Salmonella* positive and would therefore allow us to test a

larger number of samples from different plants in the context of this study. Further, the use of carcass drip samples enabled us to assess *Salmonella* in a larger number of birds, which is more reflective of the flock than a smaller number of carcasses (Line et al., 2013). It is possible that a higher *Salmonella* load at this location during processing enabled us to isolate *Salmonella* using the selective pre-enrichment conditions, and that at later points in processing (i.e., post chilling) where the quantity of *Salmonella* is reduced, that our methodology may yield false negatives. Nonetheless, for samples that may have a high *Salmonella* load (e.g., chicken cecal samples, or environmental samples collected from broiler or breeder houses), this methodology reduces the time and cost burden for *Salmonella* isolation. Future work will involve testing individual carcasses collected post chilling and will compare carcass drip sampling directly to individual carcass rinses. Future work would also compare a selective pre-enrichment with established *Salmonella* isolation protocols from carcass rinses. The concentrations of each additive to BPW were matched to the concentration used in its native media; future studies may reveal optimal concentrations. Future work would also involve plating on multiple selective media to reduce any incidence of false negatives. In this work, all 18 plant samples were positive and since serovar population analyses was performed on multiple sub-samples from each collection, we do not expect that we missed any false negatives in this work. From a technical perspective, the four-hour incubation might be challenging for technical labs who receive samples late in the day. The required concentration of selective components for successful inhibition of background flora and the resulting *Salmonella* recovery rate may change based upon the complexity and matrix of the samples.

CONCLUSIONS

In conclusion, our results suggest that addition of bile salts and novobiocin to BPW provides sufficient selection to enable *Salmonella* recovery 24 hours earlier than afforded by conventional *Salmonella* isolation that requires a pre-enrichment step followed by selective enrichment. Importantly, the serovar profiles do not alter dramatically between pre-enrichment and enrichment, for the majority of samples studied here. Though performed in poultry, the work presented here is relevant to multiple different food industries where *Salmonella* surveillance is routinely performed.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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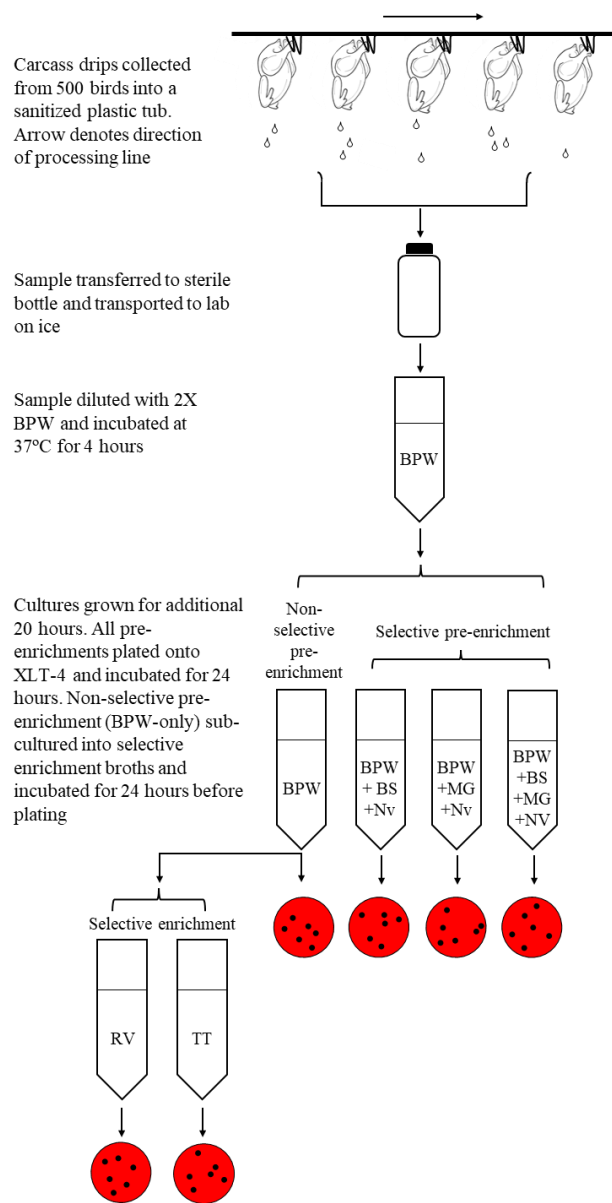


Figure 2.1. Schematic of sample collection and culture conditions. Figure shows carcass drip line sample collection and workflow of *Salmonella* isolation by both conventional and experimental culture conditions. BPW, buffered peptone water; RV, Rappaport-Vassiliadis broth; TT, tetrathionate; BS, bile salts; Nv, novobiocin; XLT-4, Xylose Lysine Tergitol-4 agar.

Table 2.1. *Salmonella* recovery after pre-enrichment in different selective conditions^a.

Culture condition ^b		Plant 1		Plant 2		Plant 3		Plant 4		Total no.of positives
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	
Non-selective pre-enrichment	BPW	O:4	-	O:8	O:8	-	O:8	-	O:8	5/8
Selective pre-enrichment	BPW + BS + Nv	-	-	O:8	O:8	O:8	O:8	O:8	O:8	6/8
Selective pre-enrichment	BPW + MG + Nv	O:4	O:4	-	-	-	-	-	O:8	3/8
Selective pre-enrichment	BPW + BS + MG + Nv	-	O:4	-	-	-	O:8	O:8	O:8	4/8
Selective enrichment	BPW into RV	-	O:7	O:8	O:8	-	-	-	-	3/8
Selective enrichment	BPW into TT	O:4	-	O:8	O:8	O:8	O:8	O:8	O:8	7/8

^aSerogroups: O:4 (B), O:7 (C₁), O:8 (C₂₋₃), O:9 (D₁).

^bBPW, buffered peptone water; RV, Rappaport-Vassiliadis broth; TT, tetrathionate; BS, bile salts; Nv, novobiocin; MG, malachite green.

Table 2.2. Comparison of Salmonella recovery after pre-enrichment with bile salts and novobiocin, individually and in combination^a.

Culture condition ^b		Plant 5		Plant 6		Plant 7		Plant 8		Plant 9 ^c		Total no.of positives
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	
Non-selective pre-enrichment	BPW	O:8	O:8	-	-	O:8	O:8	O:8	O:8	O:8	-	7/10
Selective pre-enrichment	BPW + BS + Nv	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	-	9/10
Selective pre-enrichment	BPW + MG + Nv	O:8	O:8	-	-	O:8	O:8	O:8	O:8	O:8	O:8	8/10
Selective pre-enrichment	BPW + BS + MG + Nv	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	O:8	10/10
Selective enrichment	BPW into RV	-	O:8	O:8	-	O:8	O:8	O:8	O:8	O:8	-	7/10
Selective enrichment	BPW into TT	O:7	O:8	O:8	O:9	O:8	O:8	O:4	O:7	O:8	O:4	10/10

^aSerogroups: O:4 (B), O:7 (C₁), O:8 (C₂₋₃), O:9 (D₁).

^bBPW, buffered peptone water; RV, Rappaport-Vassiliadis broth; TT, tetrathionate; BS, bile salts; Nv, novobiocin.

^cSerogroups for Plant 9 determined by CRISPR-SeroSeq.

Table 2.3. Number of CRISPR spacer reads from sequencing (Supplementary)

Sample		Number of <i>Salmonella</i> CRISPR spacer reads ^a
Plant 1	1-TT	130416
	1-MG+Nv	266920
	2-RV	9495
	2-MG+Nv	169948
Plant 2	1-RV	2707
	1-TT	50600
	1-BS+Nv	2788
	2-RV	22105
	2-TT	52823
Plant 3	2-BS+Nv	21223
	1-TT	288773
	1-BS+Nv	6180
	2-TT	405624
Plant 4	2-BS+Nv	113770
	1-TT	103203
	1-BS+Nv	7522
	2-TT	3158
Plant 5	2-MG+Nv	189966
	1-TT	172915
	1-BS+Nv	26658
	2-RV	18920
	2-TT	854552
Plant 6	2-BS+Nv	7100
	1-TT	639397
	1-BS+Nv	6564
	2-TT	124937
Plant 7	2-BS+Nv	1147
	1-RV	428028
	1-TT	400191
	1-BS+Nv	24893
	2-RV	96145
	2-TT	333189
Plant 8	2-BS+Nv	69046
	1-RV	2683
	1-TT	43253
	1-BS+Nv	183502
	2-RV	13739
	2-TT	487841
Plant 9	2-BS+Nv	20060
	1-RV	5116
	1-TT	777788
	1-BS+Nv	4071
	2-TT	137120
	2-BS+Nv	17447

^aNumber of reads remaining after reads with barcode errors were excluded

CHAPTER 4

USE OF SELECTIVE PRE-ENRICHMENT IN ASSESSING *SALMONELLA* PRESENCE
DURING BROILER PROCESSING²

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Key Words: *Salmonella*, pre-enrichment, CRISPR-SeroSeq, broilers

ABSTRACT

Conventional *Salmonella* surveillance requires a week for isolation, confirmation, and subsequent serotyping. We previously showed that this time burden could be reduced by 24 hours by combining the pre-enrichment and enrichment steps to assess *Salmonella* prevalence in broiler carcasses directly after picking. This was done by adding some selective components from the traditional Rappaport-Vassiliadis (**RV**) and tetrathionate (**TT**) selective *Salmonella* enrichment broths to the non-selective neutralizing buffered peptone water (**nBPW**) pre-enrichment after four hours of pre-enrichment in nBPW alone. We found good concordance between the two culture methods and in the present study we sought to investigate the efficacy of selective pre-enrichment to isolate *Salmonella* at five different points during broiler processing. Duplicate carcass drip samples, each representative of 500 broiler carcasses, were collected by catching processing water drip under moving carcass shackle lines in each of three commercial broiler slaughter plants. Samples were collected post-pick, post-inside-outside bird washer (**IOBW**), and post-chill; duplicate wing rinses were performed pre- and post-antimicrobial parts dip. Each processing plant was sampled six times for a total of 180 samples collected. The number of positives identified with selective pre-enrichment conditions (48/180) was similar to traditional selective enrichment culture conditions (52/180) (Fisher's exact test, $P = 0.72$). Importantly, the incidence of *Salmonella* reduced dramatically after antimicrobial intervention (post-pick 66.7% vs. post chill 8.3%). Collectively, these results suggest that a selective pre-enrichment step reduces the time required for *Salmonella* isolation without negatively affecting detection. We detected four different *Salmonella* serovars, Kentucky, Infantis, Schwarzengrund, and Typhimurium using CRISPR-SeroSeq, and their incidence rose between post-pick and post-IOBW. The relative abundance of

Infantis within individual samples increased between post-pick and post-IOBW while the relative abundance of the other three serovars decreased.

INTRODUCTION

Salmonella is a leading foodborne bacterial pathogen in the United States and is responsible for over 1 million illnesses each year (Scallan et al., 2011; Tack et al., 2019). A recent report noted that 16% of salmonellosis cases are attributed to chicken (Batz et al., 2021; IFSAC, 2021). *Salmonella* is often a commensal in the gastro-intestinal tract (GIT) of chickens and mechanical interventions during processing such as scalding, plucking, evisceration and parts cut-up might contribute to the cross-contamination of *Salmonella* (Rouger and Zagorec, 2017; Ramirez-Hernandez et al., 2019; Vaddu et al., 2021; Zeng et al., 2021), thus processing facilities could be a potential source of contamination for poultry meat (Rasschaert et al., 2007; Bourassa et al., 2015). Poultry processors usually employ multi-faceted approaches to effectively reduce *Salmonella* during processing. These include several antimicrobial interventions used at different locations in the first processing area (New York rinse, inside-outside bird washer (**IOBW**), and immersion tanks pre- and post-chilling), while in the second processing areas parts (wings, drumsticks, and thighs) are dipped in antimicrobial solution (Kataria et al., 2020; Vaddu et al., 2021). The major antimicrobial intervention during processing is immersion chilling of carcasses and the most commonly used antimicrobial solution in the poultry industry is peroxyacetic acid (**PAA**) (Kumar et al., 2020).

Salmonella isolation from carcass or parts rinses is a multi-day process that includes a 24-hour non-selective pre-enrichment step to resuscitate injured bacteria, followed by a 24-hour selective enrichment step to inhibit the growth of non-*Salmonella* cells. The pre-enrichment is often performed in neutralized buffered peptone water (**nBPW**, also known as modified BPW), which contains sodium thiosulfate to neutralize the antimicrobial effect of residual PAA on the

carcass (Gamble et al., 2017). Selective enrichment is performed in parallel in two *Salmonella* selective enrichment broths, Rappaport–Vassiliadis (**RV**) and tetrathionate (**TT**), to be able to capture *Salmonella* from high- and low-contaminated foods (Hammack et al., 1999; USDA-FSIS, 2021). Broth incubations are followed by plating onto selective indicator agar, such as Xylose lysine tergitol-4 agar (**XLT4**) and biochemical confirmation of presumptive *Salmonella* colonies. Given the time burden, there is significant interest in being able to reduce the time it takes to isolate *Salmonella*.

In our previous study, we showed that adding a selective component of RV broth (malachite green) and TT broth (bile salts), along with the antibiotic novobiocin to non-selective nBPW during the pre-enrichment step shortened the time required to isolate *Salmonella* by direct plating (Rasamsetti et al., 2021). The selective pre-enrichment conditions were able to identify all the positive samples (18/18) and the serovar profile analyzed was comparable to traditional culture conditions. Because this earlier work was a proof of principal, it was performed in broiler carcasses collected at post-pick, before any significant *Salmonella* interventions were used, so the *Salmonella* numbers were high. Also as part of that previous study, we used carcass drip sampling (Line et al., 2013) as a method of surveying hundreds of carcasses, rather than a small number (Rasamsetti et al., 2021).

Previous studies have shown that carcass samples contaminated with *Salmonella* can consist of more than one serovar (Bourassa et al., 2015; Ramirez-Hernandez et al., 2019). A new technology, called CRISPR-SeroSeq, has been used to identify multiple serovars present in individual samples from poultry (Thompson et al., 2018) and a previous study of ours identified

an average of five serovars per carcass collected pre-chill using this methodology (Cox et al., 2019).

In the present work, we sought to investigate the efficacy of selective pre-enrichment to isolate *Salmonella* at five different points during processing from high to low *Salmonella* incidence from three different processing facilities. We cultured the samples using three types of selective pre-enrichment broths and compared the outcomes to conventional culture methods to determine their efficiency in detecting *Salmonella* at different stages of processing. We also used CRISPR-SeroSeq (Thompson et al., 2018; Cox et al., 2019) to characterize the serovar profiles of the positive samples, before and after anti-microbial interventions.

MATERIALS AND METHODS

Sampling

Samples were collected from three commercial broiler processing establishments, which each ran dual processing lines (Figure 3.1). One set of samples was collected from each line, and duplicate parts samples were collected before and after post-cut-up dip treatment. Carcass drip samples were collected as previously described (Line et al., 2013; Rasamsetti et al., 2021). Briefly, sanitized plastic tubs were placed beneath the moving shackle line following feather removal (post-pick), after inside-outside bird washer (post-IOBW), and after chilling (post-chill). The plastic tubs remained in place collecting all line drips for the time needed to allow approximately 500 carcasses to pass. The drip collection time ranged from 2 min and 50 s to 4 min and 4 s., depending on the line speed, and was independently calculated at each location within each plant on each visit with a timer. Carcass drip samples were transferred to sterile Nalgene bottles and immediately diluted two-fold in 2x nBPW (Difco, Sparks, MD) containing 0.1% sodium thiosulfate (Acros Organics,

NJ). Wing rinses were collected pre- and post-antimicrobial dip by placing 4lbs of wings into a sterile bag and rinsing with 400 mL nBPW for 1 min. Wing rinse samples were placed in sterile Nalgene bottles. All samples were placed on ice and taken to the laboratory for bacterial culture on the same day.

***Salmonella* isolation and confirmation**

In the lab, samples were incubated for 4 h at 37°C. Subsequently, three aliquots of 40 ml were placed into separate sterile plastic tubes and selective ingredients were added in different combinations to the following final concentrations: novobiocin 0.015 g/L; bile Salts 1 g/L; malachite green 0.1 g/L (Hardy diagnostics, Santa Maria, CA). The following three pre-enrichment conditions were used: i) non-selective pre-enrichment with nBPW as a control, ii) selective pre-enrichment with bile salts and novobiocin, and iii) selective pre-enrichment with malachite green and novobiocin. Samples were returned to the incubator for a further 20 h. After pre-enrichment, 10µL of all pre-enrichment broths were streaked for isolation onto XLT-4 agar (Hardy Diagnostics, Santa Maria, CA) and plates were incubated for 24 h at 37°C. To compare the selective pre-enrichment protocol with conventional *Salmonella* isolation, 0.1 ml and 1 ml of nBPW-only non-selective pre-enrichment cultures were sub-inoculated into RV and TT selective enrichment broths (Hardy Diagnostics, Santa Maria, CA), respectively. These were incubated for 24 h at 37°C before plating onto XLT-4 agar. XLT-4 plates were examined after 24 h for development of typical black H₂S colonies and up to five characteristic *Salmonella* colonies were picked and streaked for isolation onto new XLT-4 plates, which were incubated as described above. Presumptive *Salmonella* colonies were confirmed by serum agglutination with poly O serum (BD Biosciences, San Jose, CA).

DNA isolation and CRISPR-SeroSeq

A total of 1 ml from each pre-enriched (selective and non-selective) and enriched broth after incubation was transferred into a sterile microcentrifuge tube and centrifuged at 21,000 x g for 2 min to pellet the bacteria. The supernatant was removed, and pellets were stored at -20°C. Genomic DNA was isolated from samples confirmed to be *Salmonella* positive using the Genome Wizard Kit (Promega, Madison, WI) according to the manufacturer's instructions. DNA pellets were resuspended in 200 µL of molecular grade water and stored at -20°C until further use. A 2 µL aliquot of DNA was used as a template in the first step CRISPR-SeroSeq PCR to amplify *Salmonella* CRISPR spacer sequences with the following primers: forward primer, 5'- tcgtcggcagcggtcagatgtgtataagagacagcgcgccagcggggataaacc-3' and reverse primer 5'- gtctcgtgggctcggagatgtgtataagagacaggctggcgcggggaacac-3' (Thompson et al., 2018). A 5 µL aliquot of each PCR product was analyzed by gel electrophoresis to confirm CRISPR amplification. The remaining ~28 µL of PCR product was purified using the Ampure Bead system (Beckman Coulter, Indianapolis, IN) according to manufacturer's protocol and resuspended in 40 µL of molecular grade water. From this, 2 µL was used as a template in the second PCR with primers containing sequences for the addition of Illumina adaptors and dual index sequences as per the Illumina Nextera protocol (Illumina, San Diego, CA). The PCR products were analyzed by gel electrophoresis before purification as above and then individual samples were pooled in approximate equimolar proportions. The pooled sample was sequenced using an Illumina NextSeq with 150 cycles, single end read. The pooled sequencing sample had two negative controls (water used as template in PCR1 and then PCR2, and water used as a template for PCR2). *Salmonella* serovar Enteritidis genomic DNA with known CRISPR content was used as positive control. The

sequencing reads without a 100% dual-indexed barcode sequence match were removed. The analysis of CRISPR-SeroSeq were performed using a R script that scans sequence reads and uses BLAST to match sequence reads to a database containing 135 serovars and wrote the output directly to Microsoft Excel (Thompson et al., 2018; Deaven et al., 2021; Siceloff et al., 2021). Serovars were confirmed only if they contained multiple CRISPR spacers unique to a specific serovar. Where we had sequence files for RV and TT enrichments from the same sample, this data was combined and presented as one selective enrichment population. Similarly, where we had sequence files following malachite green/novobiocin and bile salts/novobiocin culture, we combined the data into one selective pre-enrichment population. The relative frequency of each specific serovar was calculated based on the amount of spacer reads present in an individual sample and presented as a heatmap, which was made in Microsoft Excel.

Statistical analysis

To compare the *Salmonella* incidence between the three types of culture conditions (non-selective BPW, selective pre-enrichment and selective enrichment) McNemar's test was used. Fisher's exact test was used to compare the *Salmonella* incidence between the sample locations within each processing facility. Both of these analyses were performed using Stata (version 17.0; StataCorp LP, College Station, TX). Bray-Curtis dissimilarity metric (Bray and Curtis, 1957) was used to compare serovar populations and considered both serovar identities and the relative abundance of each serovar within a population. The Bray-Curtis metric was calculated using Qiime2 (2020.11) (Bolyen et al., 2019) and ranged between 0 (identical populations) and 1 (no serovars in common).

RESULTS

***Salmonella* recovery in BPW with selective ingredients**

The overall *Salmonella* incidence at Plant 1 was 33% (20/60) (Table 3.1). The highest *Salmonella* incidence in Plant 1 was seen at post-pick 83% (10/12), followed by post-IOBW 67% (8/12), and there was not a statistical difference in the incidence between the two locations (Fisher's exact test, $p = 0.64$) (Figure 3.2a). A single post-chill (1/12) sample was positive, and this was only detected using selective pre-enrichment with novobiocin and malachite green (Table 3.1 and Figure 3.2b). A single pre-dip wing rinse sample was *Salmonella* positive, and no *Salmonella* was detected after the dip. In total, *Salmonella* incidence at Plant 2 was lower than Plant 1, 13% (8/60) (Table 3.1 and 3.2), and no *Salmonella* was detected in post-chill samples at Plant 2 (Table 3.2). *Salmonella* incidence in Plant 2 was highest at post-pick 50% (6/12) and was slightly lower at post-IOBW 8.3% (1/12) but there was not a significant difference (Fisher's exact test, $p = 0.06$) (Figure 3.2a). As observed in Plant 1, one pre-wing dip rinse sample was *Salmonella* positive, and no post-dip wing rinses were positive (Table 3.2). At Plant 3, the overall *Salmonella* incidence was higher than Plants 1 and 2, 55% (33/60) (Tables 3.1-3.3), with 67% (8/12) incidence at post-pick and 100% (12/12) incidence at post-IOBW (Figure 3.2a). This was not a significant increase in incidence (Fisher's Exact Test, $p = 0.09$). Two post-chill (2/12) samples were positive and were only detected using selective pre-enrichment conditions (Table 3.3 and Figure 3.2b). Eight pre-dip wing rinses 67% (8/12) and three post-dip wing rinses 25% (3/12) were *Salmonella* positive (Table 3.3). There was a significant increase in incidence from post chill 17% (2/12) to pre-dip 67% (8/12) (Fisher's Exact Test, $p = 0.036$), followed by significant reduction in post-dip samples (25%) (Fisher's Exact Test, $p = 0.045$) (Figure 3.2a).

We next sought to compare the *Salmonella* incidence between selective pre-enrichment (addition of selective ingredients to the nBPW pre-enrichment step) and selective enrichment in RV and TT broths. At Plant 1, the incidence between selectively pre-enriched and selectively enriched samples was similar at 25% (15/60) and 32% (19/60) and there was no significant difference between the two culture methods used (McNemar's test, $p = 0.37$) (Table 3.1). Similar observations were made for Plant 2 ($p = 0.68$) and Plant 3 ($p = 0.75$) (Tables 3.2 and 3.3). Therefore, across the whole study, the isolation of *Salmonella* by selective pre-enrichment and selective enrichment was comparable. The *Salmonella* incidence between non-selective pre-enrichment (nBPW alone) and selective enrichment was significant at Plants 1 and 3 ($p = 0.003$ and $p = 0.004$, respectively), demonstrating that some enrichment was required for *Salmonella* detection and that the congruence between selective pre-enrichment and selective enrichment was not simply because of a higher initial *Salmonella* load in the samples. At Plant 2, the overall low incidence of *Salmonella* limited appropriate statistical analysis to compare non-selective pre-enrichment with selective enrichment.

Overall, the *Salmonella* incidence in all the samples from the processing plants was 34% (61/180) (Tables 3.1-3.3). Direct plating from the selective pre-enrichments identified 27% (48/180) while plating from the selective enrichments identified 29% (52/180), and these two approaches were not statistically different (Fisher's exact test, $P\text{-value} = 0.72$). When the culture conditions were considered individually, enrichment in TT broth yielded 26% (47/180) positive samples compared to enrichment in RV broth (23%; 41/180). No selection was provided in samples plated directly from the 24-hour nBPW culture and this resulted in 14% (25/180) *Salmonella* positive samples. For the selective pre-enrichments, addition of malachite green and

novobiocin yielded 22% (40/180) positive samples and addition of bile salts and novobiocin in 15% (28/180) positives.

We performed CRISPR SeroSeq analysis on the *Salmonella* positive samples obtained from Plant 3 because for this plant we were able to isolate *Salmonella* at each processing step, albeit over multiple sampling visits. Overall, we identified four different serovars: Infantis, Kentucky, Schwarzengrund, and Typhimurium (Figures 3.3 and 3.4). The most common serovar was Infantis, which was found in 23/33 *Salmonella* positive samples. This analysis was only performed on the selectively pre-enriched and selectively enriched samples, and not the non-selectively enriched samples (nBPW alone) as the latter often have a high background due to contaminating DNA of non-*Salmonella* that precludes a full population analyses (Cox et al., 2019). In total, we analyzed all 33 *Salmonella* positive samples, which included 22 samples where we could pair *Salmonella* profiles from selective pre-enrichment and selective enrichment, and 11 samples where we had singles of either selective pre-enrichment or selective enrichment conditions. There was one culture-positive sample [Collection 4_2 (fourth collection drip, second processing line), selective pre-enrichment condition] that we were unable to analyze by CRISPR-SeroSeq due to low read depth. To determine whether the media had any influence on the *Salmonella* serovar profiles we detected, we calculated the Bray-Curtis metric for the 22 paired samples. This provides a measure of population similarity by taking into account the presence of community members and their relative frequency. In total 68% (15/22) paired samples showed a good match (Bray Curtis <0.3), and 27% (6/22) a moderate match (Bray Curtis >0.3, <0.7) and a single paired sample had no match (Collection 2_1 pre-dip wing rinsate; Bray-Curtis = 0.79) (Supplementary material; Figure 3.5). We next compared the serovars found at post-pick with

those found at post-IOBW. All four serovars were detected at both post-pick and post-IOBW. The incidence of each serovar also increased between these two stages. For example, serovar Infantis was found in six samples at post-pick and in 11 samples at post-IOBW. Similarly, serovar Schwarzengrund was detected four times at post-pick and six times at post-IOBW. In considering the relative abundance of each serovar, serovar Infantis is particularly interesting as it was found as the dominant serovar (dark green to blue shading) in three post-pick samples (Collections 2_2, 3_1, and 5_1) but was found to be the dominant serovar in four post-IOBW samples following both selective pre-enrichment and selective enrichment (Collections 2_1, 2_2, 5_1, and 5_2) plus two of the selective pre-enrichment samples (Collection 3_2, and 4_2) and three of the selectively enriched samples (Collections 1_1, 3_1, and 4_1). For serovar Schwarzengrund, its relative abundance in each sample decreased between post-pick and post-IOBW. Both serovars Kentucky and Typhimurium also decreased in relative abundance between post-pick and post-IOBW except for in two samples (Collections 4_2 and 6_2 and Collections 4_2 and 5_1, respectively). At post-chill, *Salmonella* positive samples were identified, and these contained serovars Infantis and Typhimurium as major serovar. Both serovars were identified in the respective post-IOBW samples from the corresponding collection.

In the pre- and post-dip wing rinses, the same four serovars were also detected. Since we only had two paired pre- and post-dip samples (Collections 3_1, selective enrichment and 5_2, selective pre-enrichment), it was not possible to draw a meaningful conclusion across the dataset with respect to serovar dynamics before and after intervention. The incidence of serovars Infantis, Kentucky, and Schwarzengrund decreased between pre-dip and post-dip wing rinses. For example, serovar Schwarzengrund was found in four samples at pre-dip and in a single sample at post-dip.

In contrast, serovar Typhimurium was detected twice in pre-dip rinses and three times in post-dip rinses. Considering the relative abundance of each serovar, three serovars such as Infantis Kentucky, Schwarzengrund were found as the dominant serovar (dark green to blue shading) in six (6/8) pre-dip samples but were lost in all the relative post-dip samples. Serovar Typhimurium remained the dominant serovar in the paired pre and post dip samples. A single post-dip sample (Collection 2_2) had serovar Kentucky as dominant one with relative pre-dip sample as negative.

DISCUSSION

Nearly a fifth of all salmonellosis cases are linked to chicken (IFSAC, 2021) and there is a need to mitigate this pathogen during broiler processing. In this study we sought to address this need in two different ways. First, *Salmonella* isolation is the cornerstone of all *Salmonella* surveillance and monitoring programs but since it takes several days to complete, we sought to determine if the culture protocol could be shortened by 24 hours. Second, it is likely that future *Salmonella* monitoring programs will be focused on certain serovars, such as those most frequently linked with human salmonellosis. It is becoming clear that *Salmonella* in poultry often exists as mixed serovar populations (Thompson et al., 2018; Cox et al., 2019; Rasamsetti et al., 2021). Culture-based *Salmonella* isolation and subsequent characterization is limited to analyzing the most abundant serovars in such mixed populations, which is a challenge in terms of understanding how different serovars can respond to different antimicrobial controls used during processing. Thus, we used population analyses by CRISPR-SeroSeq to investigate differences among serovars at different stages of processing. We also used this approach as an additional comparison of the two culture methods used in this study.

In this study, we used carcass drip sampling, as previously described (Line et al., 2013) to be able to capture *Salmonella* incidence in a larger number of carcasses than is logistically possible with carcass rinses. We intended to collect drip samples for the pre- and post-dip parts samples, however, this wasn't possible at the three plants that we visited. Therefore, for the parts analysis, we used wing rinses as pre-dip and post-dip samples. Previous work has identified *Salmonella* at each critical step in processing, with significantly lower incidence post interventions (i.e., after immersion chilling and after parts were submersed into an antimicrobial dip) (Ramirez-Hernandez et al., 2019; Boubendir et al., 2021). For example, Boubendir and colleagues observed that the *Salmonella* incidence dropped from 43.6% pre-evisceration to 5.1% post-immersion chilling containing PAA identified (Boubendir et al., 2021). This and other studies show that the chiller treatments with chemical solutions such as PAA effectively reduce the *Salmonella* incidence (Nagel et al., 2013; González et al., 2019; Ramirez-Hernandez et al., 2019). At Plant 3, *Salmonella* incidence increased between post-pick and post-IOBW, which suggests cross contamination had occurred during that stage of processing, most likely during evisceration (Rivera-Pérez W, Barquero-Calvo E, 2014; Gu et al., 2020). This is supported by our serovar data, as the incidence of each serovar increased from post-pick to post-IOBW. The higher incidence following evisceration is consistent with the other studies, although our sampling pattern is different (Rivera-Pérez W, Barquero-Calvo E, 2014b; Park et al., 2015; Ramirez-Hernandez et al., 2019; Gu et al., 2020) and but in contrast, another study observed reduction in *Salmonella* incidence after evisceration (Ramirez-Hernandez et al., 2019). These discrepancies are likely driven by the use of different antimicrobials at different processing steps. A recent study suggested that the use of

chlorine at defeathering and PAA at multiple intervention steps could be most effective in reducing *Salmonella* incidence (González et al., 2019).

Overall, the *Salmonella* incidence between selective pre-enrichment and selective enrichment was similar across all three plants in our study, encompassing 18 visits and 180 total samples. CRISPR-SeroSeq analysis of samples collected from Plant 3 also demonstrated that 95% of paired selective pre-enrichment and selective enrichment had good or moderate concordance at the population level. Despite this overall congruence, there were a few individual samples where we were able to isolate *Salmonella* from the selective pre-enrichment but not the selective enrichment, and this only occurred in post chill samples (one sample from Plant 2, two samples from Plant 3) and one pre-dip sample (Plant 3). This is somewhat perplexing; a potential explanation is that the cells were damaged during antimicrobial treatment and despite the 24-hour recovery in non-selective nBPW, they were unable to withstand the stronger selective pressures provided by RV and TT broths but were able to survive the reduced selective pressure in the selective pre-enrichment broths. Cumulatively, we only had four *Salmonella* positive samples collected at post-chill, so a larger study is required to determine whether this relationship is true, and these would be important studies to perform as they would have implications on current culture methods used to monitor for *Salmonella*. Similarly, we found some reciprocal instances with isolation from selective enrichment but not selective pre-enrichment, which most notable occurred during early processing stages (post-pick at Plants 1 and 2 and post-IOBW at all three plants). It is possible that the high overall microbial load present on carcasses pre-chill required a full enrichment in RV or TT broths to limit background flora and allow *Salmonella* to be isolated and therefore the lower selective pressure provided by the selective pre-enrichment was not sufficient.

In some cases, we were able to isolate *Salmonella* following selective pre-enrichment in novobiocin and bile salts but not with novobiocin and malachite green, and vice versa. This is not unexpected given the differences sometimes seen in isolation between TT and RV broths (Singer et al., 2009; Gorski, 2012; Cox et al., 2019; Larsen et al., 2021), therefore, and similarly to current culture protocols, we suggest performing these selective pre-enrichment steps in parallel to be able to best capture *Salmonella*.

At the *Salmonella* population level, pre-intervention serovar diversity decreased from 3.00 and 2.83 serovars per sample at post-pick and post-IOBW, respectively, to 1.50 serovars per sample at post-chill, demonstrating that the interventions used effectively reduced *Salmonella* incidence and that this was accompanied by a reduction in the number of serovars found in each sample post intervention. While this trend is interesting and expected, a larger sample number is needed to confirm this relationship. A recent study showed a similar trend by identifying two different serovars on four pre-chill carcasses compared to zero carcasses post chill that contained more than one serovar (Boubendir et al., 2021).

Across our data, serovars Infantis and Kentucky were the most prevalent and were found in 69.7% and 63.6% of *Salmonella* positive samples, respectively. This is not unexpected as both these serovars are commonly found in poultry (Antunes et al., 2016; Shah et al., 2017; Ramirez-Hernandez et al., 2019; Richards et al., 2020). These serovars differ in their association with human illness: serovar Infantis is the 6th most common serovar isolated by the CDC (Tack et al., 2019), and ser. Kentucky is less clinically significant (Shah et al., 2017). Studies have demonstrated that serovar Infantis can effectively colonize chickens, has increased tolerance to antimicrobials, and that some strains are multi drug resistant (Drauch et al., 2021). Using CRISPR-SeroSeq, we

determined that serovar Infantis was present at all sampling locations. Recent studies (Park et al., 2015; Zeng et al., 2021) have also reported the persistence of serovar Infantis in processing facilities. Importantly, between post-pick and post-IOBW, serovar Infantis increased in relative abundance in most of the samples. This suggests that it might have tolerated the antimicrobials used in the New York rinse than the other serovars that were present. Studies have shown that cleaning and disinfection may not eliminate serovar Infantis (Olsen et al., 2003; Zeng et al., 2021) and FSIS *Salmonella* monitoring shows that this serovar is increasingly found in plants across the United States, which suggests that it is more tolerant to antimicrobials such as peroxyacetic acid (Mcmillan et al., 2020). While this is may be occurring here, we evaluate our data with caution because i) serotyping was only performed at one plant, ii) only a few of our post-intervention samples from all three plants were *Salmonella* positive, iii) the cross contamination between post-pick and post-IOBW confounds our understanding of serovar Infantis persistence.

Collectively, the data presented herein demonstrate that selective pre-enrichment is comparable to selective enrichment in effectively isolating *Salmonella* and does not impact the serovars that are detected. Selective pre-enrichment shortens *Salmonella* culture protocol by 24 hours, which is beneficial for any *Salmonella* monitoring system.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no competing financial interests.

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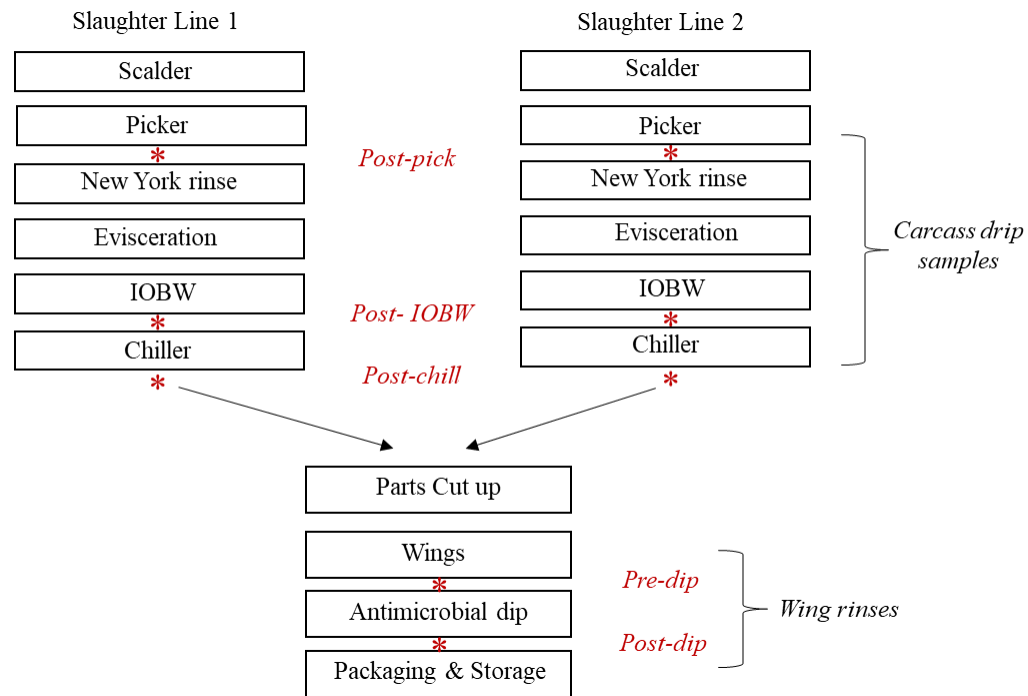


Figure 3.1. Diagram of a broiler processing establishment with relevant processing and intervention steps.

Sample locations are indicated with an asterisk. IOBW, Inside-outside Bird Washer

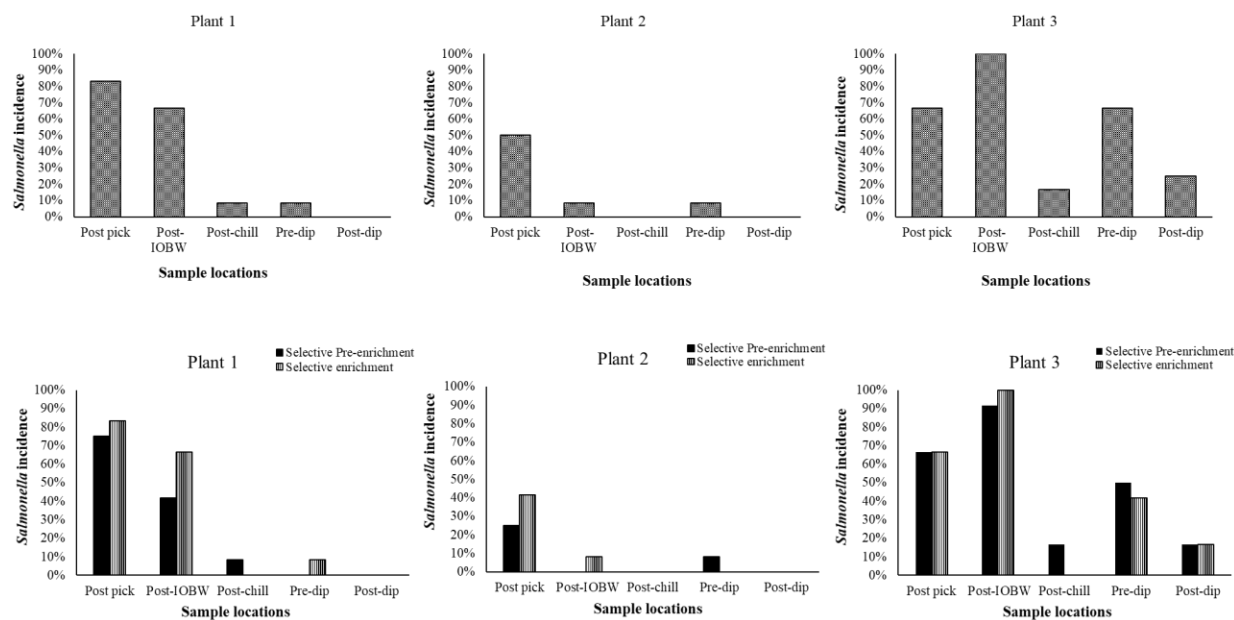


Figure 3.2. *Salmonella* incidence from Plants 1-3 and comparison of different enrichment cultures. A total of 12 samples were collected at each location in each plant and the graph indicates the overall *Salmonella* incidence (a) and *Salmonella* incidence following selective pre-enrichment and selective enrichment individually (b). IOBW, Inside-Outside Bird Washer. Different italicized letters indicate significant difference in *Salmonella* incidence determined by a Fisher's exact test

Figure 3.3

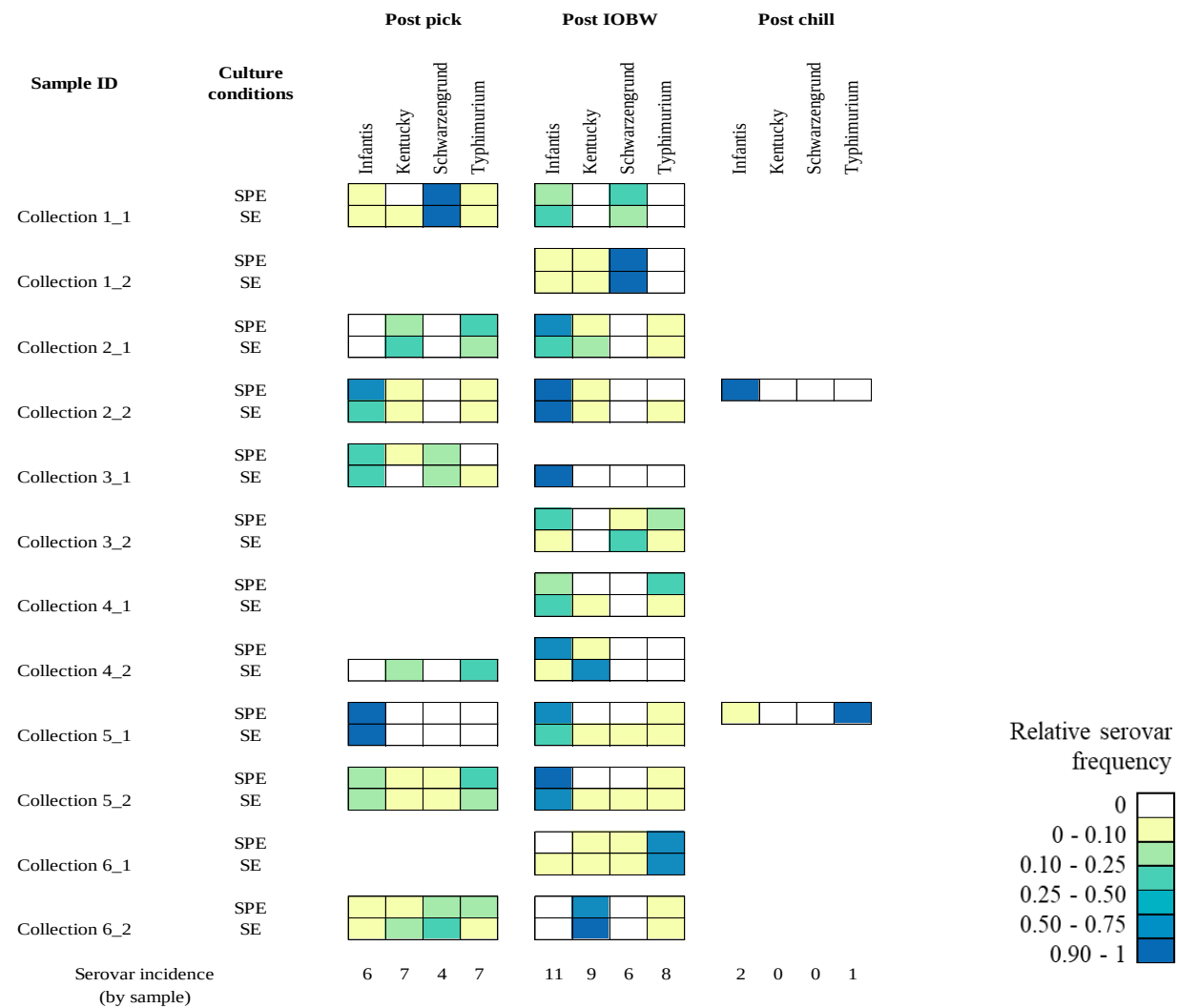


Figure 3.3. *Salmonella* serovar population profiles in carcass drip samples collected at post-pick, post-IOBW and post-chill locations in plant 3 cultured following selective pre-enrichment and enrichment. The sample ID field indicates when and where the sample was collected, for example, Collection 1_1 refers to the first of six sample collections and was performed on the first of two processing lines. IOBW, Inside-Outside Bird Washer; SPE, selective pre-enrichment; SE, selective enrichment.

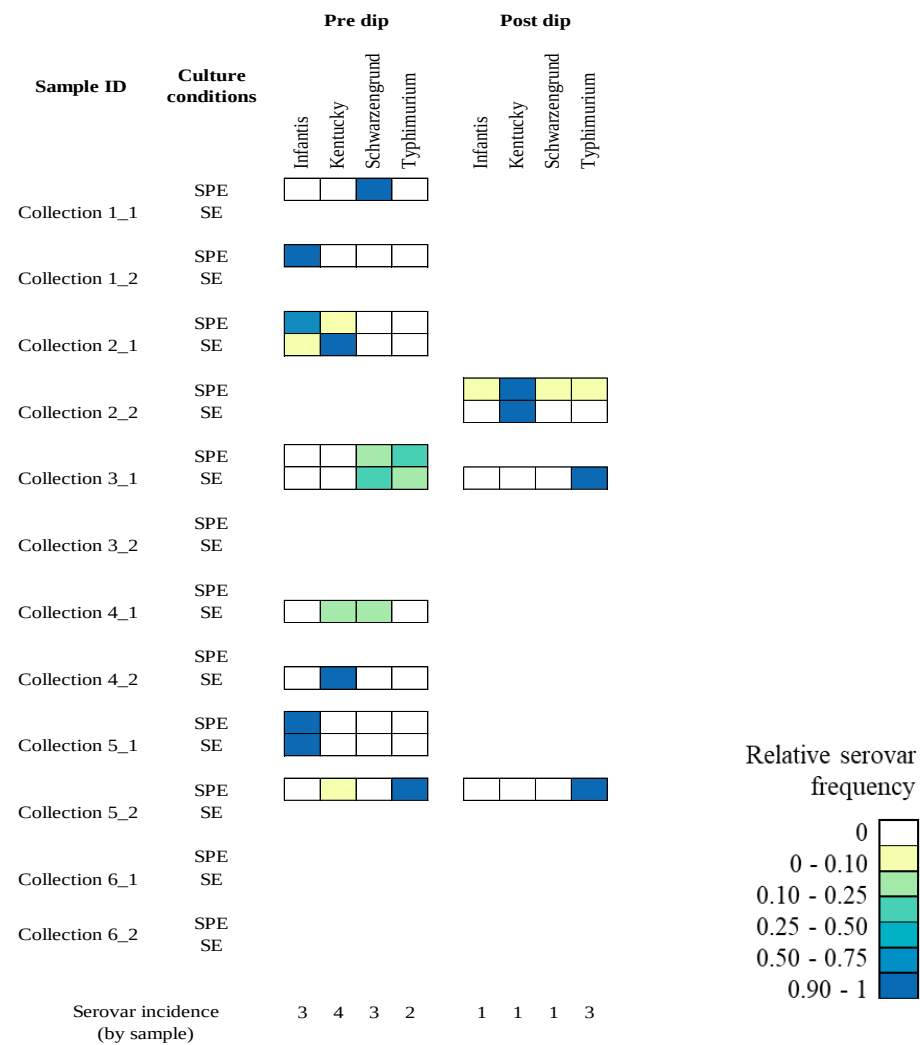


Figure 3.4

Figure 3.4. *Salmonella* serovar profiles in wing rinses collected at pre- and post-dip in plant 3 and cultured under selective pre-enrichment and enrichment conditions. The sample IDs are as explained in Figure 3. SPE, selective pre-enrichment; SE, selective enrichment

SUPPLEMENTARY FIGURE

Sample pair (SPE and SE)	Bray-Curtis metric
Collection 1_1, post-pick	0.0542
Collection 1_1, post-IOBW	0.3201
Collection 1_2, post-IOBW	0.0077
Collection 2_1, post-pick	0.1855
Collection 2_1, post-IOBW	0.3173
Collection 2_2, post-pick	0.2272
Collection 2_2, post-IOBW	0.037
Collection 3_1, post-pick	0.097
Collection 3_2, post-IOBW	0.5529
Collection 4_1, post-IOBW	0.4543
Collection 4_2, post-IOBW	0.6931
Collection 5_1, post-pick	0
Collection 5_1, post-IOBW	0.1522
Collection 5_2, post-pick	0.1604
Collection 5_2, post-IOBW	0.1401
Collection 6_1, post-IOBW	0.0224
Collection 6_2, post-pick	0.3977
Collection 6_2, post-IOBW	0.1622
Collection 2_1, pre-dip wing rinsate	0.7941
Collection 2_2, post-dip wing rinsate	0.0733
Collection 3_1, pre-dip wing rinsate	0.0766
Collection 5_1, pre-dip wing rinsate	0

 Strong match; Bray-Curtis <0.3
 Moderate match; Bray-Curtis 0.3-0.7
 Weak/no match; Bray-Curtis > 0.7

Figure 3.5

Figure 3.5. Comparison of *Salmonella* serovar diversity between selective pre-enrichment and selective enrichment. The Bray-Curtis metric was used to compare the number of serovars and the relative abundance of each serovar between two populations, in this case to compare the 22 paired samples for which we had serovar population data from both selective pre-enrichment and selective enrichment. SPE, selective pre-enrichment; SE, selective enrichment (Supplementary)

Table 3.1. *Salmonella* incidence at different locations in processing plant 1 following pre-enrichment, selective pre-enrichment, and selective enrichment.

Sample location	Pre-enrichment	Selective pre-enrichment		Selective enrichment ^a		Cumulative		Total
		Novobiocin, bile salts	Novobiocin, malachite green	RV	TT	Selective pre-enrichment	Selective enrichment	
Post pick	4/12	3/12	7/12	7/12	10/12	9/12	10/12	10/12
Post-IOBW	3/12	3/12	4/12	8/12	5/12	5/12	8/12	8/12
Post-chill	0/12	0/12	1/12	0/12	0/12	1/12	0/12	1/12
Pre-dip	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12
Post-dip	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
Total	7/60	6/60	12/60	15/60	16/60	15/60	19/60	20/60

Abbreviations: IOBW, Inside-Outside Bird Washer; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth.

^aSelective enrichment in RV and TT broth followed 24-hour incubation in non-selective neutralized buffered peptone water.

Table 3.2. *Salmonella* incidence at different locations in processing plant 2 following pre-enrichment, selective pre-enrichment, and selective enrichment.

Sample location	Pre-enrichment	Selective pre-enrichment		Selective enrichment ^a		Cumulative ^c		Total
		Novobiocin, bile salts	Novobiocin, malachite green	RV	TT	Selective pre-enrichment	Selective enrichment	
Post pick	1/12	0/12	3/12	3/12	4/12	3/12	5/12	6/12
Post-IOBW	0/12	0/12	0/12	0/12	1/12	0/12	1/12	1/12
Post-chill	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
Pre-dip	1/12	1/12	1/12	0/12	0/12	1/12	0/12	1/12
Post-dip	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
Total	2/60	1/60	4/60	3/60	5/60	4/60	6/60	8/60

Abbreviations: IOBW, Inside-outside bird washer; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth.

^aSelective enrichment in RV and TT broth followed 24-hour incubation in non-selective neutralized buffered peptone water.

Table 3.3. *Salmonella* incidence at different locations in processing plant 3 following pre-enrichment, selective pre-enrichment, and selective enrichment.

Sample location	Pre-enrichment	Selective pre-enrichment		Selective enrichment ^a		Cumulative		Total
		Novobiocin, bile salts	Novobiocin, malachite green	RV	TT	Selective pre-enrichment	Selective enrichment	
Post pick	2/12	3/12	7/12	6/12	7/12	8/12	8/12	8/12
Post-IOBW	10/12	11/12	10/12	10/12	12/12	11/12	12/12	12/12
Post-chill	0/12	1/12	1/12	0/12	0/12	2/12	0/12	2/12
Pre-dip	3/12	4/12	5/12	5/12	5/12	6/12	5/12	8/12
Post-dip	1/12	2/12	1/12	2/12	2/12	2/12	2/12	3/12
Total	16/60	21/60	24/60	23/60	26/60	29/60	27/60	33/60

Abbreviations: IOBW, Inside-outside bird washer; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth.

^aSelective enrichment in RV and TT broth followed 24-hour incubation in non-selective neutralized buffered peptone water

CHAPTER 5

SUMMARY

Salmonella is a leading cause of foodborne illnesses in the United States, with many salmonellosis cases being attributed to poultry products (IFSAC, 2021). Although there has been a reduction in *Salmonella* prevalence on raw broiler carcasses and parts in national baseline survey data, the number of illnesses associated with *Salmonella* linked to poultry has not changed (USDA-FSIS, 2021; Healthy people, 2030). Interventions at processing helps to eliminate *Salmonella* from poultry products before retail display.

In the first study, we optimized the pre-enrichment broth by adding selective ingredients used in traditional enrichment cultures at a lower concentration making it a selective pre-enrichment broth. From the first study, we collected broiler carcass drip samples (n=18) post-pick (after feather removal); all the samples were positive for *Salmonella*. Serovar Kentucky was the most common serovar identified in this study. In the second study (n=180 samples), our results showed that *Salmonella* prevalence was reduced through processing from post-pick to post-chill (post pick-67%; post-IOBW-58%; post-chill-8%). An increase in *Salmonella* prevalence was observed after portioning of carcasses followed by significant reduction due to antimicrobial dipping, where we assessed parts by collecting wing rinses before (pre antimicrobial dip) and after antimicrobial dip (post antimicrobial dip). Serovars Kentucky and Schwarzengrund both known for harboring antimicrobial resistance genes were not detected after chilling (Aarestrup et al., 2007; Shah et al., 2017). *Salmonella* prevalence was lower on post-chill carcasses in the processing plants

we tested demonstrating the effectiveness of antimicrobial interventions followed by poultry industry. Selective pre-enrichment broth is useful to assess *Salmonella* prevalence from broiler processing samples. Selective pre-enrichment broth showed similar recovery rate and serovar profile compared to standard traditional selective enrichment broth in both studies.

By reducing the isolation time by 24 hours, the new culture detection protocol from this research may help the broiler industry to track the *Salmonella* serovars more efficiently through processing, even at locations such as post-chill with lower *Salmonella* incidence. Due to addition of selection after four hours, the selective pre-enrichment broth resulted in less background bacteria making serovar determination by CRISPR-SeroSeq PCR more efficient. Using the improved *Salmonella* culture detection approach along with efficient molecular approaches such as CRISPR-SeroSeq we can accurately map *Salmonella* serovars through broiler processing and identify clinically relevant serovars.

To reduce *Salmonella* colonization in broilers, pre-harvest studies have shown that vaccination in parental breeder flocks is an effective approach (Dórea et al., 2010; Dale et al., 2015). Serovars of public health concern can be identified at the processing plant for a given integrator and those can be prioritized to prepare autogenous vaccines for use in parental breeder flocks. Previous studies have shown that enrichment broths used in standard *Salmonella* isolation, allowed serovars to grow better than others. For example, ser. Schwarzengrund grows better in RV broth and ser. Enteritidis in TT broth (Singer et al., 2009; Gorski, 2012; Cox et al., 2019; Larsen et al., 2021). The selective pre-enrichment protocol evaluated here addresses this limitation, provides reliable and effective surveillance data in a shorter time. There may be less chance of serovar bias being introduced by using selective pre-enrichment broth, as it contains selective ingredients from both RV and TT broths.

Further research with artificial inoculation of a variety *Salmonella* serovars to selective pre-enrichment broth will be needed to evaluate serovar bias. The variation in growth kinetics under different culture conditions needs to be evaluated to differentiate between *Salmonella* serovars and strains. With the lower *Salmonella* incidence noted in post-chill samples, it was a challenge to generate statistically sound data to address which serovars survived best during processing. A larger study with more visits to processing plants with higher number of samples collected through processing is required to address our second goal effectively. Nonetheless, this study demonstrates that the time required for *Salmonella* isolation can be shortened. This is also relevant to other food industries where *Salmonella* is a problem. As serovars can exhibit varied phenotypes, the *Salmonella* serovars surviving chilling process in plants should be further evaluated by *in vitro* antimicrobial assays. These types of tests would complement the population profile analyses provided by CRISPR-SeroSeq. The continuous exposure of *Salmonella* strains to sublethal concentrations of antimicrobials may contribute to higher tolerance or cross resistance to other antimicrobials. In turn, this may contribute to persistence of *Salmonella* in processing plant environments. Further experiments focused on the detection of viable but non-culturable bacteria (VBNC) *Salmonella* in samples due to use of antimicrobials in the processing and quantification of cells needs to be evaluated. Antimicrobial assay studies will be helpful in making strategies to reduce *Salmonella*.

To conclude, this study contributes to improve *Salmonella* culture detection protocol by reducing time without compromising detection ability. The CRISPR-SeroSeq analyses provides a framework for investigating *Salmonella* serovar dynamics through processing in commercial broiler processing plants. Due to the complex nature of transmission and diverse types of *Salmonella*, multi-hurdle control measures at all levels of broiler production are required to

provide safer poultry meat. Efficient surveillance is a significant piece of assessing the efficacy of *Salmonella* controls.

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