HIGH-RESOLUTION *SALMONELLA* SURVEILLANCE IN COMMERCIAL BROILER BREEDER PRODUCTION

by

AMY TAYLOR SICELOFF

(Under the Direction of Nikki W. Shariat)

ABSTRACT

A high-resolution sequencing approach, CRISPR-SeroSeq, has revealed the frequent occurrence of multiserovar *Salmonella* populations in food animals and the environment; this highlights the limitations of traditional culture-based methods, as most are reliant on isolation of a few colonies and thus underestimate serovar diversity. The work presented here relies on deep serotyping by CRISPR-SeroSeq to investigate multiserovar *Salmonella* population dynamics in domestic poultry production. Despite a decrease in *Salmonella* prevalence at broiler processing from 17% to 8% between 2016 and 2020, the rate of foodborne illnesses remains stable. This disconnect may be attributed to serovars evading detection and therefore intervention strategies. A two-year surveillance study of broiler breeder farms found 18% of *Salmonella*-positive environmental samples contained multiserovar populations, with serovar Kentucky often excluding others. Longitudinal sampling across two commercial complexes found 17% and 41% of samples collected from pullet and breeder flocks were *Salmonella*-positive, respectively, with peak prevalence around 38 weeks of age. On-farm rodents were collected and screened for *Salmonella* by composite gastrointestinal tract samples,

revealing 35% positivity with shared serovars between the corresponding flocks and underscoring the potential for on-farm transmission. Successful *Salmonella* mitigation is contingent upon robust surveillance data, which in turn requires optimal sample collection and isolation methods. The combination of selective pre-enrichment with molecular enumeration in environmental breeder and broiler farm samples demonstrated comparable serovar recovery compared to traditional enrichment while reducing the isolation process by 24 hours. Additionally, PCR assays, along with retrospective bioinformatic analyses, were used to differentiate between live attenuated serovar Typhimurium vaccine strain and field strains found at processing. Importantly, 6% of serovar Typhimurium isolates from domestic broiler products collected in 2016 to 2022 were vaccine strains, which negatively counted towards a processing establishment's ranking and may have dissuaded vaccination use. Collectively, these results emphasize the need to improve *Salmonella* detection methods while minimizing turnaround time. By leveraging high-resolution sequencing, this dissertation highlights the complexity of *Salmonella* populations in chickens, including serovar-specific interactions and on-farm transmission pathways. This work supports that effective control strategies require adherence to biosecurity measures, reliable monitoring, and tailored interventions, all of which are fundamental in pre-harvest poultry production.

INDEX WORDS: broiler breeder production, *Salmonella enterica* subsp. *enterica*, multiserovar populations, CRISPR-SeroSeq, next-generation sequencing, molecular diagnostics

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Evolution of *Salmonella* subspecies and serovars

Salmonella was first discovered in 1885, when it was presumed to be the causative agent of swine cholera (1). It has since become one of the most studied microorganisms as it is a significant contributor to foodborne disease in a wide range of hosts. The divergence of *Salmonella* and *Escherichia coli*, another leading bacterial cause of foodborne illness, occurred between 120 and 160 million years ago, and marked the acquisition of *Salmonella* pathogenicity island 1 (SPI-1)(2, 3). The SPI-1 encodes a type III secretion system (T3SS), which is considered to be the most important virulence determinant of *Salmonella*, as it supports host invasion and regulates immune responses (4). Members of the genus *Salmonella* are gram-negative, facultative anaerobic, rod-shaped bacilli divided among two species, *S. bongori* and *S. enterica*, with the latter responsible for more than 99% of human clinical infections (5–9). *S. bongori* is primarily associated with cold-blooded animals with rare instances of acute salmonellosis in humans, usually following exposure to an infected reptile (10). Importantly, this species provides context for *Salmonella enterica* evolution as the inclusion of *Salmonella* pathogenicity island 2 (SPI-2), and corresponding macrophage replication abilities, occurred following species delineation (3, 11–14).

Currently, there are six defined subspecies within *Salmonella enterica,* including *enterica* (I)*, salamae* (II)*, arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *indica* (VI)

(15, 16). In turn, each subspecies is comprised of serovars that are each defined by the unique combination of two flagellar (H) and one somatic (O) antigen(s), for a total of over 2,600 serovars (6, 15). More than half of these serovars belong to *Salmonella enterica* subsp. *enterica* (hereafter, *Salmonella*), and less than 10% regularly cause human illness (17). These serovars are further classified as typhoidal or nontyphoidal; members of these two groups have vastly different disease outcomes, with typhoidal serovars acting as specialist pathogens to cause severe diseases in a limited host range (18, 19). Interestingly, many *Salmonella* serovars are named after geographical regions or the presumed illness caused, rather than the antigenic profile naming convention applied for other subspecies or bacteria (20). As a result of this, a handful of serovars are inappropriately named, including Infantis, Typhimurium, and London; the former two serovars do not exclusively cause the illnesses described (i.e., sickness in infants or typhoid fever in mice), while the latter was actually first isolated from Reading, England but serovar Reading was already defined so it was instead inspired by the last name of the patient. Aside from some interesting origin stories, it is most important to define *Salmonella* serovars as they exhibit a range of phenotypes based on varying genotypes, including virulence factors, antimicrobial resistance, propensity to cause disease, manifestation of symptoms, and host preferences (21–32). Further variation exists at the strain level as well (33–37). The high phenotypic diversity may be attributed in part to host adaptation, as colonization of different hosts may rely on certain fitness traits and the community composition surrounding salmonellae likely varies by host, thus providing additional opportunities for divergent evolution (38–43).

One of the main limitations associated with the conventional *Salmonella* serovar naming methods is the lack of recognition of lineages (44–46). Polyphyletic serovars are characterized by shared O and H antigens but separate genomic features, such that lineages often have different disease outcomes and host associations (47–50). It is hypothesized that polyphyly may occur as a result of convergent evolution, such that the genes encoding the surface and flagellar antigens have undergone horizonal transfer, and this is supported by the lack of shared most recent common ancestors between lineages (51–57). While there may one day be a reclassification of *Salmonella* serovars or refinement of the attributes used to define them, the primary focus of surveillance and risk assessment models should be the identification of all serovars present such that appropriate controls may be enacted and the threat to public health is minimized (58, 59).

Public health implications of *Salmonella*

Salmonella is a leading bacterial cause of illness in the United States, with an estimated 1.35 million infections, 26,500 hospitalizations, 420 deaths, and cost of illness of over $4 billion annually (17, 60). As an enteric bacterium, *Salmonella* transmission occurs primarily via the fecal-oral route. Salmonellosis is typically self-limiting, with mild gastrointestinal symptoms, but some illnesses may require treatment for dehydration or prolonged infection. In the case of a doctor visit, salmonellosis is a nationally notifiable disease, with submission of clinical samples or isolates to local or state public health laboratories (61). The Centers for Disease Control and Prevention (CDC) track the frequency and/or extent of domestic *Salmonella* illnesses, along with serotyping results, to reevaluate the public health risk annually with the assistance of PulseNet, the national molecular subtyping network for foodborne disease surveillance including all 50 states

(62, 63). PulseNet was developed in 1996 in response to the major *E. coli* outbreak three years prior, and it has helped recall over 1 billion pounds of contaminated foods since its inauguration. PulseNet originally relied on Pulsed-Field Gel Electrophoresis (PFGE) as DNA fingerprinting to detect outbreak strains but now whole genome sequencing (WGS) is employed as the gold standard for identifying foodborne pathogens (64, 65). Similarly, the Foodborne Diseases Active Surveillance Network (FoodNet) conducts surveillance for eight major foodborne illnesses, including *Salmonella*; this is a collaboration between the CDC, United States Department of Agriculture – Food Safety and Inspection Service (USDA-FSIS), United States Food and Drug Administration (US-FDA), and 10 state health departments, which encompasses ~16% of the domestic population (66, 67). Outside of these two networks, the CDC can work alongside other organizations in the United States and internationally to complete epidemiological traceback. The BEAM (Bacteria, Enterics, Ameba, and Mycotics) Dashboard is a newly released, interactive tool to analyze and visualize data collected by the CDC on bacterial, viral, and parasitic agents, and other foodborne, waterborne, and fungal diseases and is publicly accessible (68).

Most (80%) *Salmonella* outbreaks can be attributed to seven specific food categories, including: chicken, fruits, pork, seeded vegetables (such as tomatoes), other produce (such as fungi, herbs, nuts, and root vegetables), beef, and turkey (69). This source diversity highlights the complexity of *Salmonella* outbreak traceback, compared to other foodborne pathogens, such as *Listeria monocytogenes* or *Escherichia coli* O157, which are attributed to fewer sources. Importantly, chicken is estimated as the largest single food contributor to *Salmonella* outbreaks (19.7%)(69). In terms of confirmed tests,

chicken accounted for 46% (88/192) of meat and poultry outbreaks reported to the CDC from 2012 to 2021 (70). In response to the high rate of foodborne salmonellosis, many regulatory policies have been instituted to control *Salmonella* contamination in food animal production. The USDA-FSIS established performance standards in processing plants in 1996 as part of the "Pathogen Reduction; Hazard Analysis and Critical Control Point (PR/HACCP) Systems Final Rule" (71). Their performance standards have been updated over the years in response to successful pathogen reduction efforts. As part of the USDA-FSIS *Salmonella* Verification Program, samples are collected from processing establishments across the United States each week (72). These are analyzed in a 52-week window to assess the public health risk from certain meat and poultry products as they are processed. *Salmonella* prevalence in raw parts (legs, breasts, wings) has decreased in the last few years, from 16.7% in 2016 to 7.8% in 2022, without a concurrent reduction of number of salmonellosis cases attributed to chicken (69, 73)(Fig. 1-1). Notably, *Salmonella* incidence is greater in parts than whole carcasses; this may be due to the release of internalized serovars within joints, cross-contamination during processing, or contaminated equipment during parts cut-up (74–82). Overall, the antimicrobial interventions applied in poultry processing plants have significantly reduced contamination on final products, but any remaining salmonellae are still causing human illness, which underscores the need for additional control measures (81, 83–95). Importantly, serovars of concern may be persisting within the processing environment or they may be continually reintroduced to the processing environment due to sustained contamination of the broiler house environment (76, 80, 96–99); thus, it is necessary to

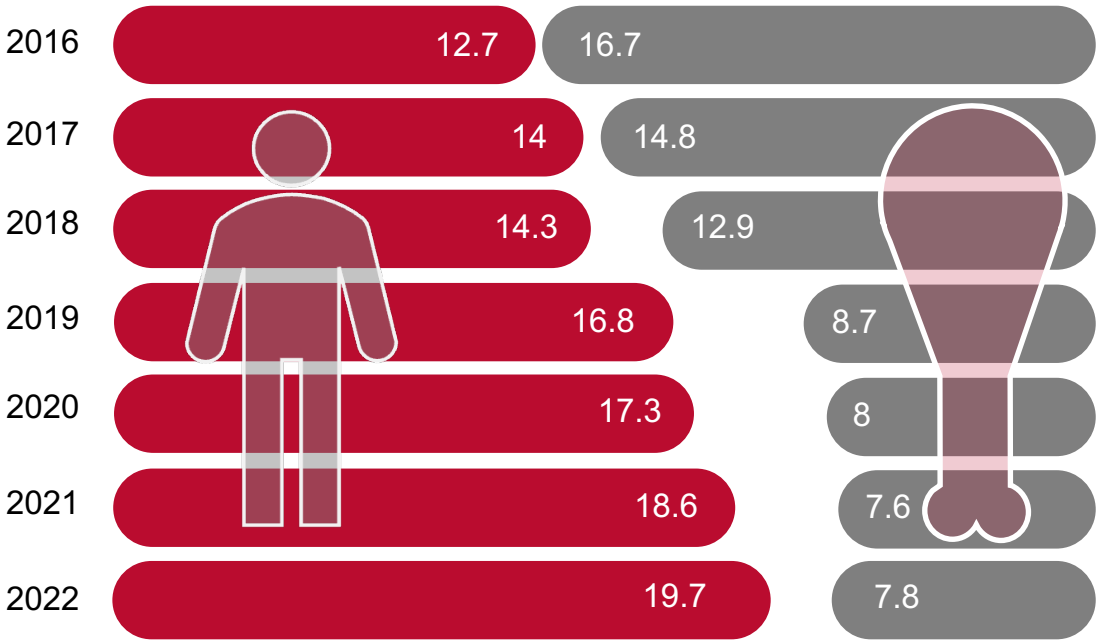


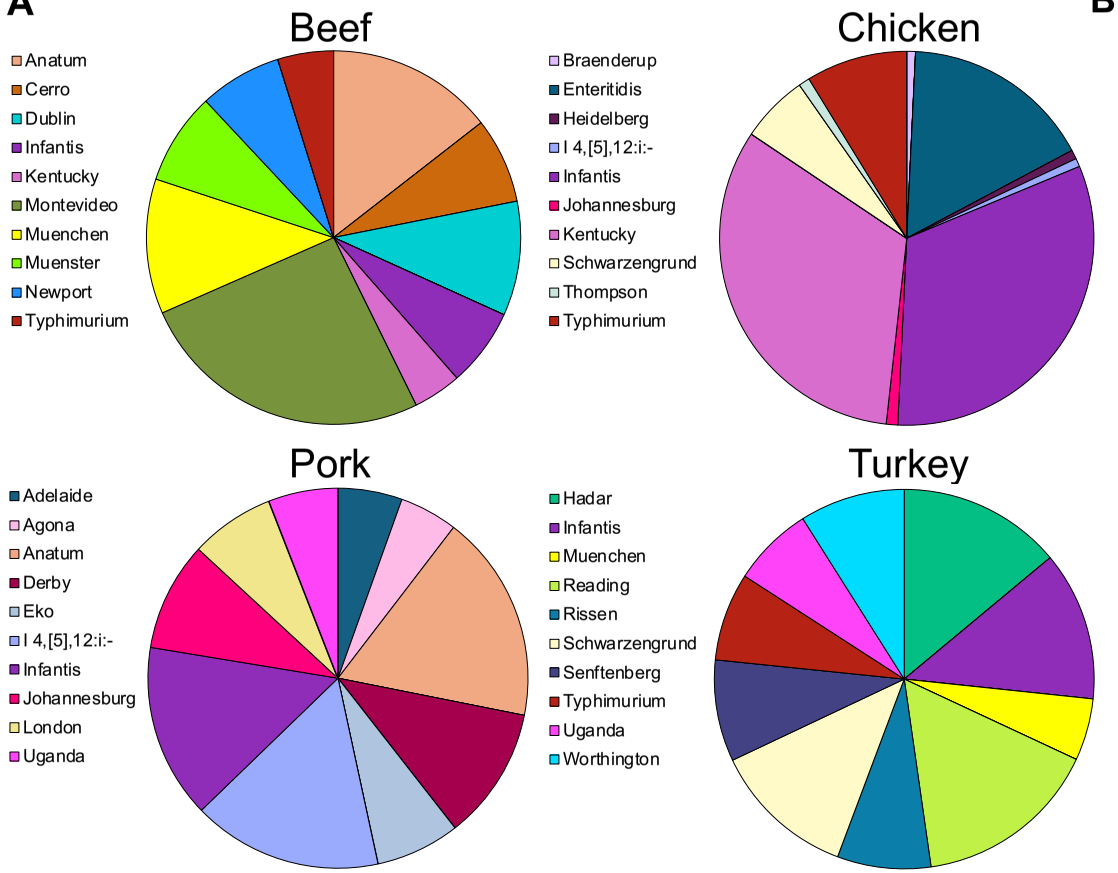
Figure 1-1. Salmonellosis cases attributed to chicken has increased while *Salmonella* contamination of final products has decreased from 2016 to 2022. The red bar graphs represent the estimated proportion of *Salmonella* illnesses attributed to chicken, with an increase from 12.7% in 2016 to 19.7% in 2022 (69). The gray bars represent *Salmonella*-positive surveillance samples collected from raw parts (legs, breasts, wings) in domestic broiler processing plants, with a 50% reduction over six years (73). Bar length is proportional to percentages listed**.**

employ high-resolution *Salmonella* surveillance to characterize present serovars and develop targeted control strategies (100, 101).

Salmonella serovars in food animal production

All *Salmonella* serovars can cause disease in humans, and many of those have multiple animal or environmental reservoirs. Some serovars are considered to be host-adapted (e.g., serovar Dublin) with even fewer as host-restricted (e.g., serovar Gallinarum), which has important implications as host adaptation may concurrently increase mortality (22, 39, 102, 103). As such, there is variation in the serovars most commonly found in the four main food animal commodities (beef, chicken, pork, turkey) according to host (Fig. 1-2). Notably, serovars Infantis is the only to be found in the top ten list of all commodities. Serovars Montevideo, Kentucky, Anatum, and Reading were the most abundant in beef (17%, 277/1634), chicken (31%, 4855/15553), pork (12%, 674/5696), and turkey (8.2%, 206/2503), respectively. Interestingly, the top serovar isolated from each food commodity differed from the serovar linked to the most outbreak illnesses over the last 10 years: serovar Newport in beef (n = 659), Typhimurium in pork (n = 480), Enteritidis in chicken (n = 1,065) and turkey (n = 661) (68). This observation underscores the importance of serovar identification, as a lower abundance of a highly pathogenic serovar poses a greater risk than a higher abundance with lower pathogenicity and the infectious dose varies between serovars (104). Between 2019-2023, serovars Kentucky and Infantis represent 62% (9,619/15,553) of *Salmonella*-positive chicken samples, which highlights the lower serovar diversity found in this commodity. Comparatively, the top two serovars in beef, pork, and turkey account for 26% (433/1,634), 23% (1,288/5,696), and 16% (388/2,503) of samples, respectively. Notably,

A



B

	Beef	Chicken	Pork	Turkey
Adelaide			**x**	
Agona			x	
Anatum	x		x	
Braenderup		**x**		
Cerro	x			
Derby			x	
Dublin	**x**			
Enteritidis		**x**		
Eko			x	
Hadar				**x**
Heidelberg		**x**		
I 4,[5],12:i:-		**x**	**x**	
Infantis	x	**x**	**x**	x
Johannesburg		x	x	
Kentucky	x	x		
London			x	
Montevideo	x			
Muenchen	x			x
Muenster	x			
Newport	**x**			
Reading				**x**
Rissen				x
Schwarzengrund		x		**x**
Senftenberg				x
Thompson		**x**		
Typhimurium	**x**	**x**		x
Uganda			x	x
Worthington				x

Figure 1-2. Top *Salmonella* serovars differ between food animals.
A) Ten of the most common serovars for each food animal (beef, chicken, pork, turkey) monitored by USDA-FSIS are displayed. Serovar data was accessed from the publicly available sampling repository (73) and included samples that were collected from 2019 to 2023. B) Comparison of top serovars between commodities, with bolded "x" to represent outbreak serovars of concern for human health (69).

chicken production is the only food production system in the United States with true vertical integration, which supports improved biosecurity as a closed system limiting external contamination. The USDA-FSIS is the best national dataset to indicate major serovars in food animal production through their *Salmonella* Verification Program (105); however, this program samples animals at processing and does not include any on-farm testing. Therefore, selection of certain serovars with enhanced antimicrobial resistance at processing may bias this dataset and not accurately reflect serovars most often found in the food animals. Additionally, the CDC periodically updates the list of serovars that are frequently attributed to foodborne outbreaks on their BEAM dashboard (68).

Both the USDA-FSIS and CDC lists may change in terms of serovar identity following shifts of prevalence in the poultry industry; these shifts may be naturally occurring as some serovars are better adapted to compete against other serovars (i.e., serovar Kentucky can colonize chickens more readily), or they may occur because of industry-wide, serovar-specific mitigations that leave a vacant niche (106–109). For example, serovar Gallinarum and its biovar Pullorum were predominant in commercial poultry production in the early 1930s but these serovars caused severe disease in chickens, making it a top priority to control transmission and avoid a food shortage (110). To avoid endemic colonization on farms, strict control measures had to be enacted such that one positive test result for serovars Gallinarum or Pullorum mandated culling the entire flock, which successfully eliminated these pathogens from the domestic poultry industry. Following this eradication, serovars Enteritidis and Heidelberg increased in frequency until targeted vaccination efforts began (109, 111–113); the efforts have not

removed these serovars entirely but have reduced their prevalence. Currently, serovar Kentucky is most abundant in the poultry industry, as it has been for over 20 years, but it is infrequently linked to human illness in the United States (48). Serovar Infantis is a potential candidate for the next serovar: it has increased considerably following the multistate outbreak in 2018 (114).

Genomic analysis may support the identification of emerging serovars through characterization of genes present in past superspreader serovars but routine, high-resolution *Salmonella* surveillance can help narrow the focus based on serovars existing within production systems (115). *Salmonella* control in poultry production is a complex undertaking due in part to the range of antimicrobial tolerance across serovars and asymptomatic colonization of chickens; both features impact final product contamination as some serovars are preferentially surviving interventions and it is challenging to design an economic monitoring or control program when extensive testing is required to characterize *Salmonella* populations in chickens. Namely, the ubiquity of *Salmonella* in commercial chicken flocks acts as a bottleneck in the implementation of effective management strategies since the risk cannot be properly attributed without determining the quantity and identity of present salmonellae. Reduction of *Salmonella* in finished chicken products has been achieved with generic antimicrobial interventions but the discordance with clinical cases demonstrates that further efforts are required to control problematic serovars. Current USDA-FSIS performance standards only regulate the *Salmonella* incidence of poultry products, so there is no differentiation of serovars, but a new framework has been proposed to consider *Salmonella* presence, quantity, and identity collectively (116, 117). Importantly, this framework highlights the impact of the

Salmonella load in the incoming flock, such that greater quantities or presence of resistant serovars lowers the efficacy of applied antimicrobials, which in turn prioritizes on-farm surveillance.

Domestic poultry production and the impact of *Salmonella*

The United States produces over nine billion meat-type chickens (broilers) each year, and Georgia is the largest broiler producer (annual production of 1.3 billion broilers)(118). As a result, Georgia has an annual profit of $4,032,731,000, which accounts for 14% of the total profit produced by the domestic poultry industry (119). In the United States, the poultry industry is vertically integrated, as each company maintains all production operations within complexes and the chickens are moved between different farms depending on the life stage (Fig. 1-3). Additionally, each complex typically has one hatchery, feed mill, and processing plant. The vertical integration model can be split between live production (pre-harvest) on farms and processing (post-harvest).

The pre-harvest stages include the maturation of young chickens (pullets) for breeding (breeders), and then the hatching of eggs for meat production (broilers). Pullet chicks are sourced from primary breeding (genetics) companies that enact strict biosecurity practices to limit pathogen introduction (including *Salmonella*). These biosecurity measures include building facilities on concrete pads and requiring shower in-shower out practices for visiting personnel. The male and female pullets are raised in single-sex houses until ~21 weeks of age when they reach sexual maturation. At this time, all the hens from one pullet farm are typically moved to one breeder farm, while the roosters may be divided among multiple breeder farms to facilitate gender ratios of 10 males per 100 females. Breeder chickens produce broilers, which are the chickens that

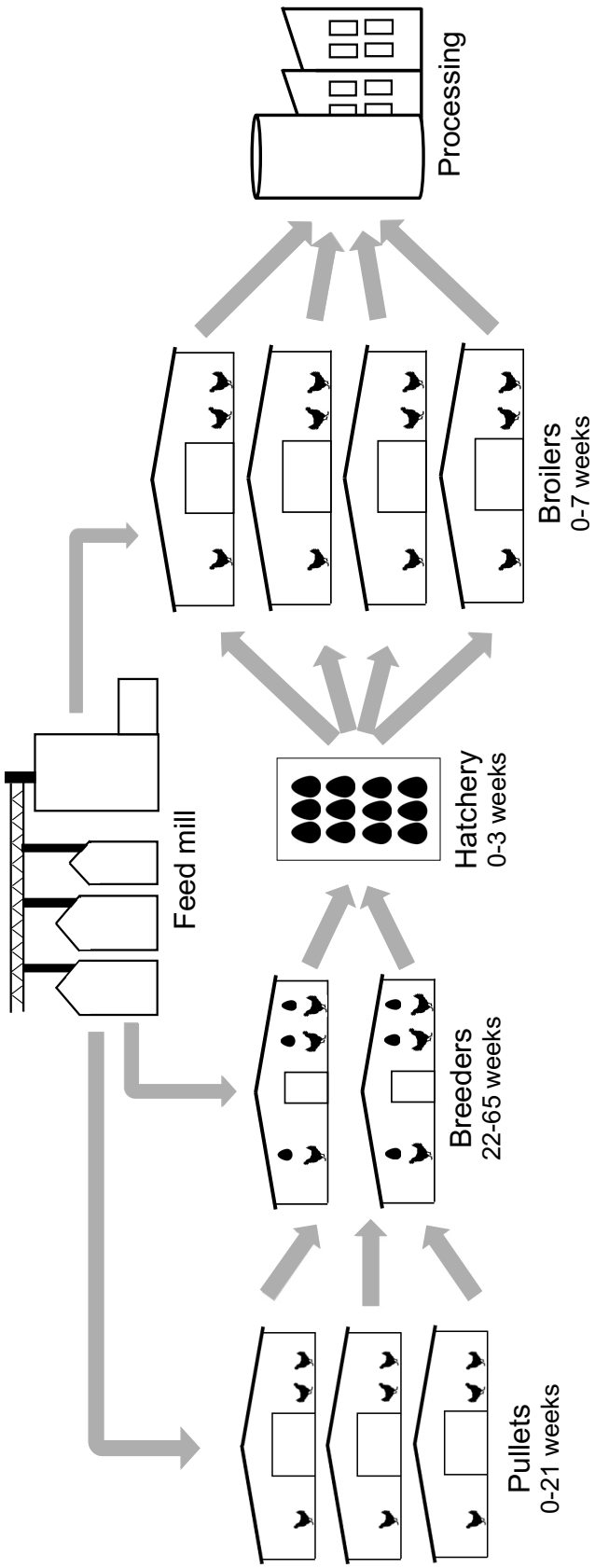


Figure 1-3. Vertical integration model of broiler breeder production. The schematic for a single commercial broiler complex is shown, with the gray arrows indicating the movement of chickens, eggs, and feed. The hatchery and processing plant act as bottlenecks in this model with all eggs and broilers going to each establishment, respectively. Total grow-out times for broilers may vary depending on ideal size for processing, with a range of five to nine weeks of age and an average of seven weeks shown.

are commercially sold. Broiler breeders continue reproducing until about 65 weeks; the average breeder hen will lay around 180 eggs, all of which are transferred to the complex hatchery (120). After hatching, the new broiler chicks are moved to broiler farms for grow-out until approximately five to nine weeks of age, depending on the broiler product being grown.

Salmonella colonization of chickens and transmission between flocks

While chickens are a significant reservoir of *Salmonella*, most serovars do not cause symptomatic illness in chickens, leading to the (unofficial) classification as a commensal bacterium; there are exceptions, of course, such as the substantial mortality caused by serovar Gallinarum and its biovar, Pullorum, or a high dosage of some serovars (e.g., serovar Enteritidis) in newly hatched chicks (121, 122). *Salmonella* colonization is typically limited to the lower gastrointestinal tract of chickens, namely the ceca, although some serovars may spread to the internal organs and tissues (123–126). There are opportunities for disease introduction and subsequent spread at each stage of poultry production within a commercial complex, including vertical transmission from parents to progeny and horizontal transmission between multiple houses on a farm. For example, the individual components of feed could be contaminated and distributed among farms, eggs covered with excess fecal content could introduce pathogens from the farm to hatchery, or personnel and surrounding wildlife populations could cause lapses in on-farm biosecurity (127–134). Additionally, the presence of rodents and insects (namely darkling beetles) has negative implications for on-farm *Salmonella* control since there is cyclical transmission between these populations and the corresponding chicken flocks (135–141). Rodents are primarily cause for concern in breeder and table egg producing

(layer) flocks since the extended production period and presence of raised slats with nest boxes for egg laying provides ample opportunity for rodents to live within the house (142–145). Rodents and insects may become colonized with *Salmonella* before or after entering a poultry house and then spread the bacteria throughout the shared environment via fecal droppings. In turn, the chickens may eat the droppings and then maintain the *Salmonella* in the house via their own shedding. Further, rodents may introduce structural damage to the poultry houses as they nest in ceilings and walls, thus creating additional opportunities for *Salmonella* introduction from external sources.

It has been demonstrated that both horizontal and vertical transmission of *Salmonella* occurs within a poultry complex, as isolated strains matched between breeder farms and subsequent broiler flocks (127, 146–153). Horizontal transmission refers to *Salmonella* spread among flocks following an initial introduction event, such as contaminated litter or feed, shared farm equipment, or improper personal protective equipment use by farm personnel (129, 154–156). Contamination from a previous flock through litter or dust is more likely to occur on broiler rather than breeder farms since the whole house environment is only emptied and sanitized between breeder flocks (157). Additionally, cross-contamination occurs in broiler flocks through the movement of chick trays, since these are used to transport chicks to the farm from the hatchery but not always disinfected in between trips (158). Importantly, some serovars, such as Enteritidis, are able to enter the fertilized egg, thus enabling vertical transmission from hen to progeny, which may then lead to subsequent spread to other chicks in the shared environment for several weeks of production (113, 126, 159–166). Vertical transmission is exclusively driven by *in ovo* colonization in chickens, and may occur between primary

breeding flocks, broiler breeders, and broilers, with breeders as the common denominator (132, 153). Across the commercial complex, *Salmonella* incidence increases through live production (pullets: 16.5%; breeders: 23.2%; broilers: 70-95%) followed by a steep reduction at processing (2.4-4.7%) (83, 99, 131, 167, 168).

Despite the regulatory programs for food safety, there is no mandatory, national surveillance system in place to record *Salmonella* incidence pre-harvest. The National Poultry Improvement Plan (NPIP) is a voluntary federal-state cooperative testing and certification program that was instituted in the 1930s to initially help the United States eradicate pullorum disease in chickens but has since expanded to include other poultry diseases (169–171). The NPIP program was established by the USDA – Animal and Plant Health Inspection Service (USDA-APHIS) and is a cooperative between the USDA, state officials and authorized laboratories, as well as industry, to conduct monitoring of commercial and backyard flocks. There are published program standards to guide best biosecurity practices, sample collection, and testing approaches, including approval of commercial assays (171). Importantly, the NPIP designated the "gold standard" on-farm, noninvasive sample collection method as boot socks, pre-moistened cotton that is worn on top of protective, plastic booties to promote adherence of fecal material while walking through the poultry house. The technical standards are developed as a joint effort by industry members, state governments, and federal officials, and are designed to promote disease control and prevention in the poultry industry. To that end, NPIP hosts a biennial conference to provide updates from the poultry industry and review new diagnostic tools. While NPIP is focused on poultry health rather than human health, it does include testing

for *Salmonella* serovar Enteritidis which frequently causes salmonellosis outbreaks and therefore supports food safety advances.

Poultry producers may opt to monitor *Salmonella* in their pullet and breeder flocks by collecting environmental samples around 16 and 42 weeks of age, respectively, and integrators can analyze their samples in-house or send them to a third party. Any results from these company samples are kept private, so the first time that commercial flocks are officially tested for *Salmonella* is at the processing plant by USDA-FSIS and these results, including product type and serovar, are made publicly available. As such, there is limited knowledge of *Salmonella* serovars routinely identified in broiler breeder production, although previous work has demonstrated that parental breeder flocks contribute to *Salmonella* colonization of progeny broiler flocks (146). There are several important questions to consider when developing a robust monitoring program, including when the best time is to collect samples, what the best sample type is, and what the limitations of the isolation method are. The vertical integration model of poultry production enables the standardization of methods, but optimal sample collection likely varies between production stages and commercial complexes.

On-farm *Salmonella* control

There are several different strategies for mitigating *Salmonella* during live production, and the implementation of these strategies also varies by production stage and commercial complex. The final rule for the Veterinary Feed Directive by the US-FDA limited the off-label application of antibiotics for increased growth and feed conversion in food animal production, which necessitated the implementation of alternative control strategies for *Salmonella* (172). There are different methods employed

between breeder and broiler production, including the use of pre-and probiotics, litter amendments, water treatments, feed withdrawal, phage cocktails, vaccines, and on-farm biosecurity measures (129, 135, 173–193). The primary *Salmonella* control in breeder flocks is vaccination, to prevent vertical, and then horizontal, transmission to broiler flocks (167, 187, 188, 194–204). Both live attenuated and killed (bacterin) vaccines are used for *Salmonella* control in the poultry industry; the former contains an avirulent strain to promote long-lasting immunity without causing a host carrier state and includes widely used commercial formulations against serovar Typhimurium, while the latter is developed using heat-inactivated bacteria and provides short-term protection (205–214). Importantly, bacterin vaccines may be developed for widespread commercial use, such as for serovar Enteritidis, with extensive efficacy testing and implementation as a long-term control strategy. Alternatively, autogenous vaccines serve as a rapid, short-term control as they are produced based on a *Salmonella* strain isolated from a complex and only allowed to be used on flocks within that complex (215). Ultimately, the purpose of all vaccines is to reduce *Salmonella* contamination on final processing, though this may occur through decreased colonization of chickens, minimized *Salmonella* shed into the environment, and provided cross-protection against nontarget serovars (204, 206, 210, 216). Vaccinations are primarily used within breeder flocks due to the importance of limiting colonization earlier in production and conferred resistance to progeny but in the case of excessive *Salmonella* contamination at processing, they may be utilized as an additional control in broiler flocks (217, 218). Only live attenuated vaccines are used in broiler flocks since they may be administered without excessive stress and handling (both which may lead to high mortality) by spraying the flocks or adding to the drinking water.

The use of this control strategy should be increasing as USDA-FSIS updated their regulatory policy to discount isolation of vaccine strains from the *Salmonella* performance categorization of processing establishments (219, 220).

Since *Salmonella* is an enteric pathogen, the addition of pre- and probiotics can support a healthy gut microbiota and therefore limit colonization through competitive exclusion (173, 201, 221–224). The gut microbiome is one of the primary defenses against *Salmonella* colonization in chickens, as the presence of other bacteria limits the resources available to salmonellae (225–229). This occurrence is especially important for *Salmonella* control in hatcheries, as the parental fecal microbiota influences the establishment of the eggshell microbiota for progeny (230, 231). In broiler production, poultry litter is reused between flocks to promote the development of a stable gut microbiome and protect against poultry pathogens, although this also contributes to continued *Salmonella* colonization of flocks (127, 155, 157, 232–236). To control *Salmonella* in the broiler house environment, litter amendments and heat treatments, water acidification, and phage cocktails are applied (178–183, 192, 234, 236–243). All of these *Salmonella* control strategies are costly for integrators to implement and have varying levels of success. Conversely, increased on-farm biosecurity may be achieved at much lower cost while still proving effective in limiting disease transmission.

Best biosecurity practices include, but are not limited to, not sharing equipment between farms or cleaning before use, disinfecting vehicles (e.g., wheels and wheel wells) before entering the property, providing disposable boot covers and sanitizing footbaths prior to entering a house, controlling rodent and insect populations, limiting moisture levels in the house and litter, removing dead chickens from the shared

environment, and ensuring that the houses are structurally intact to prevent any interactions with wildlife (100, 101, 127, 244–250). There are numerous other biosecurity components to consider, ranging from the condition of the farm environment (presence of surface water, overgrown foliage, etc.) to the house infrastructure (ventilation fans, water quality, feed delivery, etc.) and logistics (supply deliveries, egg collection, etc.). The presence of multiple houses on one production farm increases the importance of biosecurity, as any initial introduction event to one house will likely lead to colonization of the other flocks as the contamination is spread between houses. One study found that samples collected from multiple houses on one farm were almost as highly correlated as samples collected within the same house (245). Anecdotally, domestic breeder farms have fewer houses than broiler farms on average, which may further support lower levels of *Salmonella* at this stage. In addition to *Salmonella* transmission via human activity, rodents and insects may serve as disease vectors (136, 138–140, 142–144, 191, 251–256). Interestingly, serovars Enteritidis and Typhimurium were originally used as rodenticides to control rodent populations, although this practice ended following repeated *Salmonella* transmission to humans (257–259). As such, controlling rodent and insect populations is an important component of on-farm biosecurity. Ultimately, combining multiple aspects of pre-harvest control is necessary to effectively reduce the bacterial load entering the processing plant to ensure the antimicrobial interventions are able to minimize contamination of final products (90, 97, 99, 127, 217, 260).

Methods for *Salmonella* isolation and detection, quantification, and serotyping

Conventional culture-based workflow

Culture-based *Salmonella* surveillance involves a multistep process to recover *Salmonella* isolates that are then serotyped, with an average turnaround time of five days from sample collection to an isolate. One key component of this process includes a selective enrichment step that is performed in broth with various reagents to limit the growth of background organisms while still allowing salmonellae to grow. This is required as the relative levels of *Salmonella* in most matrices are quite low. Currently, two of the most commonly used selective enrichment broths are Rappaport-Vassiliadis (RV) and tetrathionate (TT), and they are often applied in parallel to increase *Salmonella* recovery as they have different modes of selection (261–263). RV broth includes magnesium chloride and malachite green to collectively decrease pH, increase osmolarity, and inhibit general coliforms (264). As indicated by its name, TT broth is dependent on the production of tetrathionate to permit *Salmonella* growth, along with bile salts to suppress gram-positive bacteria and coliforms (265). Salmonellae can use tetrathionate as a terminal electron acceptor for anaerobic respiration, which confers a fitness advantage over competing bacteria (266, 267). RV broth is more sensitive and specific than TT broth, which, in food, corresponds to increased *Salmonella* recovery from samples with a high and low microbial load in RV and TT media, respectively (268, 269).

Following selective enrichment, the cultures are struck onto differential and selective agar to determine if *Salmonella* is present, and presumptive colonies can then be confirmed and characterized with biochemical and molecular assays. There are many media available for *Salmonella* isolation with different compounds added for distinguishment, including brilliant green sulfa (BGS), double modified lysine iron

(DMLIA), Hektoen enteric (HE), bismuth sulfite (BS), xylose lysine deoxycholate (XLD), xylose lysine tergitol-4 (XLT-4), and a suite of chromogenic agars. Some agars may rely on the same indicator mechanism with unique selection, such as XLD and XLT-4 which both indicate *Salmonella* presence through hydrogen sulfide production and resulting black colonies, but background microflora is inhibited by a bile acid and a surfactant, respectively (270). Additionally, some formulations are separated by the inclusion of an antibiotic, such as novobiocin in brilliant green (BGN) agar to limit growth of gram-positive bacteria. Indicator agars relying on general biochemical reactions may have a greater rate of false positives due to metabolic similarities between closely related bacteria and subsequent shared colony morphologies; this may be overcome by the use of chromogenic agar as coloration is driven by chromogen hydrolysis following targeted bacterial uptake (271). Alternatively, biochemical-based agars may also have increased false negatives when isolating strains with atypical colony formation, such as an H_2S-negative salmonellae on XLT-4 (272). Therefore, multiple selective indicator agars with different mechanisms of action should be incorporated into any routine *Salmonella* workflows to increase sensitivity and specificity. As an alternative to biochemical tests, semi-solid agar relies on bacterial motility to indicate presence or absence (273). For example, modified semi-solid Rappaport-Vassiliadis (MSRV) contains the same base ingredients as RV broth, but the selectivity added by the motility test can eliminate the need for prior selective enrichment while improving *Salmonella* recovery (274). Alternatively, nonmotile serovars or variants would not be identified with MSRV, and these false negatives can have important public health

implications, as observed in a historical French outbreak of a nonmotile serovar Typhimurium variant (275).

While the fundamental workflow for *Salmonella* culturing is maintained across laboratories, such that the original sample is selectively enriched and plated onto differential agar, the actual media and methods used may vary widely based on the sample matrix and geographic location. Importantly, non-selective pre-enrichment may be necessary for some sample types, such as carcass rinses from the processing plant or antimicrobial-treated feed, to allow for the recovery of injured salmonellae (276–280). Alternatively, matrices with high bacterial load, such as environmental samples, may be better served by starting with selective enrichment to inhibit the growth of nontarget organisms. As such, each of the regulatory agencies have developed protocols for the isolation and identification of *Salmonella* from a range of sample types, depending on which products the agency is responsible for (Fig. 1-4).

The USDA-FSIS protocol is designed for *Salmonella* recovery from post-harvest, post-intervention samples, including chicken carcass and parts rinses, so it begins by adding neutralizing buffered peptone water (nBPW) to the sample to mitigate the bactericidal effects of applied antimicrobials and reduce the risk of false negatives (281, 282). Since the USDA-FSIS laboratories screen thousands of surveillance samples annually, it is necessary to have a streamlined workflow and a rapid turnaround, so they have implemented the use of molecular screening to determine *Salmonella* status prior to conventional culturing steps. Any *Salmonella*-positive samples are then selectively enriched with RV and TT Hajna, a variation on the standard TT formulation to increase selectivity, followed by plating on two indicator agars (BGS, DMLIA) and selecting one

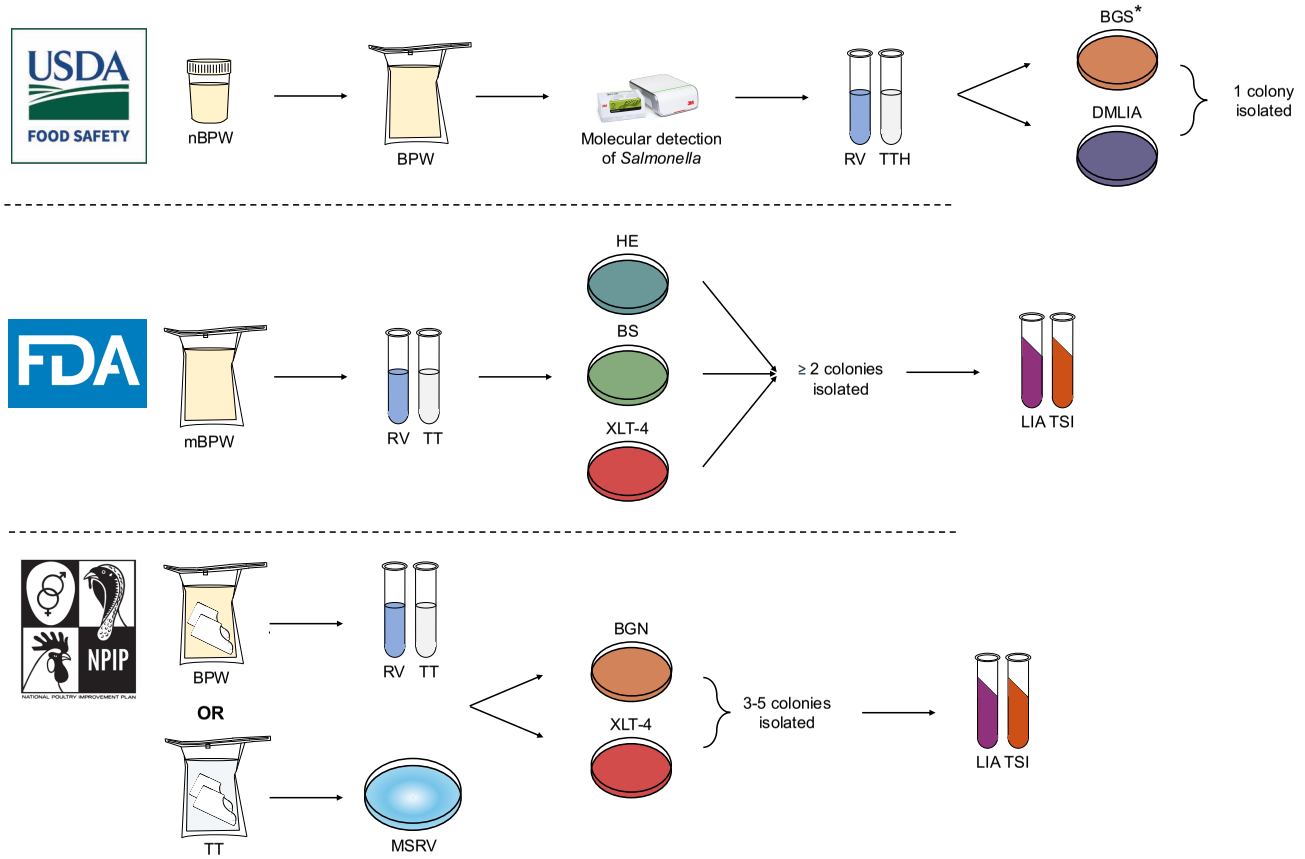


Figure 1-4. Workflow for *Salmonella* isolation varies by organization and sample type. Culturing procedures followed by the USDA-FSIS, FDA, and NPIP for post-harvest poultry products, general environmental samples, and poultry house environmental samples, respectively (282; 284; 171). All workflows result in confirmation of presumptive *Salmonella* colonies with biochemical or molecular tests (not pictured for the USDA-FSIS protocol). The FDA and NPIP protocols have an optional rapid screening step prior to enrichment. *The USDA-FSIS protocol was edited in August 2024 to replace BGS with XLD plates (283). nBPW: neutralizing buffered peptone water; BPW: buffered peptone water; RV: Rappaport-Vassiliadis broth; TT: tetrathionate broth; TTH: tetrathionate broth, Hajna formulation; BGS: brilliant green sulfa agar; DMLIA: double modified lysine iron agar; LIA: lysine iron agar slant; TSI: triple sugar iron slant; HE: Hektoen enteric agar; BS: bismuth sulfite agar; XLT-4: xylose lysine tergitol-4 agar; MSRV: modified semi-solid Rappaport-Vassiliadis agar.

presumptive colony for characterization. As of August 2024, the USDA-FSIS laboratory guidebook has been updated to remove the use of BGS and supplement with XLD plates but most samples in the current regulatory dataset were processed with the previous protocol (283). The US-FDA has an extensive manual for *Salmonella* isolation, due to their need to isolate *Salmonella* from a wide variety of matrices, including produce, egg products, spices, and other environmental samples (284). For environmental samples, the US-FDA protocol is similar to that of the USDA-FSIS except multiple presumptive colonies are picked from each of three agar plates (HE, BS, XLT-4), followed by biochemical testing for confirmation. The NPIP presents two workflows for *Salmonella* recovery from environmental poultry samples (e.g., boot socks), one including pre-enrichment (BPW) while the other starts from selective enrichment (TT) (171). Skipping the pre-enrichment step is only applicable in matrices with high microflora since the focus needs to be on reducing background bacteria to allow for *Salmonella* growth. The NPIP method prescribes successive selective enrichment, as the TT is sub-cultured onto MSRV plates, which are then used to inoculate selective and differential agar (BGN, XLT-4) following growth. For screening organs and tissues, the NPIP program recommends the use of delayed secondary enrichment (DSE) to confirm true negatives. DSE involves leaving an incubated selective enrichment culture at room temperature for five days, followed by subculture into fresh enrichment media; this has been shown to greatly improve *Salmonella* recovery, particularly as it allows for the detection of slower growing serovars (285). Both the US-FDA and NPIP protocols contain the optional step of utilizing a molecular assay to screen for *Salmonella* prior to culturing, as the USDA-FSIS does, and result in confirmation with differential agar slants.

Incubation of enrichment cultures may vary slightly by time and temperature, but it is usually in the range of 35 – 42°C and 24 – 48 hours for *Salmonella* growth. These variables may have negative or positive interactions depending on the state of the cells prior to enrichment and the presence of competing microflora (286, 287). For example, a higher temperature may inhibit nontarget organisms, but *Salmonella* growth is not concurrently increased due to the heat stress caused to previously injured cells. Alternatively, extending the time of enrichment has been suggested to increase *Salmonella* recovery but this may also allow for continued growth of other bacteria (285, 288). Any deviation outside of the optimal range may have unexpected effects, which underscores the importance of considering all the abiotic and biotic factors associated with *Salmonella* enrichment (261, 289, 290).

The composition of enrichment media is one of the most influential factors on *Salmonella* recovery as previous work has demonstrated that some serovars grow better in certain enrichment broths, which then impacts the applicability of surveillance testing (291, 292). This phenomenon is often referred to as “media bias” or “enrichment bias”, and it was discovered by comparing colony compositions on indicator plates inoculated with RV and TT culture, along with molecular population analyses (276, 277, 292–299). As such, it is important to integrate complementary methods to minimize the impact upon recovery and characterization of *Salmonella* populations; this may be achieved by comparing the mode of selectivity and optimizing culture conditions based on sample matrix (300–302). For example, it has been observed that serovar Enteritidis grows more readily in TT broth compared to RV broth, so surveillance samples collected from a commercial hatchery should utilize TT medium to prevent false negatives (294). More

work is required to define the mechanism(s) behind media bias to then better understand the assumptions of *Salmonella* enrichment. Further, identifying the determinants behind media bias can enable analyses of publicly available whole genome sequences to extrapolate results to other serovars and develop a robust enrichment protocol to account for bias. However, in the meantime, industries should institute standardized protocols to reduce the random variation introduced to risk analysis models, as current datasets may have skewed counts of serovar abundance and diversity due to the different methods used for *Salmonella* isolation (303).

The standard culture-based methods for *Salmonella* isolation typically only identify one serovar; this limitation is due in part to the similar colony morphology most serovars display, but largely due to time and resource constraints associated with characterizing multiple colonies per sample. For a 95% probability of identifying two serovars, which must exist in equal proportions, six colonies would have to be selected and characterized (304). It is not feasible to select that many colonies as part of a longitudinal surveillance study or regulatory program involving hundreds (or thousands) of samples. Additionally, most multiserovar populations contain serovars of varying relative abundances, which then changes the probability of isolation (277). Considering the range of human pathogenicity associated with different serovars, it is crucial to identify all serovars present in a *Salmonella* population to assess the public health risk. Further, salmonellae may interact directly or indirectly with a variety of other microorganisms in the environment and animals, so they are under selective pressure to not only outcompete other serovars but other genera as well. These mixed communities can serve as an opportunity for the spread of mobile genetic elements, and given overall

Salmonella sequence similarity, may also promote recombination. Transfer of genetic material can significantly impact *Salmonella* serovar evolution as phenotypes and genotypes fluctuate, which was demonstrated by the two recent outbreaks attributed to an emergent clade of serovar Reading in raw turkey products (305). Retrospective genomic investigation suggested that the emergence of the outbreak strains may be partly attributed to plasmid-mediated antimicrobial resistance acquisitions, integration of phage-like sequences encoding virulence factors, and mutations affecting a siderophore receptor that also served as a colicin receptor and beta-glucuronidase activity. Collectively, these genomic changes support the two-part hypothesis pertaining to the spread of outbreak strains following introduction to the domestic turkey industry. Firstly, resistance to colicin, an antibiotic peptide, allowed serovar Reading to survive in mixed populations including other serovars and *E. coli*. Secondly, the addition of virulence factors through plasmids and phage-like sequences promoted the persistence of strains in production and subsequent host colonization. This phenomenon underscores the importance of robust *Salmonella* surveillance programs with reliable methods of detection to monitor the rise of pathogenic serovars.

Culture-independent diagnostic tests (CIDTs) are becoming widely used in clinical settings due to the rapid turnaround time and ease of use, but these are not currently a suitable, independent alternative to conventional culturing methods for two primary reasons (306). First, most CIDTs on the market rely on polymerase chain reactions (PCR), so an overabundance of background bacteria, along with biochemical compounds, in a sample may inhibit target amplification (307, 308). However, there are mechanisms to alleviate PCR inhibition, such as additional DNA extraction steps,

inactivation of proteases, or addition of bovine serum albumin to the PCR master mix (309, 310). Additionally, some CIDT assays are highly sensitive and specific, which can allow for detection of *Salmonella* at low quantities or in mixed population, and there are products, such as antibody-integrated magnetic beads, filters, and bacteriophage-based biosorbents, that can capture *Salmonella* cells from a mixed sample (311–317). Second, the integration of these methods in existing commercial kits may overcome detection limitations but CIDTs are not sufficient for advanced surveillance systems since they do not result in an isolated colony for further characterization (318). This may be overcome as sequencing technologies continue to improve but this is currently an important restriction since epidemiological traceback investigations rely on strain WGS comparisons. For example, the 14% (1253/8825) of *Salmonella*-positive clinical samples reported by the CDC in 2022 would not have been linked to outbreaks, possibly underestimating the correspond magnitudes (319). Furthermore, reflex cultures from CIDT-positive samples have lower *Salmonella* recovery with higher costs when compared to conventional culture methods (320). Importantly, CIDTs may result in false positives due to the presence of dead cells in the sample, which would usually be excluded during the culturing process (321–323). On the other hand, this occurrence is also what enables PCR detection of viable but nonculturable cells, which refers to the survival state of bacteria when they are metabolically or physiologically active but cannot be grown in culture without prior recovery (324). As such, the viability of cells plays an important role in method selection and consideration should be given to approaches that can differentiate between live and dead cells (325, 326). Other molecular methods that are dependent on culture-based enrichment may provide better resolution for *Salmonella*

monitoring, including analysis of whole genome sequences, with some approaches having potential tradeoffs of enrichment bias, higher costs, or longer turnaround time compared to CIDTs.

Molecular-based approaches

Among molecular assays, real-time and endpoint PCR methods are most commonly employed for rapid *Salmonella* screening in food production due to the high sensitivity and specificity, reliability, and accessibility in comparison to culture-based methods (327–343). PCR has been utilized for *Salmonella* detection for 30 years, with significant improvements in specificity and sensitivity over the years as new genomic targets have been identified (344–347). For example, the introduction of internal amplification controls to real-time PCR assays reduced the incidence of false negatives, in which *Salmonella* was present but not detected due to assay failure (348, 349); limiting false negatives is critical to promote public health and prevent distribution of contaminated products (350). Additionally, it is now possible to screen for multiple gene targets within a single reaction, which simplifies testing for laboratories focused on multiple pathogens and increases confidence in detection of a single organism with multiple targets (351–358).

Recent technological advances have led to the development of PCR-based platforms for *Salmonella* detection and quantification based on amplification of genetic targets conserved among all subsp. *enterica* serovars, such as the tetrathionate reductase cluster (*ttr*) and genes encoding invasion proteins (*invA*) or transcriptional regulators (*hilA*) (334, 359–361). These commercial systems streamline *Salmonella* testing by reducing the time required for sample processing and analysis; consumables are provided

for sample lysis and PCR amplification, including a lyophilized PCR master mix and internal amplification control, and it is possible to screen 96 samples simultaneously on a preprogrammed instrument with visualization software (362). For *Salmonella* detection, each sample is reported as positive, negative, or undetermined, while quantification is based on a cycle threshold (Ct) value, which indicates how many cycles were required for the fluorescence signal from target DNA amplification to exceed a predefined threshold. The resulting Ct value can be transformed to a unit that is more easily interpreted, such as logarithmic colony-forming units per milliliter (log CFU/ml) or gram (log CFU/g), if the company provides a formula based on the predefined calibration curves for each sample matrix (363, 364).

Molecular enumeration approaches serve as an alternative to the most probable number (MPN) culture-based enumeration method, which is a time- and resource-intensive process (365); however, there are still limitations associated with the molecular assays (366). Primarily, these assays can only report results for sample types that have been validated by the companies, which includes the most used matrices for the respective industry, but this hinders the innovation of these systems since new sample types cannot easily be analyzed. Additionally, PCR efficiency may differ between the isolates used to develop and standardize the assay and the experimental isolates, leading to quantification errors (367). Importantly, some studies have demonstrated that molecular enumeration results are not replicable, and the rate of false positives ranges across manufacturers (366); this complicates the integration of these systems into routine *Salmonella* surveillance. The combination of culture- and molecular-based approaches for *Salmonella* detection can help overcome some of the associated limitations, including

the combination of MPN and qPCR tests for quantification and the repurposing of commercial PCR systems for detection of *Salmonella* above a threshold and measurement of time-to-positivity based on growth rates (366, 368–370). There are many detection methods available, including additional approaches based on molecular, immunological, and biochemical principles, all with their respective pros and cons, so it is important to consider the sample matrix, desired measurements, and laboratory constraints in method selection (371).

Salmonella serotyping and characterization

Following isolation and confirmation of a presumptive *Salmonella* colony, additional culture- and molecular-based methods may be applied to identify the present serovar. As a first pass of characterization, sera containing antibodies against the O antigens can be used in agglutination testing to determine the serogroup of an isolate (372). Similar testing may be done to recognize the H antigens, but this is more time- and labor-intensive as the cells must be captured in both antigenic phases and there are more than twice as many H antigens to O antigens. As such, typically only the O antigen is resolved with serum agglutination, and there has been an increase in use of molecular assays for serovar typing (serotyping) (373). There are several PCR targets for serotyping, including but not limited to the O antigen gene cluster (*rfb*), H antigen phase variation (*fliC*, *fljB*), intergenic spacer regions, and serovar-specific markers (374–378). Prior to the routine implementation of WGS, multilocus sequence typing (MLST) was utilized for serotyping as an improvement upon the resolution provided by PFGE (379). MLST is determined by comparing the nucleotide sequences of seven conserved housekeeping genes in *Salmonella*, such that the combination of these genes denotes a

sequence type (ST) which can be used to assess relatedness of isolates along with serovar identification.

The development of WGS has enabled increased resolution of MLST as the core (cgMLST) and whole genomes (wgMLST) can now be compared to provide greater separation between isolates based on the additional targets (380). There has been an explosion of WGS-based methods for serotyping and characterization, including single nucleotide polymorphism (SNP) analyses, identification of antimicrobial resistance and other virulence factors, phage and plasmid detection, source attribution, and exploration of evolutionary relationships (51, 381–398). While the continued advancement of next-generation sequencing technology expands accessibility to WGS, this and the aforementioned approaches for *Salmonella* characterization are limited by the isolation of a single colony for DNA extraction and downstream analyses. As an alternative, metagenomic sequencing provides insight into the microbial community composition surrounding salmonellae but there are still associated limitations (399–404). Classical metagenomics are based on unenriched bacterial populations, which, given the balance of sequencing depth and affordability, restricts identification to the genus or species level. Continued exploration of quasi-metagenomics (sequencing from enrichments) may provide the high resolution required for epidemiological investigations (405, 406).

CRISPR-SeroSeq

Salmonellae possess two arrays containing clustered regularly interspaced short palindromic repeats (CRISPRs), consisting of invariant direct repeats (29 nucleotides) and variable spacer sequences (32 nucleotides)(407). CRISPR systems serve as a prokaryotic adaptive immune response, such that spacer sequences were added into the

arrays from foreign nucleic acids (408–417). The CRISPR-associated (Cas) proteins are responsible for the generation and maintenance of spacer sequences, and in turn, the transcribed spacers guide the Cas machinery to cleave the complementary exogenous DNA introduced by invading mobile genetic elements. Importantly, while there may be redundancy of some spacer sequences due to shared environments and exposure to bacteriophages, the spacer composition in both CRISPR loci can be defined and combined to create unique profiles for different serovars (Fig. 1-5A)(418). The canonical CRISPR-based immunity is no longer active in *Salmonella:* the CRISPR arrays are still intact in *Salmonella* genomes, but there is no evidence of recent evolution(408). However, they have been utilized as a reliable genomic target for differentiating between serovars and determining relatedness of isolates in diagnostic assays as a CRISPR typing scheme (49, 419–428). Additionally, since *Salmonella* spacers are mostly organized in a chronological order, based on exposure to foreign DNA, phylogenetic analysis of the CRISPR arrays in *Salmonella* can provide insights into the evolution of serovars (46, 429, 430).

A deep serotyping method, CRISPR-SeroSeq, relies on the conservation of CRISPR arrays in *Salmonella* to identify and quantify the relative frequencies of all serovars present in a mixed bacterial population (Fig. 1-5B)(431). Importantly, CRISPR-SeroSeq is not limited to single, isolated *Salmonella* colonies and usually begins with an overnight enrichment instead, from which total genomic DNA is extracted. The resulting high-resolution population analyses have the potential to support the development of an early warning system for rising *Salmonella* serovars of concern since these would be identified earlier than with culture-based methods (Fig. 1-6). Due to the sequence

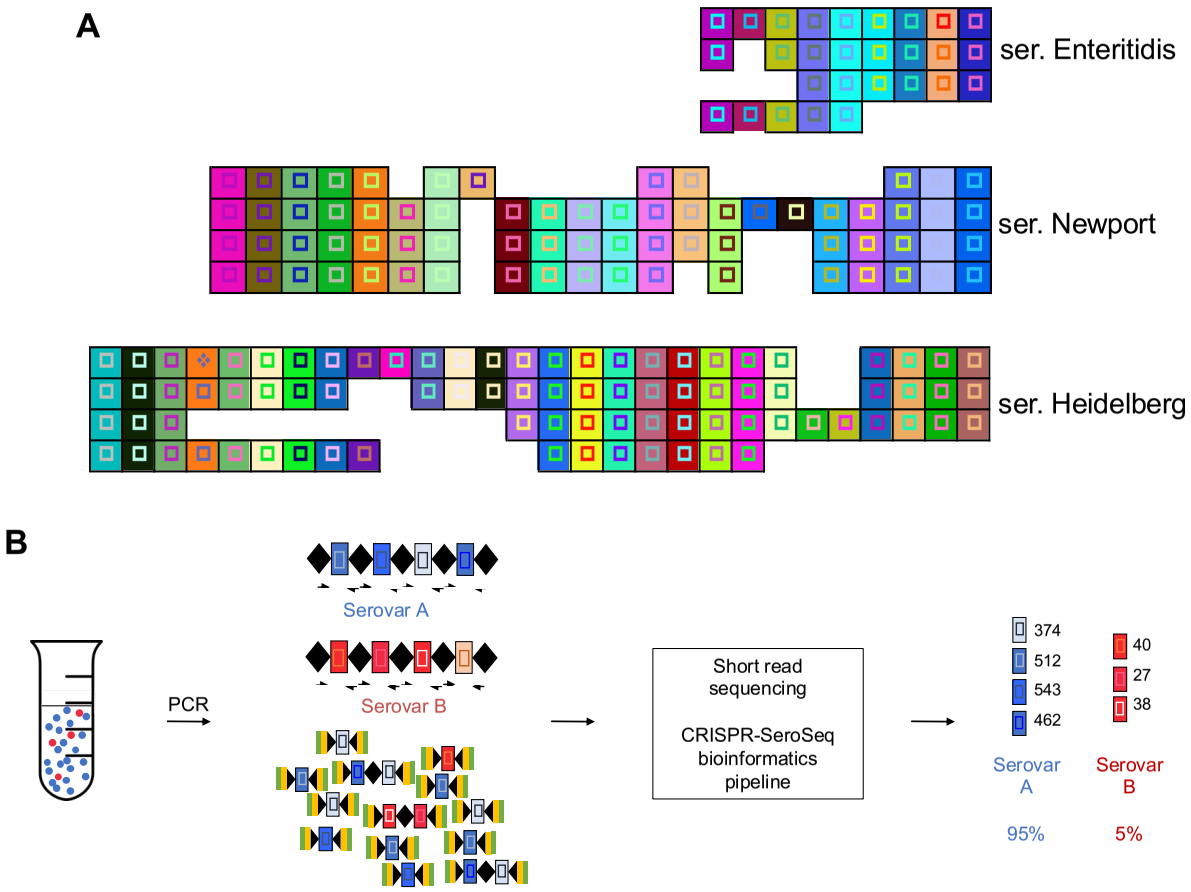


Figure 1-5. The application of *Salmonella* CRISPR arrays for deep serotyping. A) Graphical representation of spacers within one CRISPR array for serovars Enteritidis, Newport, and Heidelberg. Each box with a unique combination of colors and symbols represents one unique spacer sequence. Each row represents one isolate, with spacer deletions delineating strains. The direct repeats are not shown. Adapted from Shariat et al., 2015. B) Genomic DNA is isolated from a mixed population of *Salmonella* serovars grown in enrichment media. Direct repeat sequences (black diamonds) are conserved throughout *Salmonella* and serve as a target for primers (black arrows). Dual indexed barcodes (yellow) and Illumina adaptors (green) are added to allow sample multiplexing for next-generation sequencing. The pooled sample PCR products are sequenced, then parsed through our CRISPR-SeroSeq program. Adapted from Thompson et al., 2018.

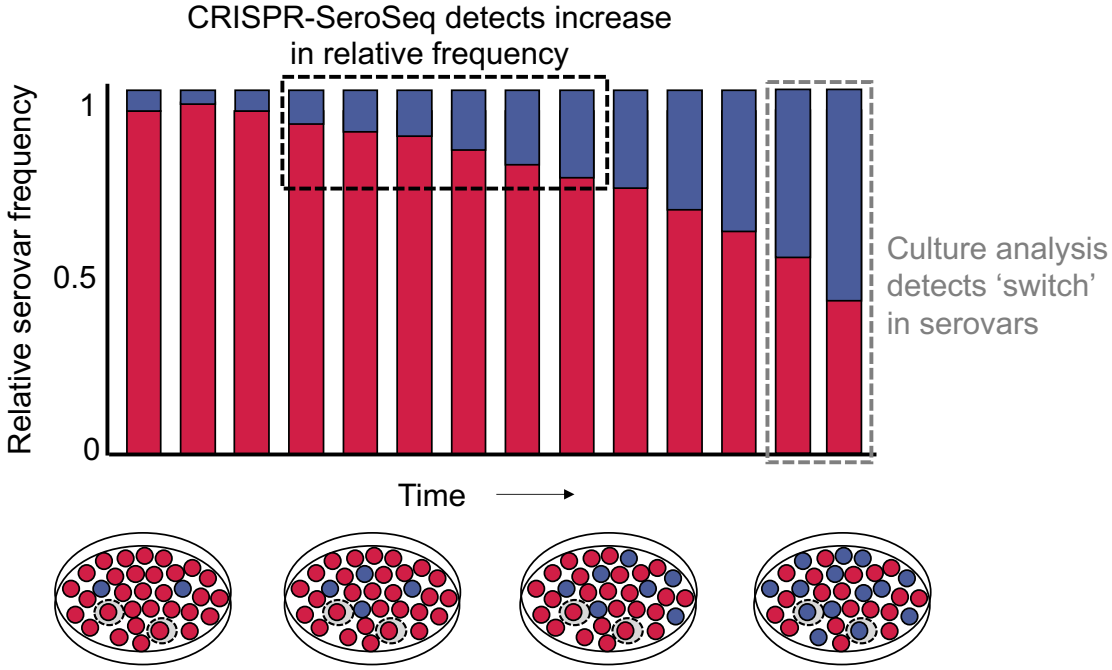


Figure 1-6. High-resolution *Salmonella* surveillance can enable early detection of serovar shifts. Graphical representation of multiserovar population with two serovars (red and blue) to visualize proportional changes over time, as measured by CRISPR-SeroSeq (bar graph) and conventional plating methods. The red serovar is decreasing in abundance, corresponding with an increase of the blue serovar. Culture-based approaches are often limited by the number of colonies picked for further characterization, which could delay response until the two serovars are almost equally represented. With CRISPR-SeroSeq, the blue serovar would be detected earlier, thus allowing more time for enacting controls to avoid an outbreak.

similarity of direct repeats and widespread presence of CRISPRs in bacteria, present in 40% of species, it is necessary to use selective enrichment media to promote *Salmonella* growth while limiting others to reduce nontarget DNA and possible inhibition (277, 432). CRISPR-SeroSeq is similar to 16S amplicon sequencing, with PCR amplification of CRISPR spacers rather than gene regions. Because the spacer content is related to the serovar identity, CRISPR-SeroSeq is more high-resolution as the spacers can differentiate between serovars of the same subspecies rather than operational taxonomic units (OTUs) (433). The PCR primers for CRISPR-SeroSeq are complementary to the conserved direct repeats to then amplify the spacer sequences. Therefore, the assay does not amplify the whole array. This was designed to overcome PCR bias as array length differs among serovars and among isolates of the same serovar (431). Illumina adapters and dual-indexed barcodes are included in the primer sequences to facilitate multiplexed sequencing. To account for media bias, deep serotyping results can be normalized across all enrichments sequenced for one positive sample, such that the final relative frequencies reflect population dynamics across different methods.

The CRISPR-SeroSeq data analysis pipeline includes a series of shell and R (434) scripts to calculate the relative *Salmonella* serovar frequency within each sample population. The first step is to convert the raw reads from the sequencing facility to a FASTA formatted file, using seqtk (435), such that a local alignment search (BLAST, (436) search may be completed with the experimental sequences as the query and a curated database of *Salmonella* spacer sequences as the subject. To start, the CRISPR-SeroSeq database was created with whole genome sequences from lab datasets and GenomeTrakr (437) accessed from NCBI Pathogen Detection and assembled using

SPAdes (438). CRISPR spacers were extracted with CRISPRFinder (439), then added to the database. To account for spacer duplication or deletion events between strains of the same serovar, CRISPR arrays from at least 10 different isolates, representing various regions, sources, and years, were used to generate a comprehensive spacer list for each serovar. The database includes serovars that are commonly associated with human illness or found in food animals and the environment, with new serovars continually being added. Following the increase in accessibility of WGS, and the transition to this as a standard practice in food safety regulation, there are now over 700,000 *Salmonella* isolates accessible on NCBI Pathogen Detection. While it is possible for users to manually annotate serotyping information for their isolate, this is not always accurate, so SeqSero2 (390) is now used to predict "computed serotypes" based off serovar-specific markers and thus improves confidence in any downstream uses of the publicly available genomes.

Deep serotyping with CRISPR-SeroSeq has been applied to a variety of matrices, including post-intervention chicken carcasses and parts, surface water, on-farm environmental samples, produce, animal feed, and wild bird feces (82, 83, 168, 277, 296, 431, 440–444). Collectively, these previous studies revealed that *Salmonella* often exists in multiserovar populations, with a range in serovar diversity across sample types. Notably, the proportion of multiserovar populations found in samples increases from breeders to broilers, with a significant reduction following antimicrobial interventions (Fig. 1-7). Additionally, members of multiserovar populations differ between sample source, which serves as an interesting observation to investigate host-associated serovars. Namely, the high-resolution population analyses provided by CRISPR-SeroSeq can help

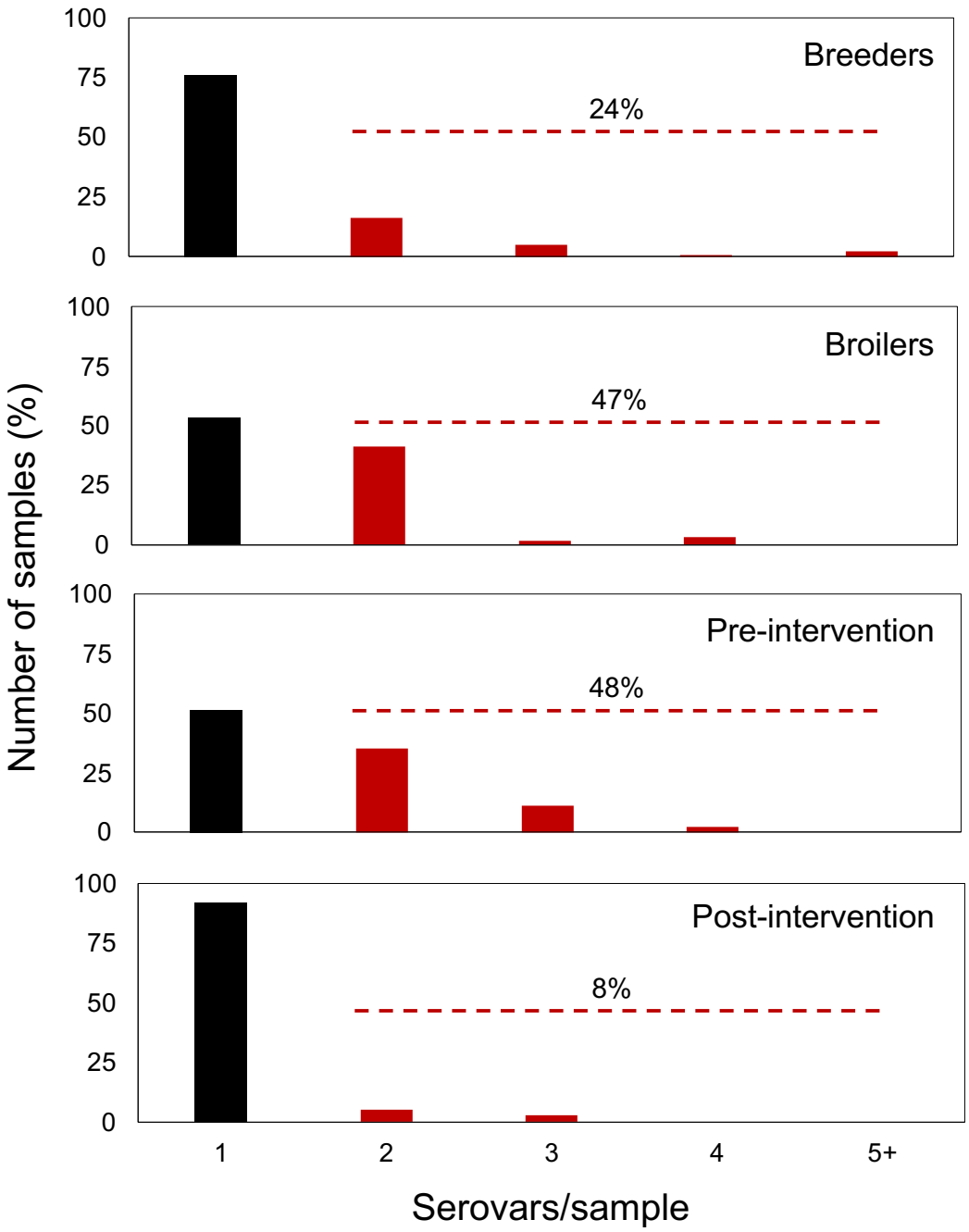


Figure 1-7. Distribution of serovars/sample varies by stage of poultry production. CRISPR-SeroSeq was used to analyze multiserovar populations (red bars) found in environmental samples collected from commercial breeder and broiler flocks and raw poultry products before and after antimicrobial interventions. The proportion of total samples containing multiserovar populations is listed above the dotted line.

reveal patterns of serovar co-occurrence and co-exclusion. It has been demonstrated that interserovar competition occurs, such that some serovars may exclude others from colonization or growth, but there may also be undefined serovar mutualism as is observed between some bacterial species (445, 446). Post-intervention, post-harvest poultry samples have the lowest complexity, while surface water samples have the greatest; this observation highlights the impact of environment and contributing sources upon *Salmonella* population dynamics. Further, multiserovar populations may be limited by carrying capacity and selective pressure, such that a chicken cannot contain as much *Salmonella* as an environmental reservoir can and different stresses are associated with both. In particular, the studies on *Salmonella* in poultry production formed the foundation for my doctoral research as the initial surveillance results provided an overview of the serovars found in broiler production. However, the incidence of *Salmonella* in broiler breeder production remained an important question mark, since it had been suggested that these flocks contribute significantly to *Salmonella* transmission in broilers and contamination on final products (146). To better understand how to control *Salmonella* in poultry, it is necessary to explore the population dynamics present at the earlier pre-harvest stages to determine if serovar presence is stable through the duration of a flock and to identify factors that could influence this stability. Additionally, the application of CRISPR-SeroSeq to breeder samples would provide insight on best management practices since the resulting serovar diversity could be compared to that samples collected from broiler farms with different management strategies.

Research objectives

Salmonella is a leading bacterial cause of foodborne illness in the United States (447), with a fifth of salmonellosis cases attributed to poultry (69). *Salmonella enterica* is comprised of over 2,600 serovars, as characterized by their lipopolysaccharide (O) and flagellar (H) antigens (6). More than half of the identified serovars are within subsp. *enterica* and collectively account for over 99% of isolated strains (6). The different serovars display various phenotypes, including host restriction (448), host adaptation (22), modes of pathogenesis (21, 32), increased development of multidrug resistance (449), and propensity to cause disease (450). Previous work in our lab has demonstrated that *Salmonella* often exists in multiserovar populations and that conventional culture approaches are limited in their identification of multiple serovars within one sample. To further complicate the issue, serovars are not always of equal abundance within a population which can lead to some, potentially more pathogenic, serovars remaining undetected. As such, to minimize the public health risk for America's most consumed protein (https://www.nationalchickencouncil.org/about-the-industry/statistics/per-capita-consumption-of-poultry-and-livestock-1965-to-estimated-2012-in-pounds/), it is necessary to not only identify when *Salmonella* is present in broiler breeder production but to differentiate between serovar diversity as well.

My doctoral research aimed to combine microbiology, molecular biology, and computational biology approaches to observe multiserovar population dynamics, characterize shifts in serovar prevalence, and highlight the importance of *Salmonella* controls in the chicken industry. In collaboration with the Georgia Poultry Laboratory Network (GPLN), I analyzed *Salmonella*-positive surveillance samples from southeastern

broiler breeder farms to measure the frequency of multiserovar populations, as well as to identify the most prevalent serovars in production and observe patterns of interserovar interactions. Additionally, I completed longitudinal surveillance sampling of pullet and breeder flocks in two commercial broiler breeder complexes to record any fluctuations in *Salmonella* population dynamics throughout production. On-farm rodent samples were also collected as part of the longitudinal study to measure transmission between rodent populations and breeder flocks based on shared *Salmonella* serovars. A subset of the longitudinal breeder flock samples collected during peak production (weeks 29-31) were used to compare *Salmonella* recovery and diversity between conventional and modified enrichment conditions. CRISPR-SeroSeq was employed for deep serotyping on all positive samples to provide both quantitative and qualitative results, including serovar identities and relative abundances within multiserovar populations, as this method promotes a reliable, high-resolution *Salmonella* surveillance system. Collectively, these data generated from both studies can enable the identification of competitive factors which impact *Salmonella* diversity in multiserovar populations as it revealed co-occurrence and co-exclusion patterns. Ultimately, understanding the ecology of multiserovar *Salmonella* populations will inform management decisions, as they can be tailored to the serovars present, as well as to the conditions of the flock (i.e., age of chickens, number of houses on farm, extent of pest control). The framework presented here can be extrapolated to other food animal production systems, as they face similar challenges with controlling *Salmonella* populations to protect consumers, and to other foodborne pathogens as well.

To harness *Salmonella* as a model organism to characterize interserovar competitive dynamics, survey the serovar diversity in broiler breeder flocks, and support improved surveillance, I employed two specific aims:

1. Assess *Salmonella* dynamics in commercial poultry production by identifying patterns of serovar co-occurrence
2. Explore the impact of control strategies and isolation methods on serovar diversity and population complexity

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CHAPTER 2

REGIONAL *SALMONELLA* DIFFERENCES IN UNITED STATES BROILER PRODUCTION FROM 2016 TO 2020 AND THE CONTRIBUTION OF MULTISEROVAR POPULATIONS TO *SALMONELLA* SURVEILLANCE[1]

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Abstract

Poultry remains a considerable source of foodborne salmonellosis despite significant reduction of *Salmonella* incidence during processing. There are multiple entry points for *Salmonella* during production that can lead to contamination during slaughter, and it is important to distinguish the serovars present between the different stages to enact appropriate controls. National *Salmonella* data from the United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) monitoring of poultry processing was analyzed from 2016-2020. The overall *Salmonella* incidence at processing in broiler carcasses and intact parts (parts) decreased from 8.9% to 6.3% over this period. The incidence in parts was higher (11.1%) than in carcasses (4.5%). Regional differences include higher proportions of serovars Infantis and Typhimurium in the Atlantic and higher proportion of serovar Schwarzengrund in the Southeast. For Georgia, the largest broiler producing state, USDA-FSIS data was compared to *Salmonella* monitoring data from breeder flocks over the same period, revealing serovar Kentucky as the major serovar in breeders (67.9%) during production, but not at processing, suggesting that it is more effectively removed during antimicrobial interventions. CRISPR-SeroSeq was performed on breeder samples collected between 2020-2021 to explain the incongruence between pre- and post-harvest and showed that 32% of samples contain multiple serovars, with up to 11 serovars found in a single flock. High-resolution sequencing identifies serovar patterns at the population level and can provide insight to develop targeted controls. The work presented may apply to other food production systems where *Salmonella* is a concern, as it overcomes limitations associated with conventional culture.

Importance

Salmonella is a leading cause of bacterial foodborne illness in the United States, with poultry as a significant *Salmonella* reservoir. We show the relative decrease in *Salmonella* over a five-year period from 2016-2020 in processed chicken parts and highlight regional differences with respect to prevalence of clinically important *Salmonella* serovars. Our results show that the discrepancy between *Salmonella* serovars found in pre- and post-harvest poultry during surveillance are due in part by the limited detection depth offered by traditional culture techniques. Despite the reduction of *Salmonella* at processing, the number of human salmonellosis cases has remained stable, which may be attributed to differences in virulence among serovars and their associated risk. When monitoring for *Salmonella*, it is imperative to identify all serovars present to appropriately assess public health risk and to implement the most effective *Salmonella* controls.

Introduction

Despite efforts to mitigate *Salmonella* during slaughter, poultry remains a significant cause of human salmonellosis and is responsible for approximately 23% of salmonellosis cases each year, 17% of which are directly linked to chicken (1). Isolates belonging to *Salmonella enterica* subps. *enterica* are most frequently associated with human illness, and this subspecies is represented by over 1,500 distinct serovars that are characterized by their lipopolysaccharide (O) and flagellar (H) antigens (2). Serovars can inhabit different niches and show host tropism with altered capacities to cause illness in humans and animals (3–6). Of serovars frequently found in poultry in the United States,

serovar Kentucky has a low association with human illness, while serovars Enteritidis, Typhimurium, and Infantis are often responsible for human salmonellosis (7, 8).

The United States produces over nine billion broilers each year (9), with the Southeast producing 5.01 billion, followed by the South Central region (~1.95 billion) and the Atlantic (~1.11 billion). A total of 1.3 billion broilers are produced in Georgia (14%), the top broiler-producing state. The poultry industry is vertically integrated into complexes, with each life stage of the chicken separated into different houses, and large integrators operate multiple complexes. Each complex typically encompasses parental breeder flocks ('breeders') whose eggs are sent to a single hatchery facility within the complex. Newly hatched broiler chicks are then disseminated to multiple broiler farms, and these are subsequently processed at a single processing plant belonging to that complex. Vertical integration is economically beneficial and allows for stricter biosecurity measures that provide greater control over pathogen spread for foodborne and avian pathogens. Vertical and horizontal transmission from breeders to broiler flocks is the largest *Salmonella* contributor to chickens at processing, making breeders the single most important target for *Salmonella* mitigation (10–12).

USDA-FSIS regularly collects surveillance samples from processing establishments, including carcass and raw part rinses, and publicly reports this data along with establishment performance standards. Given that current *Salmonella* regulation by USDA-FSIS has not led to a decrease in overall human salmonellosis, in October 2021, the agency introduced an initiative to reduce *Salmonella* in broilers (13). It is possible that this initiative may lead to the use of additional *Salmonella* controls during live production to reduce overall *Salmonella* load as birds arrive at the processing facility.

While there is no national monitoring system in place to survey *Salmonella* in broiler flocks, since 2016, with the support of the National Chicken Council, several integrators began monitoring *Salmonella* in their breeder flocks at 16 weeks (prior to egg production) and 42 weeks (after peak egg production). This monitoring is typically performed at state-supported, commercial, or academic laboratories, and is not reported.

Vaccination of breeder flocks, and sometimes broiler flocks is an effective method of *Salmonella* control in live production (14). Commercially available live attenuated vaccines targeting serovar Typhimurium and killed vaccine against serovar Enteritidis are broadly used. An additional strategy is the use of autogenous vaccines that effectively reduce intestinal *Salmonella* in parental breeders, and in their subsequent broiler progeny (12, 15–18). These killed vaccines are developed for a specific broiler complex and are generated against 1-5 *Salmonella* serovars that have been collected from those premises. Development of effective autogenous vaccines that make the greatest positive impact to food safety rely on two attributes: i) appropriate surveillance to identify and isolate serovars in both pre- and post-harvest, and ii) recognition of greatest concern serovars. Both attributes require the identification of all *Salmonella* serovars present in a population, and this is not always achieved with conventional culture methods. As demonstrated by a previous study (19), some serovars with increased antimicrobial resistance may be hiding in the background of *Salmonella* populations and are only revealed following antimicrobial treatment, which effectively reduces all susceptible serovars and allows for the growth of resistant serovars.

Current surveillance relies on *Salmonella* isolation by enrichment and characterization of a few resulting colonies that grow on indicator agar (20). This is a

serious limitation in *Salmonella* surveillance and source tracking as generally only the most abundant serovar(s) in a mixed population are detected, while the less abundant serovar(s) remain undetected (21, 22). Where clinically important serovars are undetected, traditional surveillance underestimates the presence of *Salmonella* serovars of the greatest food safety concern. High-throughput sequencing-based technologies have addressed this problem by discerning multiple serovars in a single sample and revealing serovars at orders of magnitude greater than logistically possible by picking colonies off a plate (23, 24). *Salmonella* clustered regularly interspaced short palindromic repeat (CRISPR) spacer content is tractable with serovar identity, and these sequences have been employed effectively for molecular serotyping (25–29). CRISPR-SeroSeq (serotyping by sequencing the CRISPR loci) is an amplicon-based sequencing tool that uses *Salmonella* CRISPR identities to quantify the relative frequency of multiple serovars in a single sample, down to serovars comprising as little as 0.003% of the population (19, 22, 23, 30, 31).

Prior to this study, some poultry integrators reported to us that some serovars they find during live production (pre-harvest) do not align with those found during processing (post-harvest). We initiated this study to determine whether this pattern occurred more broadly across national and regional surveillance data from processing. Using serovar population analyses by CRISPR-SeroSeq revealed that many pre-harvest samples contain multiple serovars, which explains the serovar diversity seen during processing. Although this study uses broiler production, the findings here are broadly applicable to other industries where *Salmonella* is a concern. Additionally, this study highlights the

importance of comprehensive surveillance monitoring in food production systems to identify and control pathogens prior to an outbreak.

Results

Between 2016-2020, the percentage of *Salmonella* positive broiler carcasses increased slightly from 4.1% to 4.5% across the United States (Table 2-1). For all five regions, the percentage of *Salmonella* positive carcasses peaked in 2017 or 2018. Conversely, this measure decreased significantly in raw, intact parts (herein referred to as 'parts') from 16.8% to 7.8% and peaked for most regions in 2016 (Table 2-2). During this time, the total number of carcass samples collected by USDA-FSIS increased by 10% while the number of parts samples more than doubled. Significantly, for all regions across all five years, the percentage of *Salmonella*-positive samples was greater in parts (11.1%; 9,474/85,022) than in carcasses (4.5%; 2,145/47,538) (Tables 2-1 and 2-2).

The Southeast is the largest poultry producing region in the United States with more samples collected than any other region. Therefore, expectedly, it had the highest overall number of *Salmonella* isolated from carcasses and parts, with an annual average of 4.1% (886/21,780), and 11.5% (2,140/18,614) *Salmonella*-positive parts samples. Despite this, *Salmonella* incidence in 2020 was lowest in Southeast processing establishments, at 3.6% in carcasses and 6.0% in parts. Poultry production in the Atlantic and the South Central regions is comparable, and this is reflected in their similar *Salmonella* prevalence values. In carcasses, the Atlantic region had an average *Salmonella*-positive incidence of 6.0% (407/6736), while the South Central region had an average of 3.5% (228/6581) (Table 2-1). Both these incidences were increased in parts with averages of 13.6% (815/6010) and 8.9% (812/9115) in the Atlantic and South

Table 2-1. Prevalence of *Salmonella* in broiler carcasses at processing, 2016 to 2020.

	No (%) of samples[a]											
	Southeast		South Central		Atlantic		Midwest		Mountain & West		All regions	
Yr	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP
2020	4,624	165 (3.57)	2,101	85 (4.05)	1,391	93 (6.69)	1,081	71 (6.57)	479	37 (7.72)	9,676	451 (4.66)
2019	4,439	170 (3.83)	1,982	72 (3.63)	1,372	83 (6.05)	1,016	63 (6.20)	461	29 (6.29)	9,270	417 (4.50)
2018	4,238	170 (4.01)	1,906	77 (4.04)	1,353	99 (7.32)	1,027	81 (7.89)	500	33 (6.60)	9,024	460 (5.10)
2017	4,289	230 (5.36)	1,896	115 (6.07)	1,336	68 (5.09)	1,056	61 (5.78)	491	36 (7.33)	9,068	510 (5.62)
2016	4,179	151 (3.61)	1,833	68 (3.71)	1,283	64 (4.99)	1,004	46 (4.58)	503	23 (4.57)	8,802	352 (4.00)
Total	21,769	886 (4.07)	9,718	417 (4.29)	6,735	407 (6.04)	5,184	322 (6.21)	2,434	158 (6.49)	45,840	2,190 (4.78)

[a]SP, *Salmonella* positive.

Table 2-2. Prevalence of *Salmonella* in raw intact chicken parts at processing, 2016 to 2020.

	No. (%) of samples[a]											
	Southeast		South Central		Atlantic		Midwest		Mountain & West		All regions	
Yr	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP	Total	SP
2020	5,078	306 (6.03)	2,571	192 (7.47)	1,971	209 (10.60)	1,379	149 (10.88)	1,580	127 (8.04)	12,579	1,012 (8.05)
2019	4,229	345 (8.16)	2,022	121 (5.98)	1,466	183 (12.48)	928	101 (10.87)	1,221	92 (7.53)	9,872	1,001 (10.14)
2018	3,318	462 (13.92)	1,566	131 (8.37)	920	139 (15.11)	576	95 (16.49)	781	73 (9.35)	7,161	900 (12.57)
2017	3,236	540 (16.69)	1,570	170 (10.83)	827	126 (15.24)	570	74 (12.98)	646	91 (14.09)	6,850	842 (12.29)
2016	2,743	487 (17.75)	1,382	198 (14.33)	822	159 (19.34)	507	87 (17.16)	581	81 (13.94)	6,035	983 (16.29)
Total	18,604	2,140 (11.50)	9,111	812 (8.91)	6,006	816 (13.57)	3,960	506 (12.77)	4,809	464 (9.65)	42,490	4,738 (11.15)

[a]SP, *Salmonella* positive.

Central, respectively (Table 2-2). While the Southeast has the highest overall *Salmonella* prevalence, this is the only region where the number of *Salmonella* isolated has decreased each year in both carcasses and parts since 2017 (carcasses: 230 isolates in 2017 to 165 isolates in 2020, parts: 540 isolates in 2017 to 306 isolates in 2020), whereas for other regions the annual number of *Salmonella* isolated has somewhat increased. Importantly, the total number of samples per type and year has been relatively maintained in all regions.

The USDA-FSIS also reports the serovar information, and we analyzed this data as well. Between 2016-2020, the number of serovars found in parts (59 serovars) was greater than in carcasses (37) (Supplemental Tables 2-1 and 2-2), which fits the trend observed above with overall *Salmonella* incidence between carcasses and parts. The Southeast region had the greatest diversity, with an annual average of 22 serovars in parts and 14 serovars in carcasses. This was followed by the Atlantic and South Central regions, with an annual average of 14 and 13 serovars in raw, intact parts, and 8 and 9 serovars in carcasses, respectively. For most regions, the number of different serovars found per sample type peaked in 2016 and 2017 and has since reduced. For carcasses, the annual number of serovars peaked at 18 in the Southeast (2017) while in parts, the highest annual number of serovars was 26, in the Southeast (2016).

In terms of serovar identity, there were also regional differences, as highlighted by the major serovars in Figure 1. Serovar Typhimurium was more frequently isolated from both carcasses and parts in the Atlantic and Southeast regions (light blue; Fig. 2-1). Proportionally, serovar Typhimurium was greatest in the Atlantic region, comprising 23.3% (91/390) of carcass samples and 19.7% (160/814) of parts samples from 2016-

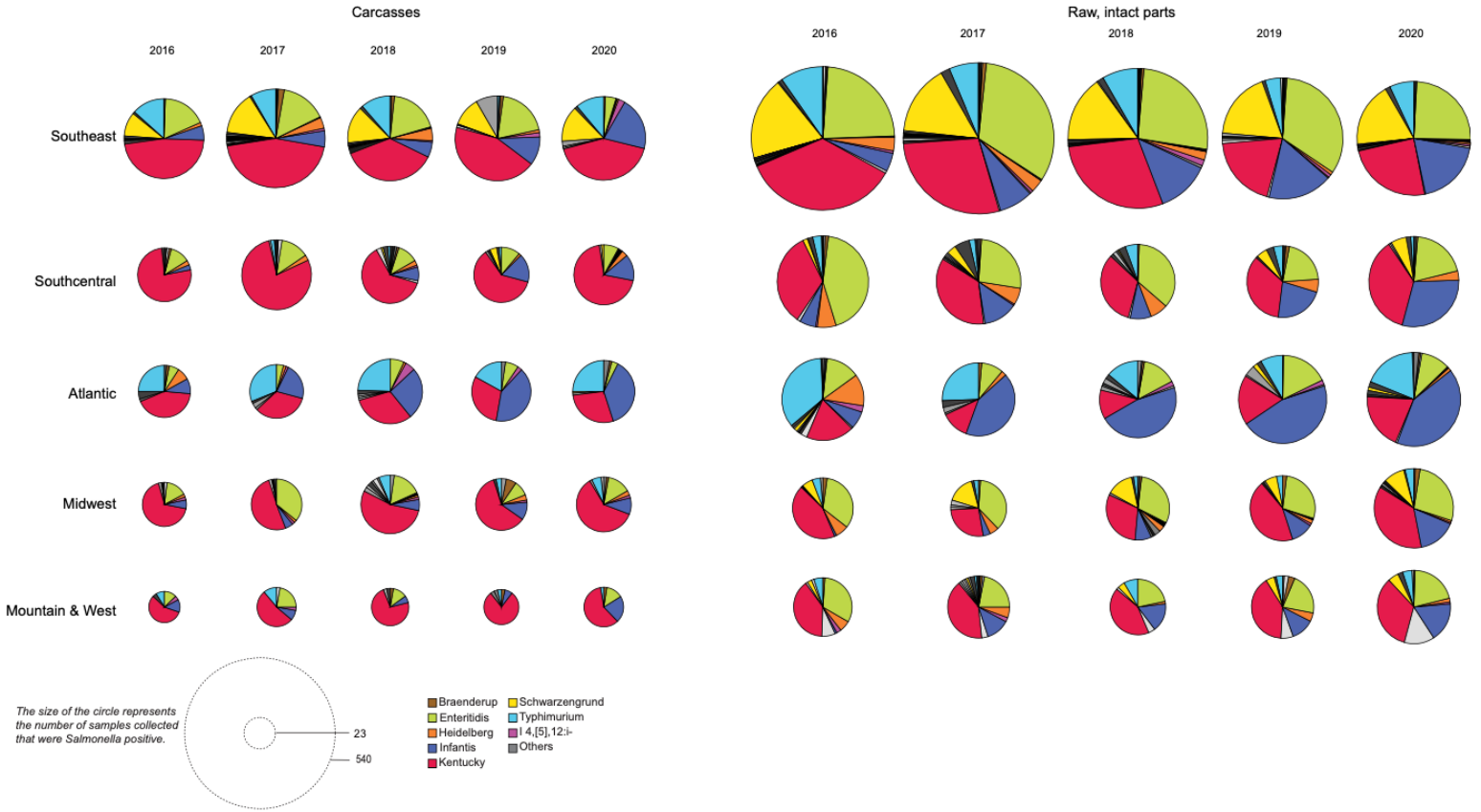


Figure 2-1. *Salmonella* serovar distribution in broiler carcasses and intact parts at processing across the United States, 2016-2020. Pie charts depicting the prevalence of different serovars in broiler processing establishments in the United States. The size of the circle reflects the number of positive samples and the regions are defined as follows: Atlantic (CT, DE, MA, MD, ME, NH, NJ, NY, PA, RI, VT, VA, and WV), Southeast (AL, GA, FL, KY, MS, NC, SC, and TN), South Central (AR, LA, OK, and TX), Midwest (IL, IN, IA, KS, MI, MN, MO, NE, ND, OH, SD, and WI), and Mountain & West (AZ, CA, CO, ID, MT, NV, NM, OR, UT, WA, and WY). Regions are ordered based on broiler production per region, according to USDA (9). Select serovars are highlighted as shown; the full serovar information is provided in Supplemental Tables 1 and 2.

2020. The relative proportion of serovar Typhimurium in the Southeast was lower, at 10.3% (90/877) and 7.3% (155/2,138) in carcass and parts samples, respectively. While serovar Infantis (dark blue) was isolated from both products in all regions each year, it was most prominent, relatively, in samples from the Atlantic. Here, it has rapidly increased from 6.9% (11/159) and 9.4% (6/64) in 2016 to 41.6% (86/207) and 34.2% (26/76) in 2020 in carcasses and parts, respectively. In the Southeast, serovar Schwarzengrund (yellow) was the third most isolated serovar from 2016-2020, with 16.8% (360/2,138) in parts and 13.0% (114/877) in carcasses. We also observed some trends that occurred across all regions. For instance, the relative proportion of serovar Kentucky (red) decreased from carcasses to parts, with an overall average of 50.5% (1,386/4,733) and 29.3% (1,093/2,164), respectively. Additionally, serovar Enteritidis (green) increased between the two sample types, as it was found in 25.2% (1,208/4,733) of parts and 13.0% (281/2,164) of carcasses.

Since our monitoring data from breeder flocks is relatively unbiased (a large number of different companies and complexes submit samples for *Salmonella* testing) and because Georgia represents the largest broiler producing state, we next sought to compare the serovars that are isolated from breeders at 16 and 42 weeks with those found during slaughter. The number of isolates peaked in 2017 for carcasses (58), raw, intact parts (185), and breeders (young = 497, old = 635) (Fig. 2-2). For non-intact parts, the number of *Salmonella* isolated peaked in 2018 (27 isolates). Following these peaks in 2017 and 2018, the overall *Salmonella* incidence in all sample types has decreased. From 2016-2020, the average number of serovars isolated each year was higher in non-intact

parts (13), compared to carcass rinses (6) and intact parts (7) (Supplemental Table 2-3). For all three processing sample types, the greatest diversity was found in 2017.

The serovar identity across the three sample types over time reveals some interesting patterns. Since 2016, serovar Kentucky has increased in prevalence, replacing serovar Enteritidis as the most common serovar isolated from carcasses (Fig. 2-2). Relative to carcasses, serovar Kentucky is proportionally reduced in both intact and non-intact parts, as was observed in the national data (Fig. 2-1). Despite not being identified in carcasses until 2019, the incidence of serovar Infantis increased dramatically since 2016, and in 2020, accounted for 18% (30/170) of *Salmonella*-positive samples. Serovar Enteritidis was proportionally higher in parts from 2016-2020 than in carcasses, though it was not identified in non-intact parts in 2020.

Strikingly, from 2016-2020 serovar Kentucky was the most common serovar isolated from young and old breeder flocks and has proportionally increased each year to account for 80% and 81% of samples, respectively. Serovars Enteritidis and Typhimurium alternate in prevalence to account for the second and third most commonly isolated serovars between 2016 and 2020, although serovar Typhimurium, and its monophasic variant, I 1,4,[5],12: i:-, are more frequently associated with young breeders while serovar Enteritidis more often isolated from older breeders (Fig. 2-2).

The discrepancy between high serovar Kentucky prevalence in breeder flocks but low prevalence at processing suggests that this serovar is effectively mitigated during slaughter. However, it does not explain the origin of other serovars such as Infantis, Enteritidis, and Schwarzengrund that are found at processing. One possible explanation is that multiple serovars exist in breeder flocks but the amount of serovar Kentucky is so

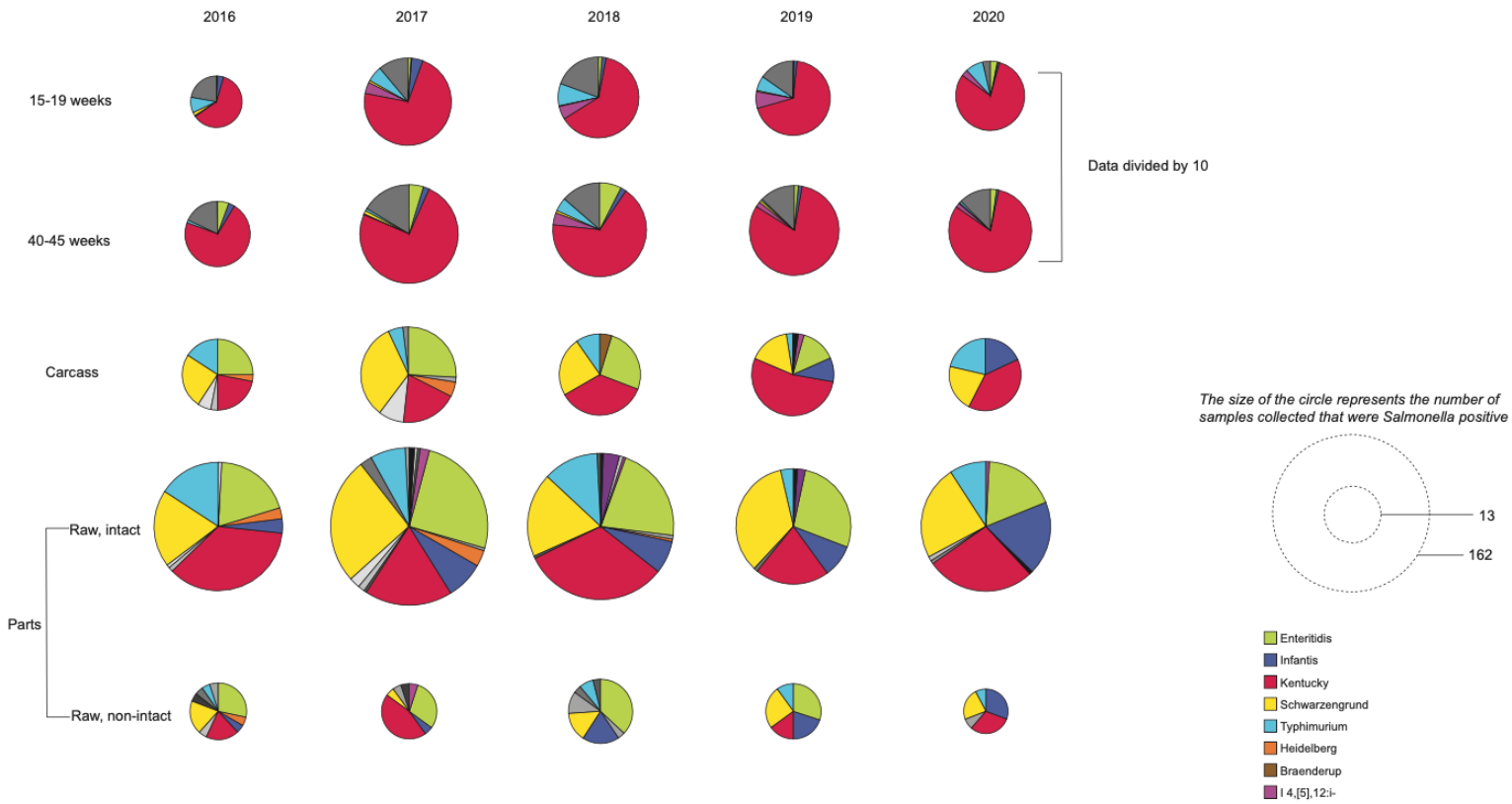
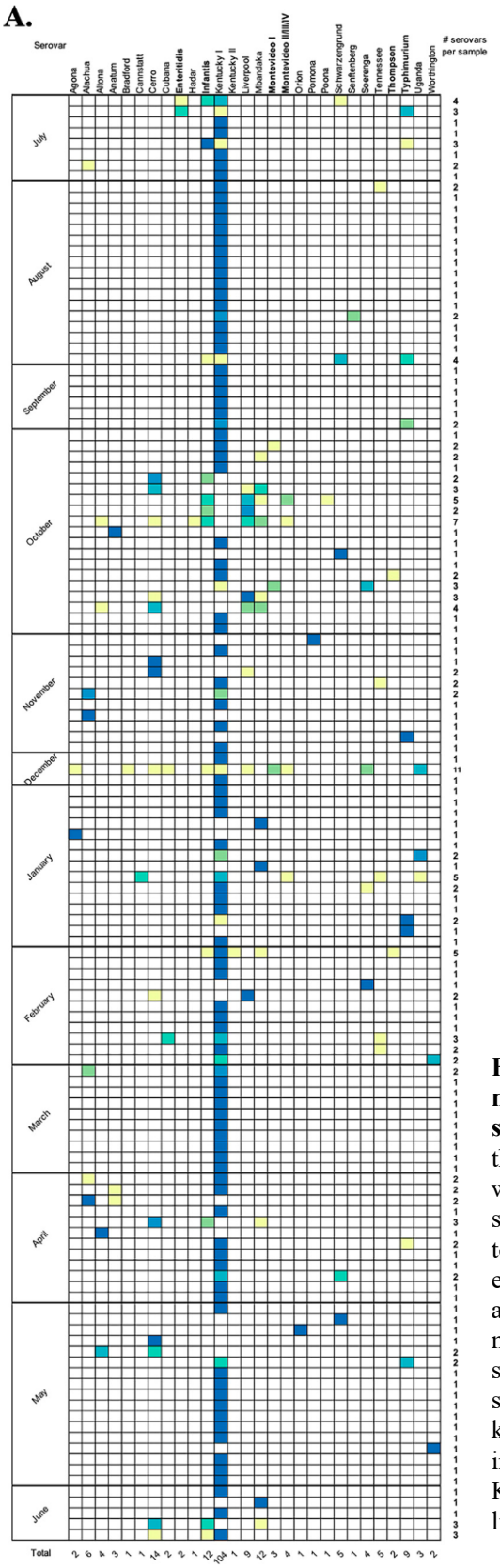


Figure 2-2. Serovar distribution for *Salmonella* positive breeder, carcass, intact parts, and non-intact parts samples collected in Georgia, 2016-2020. Pie charts depicting the prevalence of different serovars in breeder samples collected at 15-19 weeks and 40-45 weeks (top two row) and samples from processing establishments in Georgia (bottom three rows). The size of the circle reflects the number of positive samples, and the values for the breeder samples are divided by 10. Select serovars are highlighted as indicated. For the samples from processing, the full serovar data is presented in Supplemental Table 3.

high, that detecting the other serovars by traditional culture methodology (i.e. picking a small number of colonies) prevents the detection of less abundant serovars that might also be present. To determine if this was the case, we sought to apply high-resolution serovar population analyses to samples collected from breeder flocks. CRISPR-SeroSeq was performed on 134 *Salmonella* positive samples that were collected at the Georgia Poultry Lab Network from July 2020 to June 2021. There was an average of 1.6 serovars per sample, with 32.1% (43/134) samples containing more than one serovar (range 1-11), and a total of 26 serovars across the sample set (Fig. 2-3). Across the dataset, the October 2020 samples were most diverse: we identified 13 different serovars (plus two different lineages of serovar Montevideo) and 57.9% (11/19) samples contained multiple serovars. The March 2021 samples were the least diverse, with only two serovars identified and a single instance of a multi-serovar sample. Expectedly, serovar Kentucky was the most common serovar, followed by serovars Cerro and Mbandaka. Serovar Kentucky was present in 77.6% (104/134) samples, and as the majority serovar (darker blue) in 89.4% (93/104) of these samples. We identified five serovars of human importance, as denoted by the CDC Top 10 serovar list: Enteritidis, Infantis, Montevideo, Thompson, and Typhimurium. Notably, serovar Infantis was detected in 9.0% (12/134) samples, and it was present as a minority serovar in 91.7% (11/12) of these samples.

Discussion

Salmonella contamination of poultry remains a significant and complex problem (32–36). The overall number of *Salmonella* positive samples and the number of serovars identified in broiler processing plants is reflective of the size of production in the different regions. However, this is not reflected by the proportion of samples that are



B.

Serovar	Serovar prevalence across samples	Incidence of majority to minority (ratio)	Majority serovar in sample (%)	Minorityy serovar in sample (%)
Agona	2	1:1	50	50
Alachua	6	3:3	50	50
Altona	4	2:2	50	50
Anatum	3	1:2	33	67
Bradford	1	0:1	0	100
Cannstatt	1	0:1	0	100
Cerro	14	8:6	57	43
Cubana	2	0:2	0	100
Enteritidis	2	0:2	0	100
Hadar	1	0:1	0	100
Infantis	12	1:11	8	92
Kentucky I	104	94:10	90	10
Kentucky II	1	0:1	0	100
Liverpool	9	4:5	44	56
Mbandaka	12	3:9	25	75
Montevideo I	3	0:3	0	100
Montevideo II/III/IV	4	0:4	0	100
Orion	1	1:0	100	0
Pomona	1	0:1	0	100
Poona	5	3:2	60	40
Schwarzengrund	1	0:1	0	100
Senftenberg	4	2:2	50	50
Soerenga	5	0:5	0	100
Tennessee	2	0:2	0	100
Thompson	9	5:4	56	44
Typhimurium	3	2:1	67	33
Uganda	2	2:0	100	0
Worthington	1	1:0	100	0

Figure 2-3. *Salmonella*-positive breeder monitoring samples often contain multiple serovars. CRISPR-SeroSeq was used to determine the relative abundance of *Salmonella* serovars within each sample. Each column is an individual sample that was derived from the overnight tetrathionate enrichment culture from an environmental boot sock sample; these are arranged according to the date they were submitted, and the month is indicated. The individual serovars are shown on the left and the heatmap shows relative serovar abundance in each sample according to the key. Samples with more than one serovar are indicated in bold (bottom row). The two serovar Kentucky lineages and four serovar Montevideo lineages are named as previously described (72, 73).

Salmonella positive. For example, in 2020, the two highest producing regions (Southeast and South Central) had the lowest proportion of *Salmonella* positive carcasses and parts. Further, the proportional decrease of *Salmonella* positive carcasses (2017/2018-2020) and parts (2016-2020) across all five regions indicates that *Salmonella* control measures can be effective. This timeframe is concurrent with the most recent performance standards from USDA-FSIS (37) and suggests that this approach has been useful at reducing the overall *Salmonella* prevalence in poultry.

Despite the success in reducing overall *Salmonella* incidence at processing, the number of human salmonellosis cases linked to poultry has remained relatively unchanged (13). This is likely due in part to the particular serovars that are present in poultry production, their propensity to colonize poultry, and their individual association with human illness. Further investigation is warranted to identify serovar-specific capabilities to persist in poultry production. When we began this study, our intention was to analyze and present the national data together; however, we noticed the region-specific trends and decided to present the data by region. The growing proportion of serovar Infantis in the Atlantic region from 2016-2020 is particularly striking. While serovar Infantis has proportionally increased in all five regions in both carcasses and parts, in the Atlantic it has been the major serovar found in carcasses since 2019 and in parts from 2017. This may reflect climate or environmental conditions in the Atlantic that somehow promote colonization of poultry by serovar Infantis (or that suppress other serovars such as Kentucky), or that serovar Infantis has filled a vacated ecological niche (e.g. through vaccination targeting serovar Kentucky).

For several years, serovar Kentucky has been the most prevalent serovar isolated during poultry production in the United States, though in the United States serovar Kentucky does not have a high association with human illness (7, 38). The data presented here comparing *Salmonella* serovar incidence in breeder flocks with that at processing in Georgia shows that the high proportion of serovar Kentucky in flocks is reduced significantly during processing. This observation is in agreement with studies showing the competitive fitness of serovar Kentucky in colonizing chicken intestines (39) and also suggests that serovar Kentucky is susceptible to antimicrobial interventions used in the carcass chilling procedure. Further, in the national processing data, the proportion of serovar Kentucky is consistently lower in parts than on carcasses, suggesting that the additional antimicrobial intervention steps between chilling and parts cut-up also effectively removes serovar Kentucky.

Across the United States, *Salmonella* incidence increases from carcasses to parts, despite collection of similar numbers of samples. A similar increase has been noted in other studies comparing *Salmonella* prevalence in post-chill carcasses and parts (40–42). This might be due to cross-contamination of equipment used to generate parts (43). These findings suggest the need for additional interventions during processing of parts. The reduction of serovar Kentucky prevalence between carcasses and parts is accompanied by an increase of other serovars, specifically serovars Enteritidis and Infantis, which are both frequently associated with human salmonellosis (7, 8). There are a few possible, non-mutually exclusive, explanations for these observations. First, peroxyacetic acid is an effective antimicrobial commonly used in processing (44) and some serovars may tolerate this antimicrobial better than serovar Kentucky (45). Second, despite cleaning

and sanitation, these patterns may reflect serovar differences in survival in processing environments such as the ability to form strong biofilms (46–49). Third, some serovars, such as Enteritidis, can systemically infect chickens (50, 51), and separation of the carcass into parts may release internalized *Salmonella*. Significant further research is required to address the potential impact of these explanations on the serovars found in processing.

Salmonella serovars found at processing must have originated from live production, and the reduction of serovar Kentucky during processing is able to reveal the identity of additional serovars, some of which are often associated with human illness. Using CRISPR-SeroSeq, we determined that one third of samples contained more than one serovar. This type of approach overcomes the disadvantage of only detecting the most abundant serovar (or that which grows best), which occurs when only a small number of colonies are selected from a plate (52). CRISPR-SeroSeq identifies multiple serovars based on amplification of total genomic DNA in a sample, therefore allowing for a greater representation of *Salmonella* serovar diversity. This analysis explains some of the differences between the breeder samples and the processing plant samples. For example, it revealed that serovar Infantis is most often outnumbered by other serovars, including serovars Kentucky, Liverpool, and Cerro, and that serovars Enteritidis and Typhimurium were minority serovar in 100% and 33% of instances where they were detected, respectively, though the former was only detected in two samples. The high number of samples containing serovar Cerro was surprising as this serovar is most frequently associated with cattle (53). Potential explanations include the high number of cow-calf operations in Georgia (54), many of which are near broiler production farms, or

introduction via feed that contains meat and bone meal or blended animal byproducts (55, 56). Our data also suggests that there may be seasonal attributes that contribute to serovar diversity as the samples collected in October 2020 were more diverse than others and that the samples from October and November also had the lowest incidence of serovar Kentucky. The data analyzed here only represents a single year and future analyses are required to see if these trends are significant. Continuing to apply high-resolution surveillance approaches can elucidate intraspecies population dynamics, as some serovars may prove to be consistently more dominant in populations, and there may be some environmental conditions which encourage competitive exclusion.

Targeted serovar-specific *Salmonella* reduction through autogenous vaccination has increased in use in the poultry industry, as integrators try to eliminate serovars of the greatest food safety concern and to also reduce the quantity of *Salmonella* on birds arriving at their processing facilities. Generation of an autogenous vaccine requires the serovar of concern to have been isolated within a complex previously and this is subject to the limitations of *Salmonella* isolation during monitoring (i.e. the lower resolution of selecting and characterizing a small number of colonies). Population-based approaches, such as CRISPR-SeroSeq, aid in revealing the presence of serovars that maybe less fit than others in live chickens but that can persist in the processing environment and potentially cause human illness (22, 23, 31). Such serovars would be ideal candidates to be targeted by autogenous vaccines. This approach, too, will improve *Salmonella* surveillance and food safety. Historically, successful, serovar-specific industry-wide interventions have reduced or eliminated the presence of those serovars (57). This often results in another serovar taking over (e.g. serovar Kentucky replacing serovar Enteritidis

following efforts in the 1990s to eliminate the latter). The use of serovar population analyses followed by in-depth phenotypic characterization of different strains or serovars, particularly in pre-harvest poultry, may help to predict these shifts as they are occurring and allow vaccines or other interventions to be generated in a timelier manner.

There are a few caveats to our study: first, the samples analyzed here are not matched to each other, and we did not follow the same breeder flocks to broilers and then to processing. This may be why we did not identify many instances of serovar Enteritidis in our population analyses of *Salmonella*-positive breeder flocks. Further, USDA-FSIS does not sample carcasses and parts from the same processing establishment on the same day, thus it is not possible to directly compare between the samples collected at processing as different flocks are slaughtered each day. Nonetheless, the large number of samples and the unbiased nature in which they are collected has allowed us to visualize broad, industry-wide trends. A second caveat is that although *Salmonella* quantity in broilers is linked to *Salmonella* found at processing (58), we did not include broilers in this study. This was because there is no broad *Salmonella* monitoring program for broilers beyond what integrators may individually perform and any on-farm sampling we would have done would be biased to one or a small number of integrators. Colonization of breeder flocks with *Salmonella* is a major contributor of *Salmonella* found in broilers, which is one reason why interventions such as vaccination are performed in breeders (11, 59). However, this does not discount the possibility of additional *Salmonella* contamination of broiler flocks from environmental sources such as litter, insects, feed, and rodents (60–65), and this was not captured in our study. A recent study demonstrated high concordance of serovars on pre-intervention broilers (directly after kill) with those

on broilers post intervention (66), so a future study centered on assessing *Salmonella* populations in broilers directly before processing could provide some useful information. A third caveat is the *Salmonella* isolation protocols performed by USDA-FSIS differ from those performed at GPLN, as the choice of media used for enrichment and *Salmonella* isolation can impact which serovars are detected (22, 67–70) and this bias may explain some variability across sample types. We were surprised that although serovar Schwarzengrund was frequently identified at processing in the Southeast, including in Georgia, we did not often detect this serovar in breeder samples by colony isolation and serotyping, nor by CRISPR-SeroSeq analysis of enriched samples from breeder flocks. Previous work has shown that serovar Schwarzengrund is preferentially isolated following enrichment in Rappaport-Vassiliadis (RV) broth compared to tetrathionate (TT) broth (22, 70). Unlike processing plant samples where the *Salmonella* are damaged and isolation requires a non-selective pre-enrichment step to allow the *Salmonella* to recover (20), isolation from farm samples are approved by the National Poultry Improvement Plan (NPIP) to be incubated directly into selective enrichment both. In the case of this study, all breeder samples were enriched in TT broth and not in RV broth, which may explain this discrepancy.

Salmonella contamination of poultry products remains a complex issue (35, 57) and this study highlights serovar differences regionally and during processing (i.e. between carcasses and parts), which adds to this complexity. Significantly, the population analyses performed here partially explains the serovar incongruity that occurs between pre- and post-harvest by demonstrating that a third of all breeder samples contain more than one serovar and that when serovar Kentucky is present, it tends to account for a

greater proportion of the *Salmonella* that is present. There are multiple sources which may contribute to *Salmonella* presence in a poultry environment, such as cross-contamination from workers, rodent activity, and contaminated feed products (71), so it is important to expand surveillance sampling to monitor these contamination routes, as this could lead to improved *Salmonella* control. Finally, although poultry is a considerable *Salmonella* reservoir, contamination of other food animals, including cattle and swine, are also of significant food safety concern. These industries also face the pre- and post-harvest *Salmonella* challenges that have been described here; the approaches and conclusions drawn here are relevant to those and other industries where *Salmonella* is a problem.

Materials and Methods

Analysis of USDA-FSIS data

Salmonella data from January 2016-December 2020 was downloaded from the USDA-FSIS website (https://www.fsis.usda.gov/science-data/data-sets-visualizations/laboratory-sampling-data). This data includes establishment identity and location, date of isolation, sample type, and *Salmonella* serovar identity. For national analysis, regions were characterized as follows: Atlantic (Connecticut, Delaware, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia, and West Virginia), Southeast (Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee), South Central (Arkansas, Louisiana, Oklahoma, Texas), Midwest (Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin), and Mountain & West (Arizona, California, Colorado, Idaho, Montana,

Nevada, New Mexico, Oregon, Utah, Washington, Wyoming). Data for carcasses and parts were downloaded and analyzed separately. For the parts samples, the data were separated into 'raw-intact chicken' and 'raw-ground, comminuted or otherwise non-intact chicken' because these reflect different processing steps and different *Salmonella* risks/prevalence. For the national data, the non-intact category was not considered because in lower poultry production regions there were only a small number of positive samples, and this data reflected a single processor sampled multiple times or a small number of processors within that region. For the Georgia analysis, both the intact and non-intact parts data were considered.

Salmonella isolation and serotyping

Several integrators participate in the routine surveillance program through the Georgia Poultry Laboratory Network (GPLN), where breeder flocks are tested for *Salmonella* at approximately 16 weeks (pullets; pre-egg production) and 40 weeks (post-peak egg production). To condense the sample collection into two subsets, samples from weeks 15-19 (young breeders) and 40-45 (old breeders) were grouped together for analysis. Hatching egg companies maintain breeder flocks and in accordance with NPIP regulations must test their flocks for *Salmonella* every 30 days. *Salmonella* data from hatching egg company samples that were received closest to 16 and 40 weeks from these companies were included in this study. The data includes eight different hatching egg companies and six different integrators. Samples are submitted to GPLN typically as boot socks in Whirl-pak bags. Between 125-150 ml tetrathionate (TT) enrichment broth was added to each boot sock sample and these were incubated at 37°C for 20-24 hours. The bags were gently mixed and 100 µl of enrichment transferred into a modified

semisolid Rappaport Vasiliadis (MSRV) agar plate and incubated at 42°C. The plates were checked at 24 hours and 48 hours and transferred onto two types of agar: brilliant green (BG) agar containing novobiocin and xylose lysine tergitol-4 (XLT-4). These were incubated at 37°C for 20-24 hours and four presumptive *Salmonella* colonies were selected. *Salmonella* was confirmed by biochemical identification using the Vitek system (Biomeriuex), and then serotyped by conventional serum agglutination (BD Difco, Fisher Scientific, Atlanta, GA; Remel, Lenexa, KS, and SSI Diagnostics, Cederlane, Burlington, NC) and using the Luminex xMap molecular assay (Luminex, Austin, TX).

Serovar population analyses by CRISPR-SeroSeq

We selected a subset of the *Salmonella*-positive samples submitted to GPLN from July 2020 to June 2021 to complete CRISPR-SeroSeq. While the other component of the GPLN surveillance study (described above) was focused on breeder samples collected at weeks 16 and 42, this subset included samples across a range of weeks in breeder production. Our curated sample collection is once per week, shifting one day each week to reduce bias from companies who may regularly submit samples on the same day of the week. We divided the samples from each week into quadrants to have a representative dataset for each month, using the first sample in each quadrant. The number of sample collection days differs per month, and sample number is variable (higher sample numbers later in the week than earlier in the week) so our dataset is not uniform across the months but contains at least one sampling day per month. The overnight TT enrichment cultures were briefly vortexed and 1 ml of each was transferred into microcentrifuge tubes and centrifuged at 5,000 rpm for 10 minutes. The supernatant was removed, and the pellets stored at -20°C. Genomic DNA was isolated from pellets using the Promega Genome

Wizard kit (Madison, WI), according to the manufacturer's instructions, and was resuspended in 200 µl of molecular grade water and stored at -20°C. Genomic DNA was diluted 10-fold in molecular grade water and 2 µl was used as a template in the first PCR step for CRISPR-SeroSeq with primers targeting the conserved direct repeat sequences within *Salmonella* CRISPR arrays (23). PCR products were purified using the Ampure system (Beckman Coulter, Indianapolis, IN), according to the manufacturer's instructions. For the second PCR to add dual index sequences, 5 µl of the cleaned amplicon was used as a template, following the Illumina Nextera protocol (Illumina, San Diego, CA). PCR products were purified using Ampure and pooled in approximate equimolar ratios. Pooled libraries were multiplexed and sequenced on the Illumina NextSeq platform with 150 cycles, single end reads. Each sequencing run contained two negative control samples: a non-template water control from the first PCR and a non-template water control from the second PCR. A positive control containing *Salmonella* serovar Enteritidis genomic DNA with a known CRISPR profile was also included on each run. CRISPR-SeroSeq analyses were performed using a R script that scans sequence reads and uses BLAST to match sequence reads to a database of over 135 serovars, before writing the output directly to Excel. Serovars were called only if they contained multiple CRISPR spacers that were unique to that serovar.

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Author Contributions

ATS: Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization; **DW:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition; **NWS:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition

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CHAPTER 3

LONGITUDINAL STUDY HIGHLIGHTS PATTERNS OF *SALMONELLA* SEROVAR CO-OCCURRENCE AND EXCLUSION IN COMMERCIAL POULTRY PRODUCTION[1]

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Abstract

Recent advances in next-generation sequencing approaches have revealed that *Salmonella* often exists in multiserovar populations, with important implications for public health as time and resource constraints limit serovar characterization by colony-based isolation methods. It is important to characterize *Salmonella* population dynamics to then understand how the microbial ecology influences serovar evolution and thus, animal and human health outcomes. Chicken remains the leading source of foodborne *Salmonella* outbreaks in the U.S., despite reductions in contamination at the product level, underscoring the need for targeted control strategies. This study aimed to survey multiserovar *Salmonella* populations in broiler breeder flocks and monitor fluctuations throughout production. Deep serotyping was performed on environmental breeder samples collected over two years as part of a surveillance program. About 18% (104/568) of samples contained multiple serovars, with serovar Kentucky negatively associated with other serovars, often excluding them. Longitudinal sampling across two commercial complexes over 65 weeks included pullet and breeder farms. Environmental samples were collected via pre-moistened boot socks and rodent bait boxes, with on-farm rodents captured. *Salmonella* prevalence in pullet flocks was 17% (11/64), while 41% (135/330) of breeder samples were positive, peaking at 38 weeks of age. Rodents showed 35% (17/49) positivity in gastrointestinal samples and 9% (3/33) in bait station swabs, with six serovars identified, three of which were shared with flocks. Our cross-sectional and longitudinal *Salmonella* surveillance highlights the complexity of serovar interactions with further work required to elucidate the mechanisms of competitive exclusion.

Introduction

Salmonella is a leading bacterial cause of foodborne illness in the United States, with an estimated 1.35 million infections, 26,500 hospitalizations, 420 deaths, and cost of illness of over $4 billion USD annually (Centers for Disease Control and Prevention, n.d.; United States Department of Agriculture - Economic Research Service, 2021). While *Salmonella* is ubiquitous in the environment, most *Salmonella* illnesses are foodborne, with more than 75% of outbreaks attributed to seven food categories (chicken, fruits, pork, seeded vegetables, other produce, beef, turkey) (Interagency Food Safety Analytics Collaboration, 2024). Importantly, chicken is considered the largest single food contributor, accounting for 19.7% of *Salmonella* outbreaks (Interagency Food Safety Analytics Collaboration, 2024). The use of post-harvest antimicrobial interventions in domestic broiler processing plants has supported a significant decrease in *Salmonella* incidence from 8.9% in 2016 to 6.5% in 2022, based on surveillance data collected in the contiguous states by the United States Department of Agriculture – Food Safety and Inspection Service (USDA – FSIS)(United States Department of Agriculture - Food Safety and Inspection Service, 2023); however, this has not been accompanied by a reduction in attribution of poultry in human salmonellosis cases (Centers for Disease Control and Prevention, n.d.; United States Department of Agriculture - Food Safety and Inspection Service, 2022). To maximize the success of post-harvest interventions, it is necessary to reduce the load of *Salmonella* entering the plant, which in turn requires increased pre-harvest control and surveillance (Bailey, 1993).

Salmonella enterica subsp. *enterica* is responsible for 99% of human salmonellosis, and it is comprised of over 1,500 different serovars, as identified by their

lipopolysaccharide (O) and flagellar (H) antigens (Grimont and Weill, 2007; Lamas et al., 2018). In 2022, the five most commonly isolated serovars from human clinical cases in the United States were Enteritidis (2.7 cases per 100,000 population), Typhimurium (1.6), Newport (1.4), Javiana (0.9), and I 4,[5],12:i:- (0.6); these have also been the top five serovars annually since 2010 (Centers for Disease Control and Prevention, n.d.; Delahoy et al., 2022). Four of the five serovars are commonly isolated from food animal sources (poultry, beef, swine), while serovar Javiana is often attributed to fresh produce and thought to be associated with reptiles (Centers for Disease Control and Prevention, n.d.; Mukherjee et al., 2019). Different serovars pose different risks to public health based on their host restriction and adaptation (Uzzau et al., 2000), pathogenicity (Cheng et al., 2019), and propensity to carry antimicrobial resistance genes (Shah, D. H., N. C. Paul, W. C. Sischo, R. Crespo, Guard, 2016). Therefore, for meaningful food safety improvement, it is critical to identify which *Salmonella* serovars are present within a food product and to target mitigation against those that convey the greatest risk. For example, in poultry, serovars Kentucky and Enteritidis are commonly isolated; serovar Kentucky is not often responsible for human salmonellosis in the United States, while serovar Enteritidis is responsible for the largest number of cases each year (Centers for Disease Control and Prevention, n.d.).

Poultry production begins with pullet flocks, consisting of sexually immature chickens that are raised in single sex houses until ~21 weeks of age. At this point, pullet flocks are divided and transferred to breeder farms, where fertilized eggs will then become broiler chickens that are grown for five to nine weeks before slaughter. Breeder flocks remain in production until ~65 weeks, the average breeder hen will lay around 180

eggs with peak production between 28-32 weeks of age (McDaniel, 2021). In the United States, commercial chicken production is vertically integrated, with each stage of production maintained within a single complex that belongs to a single company (integrator). Poultry disease management (e.g., vaccination) is usually performed at the complex level; this also extends to *Salmonella* controls (e.g., vaccination, water acidification, or use of litter amendments or pre- and probiotics). For a single integrator, management strategies differ from complex-to-complex, depending on the *Salmonella* risks and serovars detected at processing. Each complex encompasses both live production (breeder flocks, hatchery, broiler flocks, and feed mill) and processing (slaughter and distribution) stages. This allows for greater control and coordination across the entire supply chain, leading to more efficient production and distribution, and improved food safety and quality control. Vertical integration also supports greater biosecurity control as integrators can limit pathogen introduction to flocks, but subsequently provides the opportunity for vertical transmission of existing pathogens from parent to progeny.

To add further complexity to poultry production, multiple *Salmonella* serovars can exist within a population (Thompson et al., 2018; Rasamsetti et al., 2022; Siceloff et al., 2022; Obe et al., 2023; Rasamsetti and Shariat, 2023; Richards et al., 2024). However, the conventional methods of *Salmonella* culturing typically only identify the most abundant serovar within a population or the serovar that can best outcompete others under certain enrichment conditions (Gorski et al., 2024). For many laboratories, time and resource constraints often necessitate selecting only one colony from an indicator agar plate. For a 95% probability of identifying two serovars from a sample, six colonies

must be isolated and the two serovars must exist in equal proportions (Cason et al., 2011). This limitation can be mitigated in part where careful attention is spent to select a small number of colonies that have different colony morphologies. Alternatively, molecular-based deep serotyping, such as CRISPR-SeroSeq, can provide greater resolution of *Salmonella* populations by identifying multiple serovars that co-occur within a sample. Previous studies on *Salmonella* complexity in poultry have demonstrated that 32% of *Salmonella*-positive samples from breeders and 57% of *Salmonella*-positive broiler houses contain more than one serovar (Siceloff et al., 2022; Obe et al., 2023). At processing, 48% and 7.9% of *Salmonella*-positive carcasses at hot rehang and post-chill, respectively, have multiserovar populations Richards et al., 2024). Our previous study sought to compare the serovars isolated from live production and processing operations to better understand *Salmonella* transmission dynamics in the poultry industry, but the discrepancies between serovars identified at both stages further highlighted the need for high-resolution surveillance to elucidate transmission patterns (Siceloff et al., 2022).

Previous work has demonstrated that both vertical and horizontal transmission of *Salmonella* occurs within a poultry complex, as matching subtypes were isolated from breeder farms and their subsequent broilers both on farm and at processing (Byrd et al., 1998; Liljebjelke et al., 2005; Hannah et al., 2011; Gast et al., 2014; Crabb et al., 2018; Lei et al., 2020). Some serovars, such as Enteritidis, can enter the fertilized egg, which then leads to colonization of the chicks and spread amongst flocks as the birds share a common environment for several weeks of production (Gast and Beard, 1990; Humphrey et al., 1991; Keller et al., 1995; Miyamoto et al., 1997; Guard-Petter, 2001). Additionally, *Salmonella* may be present on the exterior of the eggshell through fecal contamination

(Gantois et al., 2009). Because breeder flocks colonized with *Salmonella* can be a source of downstream *Salmonella* in broiler flocks, integrators have focused on *Salmonella* monitoring and control in their breeder flocks, with elective testing in pullet and breeder flocks around 16 and 42 weeks, respectively.

Effective *Salmonella* controls in breeders include vaccination and increased biosecurity. Vaccination provides direct and indirect protection of animals against *Salmonella* colonization There are three types of *Salmonella* vaccinations used in broiler production the United States: 1) commercial live attenuated vaccines against serovar Typhimurium; 2) a commercial killed vaccine against serovar Enteritidis; and 3) autogenous (killed) vaccines that are generated against specific serovars and are generally limited for use within a single complex. Because delivery of killed vaccines necessitates individual bird handling and the multiplication of broilers is so large, use of these vaccines is typically restricted to breeders. It has been observed that live attenuated vaccines can provide cross-protection to animal hosts against additional serovars other than the original vaccine strain, though the efficacy varies across isogenic groups and serogroups (Tennant et al., 2015; Hofacre et al., 2021; Bearson et al., 2024). Increased on-farm biosecurity can also help prevent *Salmonella* transmission; best practices include not sharing equipment between farms or cleaning equipment before use, disinfecting vehicles before entering the property, use of disposable boot covers and sanitizing footbaths prior to entering a house, controlling rodent and insect populations, maintaining dry litter, and ensuring that the houses are structurally intact to prevent any interactions with wildlife (United States Department of Agriculture - Animal and Plant Health Inspection Service, n.d.). In addition to human activity, rodents and insects may serve as

disease carriers and introduce pathogens, such as *Salmonella*, to poultry flocks (Henzler and Opitz, 1992; Davies and Wray, 1995; Goodwin and Waltman, 1996; Garber et al., 2003; Meerburg and Kijlstra, 2007; Lapuz et al., 2012; Trampel et al., 2014; Dale et al., 2015; Raufu et al., 2019; Smith et al., 2022).

The overall goal of this study was to measure the changes in *Salmonella* prevalence and serovar population dynamics during broiler breeder production and determine the incidence of multiserovar populations in breeder flocks. Additionally, we sought to assess if *Salmonella* transmission was occurring between breeder flocks and rodent populations. The study was accomplished in two parts. First, to investigate broad *Salmonella* patterns in breeder flocks, we performed deep serotyping on 568 blinded samples collected from breeder flocks over a two-year period. Second, to more finely assess *Salmonella* prevalence and serovar dynamics in breeders, we collected monthly samples from eight breeder flocks (13 different houses) and their source pullet flocks as well as rodents from the corresponding farms over one full production cycle (65 weeks) and used deep serotyping to assess *Salmonella* populations. Our findings highlight the importance of on-farm biosecurity and reveal, for the first time, patterns of serovar co-occurrence and exclusion.

Materials and methods

Longitudinal breeder flock sample collection and *Salmonella* culturing

Across two commercial complexes (Complexes 1 and 2), 15 pullet (five farms) and 13 breeder houses (seven farms) were sampled over a 65-week production period. Pullets were sampled at weeks 14 and 21, then breeders sampled monthly, apart from weekly sampling during peak production (29-31 weeks). Prior to flock placement, the

empty, cleaned out breeder houses were sampled to determine if there was any residual *Salmonella* contamination from the previous flock. Complex 2 Farm 1 (2-1) was not sampled prior to placement as the birds had been moved early but it was cleaned out previously to the same standard as the other farms. Two pre-moistened boot sock pairs (Romer Labs, Newark, DE) were collected from each house, walking between the feed and water lines on both sides of the scratch (pullets) or on the slats (breeders), and cultured for *Salmonella* (n = 394). Rodents (mice (*Mus musculus*) plus roof (*Rattus rattus*) and Norway (*Rattus norvegicus*) rats; n = 355 carcasses across 49 composite samples) were captured from breeder farms by an integrated pest management company and tested for *Salmonella*, along with bait station swabs (n = 33).

All samples were stored on ice during transportation. 200 mL of buffered peptone water (BPW; Neogen, Lansing, MI) was added to each boot sock and homogenized with a Seward stomacher (Stomacher® 400 Circulator Lab Blender, Bohemia, NY) for two minutes at 230 rpm. Following Hygiena's protocol for *Salmonella* enrichment and quantification (data not shown), 60 mL of BPW was transferred to 60 mL of pre-warmed MP media (Hygiena, Camarillo, CA) with novobiocin (40 mg/L; Thermo Scientific Chemicals, Waltham, MA) and incubated shaking at 42°C for 10 hours. Subsequently, 1 mL of culture was inoculated into 10 mL of tetrathionate (TT; Hardy Diagnostics, Santa Maria, CA), then incubated at 37°C for 20-24 hours. For the rodent samples, 200 mL BPW with novobiocin (40 mg/L) was added to the removed gastrointestinal (GI) tract, homogenized, and incubated at 42°C for 20-24 hours, then 1 mL and 0.1 mL of culture were added to 10 mL of TT and Rappaport-Vassiliadis (RV; Hardy Diagnostics, Santa Maria, CA) broth, respectively, and incubated at 37°C for 20-24 hours. Following

selective enrichment, all cultures were streaked onto xylose lysine tergitol-4 (XLT-4; Hardy Diagnostics, Santa Maria, CA) plates, then incubated at 37°C for 24-48 hours. Any presumptive *Salmonella* colonies were restreaked onto Luria-Bertani (LB; Hardy Diagnostics, Santa Maria, CA) agar, then confirmed with serum agglutination (BD Difco, Franklin Lakes, NJ). All enrichments were pelleted via centrifugation at 14,000 rpm for 3 minutes, then stored at -20°C.

GPLN sample collection

Several commercial poultry integrators participate in a routine *Salmonella* surveillance program through the Georgia Poultry Laboratory Network (GPLN), where samples are collected from breeder flocks at approximately 16 weeks (pullets; pre-egg production) and 40 weeks (post-peak egg production). In addition to integrators with conventional broiler breeder flocks, hatching egg companies maintain breeder flocks and these must be tested for *Salmonella* every 30 days in accordance with the National Poultry Improvement Plan (NPIP). The data in this study includes five different hatching egg companies, 11 different integrators, and six breeding companies. For each breeder flock, up to six samples are submitted in a single accession, typically with two boot socks in Whirl-Pak bags collected from the slats on each side of the house (left, right), two boot socks through the middle scratch area, and two miscellaneous environment samples (e.g., egg belt or ventilator fan swabs); only the four standardized samples were considered for this study. The metadata affiliated with each sample includes age of flock (if available), coded company name (to maintain blinded study), sample type, and date submitted. As part of a previous study, a subset of these samples (n = 134) was analyzed in comparison to processing plant samples and the results were published (Siceloff et al., 2022).

Between 125 and 150 mL of tetrathionate (TT) enrichment broth was added to each sample, and these were incubated at 37°C for 20 to 24 h. The bags were gently mixed, and 100 µL of enrichment was transferred into a modified semisolid Rappaport-Vassiliadis (MSRV) agar plate, followed by static incubation at 42°C. The plates were checked at 24 and 48 h and transferred onto two types of agar: brilliant green (BG) agar containing novobiocin and xylose lysine tergitol-4 (XLT-4). These were incubated at 37°C for 20 to 24 h, and four presumptive *Salmonella* colonies were selected for further characterization. From each colony, *Salmonella* was confirmed by biochemical identification using the Vitek system (BioMerieux) and serogrouped by conventional serum agglutination (BD Difco, Fisher Scientific, Atlanta, GA; Remel, Lenexa, KS; and SSI Diagnostics, Cedarlane, Burlington, NC). At least one boot sock sample per flock was then serotyped at GPLN using the Luminex xMap molecular assay (Luminex, Austin, TX) to identify the serovars belonging to representative serogroups.

For the days that we collected samples, we selected one *Salmonella*-positive boot sock sample from each breeder flock submitted to GPLN from July 2020 to June 2022 to complete CRISPR-SeroSeq. The samples for the study were collected on one day per week, shifting one day each week to avoid bias by oversampling companies who may regularly submit on the same day of the week. The number of sample collection days differs per month, and the sample number is variable (higher sample numbers later in the week than earlier in the week), so our data set is not uniform across the months but contains at least one sampling day per month over 24 months. From the main sample set, a subset of samples submitted on the same day from breeder flocks on the same farm were chosen to complete a paired house study to measure the rate of on-farm *Salmonella*

transmission. For each *Salmonella*-positive sample, the overnight TT enrichment cultures were briefly vortexed, and 1 mL of each was transferred into microcentrifuge tubes, centrifuged at 2,000 x g for 10 min, and stored at -20°C until later use.

CRISPR-SeroSeq

Total genomic DNA was isolated from the *Salmonella*-positive culture pellets using the Genome Wizard kit (Promega, Madison, WI, USA), according to the manufacturer's instructions, then DNA was resuspended in 200 µl of molecular grade water and stored at −20 °C. For the 134 samples that were previously analyzed, and for the samples from the longitudinal study, the CRISPR-SeroSeq libraries were generated using a 2-step PCR process, with the first PCR targeting the conserved CRISPR direct repeat sequences and the second PCR adding Illumina adaptors and index sequences as described (Thompson et al., 2018). For the remaining samples, a 1-step PCR was used with the Illumina adaptors and index sequences incorporated into the same primers as the target sequence, as described (Richards et al., 2024). The extracted DNA for each *Salmonella*-positive enrichment was used as template for the reaction, and the PCR products were visualized on a 2% agarose gel to confirm amplification. Following purification with AMPure XP beads (Beckman-Coulter, Indianapolis, IN, USA), the samples were pooled at approximate equimolar ratios and the resulting library was sequenced (150 cycles, single read, Illumina, San Diego, CA, USA).

Sequences were analyzed using the CRISPR-SeroSeq pipeline by means of an R script (version 4.04) that utilizes a local alignment search tool (Altschul et al., 1990) to match experimental reads to a curated database containing the complete CRISPR profiles for over 150 serovars. BLAST matches with 100% coverage and identity are recorded on

an Excel sheet and the relative frequency was calculated with unique spacer reads corresponding to each serovar (Siceloff et al., 2022). Serovars with a relative frequency greater than 0.5% were included in the analysis for all individual samples. Where a spacer was shared between two serovars present in a sample, the unique spacer read counts for each serovar were used to proportionally allocate the reads of the shared spacer to the two serovars. The CRISPR sequences are insufficient to distinguish between serovars Durban, Kokomele, Panama, Pomona, and Reading II, and in this instance, all five serovars are listed. Many *Salmonella* serovars are polyphyletic (Worley et al., 2018; Cherchame et al., 2022), and these evolutionary patterns are reflected in the CRISPR sequences (Shariat et al., 2013; Nguyen et al., 2018; Vosik et al., 2018). Where this occurs (e.g., Montevideo I and II), we have attributed a I, II, or III to indicate different lineages of a single serovar. For the longitudinal study, serovar populations were normalized across both boot socks pairs collected from one house on a single sampling visit.

Statistical analysis

All statistical analyses were conducted with R (version 4.2.3). While the longitudinal dataset includes repeated measures from the same flocks over time, analyses were conducted under the assumption of independence between observations. The paired house subset from the GLPN dataset was normalized using the DESeq2 package (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) to adjust the read counts per sample based on the size factors present.

Results

From July 2020 to June 2022, a total of 4,485 samples from 1,421 breeder flocks were submitted to the Georgia Poultry Laboratory Network (GPLN) on our sample collection days, and 35% (1581/4485) of these were *Salmonella*-positive. Flock age information was provided for 92% (4140/4485) of the samples (Fig. 3-1A). One-quarter of the submitted samples were from pullet flocks under 21 weeks of age. Within breeder flocks, most submitted samples were after peak production, between 35 – 50 weeks (34%; 1388/4485). The high proportions at these two time ranges corresponds to the participation of many companies in screening their pullet and breeder flocks around 16 and 42 weeks, respectively. *Salmonella* prevalence was highest in flocks aged 28-35 weeks (42%; 210/495), the time frame that corresponds to breeder peak production. Prevalence was lowest in flocks aged 21-28 weeks (26%; 104/396), and there was an observed relationship between age and prevalence such that the prevalence within each age class was not due to random chance (Fig. 3-1B; $p < 0.00005$, Chi-squared test).

To assess the overall serovar diversity throughout the GPLN sample set, one *Salmonella*-positive sample was selected from each flock on each collection day to complete deep serotyping using CRISPR-SeroSeq, for a total of 568 samples analyzed with 22 companies represented (Fig. S3-1). A total of 38 serovars were identified, including 16 serovars and one untypeable serovar that were each found in at least five different samples (Tables 3-1, S3-1). There was an average of 1.3 serovars per sample, with a maximum of nine serovars identified from one boot sock. About one-fifth (18%; 104/568) of samples contained more than one serovar (Fig. 3-1C). The measured frequency of multiserovar populations was the greatest in flocks aged 21-28 weeks (pre-

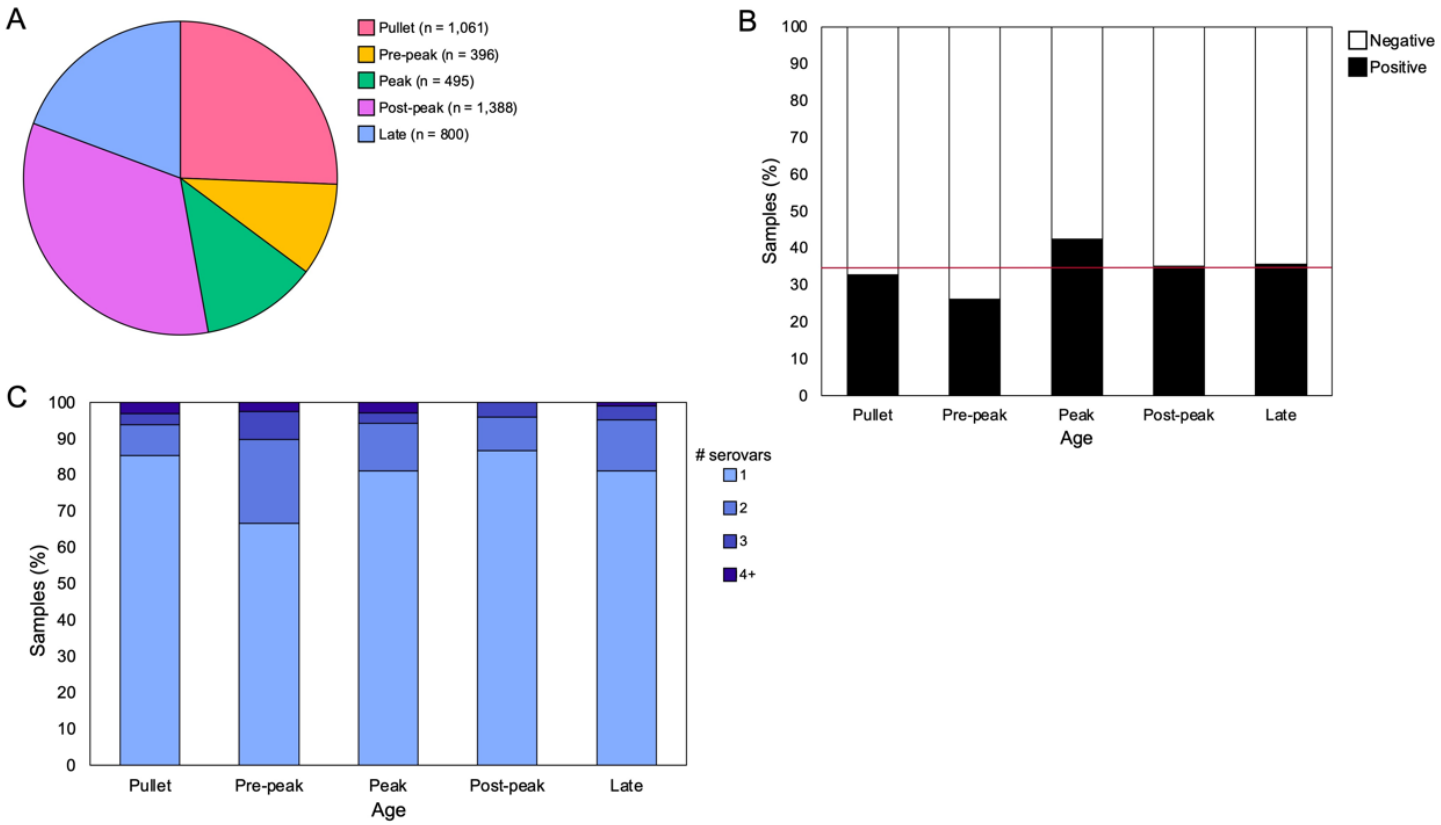

Figure 3-1. *Salmonella* prevalence and presence of multiserovar populations by age. A) Proportion of submitted breeder samples per age class from July 2020 – June 2022 on predetermined sample collection days (n = 4,140). Categories are defined as – pullet: 0 – 21 weeks, pre-peak: 21 – 28 weeks, peak: 28 – 35 weeks, post-peak: 35 – 50 weeks, late: after 50 weeks. B) Frequency of submitted samples that were positive or negative for *Salmonella* in each age class, with an observed relationship between age and prevalence ($p < 0.00005$, Chi-squared Test). The red horizontal line denotes the average prevalence across the sample set (34.7%). C) Distribution of serovars/sample identified through deep serotyping of 568 *Salmonella*-positive samples. Counts of multiserovar populations across age classes are varied but not statistically significant ($p = 0.06$, Fisher's Exact Test).

Table 3-1. CRISPR-SeroSeq summary results from GPLN sample set (n = 568).

Serovar[a]	Frequency[b]	Present (%)[c]	Alone (%)[d]	Major (%)[e]	Average relative frequency (%)[f]	Months[g]	Companies[h]
Kentucky I	462	81.3	86	65	94	23	20
Cerro	43	7.6	30	57	65	10	5
Mbandaka	34	6	12	17	30	15	7
Typhimurium	25	4.4	32	41	62	14	11
Liverpool	21	3.7	29	53	58	11	6
Infantis	19	3.3	16	12	38	11	6
Alachua	17	3	47	11	61	6	2
Senftenberg II	12	2.1	8	18	36	8	6
Tennessee	9	1.6	11	25	36	5	5
Enteritidis	8	1.4	62	0	72	5	7
Uganda	8	1.4	12	57	49	5	1
Montevideo I	6	1 .1	0	17	18	4	3
Montevideo II	6	1 .1	0	0	4	3	4
Agona	5	0.9	40	0	45	3	3
Altona	5	0.9	20	25	39	5	2
Anatum	5	0.9	20	25	42	2	3
Untypeable	5	0.9	20	0	32	3	2

[a]Only serovars present in five or more samples were included (n = 17), including eight serovars of clinical importance (bolded), that are most frequently isolated from human samples (Center for Disease Control and Prevention BEAM Dashboard). The suffixes (-I, -II, -III) for some serovars refer to polyphyletic lineages.
[b]Indicates the total number of samples each serovar was found in.
[c]Indicates the total percentage of samples each serovar was found in.
[d]Indicates how often a serovar was the single serovar in a sample.
[e]Indicates the frequency in which the serovar was present at a higher relative frequency in a mixed population of multiple serovars.
[f]This was calculated across all 'present' samples.
[g]Indicates how many months (n = 24) each serovar was identified in.
[h]Indicates how many companies (n = 22) each serovar was identified from.

peak production; 33%; 13/39), and lowest in pullet flocks (15%; 19/130) and flocks aged 35-50 weeks (post-peak production; 13%; 26/196). However, there was not a significant difference in the multiserovar populations recorded across the age classes ($p = 0.06$, Fisher's exact test).

From the deep serotyping results of the GPLN dataset, serovar Kentucky was most often identified as the major serovar within a sample (as defined by the relative frequency): in 86% (396/462) of samples where it was detected, it was the sole serovar and in samples where it co-occurred with another serovar (n = 66), it was the major serovar in 65% (43/66) of these (Fig. 3-2A). The average relative frequency of serovar Kentucky when it was present was 94%, as determined by calculating the mean of relative frequencies in each corresponding sample with deep serotyping results (Table 3-1). Although at a significantly lower incidence, serovar Cerro was the second most prevalent serovar detected (n = 43; 7.6%) followed by serovar Mbandaka (n = 34; 6.0%). When comparing the presence versus majority of the top ten serovars in our dataset, some serovars displayed a higher overall frequency across the samples but lower relative frequency within samples (Fig. 3-2A, top and middle panel). For example, serovar Mbandaka was present in 6.0% of samples (34/568) but was major or alone in 27% (9/34) of these and at an average relative frequency of 30% (Fig. 3-2A and Table 3-1).

To observe any relationships between serovar identity and overall serovar complexity within a sample, we compared the distribution of serovars per sample for the ten most abundant serovars in the dataset (Fig. 3-2A, bottom panel). Serovar Kentucky was most often found as the only serovar within a sample (red dot in Fig. 3-2A; mean = 1.2 serovars per sample when serovar Kentucky is present). Alternatively, serovars Cerro

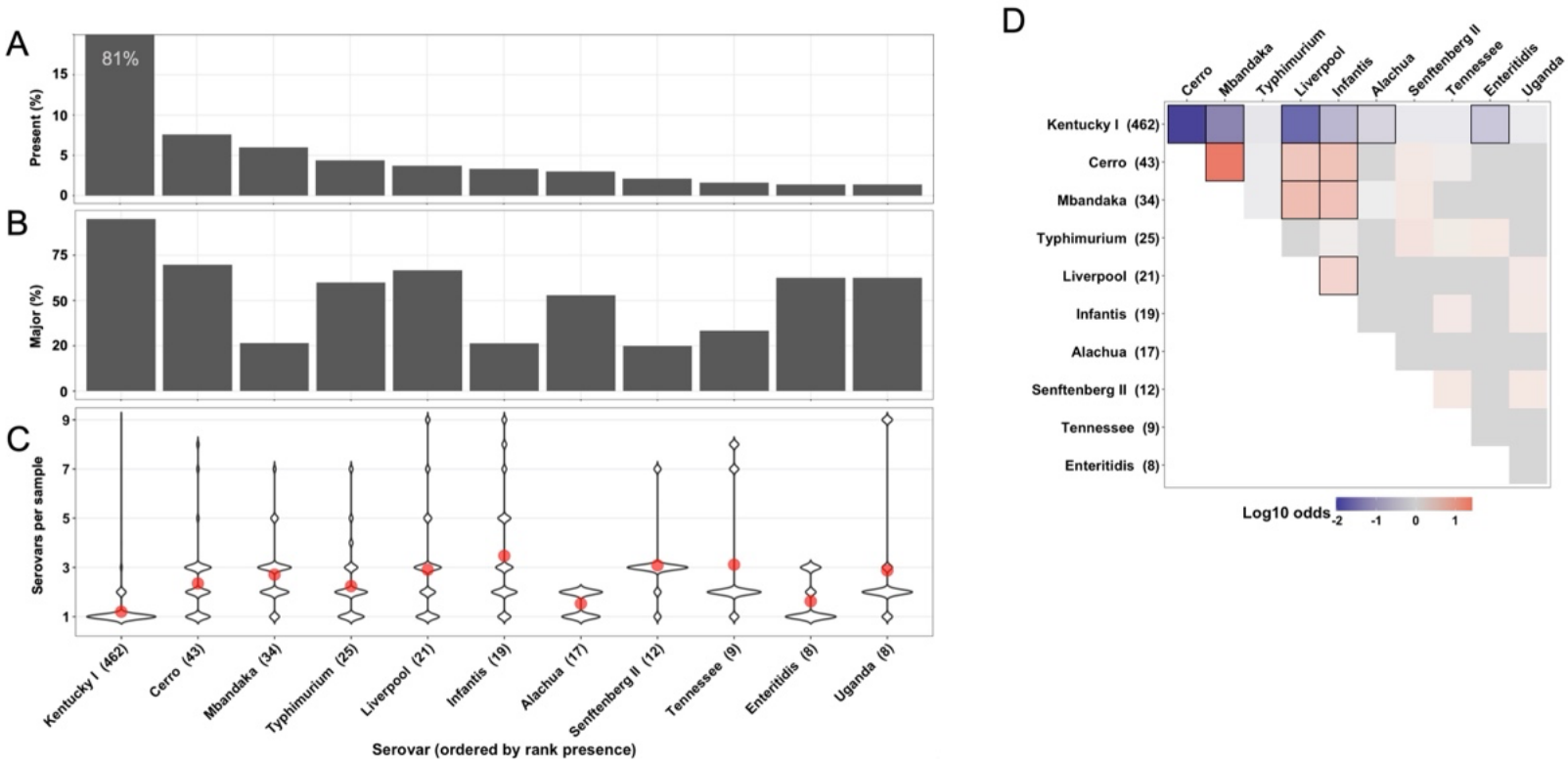

Figure 3-2. Multiserovar population dynamics vary by serovar. Only the top 10 most present serovars from the dataset are shown, with serovars Enteritidis and Uganda tied in 10th place. A) Top bar graph indicates the total sample percentage that each serovar was identified in, with only serovars present in 5 or more samples being displayed. B) Percent of samples in which each serovar was considered major, or most abundant. C) Violin plot shows number of serovars per sample (Y) when a specific serovar (X, ordered by rank presence) is present. Red dots show mean number of serovars per sample (among samples containing each indicated serovar). D) Log_{10} odds ratio of serovar co-occurrence. Row labels show number of samples where serovar was present. Red shading shows positive association while blue shading show negative association. Black outlines indicate cells with FDR < 0.05.

and Mbandaka were often found within samples containing multiple serovars (mean number of serovars per sample of 2.3 and 2.7, respectively). This trend was observed with five of the other top ten serovars as well, with the exception of serovars Alachua and Enteritidis which were most often detected in samples with low serovar complexity. Of the top 10 most frequently detected serovars, serovar Infantis was detected as a member of the most complex samples (mean number of serovars per sample = 3.5) and was infrequently found alone (16%; 3/19). To determine if there was a pattern of serovar co-occurrence, we calculated the pairwise odds ratio of co-occurrence for the top ten serovars (Fig. 3-2B). To account for multiple comparisons, we controlled the false discovery rate (FDR) at $\alpha = 0.05$ (Benjamini and Yekutieli, 2001). In concordance with previous observations, serovar Kentucky has a significantly negative odds ratio of being identified with other serovars (FDR < 0.05). Serovars Cerro, Mbandaka, Liverpool, and Infantis all had significantly positive odds ratios, indicating that they are more likely to co-occur with each other. This is consistent with the frequent finding of these four serovars in multiserovar populations.

To compare the most common serovar identities between pre- and post-harvest, we downloaded the *Salmonella* regulatory sampling results from domestic poultry processing establishments in Georgia as collected by USDA – FSIS for the same period as the study (2020 – 2022)(Fig. 3-3), and expanded the GPLN dataset to include all boot sock samples with conventional serotyping information (n = 719). Serovar Kentucky was the most abundant serovar across both GPLN and FSIS datasets, with a marked decrease of the second most abundant serovar in the GPLN dataset (serovar Cerro; 5.1% by colony serotyping) but not FSIS (serovars Infantis (24% in parts) and Typhimurium (21% in

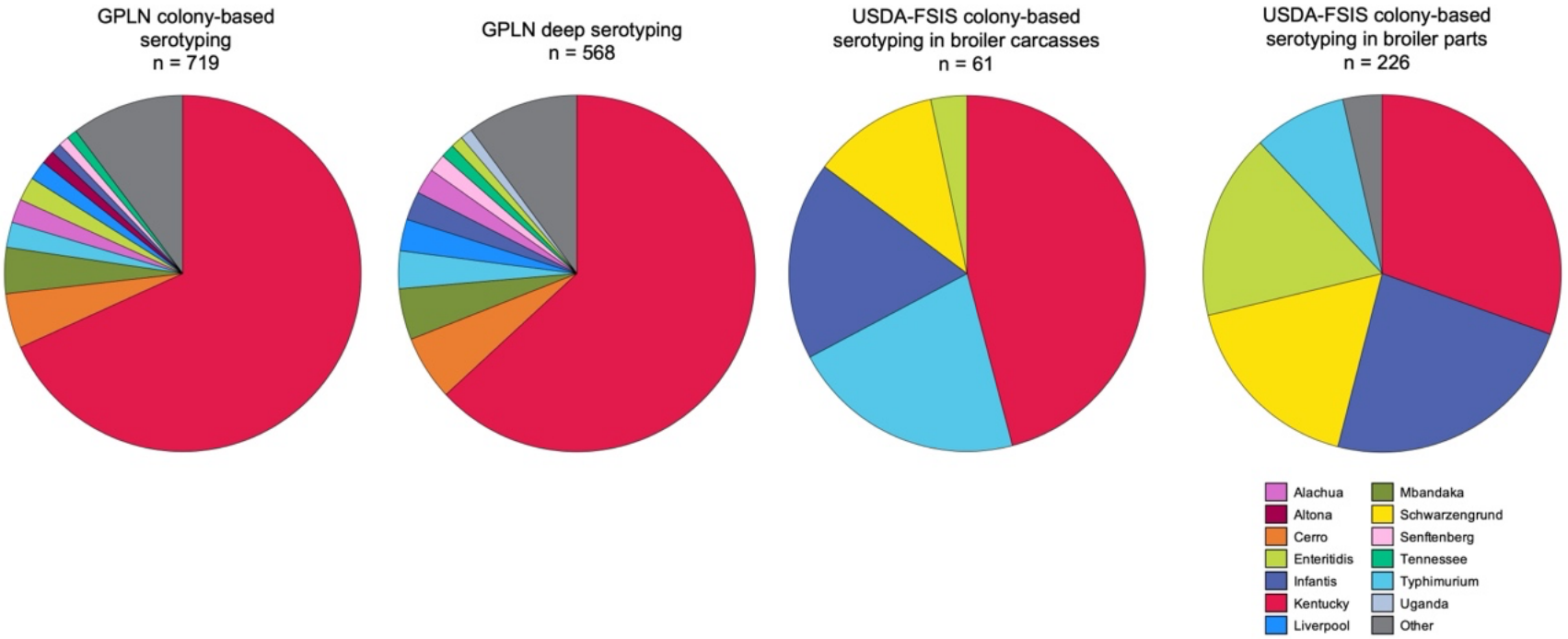

Figure 3-3. Serotyping results of breeder and broiler samples collected in Georgia from August 2020 to June 2022. The two pie charts on the left represent serotyping results from environmental samples sent to GPLN; only the 10 most frequently identified serovars are reported. The leftmost pie chart contains the CRISPR-SeroSeq results for the GPLN subset analyzed in this study, while the second pie chart includes all samples traditionally serotyped (i.e., colony-based) during the study dates. The two pie charts on the right indicate traditional serotyping results from samples routinely collected at domestic processing establishments by USDA-FSIS, separated by sample type of parts (i.e., legs, breasts, wings) and whole carcasses; only the 5 most frequently identified serovars are reported (Raw Poultry Sampling, https://www.fsis.usda.gov/news-events/publications/raw-poultry-sampling, accessed 5 April 2023.)

carcasses)). Alternatively, serovar Infantis was found 3.3% (19/568) and 0.97% (7/719) of breeder samples through deep serotyping and conventional serotyping, respectively. Similarly, serovar Typhimurium was identified in 4.4% (25/568) of samples with deep serotyping and 2.4% (17/719) of conventionally serotyped breeder samples. Notably,serovar Schwarzengrund was not present in the top 10 serovars isolated from breeder flocks while it was often found at processing. Overall serovar diversity was greater in the pre-harvest samples, with similar profiles observed from both deep serotyping and conventional serotyping due to the selection and typing of multiple colonies for isolation at GPLN, according to the National Poultry Improvement Plan (NPIP) *Salmonella* isolation protocols (United States Department of Agriculture - Animal and Plant Health Inspection Service, 2024).

To effectively control *Salmonella*, it is necessary to not only identify all serovars present but also to recognize the sources and transmission patterns of *Salmonella*. Thus, to determine the level of on-farm *Salmonella* transmission, we chose a subset of samples submitted to GPLN representing multiple breeder houses on the same farm that were collected on the same day; this subset is not mutually exclusive from the main GPLN dataset due to the instances where a paired house sample was also the representative sample for the flock accession. In total, there were 322 boot sock samples, each representing a single breeder house across 129 farms. The number of houses on each farm ranged from two to eight houses. The majority of these samples (82%; 265/322) contained only a single serovar, which, as expected, was predominantly serovar Kentucky. There was an average of 1.3 serovars per sample, with a total of 38 farms that contained at least one multiserovar population (i.e., on a single boot sock) (Fig. 3-4). In

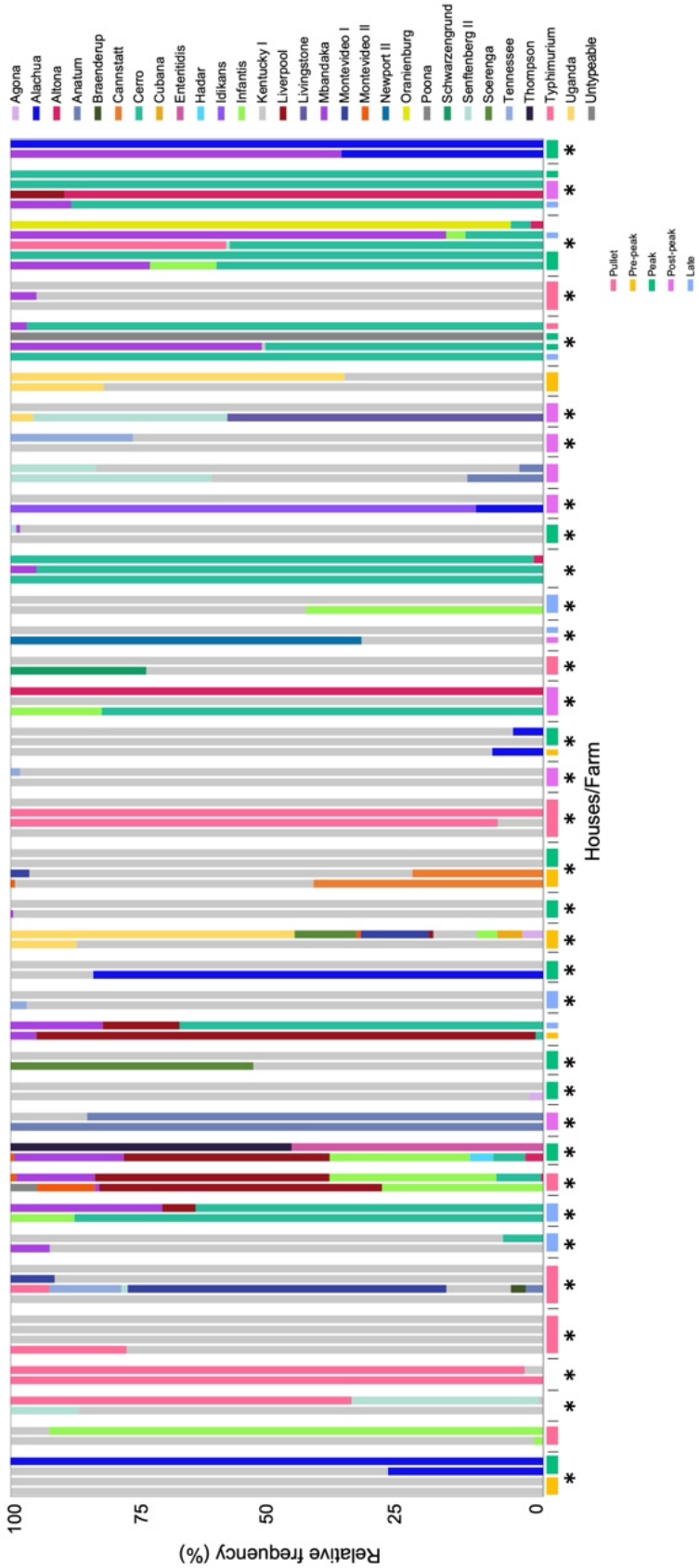

Figure 3-4. Multiserovar populations observed among multiple farms with several pullet/breeder houses. Deep serotyping results for farms containing multiserovar populations (38/129) are shown, with samples from the same farm indicated by the vertical lines, age of flock represented by the colored rectangle, and farms with at least one serovar not shared amongst all the houses are denoted with an asterisk. Flocks without provided age information are shown without a corresponding-colored rectangle. Pullet: 0 – 21 weeks, pre-peak: 21 – 28 weeks, peak: 28 – 35 weeks, post-

34 of these 38 farms, there was at least one serovar present that was absent in another house on the same farm. Additionally, there were 10 farms comprised of single serovar populations where at least one house contained a separate serovar from the rest (Fig. S3-2). From the entire paired house dataset (129 farms, 322 houses), the Bray-Curtis dissimilarity was calculated pairwise for all houses on a farm and then averaged to determine the similarity of populations; 70% (90/129) of farms contained similar populations (Bray-Curtis: 0 – 0.3), with the remaining farms consisting of moderate similarity (23/129; Bray-Curtis: 0.3 – 0.7) or dissimilar populations (16/129; Bray-Curtis: 0.7 – 1). Additionally, an ANOVA model indicated the Shannon diversity index based on present serovars varied with age class ($p < 0.005$). Collectively, these results demonstrate that serovar complexity may be influenced by the presence of multiple houses on one farm but also depends on the age of the flock.

The high-resolution viewpoint of *Salmonella* populations in breeder flocks provided above is useful to identify broad patterns but we next sought to more closely investigate whether *Salmonella* incidence and serovar population dynamics change through the lifetime of individual flocks. For this longitudinal study, 394 boot sock samples were collected from 15 pullet houses (P1-P15, across five flocks) and 13 breeder houses (B1-B13, across eight flocks; sourced from the 15 pullet houses) across two commercial broiler breeder complexes (1 and 2) over a 65-week production period (Fig. S3-3). Importantly, Complex 1 employed an integrated pest control service to control rodent and insect populations, while Complex 2 relied on farm staff. Sampling was increased during peak production (i.e., when the hens are laying the most eggs; weeks 29-31 in this study) because we hypothesized that the birds would be shedding more

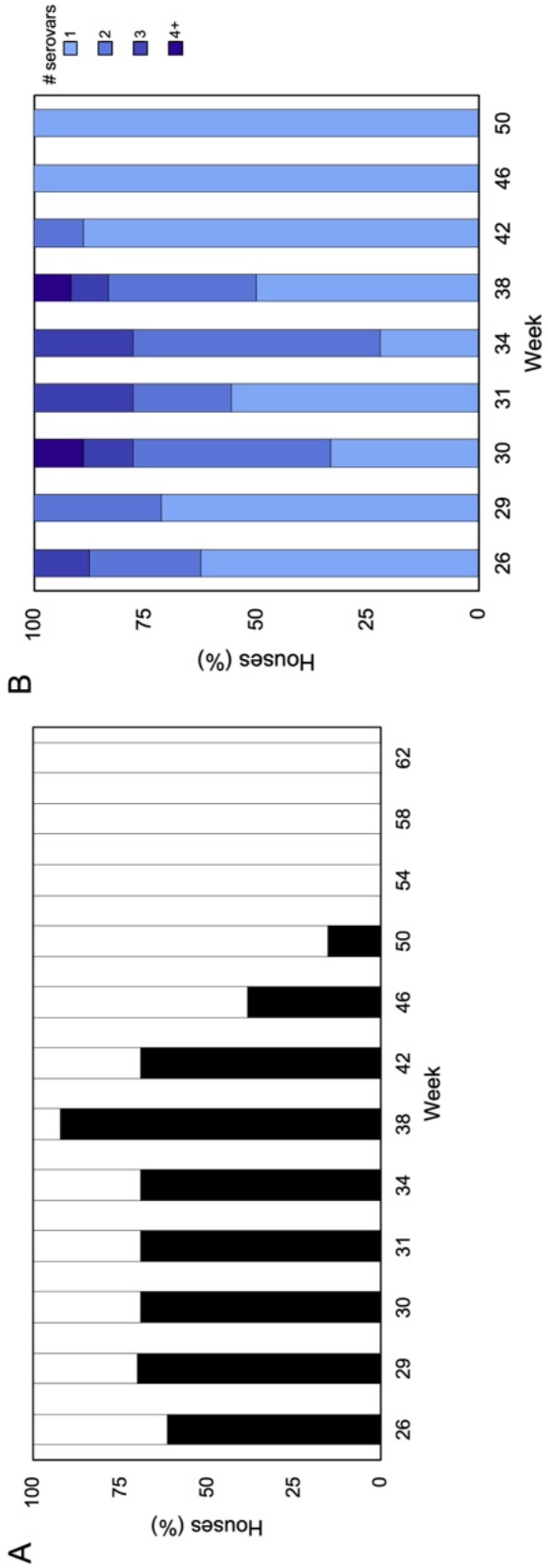

Figure 3-5. *Salmonella* prevalence and serovar complexity throughout one breeder production cycle. A) Proportion of breeder houses (n = 13) that were positive or negative for *Salmonella* during each sampling week (n = 12). One farm was inaccessible during week 29, so only 10 houses are represented at that timepoint. B) Distribution of single and multiserovar populations identified in each house. Spearman's rank correlation suggests that there is a stronger association between prevalence and complexity ($r_s = 0.79$, $p = 0.01$ (Spearman's rank correlation rho)).

Salmonella during this time due to stress; however, we found that *Salmonella* prevalence peaked at 38 weeks (Fig. 3-5A). Overall, 37% (146/394) of samples were *Salmonella*-positive, with a prevalence of 17% (11/64) and 41% (135/330) from pullet and breeder samples, respectively. Only the pullet houses in Complex 2 were positive for *Salmonella* (6/7 houses), while 92% (12/13) of breeder houses across both complexes were *Salmonella*-positive at least one sampling point (Fig. S3-3). Importantly, only two breeder flocks were positive at week 50 (flocks B1 and B2, which were on the same farm), and no flocks were positive after this time. We observed a parabolic curve of the prevalence over the duration of the study, such that the prevalence increased until week 38, and this was accompanied by a corresponding increase in multiserovar populations (Fig. 3-5B; $r_s = 0.79$; $p = 0.01$, Spearman's rank correlation r).

Deep serotyping of breeder flocks detected five serovars in Complex 1 and 15 serovars in Complex 2 (Fig. 3-6; $p < 0.00005$, Shannon diversity index with Hutcheson t-test). There was a maximum of nine serovars detected from one flock (B11, week 38), with an average of 1.6 serovars per sample. Four serovars were found in pullet flocks from Complex 2. Two out of the four serovars (Kentucky and Schwarzengrund) identified in the pullets were also found in the corresponding breeder flocks. In pullets, 18% (2/11) of boot socks contained more than one serovar, while 38% (51/135) of boot socks from breeders had multiserovar populations. Serovar Kentucky was the most predominantly identified serovar from the breeder flocks, being detected in all (n = 12) breeder flocks that were *Salmonella*-positive. Serovar Mbandaka was also frequently detected (5/6 flocks from Complex 2). Of note is that serovar Mbandaka was only detected in one source pullet flock (P13). Across the two complexes, serovar complexity

Figure 3-6. *Salmonella* prevalence and serovar distribution in pullet and breeder flocks across two complexes. Pullet (blue) and breeder (red) houses are shown; *Salmonella*-positive houses are indicated by shading. The breeder flocks that originated from shared pullet flocks are indicated by the numbering. The prevalence indicates whether or not a house was positive (+) or negative (-) for *Salmonella* on the corresponding sampling week. The relative serovar frequencies are reported as determined by deep serotyping via CRISPR-SeroSeq. *The houses on Complex 2 Farm 1 (2-1) had birds placed the morning of week 21 so the houses were not empty during sampling, but they were cleaned out prior.

was highest in samples collected during weeks 30, 31, 34, and 38 (Fig. 3-5B; $p = 0.02$, Fisher's exact test). Collectively, these data demonstrate that *Salmonella* serovar diversity differs between complexes and management strategies, namely integrated pest control, and *Salmonella* surveillance could be optimized around 34-38 weeks.

To evaluate whether *Salmonella* transmission occurs between rodents and breeder flocks, we tested rodents collected on the breeder farms, both inside and outside the houses. During the production cycle and immediately following farm depopulation, the gastrointestinal (GI) tract of 355 rodents (49 composite samples with a maximum of 10 GI tracts included for individuals of the same species that were captured from the same house) were cultured for *Salmonella*, along with 33 bait station swabs. House mice provided the majority of GI tracts (300/355; 38/49 composite samples), followed by roof rats (46/355; 8/49), and Norway rats (9/355; 3/49). In total, 35% (17/49) of composite samples and 9% (3/33) of bait station swabs were *Salmonella*-positive, and six serovars were identified (Fig. 3-7). None of the Norway rats were positive for *Salmonella*, while 50% (4/8) of the roof rat and 34% (13/38) of the house mice composite samples were positive. As observed within the breeder flocks, serovar Kentucky was most often present within the rodent samples as well. Serovar Mbandaka was only recovered in the bait station swabs although it was also identified in the boot socks collected from the breeder flocks. Interestingly, serovars Anatum, Cubana, and Enteritidis were isolated exclusively from rodents and not any flock samples, demonstrating that external factors may influence cyclical transmission and rodent populations can introduce *Salmonella* to breeder flocks.

Week	Flock	Sample	n	RV	TT	Anatum	Cubana	Enteritidis	Hadar	Kentucky	Mbandaka
pre-placement	1-1	Bait station swab	-		x						
pre-placement	1-1	Mouse	1	x							
pre-placement	1-1	Mouse	1		x						
end of placement	1-1	Mouse	10		x						
mid-placement	1-3	Mouse	4	x							
end of placement	1-3	Mouse	10		x						
end of placement	1-3	Roof rat	1	x							
end of placement	1-3	Roof rat	1		x						
pre-placement	1-5	Mouse	10	x							
pre-placement	1-5	Mouse	10		x						
pre-placement	2-1	Mouse	10		x						
pre-placement	2-1	Mouse	10		x						
mid-placement	2-1	Bait station swab	-		x						
mid-placement	2-1	Mouse	1		x						
mid-placement	2-1	Mouse	1		x						
mid-placement	2-1	Mouse	8		x						
mid-placement	2-1	Roof rat	1		x						
end of placement	2-1	Mouse	10		x						
end of placement	2-1	Mouse	7	x							
end of placement	2-1	Mouse	7		x						
pre-placement	2-2	Bait station swab	-		x						
mid-placement	2-2	Roof rat	1		x						

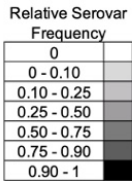

Figure 3-7. *Salmonella* serovars isolated from rodent composite samples and bait station swabs. The week column corresponds to the flock age when sampling, n includes how many individual rodents comprised the composite sample, and the RV/TT columns indicate the selective media that *Salmonella* was recovered from. The relative serovar frequencies are reported as determined by deep serotyping via CRISPR-SeroSeq. Three of the identified serovars were also found in the corresponding flock samples.

Discussion

This study, to our knowledge, is the first to characterize changes in multiserovar populations over time in breeder flocks and also to document significant patterns of *Salmonella* serovar co-occurrence in any animal production system. About 20% (104/568) of breeder flocks from this study contain multiple *Salmonella* serovars, which demonstrates the need for routine surveillance to identify all serovars present to properly assess the risk and apply mitigation strategies. Our previous work (Siceloff et al., 2022) with a subset of samples from the GPLN dataset found 32% (43/134) contained multiple serovars; this difference in multiserovar populations may be attributed to the four-fold increase in the number of isolates/samples in the dataset which may have led to a decrease in multiserovar prevalence but overall increase in serovar diversity, as the current study identified 38 serovars while the previous found 26 serovars. Serovar diversity may still be underrepresented in this study as we only analyzed one boot sock collected from each flock. Other work, albeit in broiler flocks, not breeders, has demonstrated the need to collect two boot sock pairs for a more complete understanding of the *Salmonella* population dynamics (Obe et al., 2023). In that study, which began after we started the current study, it was noted that a single boot sock pair from a broiler house was not always sufficient to capture the full serovar diversity in a single house because in 33% of instances, deep serotyping data from a second boot sock pair contained another serovar. Here, in our 15-house longitudinal study, 20% of instances required two boot socks. Nonetheless, deep serotyping identifies more serovars than isolated by culture alone (38 serovars with CRISPR-SeroSeq vs 32 serovars with colony

picking in 568 GPLN samples) and so provides a better idea of the complexity of serovar ecology within our dataset.

From surveillance sampling through GPLN, 35% of breeder flocks were *Salmonella*-positive, while 43% of samples from the longitudinal study were positive. Since GPLN receives the most samples around 16 and 42 weeks, the resulting prevalence may be an underestimation as samples from flocks around the peak and late age classes are not submitted as often. Together, the overall prevalences are comparable to a longitudinal study conducted in Australia, where 36% of breeder flocks were *Salmonella*-positive but higher than the prevalence observed from breeder flocks in Ontario, Canada (25%)(Murray et al., 2023; Willson and Chousalkar, 2023). Our results differ from the Australian study with regards to peak *Salmonella* prevalence as they found their highest number of positive samples at week 7. These results may differ due to geography and different management and production practices between the United States and Australia. Further, serovar profiles as detected by deep serotyping may not be wholly reflective of native *Salmonella* populations within hosts as selective enrichment is required prior to sequencing and may promote media bias. Previous work has demonstrated that media bias exists, such that some serovars may be preferentially enriched in one medium when compared to another, and this may be partially overcome by the use of multiple enrichment media (Gorski et al., 2024). In this study, only tetrathionate (TT) broth was used in culturing the breeder boot sock samples since we opted to follow industry standards, as prescribed by NPIP, and so, we acknowledge that the resulting serovar profiles may be skewed.

Our results from GPLN show that *Salmonella* prevalence is highest during peak production (28-35 weeks), while our longitudinal study, which was limited to two complexes, suggest that peak prevalence occurs at 38 weeks. Another difference in the two studies presented here is that in the GPLN data, 36% (286/800) of flocks older than 50 weeks were positive for *Salmonella*, while in the longitudinal study, none of the flocks were positive after 50 weeks. Additionally, some serovars were more abundant in the GPLN dataset when compared to the longitudinal study, including the frequent identification of both serovars Cerro and Mbandaka. Therefore, while this study demonstrates that broad surveillance approaches can generate strong trends with respect to *Salmonella* prevalence, integrators should consider that their complexes may differ in terms of determining the peak shedding period and the serovar profiles. This is important since optimizing *Salmonella* surveillance can lead to the development of targeted management approaches, such as vaccination.

We were intrigued by the prevalence of and the interactions between Cerro and Mbandaka from the GPLN dataset. Identifying these serovars at a relatively high frequency (7.6% and 6.0%, respectively) was unexpected as they are commonly found in cattle but not in broilers. Further, they are rarely found in poultry products; between 2016-2023, FSIS found serovars Cerro and Mbandaka in 0.045% (4/8853) and 0.21% (19/8853) *Salmonella*-positive broiler samples, respectively, and none originated from facilities in Georgia. A recent study in four broiler complexes found low prevalence of these serovars; serovar Cerro was found in one of 68 positive houses, and serovar Mbandaka in four of the houses, including the same house where Cerro was detected (Obe et al., 2023). Therefore, beyond breeders, the incidence of these two serovars in

poultry production and processing is significantly reduced. There are three potential explanations for this. First, it is possible that these serovars are entering in feed. Meat and bone meal are a common feed source for chickens, and would explain the presence of cattle-associated serovars since multiple animals may be included in the ground product. Breeders are typically fed a mash diet, while broilers are fed a pelleted diet. Importantly, extrusion during the pelleting process serves as an additional pathogen reduction step, which may explain the lower incidence in broilers. This also opens the possibility that these serovars may not be present in the birds themselves (or may not be actively shed), and that the industry standard of environmental boot sock sampling is detecting *Salmonella* in feed that has fallen on the slats/floor. This is also supported by the serovar profile observed in house B11 at week 38, which includes serovars Mbandaka and Cerro, as well as serovar Rissen (most often found in swine). At the following sampling (42 weeks), the house was *Salmonella* negative. Second, since many poultry growers also have cow-calf operations, this practice may serve as a potential entry source for these serovars. Given that we observed the co-occurrence of serovar Cerro and Mbandaka in 19 different flocks, we think this is unlikely. The third explanation is that the application of *Salmonella* vaccines in breeders is suppressing specific serovars (i.e., serovars Typhimurium, Enteritidis, and Infantis) and that this provides the opportunity for less competitive serovars such as Cerro and Mbandaka to colonize breeders. Where vaccine pressure is subsequently reduced in broilers, these serovars could then be replaced by those that are better adapted to poultry. Vaccine pressure could also explain the low incidence in our study of serovars that are often found at processing, including Typhimurium (4.4%; 25/568), Enteritidis (1.4%; 8/568), and Infantis (3.3%; 19/568). For

example, during the same time frame, serovar Infantis was found in 18% (11/61) and 23% (53/226) of regulatory carcass and parts samples, respectively (Fig. 2). Two potential but non-exclusive explanations are that i) broiler flocks become more broadly colonized by serovar Infantis due to reduced vaccine pressure in broilers and increased environmental presence of serovar Infantis in broiler houses allows broilers to become colonized; and ii) there is some selection for serovar Infantis during processing, perhaps during chilling, though a recent paper did not find evidence of selection for serovar Infantis (Richards et al., 2024).

There has been a substantial amount of work conducted to explore the physiological traits of select serovars of animal or human clinical importance, namely serovars Enteritidis and Typhimurium. However, studying the growth dynamics within multiserovar populations are a more recent consideration. In one elegant study, the fitness of two serovars (Kentucky and Typhimurium) did not differ when grown individually in chicken cecal contents. Rather, limited growth of serovar Typhimurium only manifested when co-cultured alongside serovar Kentucky (Y. Cheng et al., 2015). Further, a cell invasion-deficient serovar Kentucky strain did not have reduced colonization in chickens compared to a cell invasion-proficient strain of serovar Typhimurium, which supports the finding that differential growth rates in host can be driven by stress response pathways rather than virulence factors (Cheng et al., 2015). An additional study found that serovar dominance in mixed populations may simply be dependent on which serovar colonized the host first (Yang et al., 2018). Competitive exclusion has been utilized to inhibit *Salmonella* colonization in poultry production, but additional work is required to characterize this phenomenon in multiserovar populations and identify the driving forces

in serovar dominance (Bailey et al., n.d.; Soerjadi et al., 1981; Nisbet et al., 1998; Nava et al., 2005; Methner et al., 2011; Micciche et al., 2018; Bucher et al., 2020; Pineda et al., 2021; Maurer et al., 2024). Similarly to other control strategies, such as vaccination, competitive exclusion may have unexpected consequences as the removal of one serovar from a system leaves an open niche for another, potentially higher risk, serovar (Rabsch et al., 2000; Foley et al., 2011).

There are opportunities for pathogen colonization at each stage of poultry production, as the individual components of feed could be contaminated and distributed among farms, eggs with excess fecal content could spread pathogens from farm to hatchery, or any lapse in on-farm biosecurity could serve as an introduction event (Dale et al., 2015; English et al., 2015; Rajan et al., 2017; Vinueza-Burgos et al., 2019; Machado Junior et al., 2020; Wang et al., 2023). We observed similar serovar profiles between multiple houses on one farm, as 70% (90/129) of farms contained similar populations (Bray-Curtis: 0 – 0.3), emphasizing the need to ensure that on-farm biosecurity is promoted to prevent *Salmonella* introduction and transmission. Rodents may act to introduce *Salmonella* to flocks since they are known to be vectors, and this observation was supported in our study as we found several matching serovars between rodent and breeder boot sock samples. However, further characterization to the strain level is required to confirm transmission in this study. One previous study isolated the same strain of serovars Enteritidis and Typhimurium (Liljebjelke et al., 2005) between rodents and flocks. This finding underscores the importance of pest control on farm towards reducing *Salmonella* in the flocks. There have been limited studies conducted on the role of rodents in on-farm *Salmonella* transmission, and most have focused on layer

flocks, so future work is required to understand the impact of rodents upon *Salmonella* diversity (Henzler and Opitz, 1992; Davies and Wray, 1995; Guard-Petter et al., 1997; Garber et al., 2003; Meerburg and Kijlstra, 2007; Lapuz et al., 2012; Guard et al., 2018; Camba et al., 2020). Additionally, we noted higher *Salmonella* prevalence and greater serovar complexity in breeder flocks that were positive as pullets. This may indicate that early monitoring and response in pullets is a good strategy for reducing *Salmonella* in breeders. Since we collected noninvasive environmental samples, there is a possibility that these serovars were present in the pullet flocks but remained undetected due to low quantity or lack of shedding at the time of sampling. However, since we began sampling after the pullets had been in the houses for 14 weeks, we would expect to find evidence of *Salmonella* colonization in the litter. As the longitudinal study was only across two complexes, further studies would be needed to confirm the impact of *Salmonella* incidence from pullets to breeders.

While this study only included breeder flocks from the southeast, the framework presented here provides support to develop robust *Salmonella* surveillance at any stage of live production. The results are broadly applicable to the domestic poultry industry as Georgia contributes 14% of broilers to the national poultry production (United States Department of Agriculture - National Agriculture Statistics Service, 2023). The observed population dynamics demonstrate that select serovars can impact the presence of others, underscoring the importance of future work to explore interserovar relationships and physiological mechanisms behind competitive exclusion of serovars. To that end, this study also demonstrates the need for high-resolution surveillance approaches, as characterizing serovar interactions and developing targeted solutions requires the reliable

and robust detection and relative quantification of all present serovars. The pre-harvest reduction of *Salmonella* in of all types of food animal production systems supports further reductions at processing, so it is critical to understand the driving factors behind population dynamics in food animal production and enact effective control strategies. The framework presented here can be applied to other food animal production systems where *Salmonella* is a problem. Finally, amplicon-based approaches can be extended to other infectious organisms that occur in mixed populations to investigate relationships among bacterial subtypes or among viral variants.

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Author Contributions

ATS: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization; **DW:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition; **SPN:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration; **CEG:** Methodology, Software, Validation, Formal analysis, Data curation, Writing – review & editing, Visualization;

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CHAPTER 4

OPTIMIZING *SALMONELLA* RECOVERY FROM COMMERCIAL POULTRY ENVIRONMENTAL SAMPLES WITH SELECTIVE PRE-ENRICHMENT[1]

[1]Siceloff, AT and Shariat, NW. To be submitted to the Journal of Food Protection.

Abstract

The current, culture-based method for detecting *Salmonella* is time and resource intensive, as it can take between three to five days with pre-enrichment and selective enrichment steps. Previous work by our group has shortened this process by combining novobiocin and selective ingredients from Rappaport-Vassiliadis (RV) (malachite green; 0.1 g/L) and tetrathionate (TT) (bile salts; 1 g/L) to BPW in parallel, creating an all-encompassing selective pre-enrichment step. In this study, we sought to validate the use of selective pre-enrichment on commercial poultry live production samples, as the increased presence of background bacteria may limit *Salmonella* recovery. Two pairs of boot sock samples were collected from 35 houses, representing 17 different commercial broiler or breeder farms (n = 70 samples). The samples were cultured under selective pre-enrichment conditions in parallel with standard non-selective pre-enrichment (BPW) followed by selective enrichment (RV, TT). Additionally, molecular enumeration was performed to quantify the amount of *Salmonella* present in each sample. Overall, *Salmonella* was found in 74% (52/70) of samples collected, and selective pre-enrichment and selective enrichment conditions each recovered *Salmonella* in 14/17 farms. There was no significant difference in the *Salmonella* recovery between selective pre-enrichment (n = 41 positives) and selective enrichment (n = 52 positives) (p = 0.07, Chi-squared test). However, the average quantity was greater in *Salmonella*-positive samples recovered with selective pre-enrichment (5.2 $\log_{10}$ CFU/sample) than those that were not recovered (3.0 $\log_{10}$ CFU/sample) (p = 0.01, Welch two sample t-test). CRISPR-SeroSeq was employed to quantify the relative frequency of *Salmonella* serovars in each sample and culture condition. An ANOVA model indicated the Shannon diversity index based on

present serovars did not vary between culture conditions ($p = 0.1$). These findings suggest that increasing the selectivity of the *Salmonella* pre-enrichment step could eliminate the need for a separate selective enrichment step without compromising serovar diversity, thus reducing the time to *Salmonella* isolation by 24 hours.

Introduction

Salmonella is a leading bacterial cause of foodborne illness, with an estimated 1.35 million cases and 420 deaths annually in the United States, resulting in an estimated annual economic burden of over $4 billion (Centers for Disease Control and Prevention, n.d.-b; United States Department of Agriculture - Economic Research Service, 2021). Poultry is a considerable source of these outbreaks, with one in five cases attributed to chicken products (Interagency Food Safety Analytics Collaboration, 2024). Despite improvements in *Salmonella* mitigation during commercial poultry processing, there has not been a corresponding decrease in the number of cases linked to these products (Interagency Food Safety Analytics Collaboration, 2024; United States Department of Agriculture - Food Safety and Inspection Service, n.d.). The United States Department of Agriculture – Food Safety and Inspection Service (USDA – FSIS) has issued a directive to reduce *Salmonella* illnesses by 25% (“Healthy People 2030”), which encourages the development of preharvest controls to decrease postharvest *Salmonella* contamination. To accomplish this, accurate and rapid *Salmonella* surveillance is required to support the development of appropriate on-farm management strategies.

Conventional *Salmonella* isolation relies on culture-based approaches, which include separate recovery and selective enrichment steps, and takes several days to complete (International Organization for Standardization, 2017). The culturing process

typically begins by homogenizing the sample with a non-selective media, such as buffered peptone water (BPW) or universal pre-enrichment broth (UPB), followed by a 24-hour incubation to allow for recovery of any injured salmonellae. The enriched BPW culture is then aliquoted into selective enrichment media, such as Rappaport-Vassiliadis (RV) and tetrathionate (TT) broths. Collectively, the pre-enrichment and selective enrichment steps take around 48 hours to complete. Following selective enrichment, the cultures are plated onto selective and differential agar plates, such as xylose lysine tergitol-4 (XLT-4) or brilliant green sulfa (BGS), and incubated for 24-48 hours. Typically, one to three presumptive *Salmonella* colonies are selected and confirmed using various molecular, biochemical, or serological tests (Andrews et al., 2018; United States Department of Agriculture - Food Safety and Inspection Service, 2024). In total, conventional culturing protocols can take up to five days to isolate and confirm *Salmonella*. Some workflows may reduce time required as certain matrices enable bypassing of pre-enrichment if the salmonellae are not presumed to be injured, such as on-farm environmental samples that follow the National Poultry Improvement Plan (NPIP) protocol (United States Department of Agriculture - Animal and Plant Health Inspection Service, 2024). However, previous work has demonstrated that some serovars are preferentially enriched in different media, so starting the isolation process with selective enrichment broth may save 24 hours but observed serovar diversity and abundance may be impacted (Gorski, 2012; Gorski et al., 2024; Pettengill et al., 2012). Additionally, molecular-based detection methods may reduce time to a confirmed *Salmonella*-positive sample, but these do not culminate in an isolate, which is required for further characterization and also to demonstrate cellular viability (Centers for Disease

Control and Prevention, 2024). As such, it is critical to shorten the time to a confirmed *Salmonella* isolate, so that corrective measures may be taken sooner rather than later and the risks associated with potential outbreaks can be minimized.

Aside from the time required, the bias for improved growth of some serovars in one selective enrichment broth over another is also of concern (Cox et al., 2019; Obe et al., 2021; Rasamsetti et al., 2022). For example, serovar Enteritidis seems to grow more readily in TT broth than RV broth (Gorski et al., 2024). This occurrence may be attributed to the different metabolic capacity of serovars, but the mechanisms of selective media bias have not been broadly studied. Additionally, some serovars do not produce hydrogen sulfide, which is an important phenotypic characteristic used to identify *Salmonella* on indicator agar such as XLT-4 and double modified lysine iron agar (DMLIA) (Mallinson et al., 2000). To address these media biases, some protocols include the use of two selective enrichment media in parallel, as well as multiple agar plates. To further complicate the issue, multiple *Salmonella* serovars may be found within a sample but typically only the most abundant serovar is identified through standard isolation, as only a few colonies are picked. For a 95% probability of detecting two serovars that share the same colony morphology, the serovars must exist in equal abundances and six colonies must be picked for characterization (J. Cason et al., 2011); this is not feasible on a larger scale due to time and resource constraints. As such, some serovars may evade detection due to fluctuating population dynamics during selective enrichment and conventional method limitations. Ultimately, accurate and reliable *Salmonella* screening requires an approach that can detect all serovars present within a population, irrespective of culturing conditions.

CRISPR-SeroSeq is a next-generation sequencing approach to quantify the relative abundances of multiple serovars present in a sample based on the native CRISPR arrays (Thompson et al., 2018). *Salmonella* contains two conserved CRISPR arrays with conserved direct repeats (29 nucleotides) and variable spacer sequences (32 nucleotides); this system formerly served as a prokaryotic adaptive immune response, such that spacer sequences were added onto the arrays from foreign nucleic acids, but this is no longer active (Touchon & Rocha, 2010). However, the CRISPR arrays are still intact in *Salmonella* genomes and can provide a metric to differentiate between serovars as each has a unique spacer profile (Bugarel et al., 2018; Fabre et al., 2012; Kushwaha et al., 2020; Pettengill et al., 2014; Shariat et al., 2015). We have applied CRISPR-SeroSeq for deep serotyping in a variety of sample matrices, such as poultry carcass and parts rinses, bird and cattle feces, environmental boot socks, and freshwater, (E. E. Cason et al., 2024; Deaven et al., 2021; Obe et al., 2023; Rasamsetti & Shariat, 2023; Siceloff et al., 2021; Smith et al., 2023). In each of these cases, CRISPR-SeroSeq has provided a higher resolution profile of complex *Salmonella* populations than by colony isolation alone, demonstrating its utility for improved *Salmonella* surveillance.

Increasing the selectivity of the pre-enrichment step can reduce time required for isolation by 24 hours while still promoting *Salmonella* growth. This can be accomplished by adding in selective ingredients from standard enrichment media to limit competing gram-negative bacteria, along with an antibiotic (novobiocin) to remove any gram-positive bacteria. By condensing the conventional process of *Salmonella* isolation into a single selective pre-enrichment step, it is possible to directly streak enriched culture onto indicator plates and determine if *Salmonella* is present within two days of sample

collection. Critically, there must be a balance of selective ingredients in the pre-enrichment conditions to generate a harsh environment to minimize background flora proliferation without also inhibiting *Salmonella* growth. Previous work by our group successfully developed a selective pre-enrichment protocol with validation testing on poultry carcass rinses (Rasamsetti et al., 2021). This sample type provided an opportunity to determine the optimal concentration of selective ingredients for recovery of potentially injured *Salmonella.* However, there were not significant levels of background coliforms which compete against *Salmonella*, such as various *Campylobacter* and *Escherichia* species, since they are typically reduced following antimicrobial interventions during processing (De Villena et al., 2022). As such, we sought to validate the use of selective pre-enrichment conditions for *Salmonella* recovery from complex on-farm environmental samples. We hypothesized that the harsh conditions of a commercial poultry processing plant may reflect different dynamics of *Salmonella* recovery and growth than the conditions at commercial poultry farms, and so, selective pre-enrichment may not be sufficient for *Salmonella* isolation from environmental farm samples. Additionally, we evaluated if quantity of *Salmonella* is a limiting factor in the use of selective pre-enrichment methods.

Materials and methods

Sample collection

Boot socks were used to collect environmental samples from 17 different commercial broiler or breeder farms (n = 35 houses). Two pairs of boot socks pre-moistened in buffered peptone water (BPW) (Romer Labs, Newark, DE) were used for sampling each house, resulting in a total of 70 samples. One pair was used to walk along

the right side of the house and the other on the left side. Samples were collected by walking the entire length of the house between the feed and water lines (Fig. 4-1A). For samples from breeder houses, boot socks were collected by walking on the slats. The samples were kept on ice and transported to the laboratory for processing.

Salmonella isolation and quantification

A total of 200 mL of BPW (Neogen, Lansing, MI) was added to each pair of boot socks and these were stomached at 230 rpm for 2 min using the Stomacher 400 Circulator (Seward, Bohemia, NY)(Fig. 4-1B). For the selective pre-enrichment conditions, the homogenized culture was aliquoted into three separate sterile conical tubes and incubated for four hours at 37°C. Following this initial *Salmonella* recovery period, selective ingredients were added in different combinations with the following final concentrations: novobiocin 0.015 g/L (Thermo Scientific Chemicals, Waltham, MA); bile salts 1 g/L (Ward's Science, Rochester, NY); malachite green 0.1 g/L (Ward's Science, Rochester, NY). Condition 1 was a non-selective BPW control (BPW), Condition 2 contained bile salts and novobiocin (BPW + BS + Nv), and Condition 3 contained malachite green and novobiocin (BPW + MG + Nv). When BPW is used alone, the condition was referred to as non-selective pre-enrichment in the manuscript, while the addition of malachite green, bile salts, and novobiocin in different combinations to the BPW were denoted as selective pre-enrichment. All enrichments continued to incubate for an additional 20 h at 37°C. Following incubation, all broths were streaked for isolation onto xylose lysine tergitol-4 agar (XLT-4; Hardy Diagnostics, Santa Maria, CA) and plates were incubated for 24-48 h at 37°C. Additionally after the initial 24 hours, 0.1 and 1 mL of each BPW-only control sample were subinoculated into Rappaport-Vassiliadis (RV) and tetrathionate (TT)

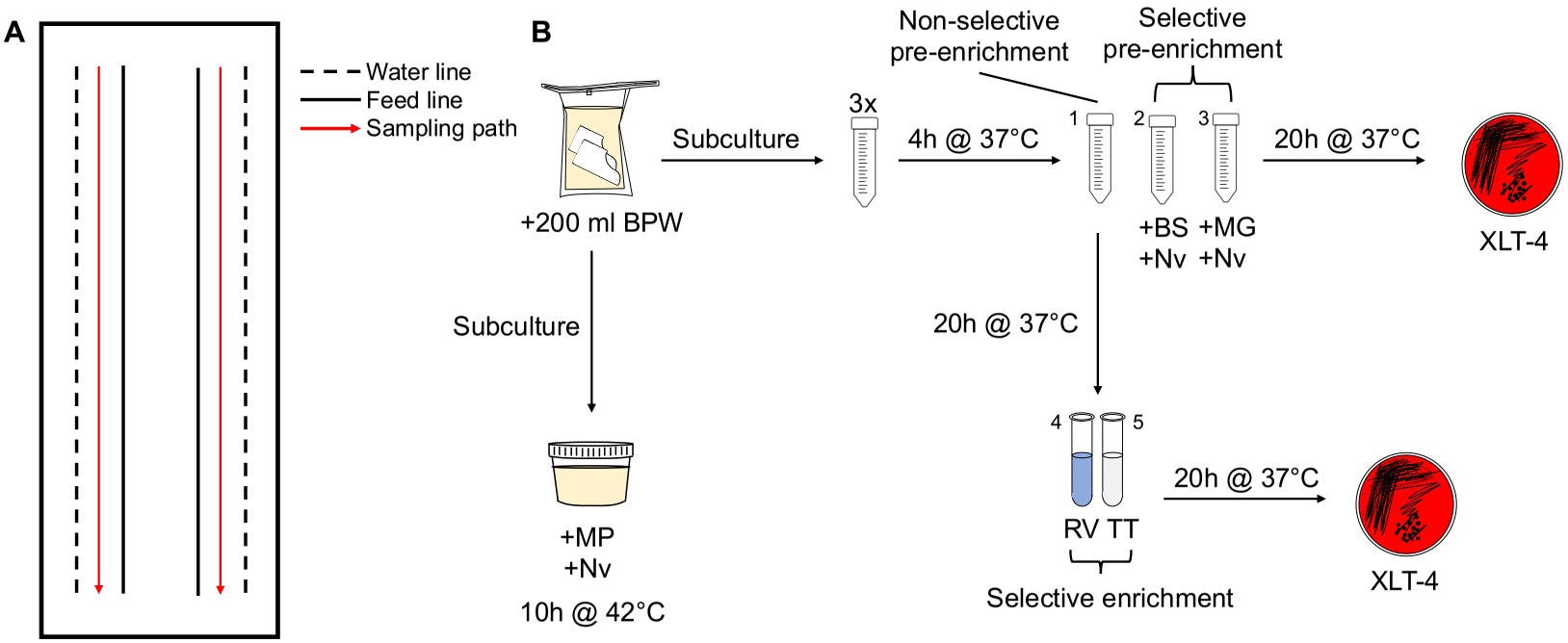

Figure 4-1. A) Sampling schematic. Two pairs of boot socks were collected between the feed and water lines on both sides of the house (n = 35 houses, 70 samples).
B) Workflow for culture conditions. Each boot sock was homogenized with 200 ml BPW, then subcultured into the selective pre-enrichment media used for molecular enumeration (MP + Nv). In parallel, aliquots from each sample were cultured in three different conditions: non-selective enrichment (1), selective pre-enrichment (2,3), and selective enrichment (4,5). 1 – BPW, 2 – BPW + BS + Nv, 3 – BPW + MG + Nv, 4 – BPW into RV, 5 – BPW into TT. All enrichments were plated on XLT-4 for *Salmonella* isolation and confirmation.

BPW, buffered peptone water; Nv, novobiocin; BS, bile salts; MG, malachite green; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth; XLT-4, xylose lysine tergitol-4.

selective enrichment broths (Hardy Diagnostics, Santa Maria, CA), respectively, and incubated at 37°C for 24 h and then streaked on XLT-4. The samples from non-selective BPW, when inoculated into either RV or TT broth represent selective enrichment in the manuscript. XLT-4 plates were examined for development of typical black H_2S colonies, followed by confirmation with serum agglutination (BD Difco, Franklin Lakes, NJ). For *Salmonella* quantification, a 60 mL aliquot of the unenriched BPW sample was transferred to 60 ml of pre-warmed MP media (Hygiena, Camarillo, CA) containing 40 mg/L novobiocin (Thermo Scientific Chemicals, Waltham, MA) and incubated at 42°C for 10 h. At this time, *Salmonella* lysates were prepared following the manufacturer's protocol and stored at 4°C prior to enumeration using the BAX® System SalQuant® (Hygiena, n.d.). Per-sample *Salmonella* quantity was calculated using the provided formulas for boot socks (version 3.6). The limit of quantification (LOQ) is reported to be 1 $\log_{10}$ CFU/sample and culture-positive samples that did not yield a value were scored as 0 $\log_{10}$ CFU/sample.

DNA isolation and CRISPR-SeroSeq.

The overnight cultures for each condition were briefly vortexed, and 1 mL of each was transferred into microcentrifuge tubes and centrifuged at 14,000 rpm for 3 min. The supernatant was removed, and the pellets were stored at 20°C. Total genomic DNA was isolated from pellets using the Promega Genome Wizard kit (Madison, WI) following manufacturer's instructions and then resuspended in 200 mL of molecular-grade water and stored at 20°C prior to use as the template for CRISPR-SeroSeq as described previously (Richards et al., 2024; Siceloff et al., 2022). Serovars were called only if they

contained multiple CRISPR spacers that were unique to that serovar in the sample and had cumulative reads greater than 0.5% of the total population for that sample.

Statistical analysis.

All statistical analyses were conducted with R (version 4.2.3). CRISPR-SeroSeq reads were normalized across culture conditions using the DESeq2 package (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) to adjust the read counts per sample based on the size factors present.

Results and Discussion

In this study, 35 commercial poultry houses (flocks) from 17 farms across multiple integrator complexes were sampled to validate the use of selective pre-enrichment culture conditions for quicker *Salmonella* recovery from a matrix with high microflora. Two pairs of boot sock samples were collected from each house and independently cultured in the various conditions: non-selective enrichment, selective pre-enrichment, and selective enrichment (Fig. 4-1). *Salmonella* presence was confirmed by colony isolation after streaking on an indicator plate and *Salmonella* quantity was enumerated using a commercial kit (BAX® System SalQuant®, Hygiena). A house was considered *Salmonella*-positive when at least one pair of boot socks tested positive and subsequently, a farm was positive if at least one house was. In total, 74% (52/70) samples were *Salmonella*-positive, as determined by the traditional culture conditions (BPW into RV or TT; bottom two rows in Fig. 4-2). In comparison, 59% (41/70) were positive by selective pre-enrichment ($p = 0.07$, Chi-squared test) and 33% (23/70) with BPW alone. Both selective conditions recovered *Salmonella* in 14/17 farms, and on the house level, *Salmonella* was detected in 69% (24/35) and 77% (27/35) of houses using selective pre-

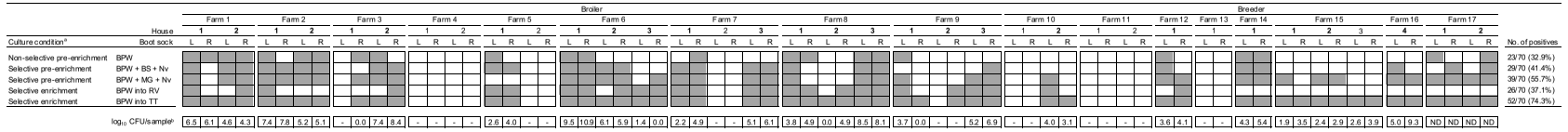

Figure 4-2. Comparison of *Salmonella* recovery among different culture conditions. The farms are separated by broiler and broiler breeder production, with total recovery in 77% (34/44) and 69% (18/26) of samples, respectively. Gray coloration indicates a *Salmonella*-positive enrichment, while white indicates a negative. The house number is bolded to reflect positivity. *Salmonella* quantity, as determined by a commercial molecular enumeration assay, is listed below each sample.

[a] BPW, buffered peptone water; Nv, novobiocin; BS, bile salts; MG, malachite green; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth

[b] -, culture negative sample; 0, below the limit of quantification (LOQ); ND, no data available

enrichment or selective enrichment, respectively. The two best recovery conditions were with malachite green and in TT broth. Notably, malachite green is the primary ingredient in RV, which conflicts with the industry-wide preference of TT enrichment for environmental farm samples. The performance of culture conditions varied between broiler and breeder flocks, such that 7.7% (2/26) of breeder samples had *Salmonella* recovery in RV compared to 55% (24/44) of broiler samples (Fig. 4-3A). This discrepancy is not explained by differences in *Salmonella* quantity between the two production systems, since there was an average of 4.0 and 5.1 $\log_{10}$ CFU/sample for broiler and breeders, respectively ($p = 0.1$, Welch two sample t-test; Fig. 4-3B). However, *Salmonella* quantity affected overall recovery, as the average of *Salmonella*-positive samples recovered in selective pre-enrichment was 3.0 $\log_{10}$ CFU/sample, compared to 5.2 $\log_{10}$ CFU/sample for those recovered only in selective enrichment ($p = 0.01$, Welch two sample t-test; Fig. 4-3C).

Boot socks have been indicated as the best sample type for capturing *Salmonella* within a poultry house, with greater recovery observed than from fecal, organ, or litter grab samples (Buhr et al., 2007; United States Department of Agriculture - Animal and Plant Health Inspection Service, 2024). However, recent work has revealed that boot socks do not provide high levels of reproducibility for *Salmonella* quantification, such that two pairs from a single house can have different quantities (Obe et al., 2023). Additionally, the same study highlighted the importance of collecting two pairs of boot sock per house for a more robust characterization of the *Salmonella* population dynamics. Namely, it was determined that one-third of boot socks collected in a single house had at

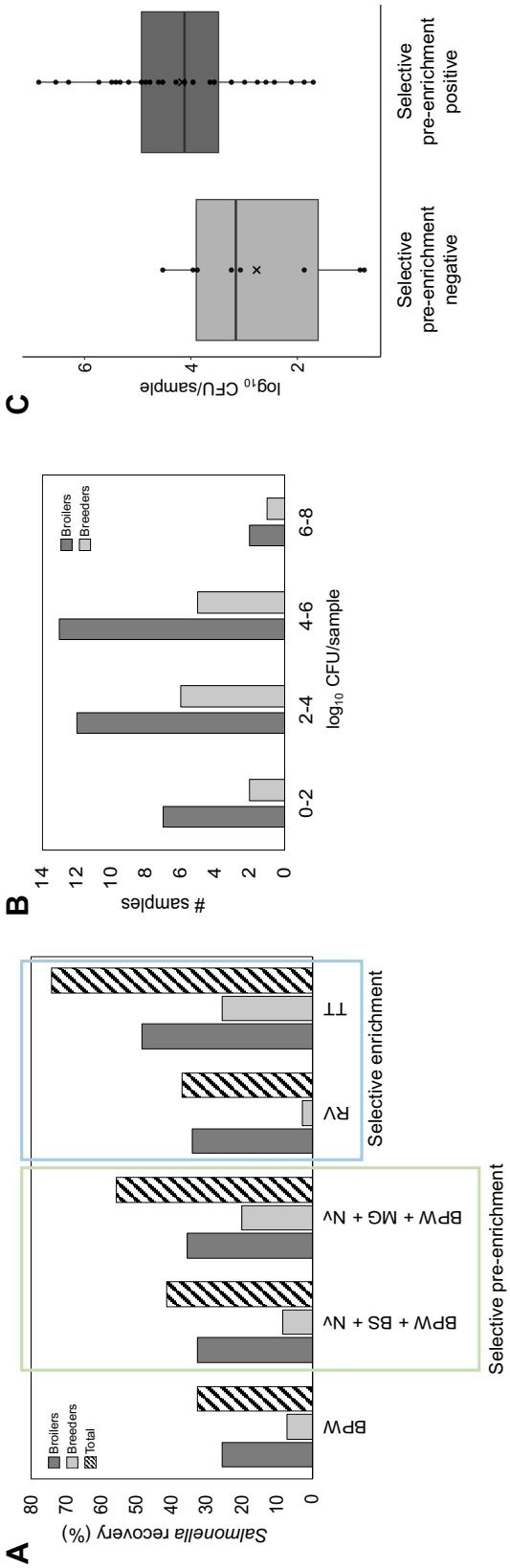

Figure 4-3. A) *Salmonella* recovery varies between broiler and breeder flocks. Selective pre-enrichment recovered *Salmonella* in 41 samples, compared to 52 by selective enrichment ($p = 0.07$, Chi-squared test). **B) Histogram of *Salmonella* quantity in broiler and breeder flocks.** There was an average of 4.0 and 5.1 $\log_{10}$ CFU/sample for broiler and breeders, respectively ($p = 0.1$, Welch two sample t-test). **C) *Salmonella* quantity impacts recovery with selective pre-enrichment.** Quantity distribution is different for samples that were *Salmonella*-positive and were recovered in selective pre-enrichment conditions or not ($p = 0.01$, Welch two sample t-test).

least one unique serovar between the two pairs. Thus, it may be necessary to regularly use two pairs of boot socks to collect environmental samples and screen for *Salmonella* in poultry houses. When combining the results of boot sock pairs, *Salmonella* recovery with selective pre-enrichment and selective enrichment were much more comparable, with a difference of three houses ($p = 0.6$, Chi-squared test). Therefore, if multiple boot socks are collected, selective pre-enrichment may be utilized to reduce turnaround time required to screen for *Salmonella* within commercial poultry flocks.

It is important to consider the average *Salmonella* quantity of a sample type prior to the implementation of selective pre-enrichment, since there was a difference in recovery based on enumeration values. However, there are likely multiple contributing factors which influence the performance of selective pre-enrichment methods, such as composition of background microflora, viability of salmonellae, and presence of external physical or chemical contaminants; this is reflected by the range of quantities in the *Salmonella*-positive, selective pre-enrichment negative samples (1.4 $\log_{10}$ CFU/sample – 6.1 $\log_{10}$ CFU/sample). Further, there were two samples below the limit of detection from which *Salmonella* was recovered by selective pre-enrichment. Importantly, our previous study with processing plant samples, with expected lower *Salmonella* quantities due to the use of antimicrobial interventions, found comparable levels of recovery between selective pre-enrichment and enrichment methods. Future work elucidating the physiology behind differential *Salmonella* growth in selective enrichment media can support the optimization of the selective pre-enrichment conditions as the exact limiting factors for various sample matrices can be determined. Overall, the ability of selective pre-enrichment to simultaneously inhibit the growth of background bacteria and recover

Salmonella at various levels, while reducing the time required for isolation, demonstrates the efficacy and feasibility of this approach.

To assess the potential impact of media bias in selective pre-enrichment and selective enrichment conditions, CRISPR-SeroSeq was used to analyze the serovar populations within samples, with enrichment in BPW serving as the control (Fig. 4-4). There were 28 enrichments that were excluded from the analysis due to poor amplification, but each *Salmonella*-positive sample was reflected in the results. In total, we identified 12 different serovars, including one untypeable. There was an average of 1.6 and 1.7 serovars per sample from selective pre-enrichment and enrichment cultures, respectively; this indicates that there is not a loss of population complexity with selective pre-enrichment ($p = 0.5$, Kruskal-Wallis test with post-hoc Dunn's test). Overall, there were differences in serovar profiles between broiler and breeder flocks but alignment among the culture conditions for each sample. Serovar Kentucky was the most abundant in breeder flocks, as it was found in 97% (36/37) of enrichments, while serovar Typhimurium was found in 63% (65/104) of broiler sample enrichments. There was greater serovar diversity observed in broiler flocks, with varied profiles between farms and the presence of five serovars of clinical importance (Centers for Disease Control and Prevention, n.d.-a). Serovar Infantis was the third most abundant, identified solely in broiler flocks (26/141). There were a few outliers with different serovars identified between the culture conditions, such as the absence of serovar Typhimurium in the TT enrichment for Farm 1, House 1 (left side) and the detection of serovar Enteritidis only from the selective pre-enrichment for Farm 2, House 2 (right side). Serovars Mbandaka, Minnesota, Montevideo II, and Rissen were only found in one house each, suggesting

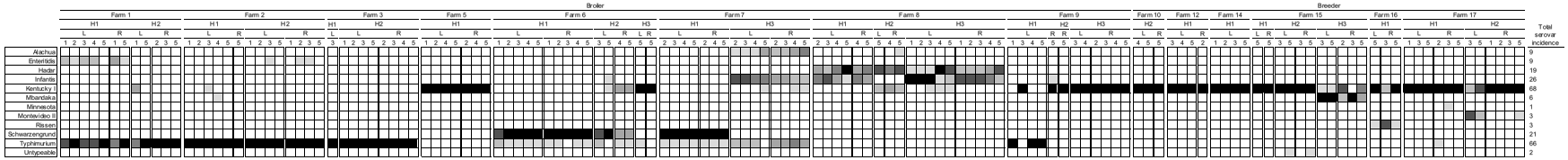

Figure 4-4. Deep serotyping results for each *Salmonella*-positive enrichment. Eight serovars were identified across nine broiler farms, while seven serovars, including one untypeable, were detected on six breeder farms. All samples within a farm are grouped together, with house (H) and side of house (L/R) indicated. Culture conditions are listed as follows: 1 – BPW, 2 – BPW + BS + Nv, 3 – BPW + MG + Nv, 4 – BPW into RV, 5 – BPW into TT. 28 enrichments were not included in the CRISPR-SeroSeq analysis due to poor amplification.

that there was an introduction event but there was not *Salmonella* transmission between the other houses on the farms yet.

The issue of *Salmonella* contamination in poultry remains a significant and complicated problem. Although overall *Salmonella* incidence has decreased during processing, the number of human salmonellosis cases linked to poultry has remained largely unchanged (Centers for Disease Control and Prevention, n.d.-a; United States Department of Agriculture - Food Safety and Inspection Service, n.d.). This is likely due in part to specific serovars present in poultry production, their ability to colonize poultry and survive antimicrobial interventions, and their association with human illness. To develop serovar-specific mitigation strategies and promote the reduction of salmonellosis, surveillance methods must provide accurate results in a timely manner. While there are commercial products available for rapid *Salmonella* detection, there is still a need to characterize all serovars present, particularly during an outbreak when strain typing can aid in source tracking. Our study shows that adding selective ingredients during the pre-enrichment step supports earlier isolation of *Salmonella* without compromising serovar diversity. Namely, the inclusion of bile salts or malachite green and novobiocin in BPW can reduce the turnaround time by 24 h, compared to the conventional method which involves a non-selective pre-enrichment step followed by selective enrichment. Although our study was conducted in the context of poultry production, our results have broader implications for other food industries, including meat production, that routinely conduct *Salmonella* surveillance. Future work may involve comparison of results from selective pre-enrichment in additional matrices, such as tissue or organ samples.

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Author Contributions

ATS: Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization; **NWS:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition

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CHAPTER 5

IN SILICO AND PCR SCREENING FOR A LIVE ATTENUATED *SALMONELLA* TYPHIMURIUM VACCINE STRAIN[1]

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Summary

The application of live attenuated *Salmonella* Typhimurium vaccines has significantly helped control *Salmonella* in poultry products. Because the U.S. Department of Agriculture–Food Safety Inspection Service (USDA-FSIS) scores all *Salmonella* as positive, regardless of serovar, attenuated vaccine strains that are identified at processing contribute negatively toward *Salmonella* performance standards. This study was designed to determine the incidence of a live attenuated *Salmonella* serovar Typhimurium vaccine identified in broiler products by FSIS and to develop a PCR assay for screening of isolates. *Salmonella* Typhimurium short-read sequences from broiler samples uploaded to the National Center for Biotechnology Information (NCBI) Pathogen Detection database by the USDA-FSIS from 2016 to 2022 were downloaded and assembled. These were analyzed using the Basic Local Alignment Search Tool with a sequence unique to field strains, followed by a sequence unique to the vaccine strain. The PCR assays were developed against field and vaccine strains by targeting transposition events in the *crp* and *cya* genes and validated by screening *Salmonella* serovar Typhimurium isolates. Between 2016 and 2022, 1,708 *Salmonella* Typhimurium isolates of chicken origin were found in the NCBI Pathogen Detection database, corresponding to 7.99% of all *Salmonella* identified. Of these, 104 (5.97%) were identified as the vaccine strain. The PCR assay differentiated field strains from the vaccine strain when applied to isolates and was also able to detect the vaccine strain from DNA isolated from mixed serovar overnight *Salmonella* enrichment cultures. Live attenuated *Salmonella* vaccines are a critical preharvest tool for *Salmonella* control and are widely used in industry. With forthcoming regulations that will likely focus on *Salmonella* Typhimurium, along with

other serovars, there is a need to distinguish between isolates belonging to the vaccine strain and those that are responsible for causing human illness.

Introduction

Salmonella is a leading causative agent of bacterial foodborne illness in the United States and is responsible for over one million human cases each year (1, 2). In total, 17% of salmonellosis cases are linked to the consumption of contaminated chicken products (3, 4) There are over 2600 different *Salmonella* serovars, though a small subset of these are responsible for most clinical infections, including *Salmonella* serovars Enteritidis, Typhimurium, and Infantis, which are commonly found in poultry (5–7). Conversely, *Salmonella enterica* serovar Kentucky, the serovar most frequently isolated from broilers at pre- and postharvest, is not associated with significant human illness in the United States (5–7).

As part of the *Salmonella* verification program, the U.S. Department of Agriculture–Food Safety Inspection Service (USDA-FSIS) performs weekly *Salmonella* monitoring at processing establishments and publicly reports these data, along with establishment performance standards (8). The workflow for *Salmonella* culturing involves selection of a single colony from an agar plate. After confirmation that this colony is *Salmonella* positive, the isolate is then analyzed by whole-genome sequencing (WGS). The *Salmonella* serovar is computationally inferred from the WGS as part of the FSIS analysis, and the sequence and serovar information is made publicly available. The current performance standards are based on *Salmonella* prevalence, and there are three different categories. Establishments in Category 1 have the lowest *Salmonella* prevalence

over a 52-wk moving window, while establishments listed as Category 3 have the highest and are considered to be failing the performance standards.

Broiler integrators use a variety of interventions to effectively reduce *Salmonella* during production and processing. In addition to proper on-farm biosecurity, the application of *Salmonella* vaccines is an important tool to reduce *Salmonella* during production (9–21). In the United States, there are three different types of vaccines available: live attenuated; commercial killed vaccine; and autogenous killed vaccine. Live attenuated vaccines consist of mutant *Salmonella* Typhimurium strains (18, 22–29) and across the industry are broadly applied to pullets and breeder flocks. The commercial killed vaccine is a *Salmonella enterica* serovar Enteritidis bacterin and is applied to pullets (17, 30–33). Autogenous killed vaccines contain the serovars that are producing problems in a particular company or complex (21, 34). Notably, when integrators have high *Salmonella* prevalence at processing (i.e., are in Category 3), they may apply live attenuated vaccines to broilers (at the hatchery or farm) with the intention of reducing the prevalence at processing plants (in this instance, killed or autogenous vaccines are not applied to broilers). When a live attenuated *Salmonella enterica* serovar Typhimurium vaccine persists, it can be isolated by the USDA-FSIS during the verification program. Because the current performance standards are based on *Salmonella* prevalence, isolation of the vaccine strain at processing counts negatively toward an establishment's performance standard.

One commercially available *Salmonella* Typhimurium vaccine (Megan Vac 1) has two transposon (Tn*10*) insertions in genes Δ*cya* and Δ*crp* (Fig. 5-1), which eliminate the ability to synthesize adenylate cyclase (CYA) and the cyclic adenosine

monophosphate receptor protein (CRP), respectively (22, 29). The *cya* and *crp* genes play a role in global regulation of many *Salmonella* functions (35, 36). This vaccine is avirulent, highly immunogenic, and is easy to grow and store (25, 28). From a diagnostic perspective, there are several indications that a group B (O:4) *Salmonella* is the vaccine strain rather than a *Salmonella* Typhimurium field strain; these include its H_2S-negative phenotype on xylose lysine tergitol-4 (XLT-4) agar among other phenotypes. With the increasing use of molecular diagnostics, there is a need for a rapid PCR-based screen that distinguishes nonvaccine from vaccine strains. Such an assay, which is straightforward to implement and affordable, would provide a standardized method across diagnostic facilities.

Given the introduction of new key performance indicators (KPIs) from the USDA-FSIS that include the specific reduction of *Salmonella* Typhimurium in broilers, it is particularly important to the commercial poultry industry to be able to determine whether *Salmonella* Typhimurium isolates found in their production systems are field strains or vaccine strain (37). It is becoming increasingly evident that *Salmonella* exists in poultry as mixed serovar populations that are not resolved when a single colony is selected off an agar plate (7, 38–43). Because the vaccine isolate is attenuated and also appears as smaller colonies than other salmonellae, it is likely that in a mixed population of multiple serovars, it will be outnumbered by other salmonellae and thus would not be selected when a colony is picked from agar. However, if the USDA-FSIS moves to the use of assays that screen enrichment cultures specifically for *Salmonella* Typhimurium (e.g., by PCR), there will be a need for a complementary PCR assay that can determine

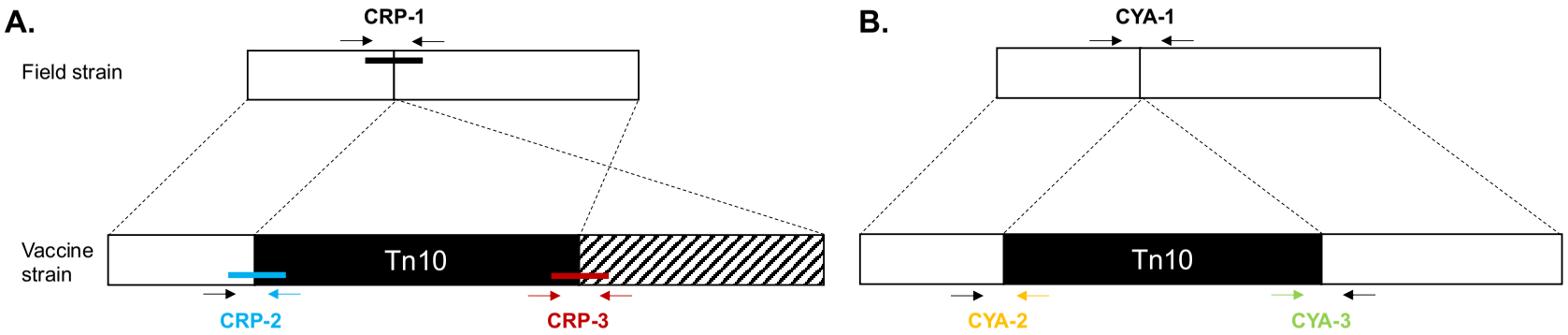
whether *Salmonella* Typhimurium positive samples represent presence of the vaccine or a field strain.

This study was developed with two objectives. The first was to determine the prevalence of a commercial live attenuated vaccine by the USDA-FSIS at processing. The second objective was to develop a PCR assay to distinguish between vaccine and nonvaccine *Salmonella* Typhimurium isolates and to be able to detect the presence of vaccine strain in a mixed *Salmonella* population. From a regulatory perspective, the bioinformatic assay provides a method to be able to distinguish between vaccine strain and field isolates. From an industry perspective, the PCR assay presented enables rapid identification of vaccine strain that may not be detected by traditional culture methodology (i.e., if it is outnumbered by additional, nonvaccine salmonellae), which will aid in *Salmonella* monitoring systems. Further, the assay could also be used by integrators to assess vaccine takes in birds after application.

Materials and methods

In silico analysis of FSIS *Salmonella* Typhimurium genomes

The National Center for Biotechnology Information (NCBI) Pathogen Detection database (44) was searched for *Salmonella* Typhimurium genomes corresponding to the USDA-FSIS isolates from all chicken-related samples from January 2016 to December 2022. Raw sequence reads were downloaded, and genomes were assembled using SPAdes (45). The Basic Local Alignment Search Tool (46) was used to align sequences of 125–126 nucleotides that correspond to the *crp* gene in native *Salmonella* Typhimurium (i.e., field isolates) and the vaccine strain (Fig. 5-1C) sequences, respectively. The accession number for the Δ*crp* mutant is MT900624.1.



C.

Target	Bait sequence (5'-3')
CRP-1	ttattggtgaactgggcctgtttgaagaaggccaggaacgcagcgcctgggtacgtgcgaaaaccgcatgtgaggtcgctgaaatttcctacaaaaaatttcgccaattaatccaggtcaacccgg
CRP-2	ggtgattttattggtgaactgggcctgtttgaagaaggccaggaacgcagcgcc**ctgatgaatcccctaatgattttggtaaaaatcattaagttaaggtggatacacatcttgtcatatgatc**
CRP-3	**gggaagacttactcgtggctgcaaccctactagctcaaaatttattcacacatggttacgctttggggaaattatgaggggatctctcag**ggcacagcaccagcgtttgccgtagtgcgtaatta

Figure 5-1. Schematic showing transposition of *crp* and *cya* genes and design of molecular assays. The vaccine strain was generated by P22 transduction of Δ*crp*::Tn*10* (A) or Δ*cya*::Tn*10* (B) and subsequent fusaric acid selection for tetracycline-sensitive isolates (18). Tn*10* is 9147 nucleotides in length (GenBank accession no.: AF162223). For both Δ*crp* and Δ*cya* mutants, the central portion of the transposon containing the *jem* operon (*jemA, jemB,* and *jemC*) and the *tet* operon (*terR*, *tetA*, *tetC*, and *tetD*) is removed. The left and right transposase elements remain. The junction where the transposon was inserted generates a sequence that is unique to the vaccine strain. Three PCR assays were developed for each gene, as indicated. The arrows indicate the primer sequences and direction of PCR amplification. The diagonal lines on the 3′ end of *crp* indicate that this portion of the sequence is in the reverse orientation compared with the native *crp* in *Salmonella* Typhimurium. (C) Bait sequences corresponding to the field strain (black horizontal bar) and either end of the transposon insertion site (blue and red bars) were used to screen WGS. The portion of the sequences that target Tn*10* are shown in bold.

Salmonella isolates and mixed enrichment samples

Four *Salmonella* Typhimurium isolates were obtained from the Diagnostic Laboratory at the Poultry Diagnostic and Research Center at the University of Georgia (Athens). Two had been typed as the vaccine strain based on three criteria: 1) an H_2S-negative morphology on XLT-4 agar; 2) agglutination with O:4 antiserum (BD Difco, Franklin Lakes, NJ); and 3) phenotype on an API 20E strip (BioMérieux, Marcy-l'Etoile, France). The remaining two isolates were typed as *Salmonella* Typhimurium field isolates. A single *Salmonella* colony from each isolate was propagated in 5 ml of Luria-Bertani broth and grown overnight at 37 C. Total genomic DNA was isolated from 600 µl of these cultures using the Promega Genome Wizard kit (Madison, WI) according to the manufacturer's protocol and was resuspended in 200 µl of molecular grade water and stored at −20 C until use. For the blinded study, 18 *Salmonella* Typhimurium isolates were provided to us from the Georgia Poultry Lab Network. Here, the *Salmonella* Typhimurium isolates were characterized as the vaccine strain based on H_2S-negative reading on triple sugar iron agar slants and the phenotype on a Vitek-2 gram-negative card. Colonies were scraped from the Luria-Bertani agar plate, and resuspended in 600 µl of nuclei lysis solution, and the genomic DNA was isolated as mentioned previously. As part of two independent projects, deep serotyping was performed on *Salmonella*-positive swabs from a commercial hatchery and on *Salmonella*-positive boot sock samples from commercial breeder flocks. Total genomic DNA was isolated from overnight tetrathionate enrichments, and deep serotyping was performed (M. Rothrock, unpubl. data; Shariat, unpubl. data). The isolated DNA from 11 hatchery samples and 21 breeder

samples that were shown to contain *Salmonella* Typhimurium were used as a template for the PCR assay.

PCR assay

The primers used for the *cya* and *crp* assays are shown in Table 1 and were designed based on the vaccine strain sequences that were publicly available through NCBI for Δ*cya* (accession no.: MT900625.1) and Δ*crp* (accession no.: MT900624.1). The expected size of each product is also shown in the table. Two microliters of DNA template (with a concentration between 10 and 35 ng/µl) was combined with 1 U/µl *Taq* polymerase (New England BioLabs, Ipswich, MA), 2 nmol deoxynucleoside triphosphate (New England BioLabs), 1× *Taq* polymerase buffer (New England BioLabs), and 10 mM of each primer for a total reaction volume of 25 µl. The PCR included denaturation for 3 min at 95 C, followed by 30 cycles of 95 C for 30 sec, 65 C for 30 sec, and 68 C for 30 sec. This was followed with a final extension step of 68 C for 3 min. A total of 5 µl of each PCR product was visualized on a 1.5% agarose gel run at 110 V. For the PCR screen of enrichment samples containing mixed serovars, the same PCR parameters were used, with the exception that it was run for 40 cycles. The PCR products for all six assays (CRP-1 to CYP-3 and CYA-1 to CYA-3) were treated with 20 U/µl of Exonuclease (New England BioLabs) and 1.25 U/µl of Antarctic alkaline phosphatase (New England BioLabs). The mixture was incubated for 30 min at 37°C to remove remaining primers and unincorporated deoxynucleoside triphosphate. The enzymes were inactivated by incubating the samples at 85 C for 15 min. Purified PCR products were sequenced at Eton Biosciences (North Carolina Branch; Research Triangle Park, NC), and the sequences were aligned using MegAlign, version 17.3.3 (47).

Table 5-1. Primer sequences used for the study.

Gene	PCR assay	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product size (nucleotides)
crp	CRP-1	ctccgtggcagtgctgatcaaag	cgtcaaggaaggcgaggttacctac	281
	CRP-2	ctccgtggcagtgctgatcaaag	gttctcgctttggttggcaggttac	386
	CRP-3	cgtcacctaaaatctactcagcgtcgg	attacgcactacggcaaacgctg	501
cya	CYA-1	gcgatatctgcgggttcaccag	cgataatcgcgcaaactggaaaatcgatc	221
	CYA-2	gcgatatctgcgggttcaccag	tcaagtaatgcgtggcaagccaac	364
	CYA-3	gctcacggatatcagaccagtcaacaag	cgataatcgcgcaaactggaaaatcgatc	494

Results

Bioinformatic analyses of FSIS *Salmonella* Typhimurium genomes

Between January 2016 and December 2022, 1,708 *Salmonella* Typhimurium genomes were uploaded to the NCBI Pathogen Detection database by the USDA-FSIS. These accounted for 7.99% of all *Salmonella* genomes belonging to FSIS isolates from chicken-related samples, including postchill carcass rinses, parts, and comminuted products. Using the publicly available sequences for the Δ*cya* (accession: no. MT900625.1) and Δ*crp* (accession no.: MT900624.1) *Salmonella* Typhimurium sequences, we identified sequences that would differentiate between field strains and the vaccine strain. The mutants were generated by P22 transduction of Δ*crp*::Tn*10* and Δ*cya*::Tn*10*. In the vaccine strain, much of Tn*10* has been deleted, leaving the left and right transposase sequences. The junctions where the transposon was inserted into the *cya* and the *crp* genes provides unique sequences to the vaccine strains (Fig. 5-1). These query sequences corresponding to the Tn*10* junction were used to screen the 1,708 assembled *Salmonella* serovar Typhimurium genomes. A total of 104 (5.97%) of these genomes were identified as the vaccine strain (Fig. 5-2A). There were no vaccine strains present in the 2016 dataset, though this subsequently increased each year to 29 instances in 2022 (Fig. 5-2B).

The analysis presented here was based on assembly of the genomic short-read sequences available at NCBI. Because this can be computationally intensive, we also developed a protocol for screening the short-read sequences themselves into WGS of the *Salmonella* genomes. The query sequences are less than 130 nucleotides; this facilitates

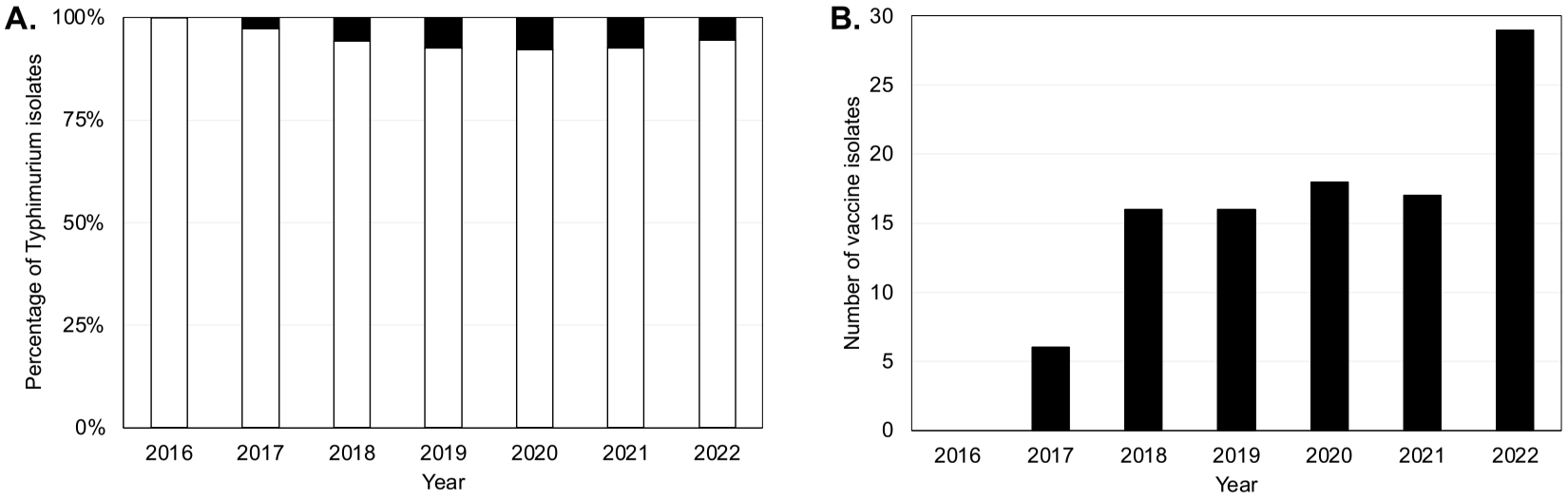


Figure 5-2. Incidence of *Salmonella* Typhimurium vaccine strain isolated from broiler products by USDA-FSIS between 2016 and 2022. (A) Proportion of 1,708 *Salmonella* Typhimurium isolates corresponding to the field strain (white) and vaccine strain (black). (B) The number of vaccine strain isolates detected each year.

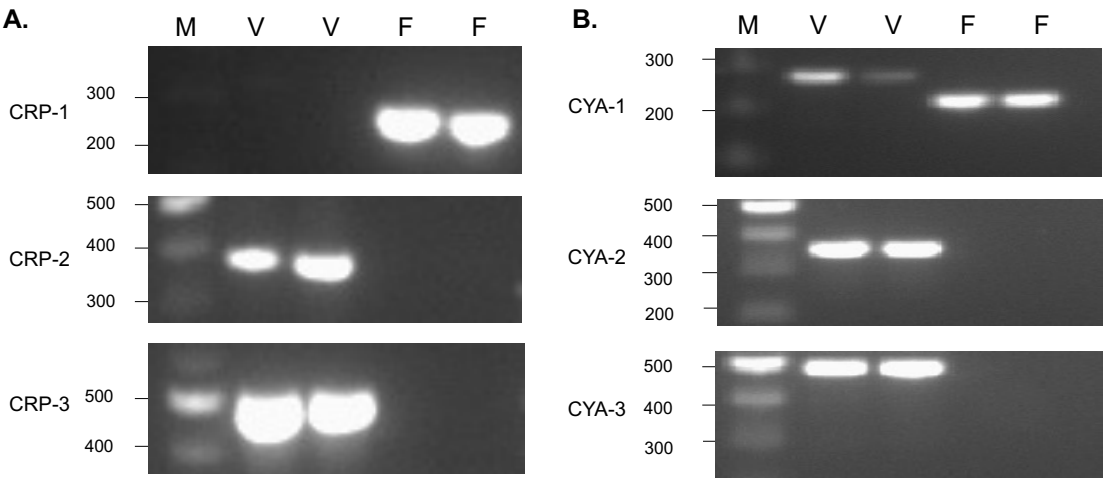


Figure 5-3. PCR assays distinguish between field isolates and vaccine strains. (A) Three *crp* PCR assays were used to screen two *Salmonella* Typhimurium field isolates (F) and two isolates of the vaccine strain (V) using the primer pairs listed in Table 1. PCR products were analyzed by gel electrophoresis. (B) The same isolates were screened with three *cya* PCR assays. Molecular marker (M), with sizes shown to the left of each gel.

using these to directly screen the short-read sequences and avoid having to assemble the genome.

PCR assays to differentiate *Salmonella* Typhimurium vaccine strain from nonvaccine strains

Six PCR assays were used to screen vaccine and nonvaccine *Salmonella* Typhimurium isolates. Three assays were designed against the *crp* gene, with one targeting the field strain sequence and two targeting the transposon insertion junctions in *crp* (Fig. 5-1). Three similar assays were designed against the *cya* gene. The PCR assays were able to successfully differentiate between the field and vaccine strains (Fig. 5-3) and were confirmed by Sanger sequencing of the amplicons for all six assays (data not shown). The primers for each assay were designed to generate products of differing sizes to facilitate the use of a multiplex PCR (Table 5-1). We demonstrated that a multiplex PCR approach would work for the Δ*crp* mutation using CRP-1 (targets the field strain) and CRP-3 (targets the vaccine strain) assays (Fig. S5-1). Next, a blinded study was performed. The Georgia Poultry Lab Network provided 18 *Salmonella* Typhimurium isolates, and these were screened using the multiplex CRP-1 and CRP-3 PCR assay. We correctly attributed nine vaccine isolates and nine field isolates (Fig. 5-4).

Detection of vaccine strain from mixed *Salmonella* cultures

As part of a separate project, we performed deep serotyping on *Salmonella* enrichment cultures that were collected at a commercial hatchery. We detected *Salmonella* Typhimurium in a number of these samples. The integrator informed us that they recently applied the vaccine at the hatchery. We used the *crp* PCR assay (CRP-1 and CRP-3) to screen 11 of these samples (H1–H11) and detected the vaccine isolate in nine

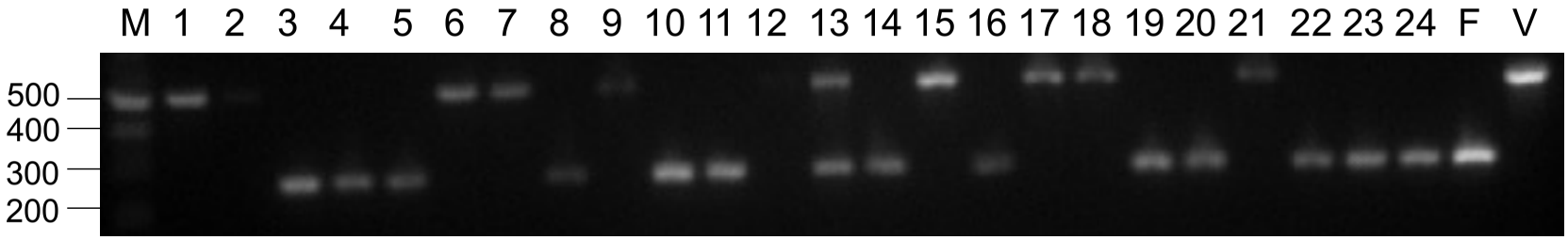


Figure 5-4. Multiplex PCR assay to distinguish between field isolates and vaccine strains. A multiplex PCR using the primer pairs from assay CRP-1 and CRP-3 were used to screen three vaccine strain isolates and two field isolates of *Salmonella* serovar Typhimurium. Molecular marker (M), with sizes shown to the left of the gel.

of them (Fig. 5-5). Eight of these had PCR products for CRP-1 and CRP-3, showing that they contained the vaccine strain and another *Salmonella.* In sample H4, only the vaccine isolate was detected. In another independent project, 21 *Salmonella*-positive enrichments from environmental breeder samples that had been shown to contain *Salmonella* Typhimurium were screened using our assay. Three samples (B3, B5, and B16) had positive amplification of the CRP-3 assay, demonstrating presence of the vaccine isolate in these samples (Fig. 5-6). All 21 samples had positive amplification from the CRP-1 assay, showing that these were mixed cultures. We note that the PCR assay targeting the field strain does not distinguish between *Salmonella* Typhimurium and other serovars, given the high conservation of this gene across *Salmonella*.

Discussion

Although the broiler industry has decreased *Salmonella* prevalence by over 50% in the last few years, there has not been a reduction in human cases attributed to *Salmonella* from poultry products in the United States (5, 49, 50). This may, in part, be due to the successful reduction of *Salmonella* Kentucky, which is the most prevalent serovar isolated from chickens but is not associated with significant human illness in the United States (5–7). In October 2021, the USDA-FSIS announced a new initiative to reduce *Salmonella* in broilers (51). This was followed by an announcement of KPIs to achieve this goal (37). Specifically, KPIs measure the percentage of reduction in raw poultry samples contaminated with the *Salmonella* serovars commonly associated with human illness, which includes *Salmonella* Typhimurium.

This new initiative highlights the importance of the different preharvest *Salmonella* control strategies such as biosecurity (52–57), pest control (53, 58–62), litter

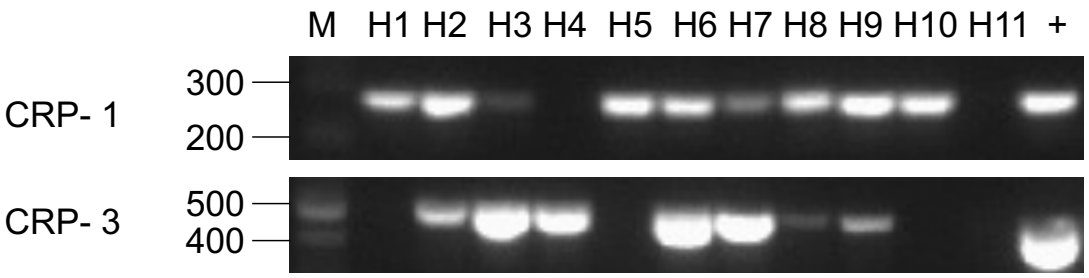


Figure 5-5. PCR screen can detect presence of the vaccine isolate in mixed *Salmonella* cultures. The CRP-1 (field strain) and CRP-3 (vaccine strain) assays were used to screen six mixed cultures containing *Salmonella* serovar Typhimurium. These samples were collected from hatchery environments and are labeled 1-6, with those containing the vaccine strain highlighted in bold. Molecular marker (M), with sizes shown to the left of the gel. For the positive controls, two isolates from our diagnostic laboratory were used; one was a field strain of *Salmonella* serovar Typhimurium (used as a positive control for CRP-1), and the other was a *Salmonella* serovar Typhimurium that had been typed as the vaccine strain (used as a positive control for CRP-3).

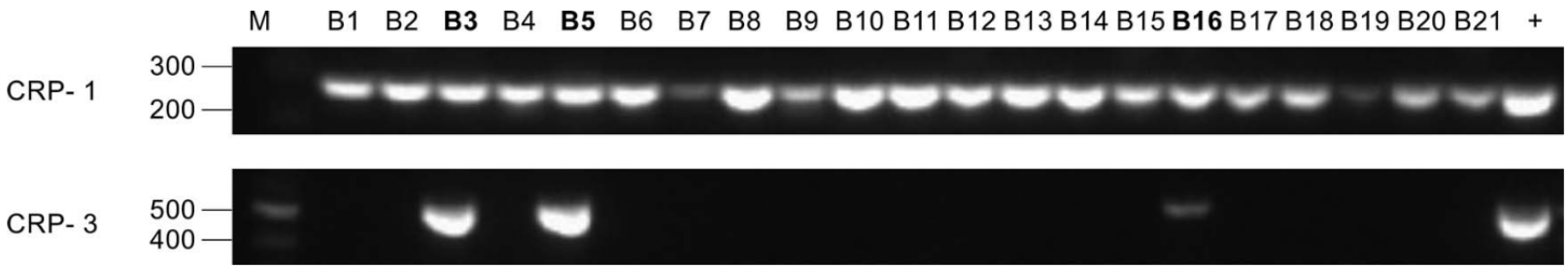


Figure 5-6. PCR screen can detect presence of the vaccine isolate in mixed *Salmonella* cultures from breeder flocks. The CRP-1 (field assay) and CRP-3 (vaccine assay) assays were used to screen 21 mixed cultures containing serovar Typhimurium (B1-B21). Samples containing the vaccine strain are highlighted in bold. M; molecular marker, with sizes shown to the left of the gel. As a positive control for the CRP-1 assay, a field isolate of serovar Typhimurium was used and for CRP-3, an isolate of the vaccine strain was used.

amendments (63–68), and treatment of feed (69–77) and water (72, 75, 77, 78). Vaccination, particularly the use of the live attenuated vaccine against *Salmonella* Typhimurium, has also shown to be effective at reducing *Salmonella* (9, 15, 18, 25, 27, 29, 79). Live attenuated vaccines are used by a large proportion of broiler integrators in the United States as the main method of *Salmonella* control in breeders (11,13,14,80). The analysis presented here shows that 6% of *Salmonella* Typhimurium isolates found by the USDA-FSIS from broiler processing establishments belong to a vaccine strain. These are negatively impacting establishment performance standards. Performance standards are available to the public, and customers can use these data to make the decision to continue or change providers. When a processing establishment falls into Category 3, the integrator may then choose to apply a live attenuated *Salmonella* vaccine to the broiler flocks. Because the vaccine strain discussed here persists in chickens, this corrective action actually exacerbates the problem, as more of it will be isolated by the USDA-FSIS during verification sampling. In light of *Salmonella* Typhimurium being designated as a KPI, isolation of the live attenuated *Salmonella* Typhimurium vaccine strain during processing may continue to count negatively toward performance standards. The bioinformatic approach outlined here provides a straightforward way that regulators could screen WGS information from *Salmonella* Typhimurium isolates to determine whether or not they belong to the vaccine strain. Furthermore, should the USDA-FSIS move to include some kind of *Salmonella* Typhimurium PCR to screen enrichment cultures that potentially contain mixed serovars (as opposed to picking and sequencing an individual colony), the PCR assay detailed here could be used in parallel to determine whether those samples are vaccine strains.

Because the USDA-FSIS is now focusing on this serovar, integrators and regulators are keen to use rapid diagnostics that can specifically detect these serovars. Rapid diagnostics will likely be used directly on a sample prior to culture or on some kind of enriched or pre-enriched sample. When *Salmonella* Typhimurium is detected in these instances, there will be a need to determine whether it is vaccine strain or not, which can be accomplished by the PCR assay presented here.

Vaccination is a valuable tool for food safety and also for animal health regarding protection against poultry pathogens. The work presented here highlights the importance of distinguishing vaccine and field strains from each other to facilitate improved *Salmonella* surveillance in the poultry industry.

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Author Contributions

ATS: Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization; **MR:** Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization; **NWS:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Salmonella is a leading bacterial cause of foodborne illness, with substantial global morbidity and mortality rates (3). This dissertation has focused on nontyphoidal serovars commonly found in chickens, but the challenges and results presented may apply to other production systems or foodborne pathogens. While 80% of *Salmonella* illnesses are attributed to seven food categories, including meat, poultry, and produce, chicken is the most significant single contributor (19.7%) (30). Concerted efforts have been made towards reducing *Salmonella* contamination on raw products, resulting in a 50% decrease from 2016 to 2022, but the fact that chicken remains a significant contributor reflects that it is a complex, multifaceted problem to solve (2, 46, 49, 67). Some major hurdles to overcome for *Salmonella* mitigation include the largely asymptomatic colonization in chickens, limitations in detection methods, and phenotypic differences among serovars. Additionally, the substantial production volume of chicken in the United States may compound the problem, as the estimated per capita chicken consumption (102.3 lbs) in 2024 was almost double that of beef and pork together (111.6 lbs).

Most *Salmonella* control has focused on post-harvest processing, where the combination of antimicrobials, such as chlorine and peracetic acid, and temperature controls, such as rapid heating and extended cooling, have been effective in reducing contamination (20, 51, 62). However, the unchanging rate of salmonellosis attributed to

chicken demonstrates that further reductions are necessary, which may be accomplished by minimizing the *Salmonella* load entering the processing plant to support greater efficacy of antimicrobial interventions. While all *Salmonella* serovars are capable of causing human illness, due to the inclusion of *Salmonella* Pathogenicity Islands 1 and 2, it is estimated that less than 100 are routinely isolated from human clinical cases (11). As such, it is essential to focus on serovar identity in addition to prevalence. Targeted control strategies, namely vaccination, have demonstrated considerable success in *Salmonella* mitigation, but these can be costly to maintain in broiler production (7, 22, 57, 69, 70). Alternatively, increased on-farm biosecurity can be a little to no cost approach with noticeable improvements in *Salmonella* prevalence (39, 65). Since *Salmonella* colonization in chickens is most often asymptomatic, it is vital to integrate high-resolution surveillance to ensure that applied controls are effective in *Salmonella* mitigation. Conventional *Salmonella* isolation is limited in the observed diversity, as multiple serovars may exist within a host but colony picking is often restricted by time and resources. Molecular-based approaches may enable the identification of multiple serovars, but these are in turn limited by the lack of a single isolate for further characterization. Evaluating all present serovars within a sample is critical to promoting public health, as serovars pose unique risks based on their individual genotypes and phenotypes. For example, some serovars may have increased antimicrobial resistance, host pathogenicity, or stress response mechanisms (13, 23, 24, 26, 58, 59, 71).

The second chapter of this dissertation detailed a study analyzing trends in *Salmonella* serovars routinely identified in commercial chicken processing plants in the United States. The study's original design considered the national data collectively, but it

was divided among five areas of the country due to the observation of region-specific trends. For example, there was an increased abundance of serovars Infantis and Schwarzengrund in the Atlantic and Southeast regions, respectively. While the total *Salmonella* prevalence and serovar diversity are reflective of the scale of production in each region, the proportion of *Salmonella*-positive samples is not in alignment. The two regions with the greatest production (Southeast and South Central) had the least proportion of positive carcasses and parts. Interestingly, the *Salmonella* incidence is higher in parts than carcasses, further supporting previous findings that cross-contamination may occur during parts cut-up and there are some invasive serovars in joints or other harborage sites (41, 52–54, 56). For the last two decades, serovar Kentucky has been the most predominant serovar isolated from domestic poultry production yet rarely causes human illness in the United States, with only 258 culture-confirmed cases by the CDC (10, 64); this observation emphasizes the importance of identifying which serovars are present in production. To determine the contribution of *Salmonella* in breeders to that found on final products, we next sought to apply deep serotyping on a subset of on-farm surveillance samples. The serovar profiles of pre-harvest breeder samples and post-harvest broiler samples were discordant, as serovar Schwarzengrund was detected primarily at processing with a concurrent reduction of serovar Kentucky. This discrepancy may be explained in two ways, or perhaps a combination of both: 1) antimicrobial interventions used as hazard control in processing plants likely reduce the majority of resistant serovars, including Kentucky, causing a shift in population dynamics; 2) there are additional contributing factors to *Salmonella*

contamination of final products, including persistent serovars on the broiler farms and within processing plants.

Biomapping in processing plants may help to recognize shortcomings in sanitization at specific steps, such as biofilms remaining on equipment used for parts cut-up or in chill tanks, but serovars found at post-harvest must have originated from pre-harvest stages; therefore, it is necessary to focus on removing primary sources of *Salmonella* introduction. Importantly, this study revealed that one-third of pre-harvest breeder samples contained more than one serovar, further complicating control strategies. For example, autogenous vaccines can only be developed following serovar isolation within a complex, and other commercial vaccines will not be applied in broiler flocks unless there is a demonstrated need. Additionally, the presence of multiple serovars may explain some of the incongruence of those identified at pre- and post-harvest as some serovars, such as Infantis, were often present in lower relative frequencies. This high-resolution analysis of multiserovar populations also revealed some interesting intraspecies dynamics, including the presence of serovars Cerro and Mbandaka, which are typically associated with cattle, and possible seasonal effects on serovar diversity, with the greatest diversity observed in October. Since this study only included one year of breeder surveillance samples, future work was required to further elucidate mechanisms driving serovar diversity and multiserovar populations. Additionally, the dataset did not include paired samples which limited comparisons to the state level, rather than complexes or flocks, and only post-harvest broiler samples were analyzed. However, this study provided the first high-resolution viewpoint of *Salmonella* populations in

breeder production and identified regional trends in serovars found at broiler processing plants across the United States.

The study presented in Chapter 3 was designed to further investigate the integrated farm continuum, with a focus on breeder production, by deep serotyping of blinded surveillance samples and monthly sampling of eight flocks throughout one production cycle, starting at the pullet farms. Importantly, this was the first longitudinal characterization of multiserovar population dynamics in broiler breeders in the United States, and the generated data can be used to inform management decisions to reduce *Salmonella* contamination in chicken; namely, the heightened biosecurity in breeder production, as compared to broilers, helps to limit serovars of concern. However, the deep serotyping of 129 farms with multiple houses revealed that 70% contained similar populations, as determined by the Bray-Curtis dissimilarity matrix, so any slip in biosecurity can enable on-farm transmission. Additionally, the high-resolution deep serotyping results provide new insight on *Salmonella* population dynamics, with observed patterns of serovar co-occurrence, including positive and negative associations. The study findings may serve as a steppingstone towards improved *Salmonella* control in commercial broiler production, as serovar-specific mitigation strategies can be developed based on inter- and intraserovar interactions, and a framework is provided for routine *Salmonella* monitoring in live production.

Since this was a follow up to data analyses presented in Chapter 2, some of the surveillance samples collected by GPLN between 2020 and 2021 are also included, although the total prevalence of multiserovar populations was lower in this study (~20%); this may be attributed to the quadrupled sample size and the inclusion of additional

samples containing single serovar populations, or the inclusion of a single boot sock representing a flock, rather than two pairs. However, the overall diversity increased in the set of samples collected between 2020 and 2022, with 38 serovars identified. Comparatively, the 35% *Salmonella* prevalence in the GPLN surveillance samples is equal to or greater than that found in previous studies, including a longitudinal study in Australia and breeder flocks in Ontario, Canada (42, 68). An additional study found varying proportions in breeder flocks, with 55% and 40% of boot sock and drag swabs being *Salmonella*-positive, respectively (7); this is in closer alignment with the prevalence of samples from the longitudinal study (43%). Notably, the prevalence was lower on pullet farms, with 15% and 17% prevalence in the submitted GPLN samples and the longitudinal study, respectively. From the repeated sampling, *Salmonella*-positive pullet farms were in a single complex, namely the one lacking integrated pest control; this supports the finding that biosecurity plays a critical role in *Salmonella* introduction. Furthermore, captured on-farm rodents contained serovars that matched those found in the corresponding breeder flocks, highlighting the potential of cyclical transmission with these vectors. Future work should evaluate the extent of *Salmonella* transmission by rodents, as they can move between farms, and regularly monitor rodent colonies to characterize *Salmonella* colonization, including the duration and stability of multiserovar populations.

There was greater serovar diversity observed in breeder surveillance samples compared to post-intervention broiler carcass and parts rinses, which exemplifies the efficacy of antimicrobial interventions during processing to reduce contamination. Importantly, vaccination efforts in breeder flocks are successfully managing serovars of

concerns, including serovars Enteritidis and Typhimurium which are both often found in broiler flocks. Serovar Kentucky is the most prevalent serovar in breeder flocks and has been dominant in the domestic poultry industry for over 20 years with no significant association with human illness (12, 25). This phenomenon may be explained by the increased fitness of serovar Kentucky in colonizing chickens, as conferred by several stress response-regulated genes, along with the absence of key virulence genes for host cell invasion and survival (14). Unsurprisingly, we found that serovar Kentucky is negatively associated with other serovars, such that it is most often isolated from single serovar populations. Interestingly, the cattle-associated serovars Cerro and Mbandaka were the second and third most abundant serovars in the GPLN dataset, respectively; their presence may be explained by the inclusion of meat and bone meal in the mash diet of breeders. Deep serotyping also revealed some serovars primarily co-exist in multiserovar populations, including the repeated detection of serovar Liverpool with serovars Cerro and Mbandaka. Further analysis determined that overall serovar prevalence is not correlated with relative serovar abundance, such that serovar Typhimurium is not widely present but is often the major serovar in a mixed population. Alternatively, serovar Infantis is typically found with a lower relative abundance. These observations underscore the limitations of conventional culture-based methods for *Salmonella* detection and isolation, since it is unlikely to identify multiple serovars when selecting a few colonies from an indicator plate. To protect consumers, it is critical to characterize all serovars present in a contaminated chicken sample, and the framework presented here supports the integration of robust *Salmonella* monitoring at any stage of live production. Additionally, future studies in commercial chicken production should

focus on further exploring interserovar dynamics, including elucidation of the mechanisms behind serovar diversity and population complexity.

The following two dissertation chapters included applied research projects to improve *Salmonella* detection in the poultry industry. In Chapter 4, a novel PCR assay was developed to separate serovar Typhimurium vaccine and field strains, which is an important distinction since the vaccine strain is attenuated and thus poses a relatively lower public health risk. In the United States, there are two primary commercial formulations of live serovar Typhimurium vaccines with attenuation by mutations in the *cya*/*crp* or *aroA* genes affecting metabolism (1, 18); this study focused on the former and its application in broiler flocks. Previous work has determined that the *cya*/*crp* mutant strains induce a stronger and long-lasting host immune response, and the ease of application through spray or water treatment further make this an attractive control strategy (19, 21). However, it is important to note that it is possible, although unlikely, that the vaccine strain may revert to a virulent phenotype following recombination, so *Salmonella* populations should be monitored following application (21). Vaccination has been proven to reduce *Salmonella* contamination on final products and in some cases offers cross-protection against other serovars, either through an induced immune response or maternally provided innate immunity (5–7, 15, 17, 22, 27, 28, 33, 35, 57, 61, 70).

Despite the conferred benefits, vaccination is not routinely used in broiler production due to the cost-benefit tradeoffs, namely, the expenses generated by required personnel and consumables for administering vaccines in hundreds of flocks and the concern of prolonged vaccine strain shedding. While the vaccine strain in this study is

non-H_2S producing, enabling differentiation from field strains, this is only applicable for certain indicator media, including XLT-4, XLD, and DMLIA. As such, vaccine strains may not be identified accordingly on alternative indicator media, such as BGS, thus negatively impacting a processing establishment's performance standards as defined by current USDA-FSIS regulations. Vaccination in broiler flocks is critical to reduce the incidence of serovars of concern in production; in particular, the results presented in Chapter 2 suggest that the selective pressure generated by vaccine use in parental flocks is relieved without additional vaccinations and allows for the return of pathogenic serovars. To encourage vaccination in broiler flocks, it was imperative to develop a reliable, rapid diagnostic test for serovar Typhimurium vaccine strains. For example, retrospective bioinformatic analyses of regulatory samples collected between 2016 and 2022 revealed that 6% of serovar Typhimurium isolates were the vaccine strain, yet these still counted negatively against a processing establishment. The presented PCR assays were validated with a blinded study of field and vaccine isolates and screening environmental poultry farm samples. Importantly, the PCR assays can be used in parallel to detect vaccine presence in mixed serovar populations, thus facilitating improved *Salmonella* surveillance in the poultry industry and promoting food safety with routine vaccine use.

The final research chapter validated the use of selective pre-enrichment to facilitate *Salmonella* recovery two days after sample collection. Selective pre-enrichment refers to the inclusion of individual selective ingredients (malachite green, bile salts, novobiocin) with non-selective pre-enrichment broth, thus encompassing the primary and secondary enrichment steps in one 24 h period. The former two ingredients are from the

widely used enrichment media, RV and TT, respectively, while the latter is a bacteriostatic antibiotic targeting gram-positive bacteria. Collectively, these components create a harsh environment (low pH, high osmotic pressure) to allow for *Salmonella* growth while minimizing the proliferation of non-target organisms (32, 40, 55, 66). The selective pre-enrichment conditions were first developed by our group during a broiler processing plant sampling project, with comparable recovery in traditional selective enrichment methods (50). In this study, 35 commercial poultry houses from 17 farms across multiple integrator complexes were sampled to validate the use of selective pre-enrichment culture conditions for quicker *Salmonella* recovery with high background microflora. Both selective pre-enrichment and enrichment recovered *Salmonella* in 14/17 farms, with no significant difference on a per-sample basis ($p = 0.07$, Chi-squared test). Similarly, the culture condition did not impact *Salmonella* diversity ($p = 0.1$, ANOVA test of Shannon diversity indices) or multiserovar population complexity ($p = 0.5$, Kruskal-Wallis test with post-hoc Dunn's test), as measured by deep serotyping with CRISPR-SeroSeq. Alternatively, *Salmonella* quantity seems to influence recovery with selective pre-enrichment, as the average amount ($\log_{10}$ CFU/sample) was greater in the positive pre-enrichment cultures than the negatives ($p = 0.01$, Welch two sample t-test). However, the substantial variance of *Salmonella* quantity in negative pre-enrichment samples (range: 1.4 – 6.1 $\log_{10}$ CFU/sample) suggests that other variables likely impact recovery as well and additional work is required to identify them. In short, selective pre-enrichment can serve as a shortened approach for *Salmonella* recovery without compromising resulting serovar profiles.

A critical finding of this study was that enrichment media efficiency varies by sample type, as we observed lower *Salmonella* recovery from breeder samples cultured in RV in comparison to broiler samples; this may be explained by the different gut microbiome composition of breeders and broilers, along with varying metabolic capacity. For example, a previous study applying metagenomics to evaluate the microbial dynamics of various culture conditions found that the taxa comprising the tomato phyllosphere varied substantially by enrichment media (45). Additional work has confirmed that enrichment media influence microbiome profiles, which denotes the importance of defining the physiological response of different serovars to selective ingredients to avoid false negatives (31, 43). Notably, there have been many studies on the gut microbiota in broilers, but this is not complemented by similar work in breeders (4, 16, 34, 38, 44, 48). It has been established that the gut microbiome development in newly hatched chicks is dependent on microbial exposure and food sources during the first few days, which has important implications for *Salmonella* control (9, 29, 36, 47, 63). Future work should define the community composition in breeders to provide insights on *Salmonella* colonization and recovery. Ultimately, it is necessary to characterize the community dynamics within the gut microbiota to optimize enrichment media and develop new or refine existing mitigation strategies, such as probiotics and competitive exclusion products (5, 8, 37, 60).

Collectively, the research conducted during my graduate program provides new insights into *Salmonella* population dynamics, including regional serovar trends and patterns of serovar co-occurrence, and supports optimized sample collection and enrichment. Most importantly, my work can be used to inform management decisions and

aid in the development of targeted mitigation strategies, thus promoting increased food safety in the poultry industry. There are many questions remaining on *Salmonella* transmission and control in chickens; still, the high-resolution deep serotyping data included in this dissertation may serve as preliminary data to design experiments and further elucidate contributing factors to serovar diversity and population complexity.

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Supplemental Table 2-1. Serovar incidence in broiler carcasses in the United States by region, 2016-2020.

Serovar	Southeast					South Central					Atlantic					Midwest					Mountain & West				
	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020
Agona																		2	2	1					
Alachua		1				1	1	1																	
Anatum			1	1				1			1														
Berta						1	2									1						1			
Blockley														2	3										
Braenderup	1	5	2	2	1	1		1			1				1				4	1			1	1	1
Enteritidis	27	34	32	33	7	8	15	9	8	7	4	3	7	6	3	7	22	13	6	10	3	8	4		5
Hadar		1	1		1					2								1							
Heidelberg	2	8	8	2	1	2	3	2	1	3	5	1	1			1	1	1	2	2					
Infantis	9	12	10	18	34	2		6	12	12	6	15	26	34	35	3	3	5	7	7	3	3	2	2	8
Johannesburg								1																	
Kentucky	71	103	63	75	69	52	90	48	44	59	27	23	31	25	26	31	31	44	38	43	13	19	24	23	22
Litchfield		1			1							1	1		1	1		2					1		
Liverpool			3																						
Manhattan		1	2																						
Mbandaka		1		1	1			2					1					1							
Montevideo	2		1			1												2	1		1		1		
Muenchen			1		3													1		1					
Newport							1			1															
Ohio	2	5		1												1									
Oranienburg																	1	2							
Orion		1																							
Rubislaw									1																
Saintpaul	1										1														
Schwarzengrund	15	33	24	19	23			1	3	1					1										
Tennessee																		1						1	
Thompson	1	1	2		2			2	1		3	1	2				1								
Typhimurium	20	19	19	14	18		2	1	1		16	21	24	14	23			5	2	4	2	4		1	1
Uganda							1																		
Worthington																				1					
4,[5],12:d:-				1																					
8,20:i:-		1						1																	
I 4,[5],12:i:-		2	1	3	4			1				1	5	2		1	1	1	1	1	1	1			
Rough O:k:-																	1								
Rough_O:e,h:e,n,z15																								1	
Rough_O:gms:-		1																							
Rough_O:r:1,5									1			2	1												
Total	151	230	170	170	165	68	115	77	72	85	64	68	99	83	93	46	61	81	63	71	23	36	33	29	37

Supplemental Table 2-2. Serovar incidence in raw intact chicken parts in the United States by region, 2016-2020.

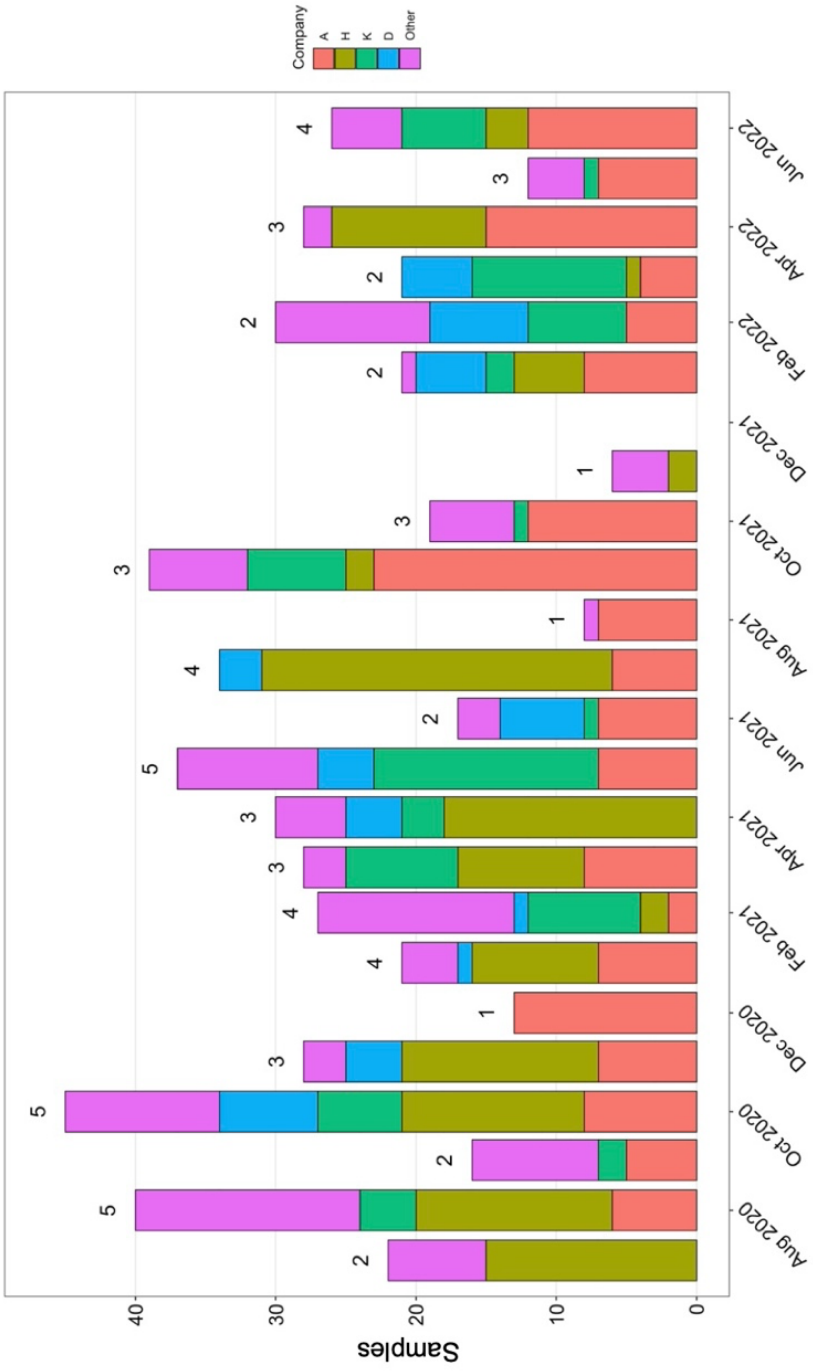
Serovars	Southeast					South Central					Atlantic					Midwest					Mountain & West				
	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020
Agona					1								2												
Alachua			1	1		1			1																
Altona	1										1														
Anatum		1																1		1				1	
Barranquilla																						1			
Berta	2			1	1	1	1			1														2	
Blockley		1											1	1	4										
Braenderup	1	5	3	1	1	2	1	1	2	1		2	1		1	2	1	1	2	3	1	1		3	1
Brandenburg	1										1											1			
Cerro	1										1														
Eko															1										
Enteritidis	113	175	121	115	75	86	45	47	25	38	21	12	20	32	21	29	27	29	28	41	26	20	16	20	26
Hadar	2	2	2		2										1			2	1						
Heidelberg	15	15	9	3	2	13	11	10	7	6	20	3			2	6	4	3	2	1	5	5	1	4	2
Infantis	20	39	57	59	58	12	22	12	27	57	11	53	65	83	86	1	3	8	11	23	1	11	12	11	22
Johannesburg	2	1		2	1	2	1	1			1				1						6	3	3	6	17
Kentucky	173	153	133	67	75	66	61	43	42	70	30	16	17	34	40	38	20	29	44	55	32	37	31	37	43
Kiambu			1		1																				
Litchfield		1	1				1						2		1							1			
Liverpool	1	1					1																		
Livingstone	1																								
London		1													1										
Manhattan	1																								
Mbandaka	1	3		3						1	4		1				1				1		1		
Minnesota																				1					
Montevideo	1	2	1		1			2	1		1		1		3					1					
Muenchen	1			2	1												1								
Muenster			1								1														
Newport	1						1	2						1						1		1			
Nima				1																					
Ohio	1	4			1											1									
Oranienburg					1		1																		
Orion												1													
Ouakam			2																						
Putten	1																								
Reading																			1	1					
Rissen																	2								
Rubislaw																				1					
Ruiru											1														
Schwarzengrund	90	81	71	62	56	3	6	1	6	11	2			3	2	5	12	13	6	13	2	1	3	4	6
senftenberg			1			1					1										1				
Tennessee	1																					1			
Thompson	4	10	6	3	4	3	10	4	4	3	2	4	3	3	4		1	1		1		2		1	3
Typhimurium	47	34	38	15	21	6	4	7	5	2	56	32	19	15	38	4	2	2	3	5	4	1	6	3	5
Uganda						1	2				1				1							1			
Worthington				1												1									1
4,[5],12:d:-		1	3	1																					
6,7:b:-		1																							
8,20:-:z6									1	1															
8,20:i:-		1																							
I 4,[5],12:i:-	3	3	7	3	2	1	1				4		3	3				3	1		2	2			1
I 4:b:-																				1					
Rough O:d:e,n,x				1				1																	
Rough O:k:1,5		1																				1			
Rough_O:gms:-																									
Rough_O:r:1,5	1	2	2	3	1		1			1		3	3	7				1	1			1			
III 13,23:gz51:-		1	2	1										1											
III_40:z4,z24:-																		1							
III_48:g,z51:-					1								1												
III_48:z4,z24:-																		1							
Total	486	539	462	345	306	198	170	131	121	192	159	126	139	183	207	87	74	95	100	149	81	91	73	92	127

Supplemental Table 2-3. *Salmonella* serovars found at processing in Georgia, 2016-2020.

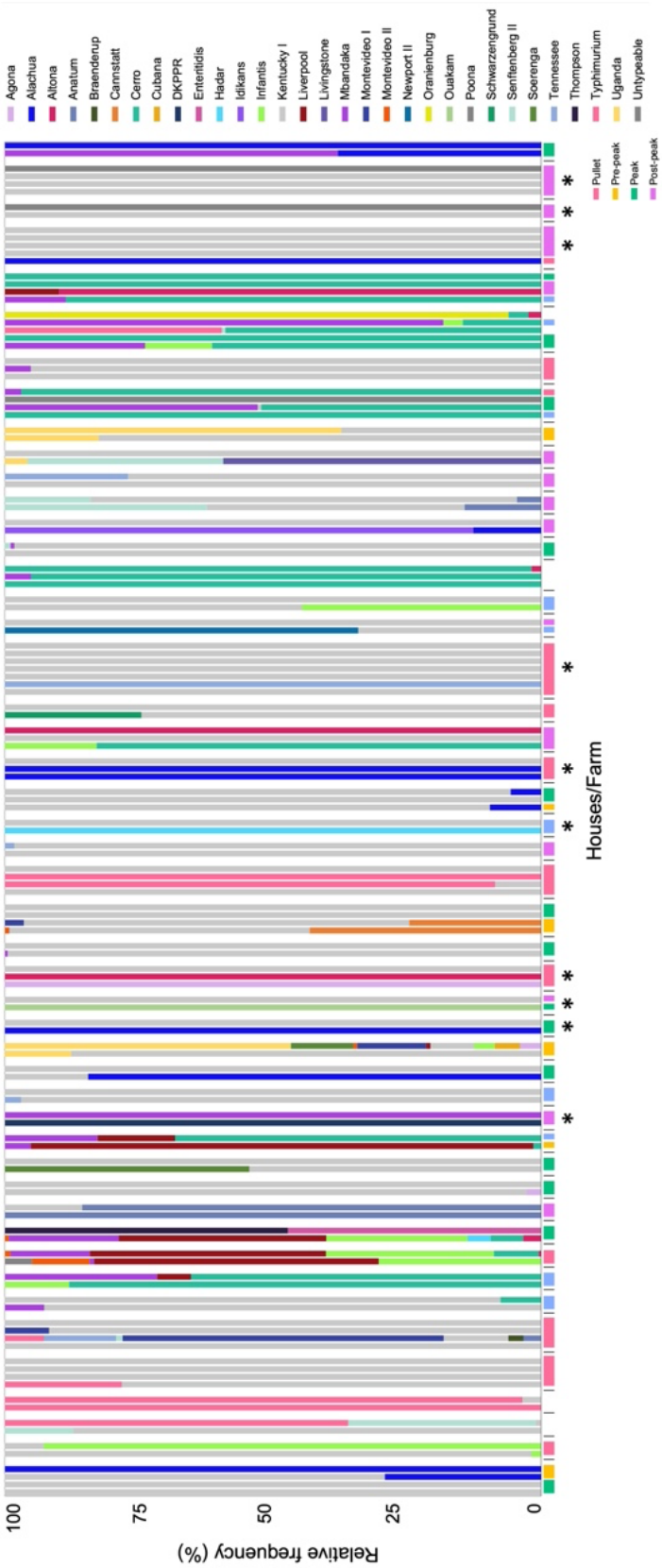
	Carcasses					Raw, intact					Raw, ground, non-intact				
Serovar	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020	2016	2017	2018	2019	2020
4,[5],12:d:-				1			2	1	1						
6,7:b:-							1								
8,20:i:-							1								
I 4,[5],12:i:-				1				5	2						
Alachua								1							
Berta						1									
Braenderup			2				3	1		1		1			
Enteritidis	8	15	11	6		21	41	30	24	20	6	6	10	6	
Hadar		1					1	1					1		
Heidelberg	1	3				3	5	1			1				
Infantis				4	6	4	13	10	8	20	1	1	5	4	4
Johannesburg										1					
Kentucky	7	11	15	23	13	39	29	45	18	30	4	9	4	3	4
Kiambu										1					
Liverpool							1								
Manhattan															1
Montevideo	1					1	2			1	1				
Muenchen									1						
Ohio	2	5				1	4								
Ouakam								1							
Schwarzengrund	8	19	10	7	7	21	42	26	30	26	4	1	3	5	3
Senftenberg											1				
Thompson							4				1		1		
Typhimurium	5	3	4	1	7	17	12	17	3	10	1		2	2	1
Rough_O:gms:-		1													
Rough_O:r:1,5								1					1		
III 13,23:g,z51:-							1				1	1			
III_48:g,z51:-												1			
Total	32	58	42	43	33	108	162	140	87	110	21	20	27	20	13

APPENDIX B

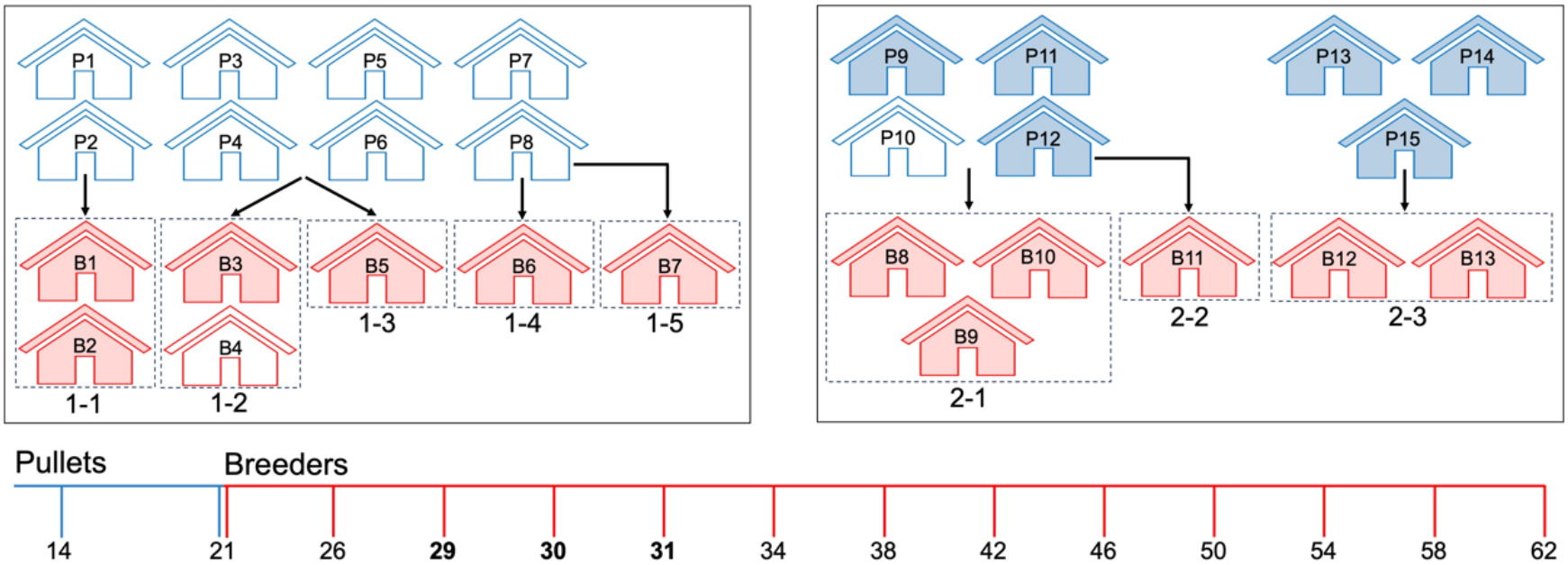
SUPPLEMENTAL MATERIAL FOR CHAPTER 3



Supplemental Figure 3-1. Distribution of *Salmonella*-positive samples across months and companies. From August 2020 to June 2022, a total of 568 breeder slat samples from 22 companies were analyzed with CRISPR-SeroSeq. The top 4 companies from the dataset (based on total number of samples submitted) are shown with individual colors, while the remaining 18 companies are grouped as "other". Numbers above bars indicate how many collection days were included in that month.



Supplemental Figure 3-2. Diverse populations observed among multiple farms with several pullet/breeder houses. Deep serotyping results for farms containing multiserovar populations or single serovar populations with different identities (48/129) are shown, with samples from the same farm indicated by the vertical lines, age of flock represented by the colored rectangle, and farms with only one serovar are denoted with an asterisk. Flocks without provided age information are shown without a corresponding-colored rectangle. Pullet: 0 – 21 weeks, pre-peak: 21 – 28 weeks, peak: 28 – 35 weeks, post-peak: 35 – 50 weeks, late: after 50 weeks.



Supplemental Figure 3-3. Longitudinal *Salmonella* sampling schematic. Pullet (blue) and breeder (red) houses for two complexes are shown; *Salmonella*-positive houses are indicated by shading. The breeder flocks that originated from shared pullet flocks are indicated by the numbering, with the farm information included below. Pullets were sampling at weeks 14 and 21. Empty, cleaned out breeder houses were sampled at week 21, then sampling occurred monthly, with the exception of weekly sampling during peak production (bolded weeks).

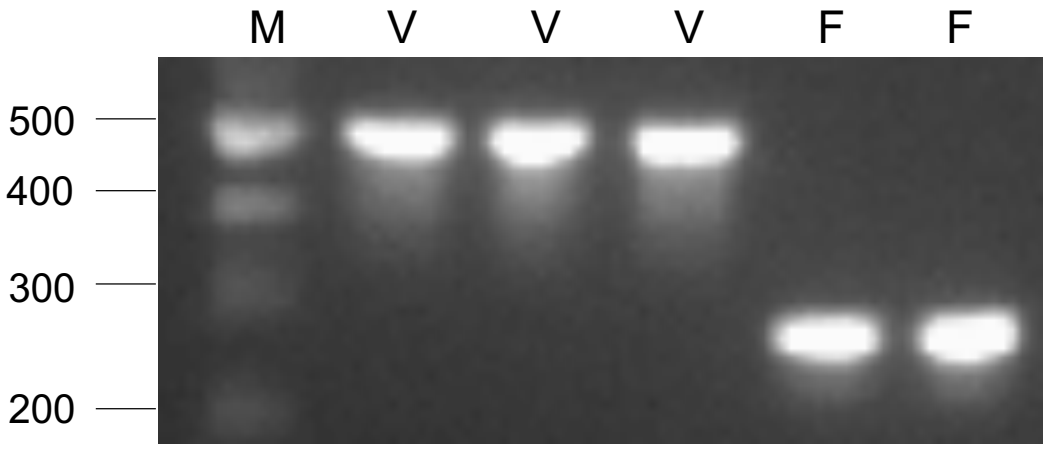
Supplemental Table 3-1. CRISPR-SeroSeq summary results from complete GPLN dataset (n = 568).

Serovar[a]	Frequency[b]	Present (%)[c]	Alone (%)[d]	Major (%)[e]	Average relative frequency (%)[f]	Months[g]	Companies[h]
Kentucky I	462	81.3	86	65	94	23	20
Cerro	43	7.6	30	57	65	10	5
Mbandaka	34	6	12	17	30	15	7
Typhimurium	25	4.4	32	41	62	14	11
Liverpool	21	3.7	29	53	58	11	6
Infantis	19	3.3	16	12	38	11	6
Alachua	17	3	47	11	61	6	2
Senftenberg II	12	2.1	8	18	36	8	6
Tennessee	9	1.6	11	25	36	5	5
Enteritidis	8	1.4	62	0	72	5	7
Uganda	8	1.4	12	57	49	5	1
Montevideo I	6	1.1	0	17	18	4	3
Montevideo II	6	1.1	0	0	4	3	4
Agona	5	0.9	40	0	45	3	3
Altona	5	0.9	20	25	39	5	2
Anatum	5	0.9	20	25	42	2	3
Untypeable	5	0.9	20	0	32	3	2
Hadar	4	0.7	75	0	76	4	1
Cubana	3	0.5	0	0	3	3	2
Minnesota	3	0.5	0	33	48	2	2
Schwarzengrund	3	0.5	67	0	75	3	3
Soerenga	3	0.5	33	0	53	3	1
Thompson	3	0.5	0	67	52	3	2
Worthington	3	0.5	67	100	84	2	2
Cannstatt	2	0.4	0	0	34	1	1
DKPPR	2	0.4	50	0	60	2	1
Idikans	2	0.4	0	50	48	2	1
Oranienburg	2	0.4	0	50	48	2	2
Orion	2	0.4	0	50	29	2	2
Poona	2	0.4	0	0	8	1	2
Braenderup	1	0.2	0	0	3	1	1
Give	1	0.2	0	100	69	1	1
Livingstone	1	0.2	0	100	59	1	1
Muenchen I	1	0.2	100	-	100	1	1
Muenster	1	0.2	100	-	100	1	1
Newport II	1	0.2	0	100	66	1	1
Ouakam	1	0.2	100	-	100	1	1
Saintpaul II	1	0.2	100	-	100	1	1

[a]Serovars of clinical importance (bolded) are most frequently isolated from human samples (Center for Disease Control and Prevention BEAM dashboard). The suffixes (-I, -II, -III) for some serovars refer to polyphyletic lineages. The abbreviation DKPPR represents serovars Durban, Kokomele, Panama, Pomona, and Reading II.
[b]Indicates the total number of samples each serovar was found in.
[c]Indicates the total percentage of samples each serovar was found in.
[d]Indicates how often a serovar was the single serovar in a sample.
[e]Indicates the frequency in which the serovar was present at a higher relative frequency in a mixed population of multiple serovars.
[f]This was calculated across all 'present' samples.
[g]Indicates how many months (n = 24) each serovar was identified in.
[h]Indicates how many companies (n = 22) each serovar was identified from.

APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER 5



Supplemental Figure 5-1. Multiplex PCR assay to distinguish between field isolates and vaccine strains. A multiplex PCR using the primer pairs from assay CRP-1 and CRP-3 were used to screen three vaccine strain isolates and two field isolates of serovar Typhimurium. M; molecular marker, with sizes shown to the left of the gel.