

The effect of *Salmonella* autogenous vaccination on serotype surveillance, recovery, and phenotypic characteristics

by

Emily Robinett

B.S., William Woods University, 2013

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2022

Approved by:

Major Professor
A. Paige Adams

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Abstract

Salmonella enterica is a major cause of foodborne illnesses around the world and is commonly caused by contaminated poultry food products. Currently, the poultry industry utilizes numerous protocols to mitigate the control of *Salmonella* prevalence and transmission within various operations. Autogenous vaccines allow for the customized vaccination of poultry in different farms, but the selection of an appropriate vaccine formulation can be challenging to narrow down based on the isolates derived from each location. *Salmonella* surveillance studies can aid in the identification of *Salmonella* serotypes that are prevalent throughout a company or farm, and the use of various phenotyping methods can aid in identifying variations between isolates. For this study, four large poultry operations in the US were analyzed for changes in the recovery, prevalence, and phenotype of *Salmonella* over a four-year period following the implementation of autogenous vaccination program. Bacterial phenotypic characteristics used in the study were arginine dihydrolase, citrate utilization, acetoin production, d-sorbitol fermentation, inositol utilization, and biofilm formation for the selection of the autogenous vaccine formulations. In the four-year study period, the overall prevalence of *Salmonella* decreased in both the hatcheries and the processing plants of the study sites. Serotype S. Heidelberg represented 6.7% of isolates in the processing plants in 2017, but within a year after the implementation of the autogenous vaccination, it was not isolated in any of the study sites. The prevalence of serotypes S. Enteritidis and S. Typhimurium decreased between 2017 and 2020, but S. Infantis and S. Kentucky increased. *Salmonella* Kentucky was not included in the autogenous vaccine formulations, which could explain the increased prevalence. There was no difference in the prevalence of phenotypic indicator, arginine dihydrolase, suggesting that it would not be useful when monitoring the efficacy of autogenous vaccines. However, the prevalence of phenotypic indicators citrate utilization, d-sorbitol fermentation, inositol utilization and biofilm formation varied throughout the study in response to the vaccine formulation, indicating that they may be useful when monitoring the efficacy of autogenous vaccines. Future studies should be conducted to determine whether the phenotypic characteristics of autogenous *Salmonella* vaccines are related to the vaccine efficacy.

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Acknowledgements

I would like to acknowledge my husband and family who have all been very supportive as I've gone back to school and pursued my Master's. I would also like to acknowledge Ceva Animal Health for allowing me the use of the data I have gathered during my employment and my co-workers for supporting and helping me along the way. I would also like to acknowledge my advisor and committee for all of your wonderful remarks and for taking the time to help me achieve my degree. Finally, thank you to the poultry company who let me use the data I've gathered in my thesis.

Dedication

I dedicate this thesis to my husband and my family for supporting me all the way through.

Chapter 1 - Introduction

Bacteria belonging to the genus *Salmonella* have the capacity to survive in a variety of environments throughout the world. *Salmonella* contamination within these various environments can result in food poisoning from many different food sources, with poultry representing a large percentage of the source of these illnesses globally. *Salmonella* is ubiquitous in poultry farms and results in the transmission of the bacteria from poultry to humans through meat and egg production. There are many procedures in place to aid in the prevention and spread of *Salmonella* and the associated diseases. This study focused on the effect of implementing an autogenous vaccination program within a large Southern United States poultry company on changes in *Salmonella* prevalence and biochemical characteristics of the recovered isolates during a four-year period.

Salmonella is as a Gram negative, motile, facultative anaerobic bacterium belonging to the family *Enterobacteriaceae*. It is a hardy organism that can thrive in a variety of environments, including soil, water, gut, manure and in poultry litter, either dry or wet conditions, various internal organs, and in the harsh chemical conditions of the poultry processing plant. The zoonotic pathogen includes two species: *Salmonella enterica* and *Salmonella bongorii* (V). *Salmonella enterica* is further classified into six (6) subspecies; *S. enterica subsp. enterica* (I), *S. enterica subsp. salamae* (II), *S. enterica subsp. arizonae* (IIIa), *S. enterica subsp. diarizonae* (IIIb), *S. enterica subsp. houtenae* (IV), and *S. enterica subsp. indica* (VI) (Brenner et al., 2000). The species are further classified into serogroups and serotypes based on the antigens present on their cell surface and flagellum. *Salmonella* serotypes associated with poultry are all classified as *S. enterica subsp. enterica*.

History of *Salmonella*

Salmonella was first discovered in 1885 by Dr. Daniel Elmer Salmon and his lab assistant, Theobald Smith, while they were working with the intestines of pigs infected with classical swine fever (Eng, et al., 2015). Dr. Salmon was a veterinary pathologist and the head of the microorganism research program at the United States Department of Agriculture in the 1800s and named the bacteria *Salmonella cholerae suis*, believing it to be the causative agent in hog cholera. Prior to Dr. Salmon's discovery in 1885, *Salmonella enterica* serovar Typhi was isolated

by Dr. Karl Joseph Eberth from the abdominal lymph nodes and spleen in 1875, but it was called *Eberthella typhosa* in his findings. Various other scientists confirmed Dr. Eberth's identification of this bacillus as the cause of typhoid fever (Monte & Sellera, 2020).

Salmonella enterica has been identified throughout history in various outbreaks and bioterrorism. During the Peloponnesian War (430-426 BC), the Spartans laid siege to Athens. In addition to cutting off access to food and weapons, the Spartans were believed to have poisoned Athens's water reservoirs as one of the earliest examples of bioterrorism (Papagrigorakis, et al., 2013). The Athenian historian, Thucydides, recounted the incident in his *History of the Peloponnesian War* (Gomme, 2020) but the causative agent was not identified until recent years when the skeletal material from a Peloponnesian mass grave provided ancient DNA for analysis. Based on the findings, *Salmonella enterica* serovar Typhi was implicated as a primary cause of the epidemic (Papagrigorakis, et al., 2013). The symptoms described by Thucydides in his *History* align with the known symptoms of typhoid fever. Once the bacteria were introduced to the Athenian water sources, the cramped and unsanitary living conditions within the city allowed for the rapid spread of typhoid fever. The plague killed nearly two-thirds of the population; however, the Athenians did not give up easily, and the first Peloponnesian War ended in a truce.

In more recent years, *Salmonella* Typhi has been the cause of multiple epidemics. From 1607 to 1624, the settlers of Jamestown, Virginia experienced an outbreak of typhoid fever that took the lives of over 6,000 settlers; this "burning fever" was America's first epidemic (Earle, 1979). Since the indigenous populations had not encountered typhoid fever previously, their population was also decimated along with the settlers.

The Spanish-American War lasted only four months in 1898, but 87% of the total deaths were attributed to typhoid fever, making the assembly camps more dangerous than the frontline. While the causative agent of typhoid fever had been identified prior to the war, conditions for the spread of the fever were still under investigation. The epidemic became a national scandal since soldiers who had never been on a battlefield were dying at alarming rates. The Typhoid Board was created to determine why the conditions during the Spanish-American War allowed for a higher rate of transmission than that seen in the Civil War. The Typhoid Board consisted of the three (3) medical officers; Majors Walter Reed, Surgeon, Regular Army (chairman); Victor C. Vaughan, Division Surgeon, U.S. Volunteers; and Edward O. Shakespeare, Brigade Surgeon, U.S. Volunteers. The three men visited all the cantonments and secondary camps, and then, they

analyzed the various hospital reports over the next twenty-one months. The analysis led to the realization that the fevers were misdiagnosed as malarial fever at the time of the war, and this misdiagnosis resulted in the spread of the fever, improper treatments, and an increased number of deaths. Overall, the Board placed a large part of the responsibility on the prevailing ignorance of the current medical schools. Due to the lack of proper laboratory equipment, they were releasing physicians without giving them the necessary knowledge regarding typhoid fever. They also concluded that camp pollution was to blame for the rapid spread of the disease and that camp officers were at fault for allowing the unsanitary conditions. The epidemic of the Spanish-American War resulted in drastic military health reforms, the organization of the first female nursing corps, and increased research opportunities that focused on bacteria and the transmission of diseases (Cirillo, 2000).

A few years after the epidemic surrounding the Spanish-American War, another outbreak took over New York. Mary Mallon was an Irish immigrant who worked as an in-home cook to multiple wealthy families in the New York City area in 1906. When members of the families came down with typhoid fever the link was uncertain for a while and so the sanitary engineer, George Sober, was hired by the Warren family to investigate the source of infection. Mary had come down with a mild case of the fever but was not yet considered the host. Over the next year, Sober continued to investigate Mary and eventually discovered that she was a “healthy carrier” of typhoid fever. Throughout his investigation, he determined that out of the eight families Mary cooked for, seven of them contracted typhoid fever. In 1907, approximately 3,000 New Yorkers were infected, and it is believed that Mary was the source of the outbreak. Sober was able to get the New York Department of Health involved in the investigation and after Mary’s stools tested positive for *S. typhi*, she was transferred to Riverside Hospital on North Brother Island for quarantine. In 1910, she was released with the understanding that she would not work as a cook again. She went by the name Mary Brown and did not follow the agreement. Within three months, 25 more people were sick and two were dead. At this point she was known as “Typhoid Mary”. She was retained once again in the Riverside Hospital until her death in 1938. There are reports that she shed the bacteria from gallstones throughout the entirety of her adult life (Marineli, et al., 2013).

Throughout history, the presence of *Salmonella* serotypes, such as *S. Typhi*, has altered the outcomes of wars and civilizations. It has created opportunities for the advancement of

science, changes in military regulations, and the understanding of how one person can make a difference in the progression of an epidemic. With the typhoid vaccine coming onto the scene in 1911 (Fanning, 1997) and antibiotic treatments available in 1948, typhoid fever has dramatically decreased in developed countries. In more recent years, *Salmonella* infections typically involve the non-typhoidal *Salmonella* serotypes that are transmitted as food-borne illnesses.

Salmonella in Poultry

Poultry is the source of the second most consumed meat with a 2019 global market worth \$231.5 billion. The top consumers of poultry products are China, the United States and Brazil with 20 million tons, 19 million tons and 12 million tons, respectively (Poultry World, 2020). In July 2021, 80,727 metric tons (178,003,035 pounds) of chicken meat were produced in foreign countries and 20,296 metric tons (44,752,680 pounds) in the United States for a total of 101,023 metric tons (222,755,715 pounds).

In the United States, the poultry industry includes over 9.22 billion chickens, 224 million turkeys, 185 million chickens (excluding broilers) and 112 billion eggs. It was valued at over 40 billion dollars in 2019. The breakdown of the Poultry Value of Production for 2019 and 2020 is detailed in Table 1.1 (United States Department of Agriculture. National Agricultural Statistics Service, 2021).

With the considerable number of birds in the poultry industry, the spread of various diseases is inevitable, such as the bacterial infections colibacillosis, mycoplasmosis, necrotic enteritis, pullorum disease, fowl typhoid, infectious coryza and fowl cholera (University of Mississippi, 2021). *Salmonella* is the causative agent for both pullorum disease and fowl typhoid

Table 1.1 Poultry Value of Production - United States: 2019 and 2020

Year	Broilers ¹	Turkeys	Chickens ²	Eggs	Total
	(1,000 dollars)	(1,000 dollars)	(1,000 dollars)	(1,000 dollars)	(1,000 dollars)
2019.....	28,313,919	4,323,977	37,560	7,334,686	40,010,142
2020.....	21,683,052	5,189,938	18,777	8,655,581	35,547,348

¹ Excludes States which produced less than 500,000 broilers.

² Value of sales

Pullorum Disease in Poultry

Pullorum disease, previously known as Bacillary White diarrhea, is caused by the host adapted, non-motile *Salmonella enterica* serovar Pullorum that affects young chickens and turkeys. It is characterized by high mortality, anorexic, weak and depressed birds that tend to congregate around a heat source and have white fecal diarrhea adhered to their vent areas. These symptoms may also appear in conjunction with blindness, swollen joints, and respiratory disease. Most affected birds fall ill within the first two to three weeks of age, although it can also affect adult birds with little to no clinical signs (Merck Veterinary Manual, 2021). In young chicks, it has a high mortality, nearly 100% in some cases, but in adults, it may result in a drop in egg production, decreased weight, and fertility in hens due to infected ovaries (Broerman). It does not infect humans, but if massively contaminated food is consumed, it can cause acute, self-limiting enteritis in humans (Markos & Abdela, 2016).

Due to the high mortality and morbidity rates associated with pullorum disease, treatment and prevention are very important. Antibiotics, such as neomycin, and oxytetracycline are the most effective drugs against *Salmonella* Pullorum, although the bacteria are generally susceptible to other antibiotics, such as ampicillin, kanamycin, streptomycin, gentamicin (Rzedzicki & Glinski, 1975) and ciprofloxacin (Parvej, et al., 2016). Prior to treatment with any antibiotic, an antibiotic susceptibility test should be performed due to the high patterns of antibiotic resistance in *S. Pullorum*. In a 2016 study involving 150 samples, “81.81% [of] isolates were resistant to amoxicillin, doxycycline, kanamycin, gentamicin, and tetracycline” (Parvej, et al., 2016).

Therefore, treatment is no longer recommended due to the risks associated with resistant bacteria and the effect of the eradication programs.

With the ability to treat the disease with known antibiotics dwindling, the pressure remains to prevent outbreaks of the disease. Vaccination is a proven way to aid in the prevention of pullorum disease. A ghost vaccine - a vaccine created with a bacterium that has no bacterial genome or cytoplasmic contents but retains the functional and antigenic determinants on the outside of the bacteria - is very effective in inducing “significant humoral and cellular immune responses, resulting in effective immunoprotection” (Guo, et al., 2016) and may be useful in the future against *Salmonella* Pullorum. Vaccines such as autogenous vaccines will be discussed in the following chapters of this thesis as they are pivotal to the research discussed in Chapter 3. Finally, control efforts put forth, such as the National Poultry Improvement Plan (NPIP), aim at eliminating the infected birds from the industry, ensuring that they do not pass along the bacteria through their eggs. When pullorum disease is confirmed on a farm, the NPIP protocol mandates that all affected flocks are to be quarantined, depopulated, and go through a thorough cleanup process. After the extensive cleaning and disinfection procedures are completed, the farm will continue to monitor the environment and rodent population to ensure that there are no remaining traces of the bacteria. Prior to placement of new birds, negative environmental swabs will be confirmed (Anderson, et al., 2006).

In the United States and other developed countries, pullorum disease has been nearly eradicated due to the various treatments and prevention methods put forth; however, it remains a huge impact on the poultry industry in developing countries, such as Africa, Latin America, and the Middle East (Markos & Abdela, 2016). The last commercial outbreak in the United States occurred from 1990 to 1991 (Shivaprasad, 2000). In Nigeria, it is widespread throughout the country and can be isolated from chicks that are only a few hours old (Ezeibe, et al., 2019). While many developing countries have some sort of surveillance system in place to monitor the outbreaks of pullorum disease, other countries use passive systems where underreporting is a large problem. Some countries have active reporting systems, while others may have no information on the prevalence within their country. Many countries have few resources available for the eradication of the bacteria, and so, they use antibiotics as the sole method of control. With the increase in resistance to these available antibiotics, they are perpetuating the cycle of increased resistance, increased asymptomatic carriers, residues in egg production and an

increased prevalence of the bacteria (Revolledo, 2018). The implementation of routine vaccination in these developing countries could be significantly impactful. The future use of an effective and affordable vaccine may be the tool to help reduce the prevalence of pullorum disease worldwide.

Fowl Typhoid in Poultry

The causative agent for fowl typhoid is the host adapted, non-motile *Salmonella enterica* serovar Gallinarum. It manifests mostly in older birds with both acute and chronic infections. Clinical signs include pale, dehydrated birds with diarrhea. Upon necropsy, the birds have swollen, bile-stained livers with or without necrotic foci, anemia, enteritis and enlarged spleen and kidneys. While *Salmonella* Gallinarum can be spread through eggs like *Salmonella* Pullorum, mechanical transmission between birds is more common (University of Mississippi, 2021). Bird-to-bird transmission, including wound contamination, cannibalism of infected carcasses, and fecal contamination of water, feed, and litter, allows the spread throughout a flock (Center for Food Security & Public Health, 2019). Diagnosis of fowl typhoid is similar to pullorum disease with isolation and identification from lesions.

Treatments using antibiotics are no longer recommended due to the high patterns of antibiotic resistance; therefore, prevention and control methods are preferred (Velge, Cloeckert, & Barrow, 2005). This includes ensuring that all chicks are hatched from disease-free flocks, enforcing strict sanitation management guidelines, providing clean feed and water, and properly disposing of deceased birds (University of Mississippi, 2021). There is a vaccine that has been used in the past, and while it may reduce flock losses, it does not prevent further infection with field strains (OIE, 2018). Similar to pullorum disease, when an outbreak occurs the entire flock must be depopulated, and the premises thoroughly disinfected and cleaned. Negative environmental swabs should be confirmed prior to the presence of more birds (OIE, 2018).

Fowl typhoid was last reported in the United States in 1980, and it has been eradicated from commercial poultry in developed countries, such as Australia, Canada, Japan, and most Western European countries, but remains a common pathogen in developing countries, such as Central and South America, Mexico, the Indian subcontinent, and Africa (Shivaprasad, 2000). In Brazil, there have been outbreaks every year, with 14 outbreaks documented in 2012. These outbreaks are believed to be linked to failure in executing proper biosecurity measures and disease monitoring programs (Rizzo, et al., 2020). In the United States, programs, such as the

National Poultry Improvement Plan, monitor and report the prevalence of various *Salmonella* infections including *Salmonella* Gallinarum (Animal and Plant Health Inspection Service, 2020).

Poultry as a source of *Salmonella* in Food-borne illnesses

According to the Centers for Disease Control (CDC), non-typhoidal *Salmonella* infections cause approximately “1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year” (Centers for Disease Control, 2021). The World Health Organization (WHO) reports that approximately 550 million people become ill to foodborne diseases and that “*Salmonella* is 1 of the 4 global causes of diarrheal diseases” (World Health Organization, 2018). Salmonellosis is characterized by abdominal pain, diarrhea, acute onset of fever, nausea and vomiting that can occur 6-72 hours post ingestion; illness lasts 2-7 days. Most cases of Salmonellosis are mild and replacing electrolytes is the only treatment required. Antibiotics are rarely used due to the high prevalence of antibiotic resistance. In some children and elderly persons, the symptoms may become life-threatening and require hospitalization (World Health Organization, 2018).

Salmonella can be found in food animals, such as pigs, poultry, and cattle, and in pets, such as dogs, cats, reptiles, and birds. It can pass through each phase of the food chain from the animal feed, the grow-out houses, processing plants and into the households of the product consumers where it may become consumed by humans and cause gastroenteritis. Vegetables and other foods can become contaminated by *Salmonella* in multiple different ways; contamination of the soil by water and waste run-off, contamination during harvest and during food preparation near uncooked meat which then passes it along to humans who ingest the items (World Health Organization, 2018). Precautions, such as proper hand-washing, keeping raw meat and poultry separate from other foods when shopping and storing groceries, storing raw meats at appropriate temperatures, washing utensils after handling food, washing raw fruits and vegetables prior to consumption, avoiding unpasteurized foods such as raw milk, and avoiding the consumption of raw or undercooked eggs, can all limit exposure to *Salmonella* and ensure safe eating habits (Minnesota Department of Health, 2019).

Antimicrobial resistance of *Salmonella* worldwide is considered a major public health threat. There is circulation of “the same multidrug-resistant *Salmonella* clones and/or identical mobile genetic elements encoding antibiotic resistance genes from poultry to humans” (Antunes,

et al., 2016). The original treatments for *Salmonella* included ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol, but due to high resistance, the current recommendations include fluoroquinolones, such as ciprofloxacin and extended-spectrum cephalosporins. With the resistance to the current recommendations increasing, antimicrobials, such as carbapenems, are reserved as a last resort for human health (World Health Organization, 2012). The increase in *Salmonella* resistance to human antibiotic treatments is driven by antibiotic use in the food animal industry. Globalization of the food animal industry has increased the rate at which antibiotic resistance occurs worldwide (Aarestrup, 2015). *Salmonella* control programs targeting poultry production are necessary on a global scale for a significant reduction in non-typhoidal *Salmonella* serotypes to occur (Antunes, et al., 2016).

Salmonella Serotypes

Within the species *Salmonella enterica*, the bacteria are further classified into serotypes based on their somatic O antigens and flagellar H antigens. There are over 2500 serotypes in the genus *Salmonella* with some found in only one location or host and others located in a variety of hosts and locations worldwide (Centers for Disease Control, 2020). Some serotypes, such as Anatum and Derby, are more prevalent in pork, whereas others such as Weltevreden, is more prevalent in seafood, and Enteritidis and Typhimurium are most commonly found in poultry (Ferrari, et al., 2019). Serotypes, such as Kentucky, are the most prevalent in poultry products, but they haven't been reported to cause any outbreaks (Hendriksen, et al., 2011). *Salmonella* Typhimurium has been reported in numerous outbreaks worldwide with contamination of poultry, beef, and pork products (Centers for Disease Control, 2021).

Serotypes are determined based on the chemical makeup of the surface somatic O antigens and the protein content of the flagellar H antigens (Centers for Disease Control, 2020). *Salmonella* and other Gram-negative bacteria contain lipopolysaccharides, commonly referred to as endotoxins that are large molecules consisting of a lipid and a polysaccharide chain (see Figure 1.1). In O antigens, the polysaccharide is made up of an inner core, outer core, and an O antigen. In *Salmonella*, lipid A is responsible for anchoring the polysaccharide to the cell wall, providing resistance to bile salts, hydrophobic antimicrobial agents, and environmental stressors, and protects the pathogen from death by macrophages. It “stimulates a strong innate immune response via the Toll-like receptor 4 (TLR4)-MD2-CD14 pathway resulting in the activation of

nuclear factor kB (NF-kB) and upregulation of costimulatory molecules and inflammatory cytokines" which can lead to endotoxic shock or death in the host (Kong, et al., 2012). The inner and outer cores make up the core oligosaccharide, which is a short chain of sugar residues that can be diverse between bacterial species. While it is not typically considered a virulence factor, the core oligosaccharide may aid in the adhesion of the bacteria to the host cells (Jacques, 1996) and provides an attachment site to the O polysaccharide.

The polysaccharide part of the LPS, commonly referred to as the O antigen, is a highly variable glycopolymer that determines the serotype of the bacteria. The specific combination of glycan repeating units determine the individual serotype structures (Seif, et al., 2019). These combinations can be anywhere from two to eight sugar residues and vary in the order they are linked, the type of linkage between O units, and the type of linkage between each individual residue (Liu, et al., 2008). There are 46 known O antigens for *Salmonella*, resulting in 46 recognized serogroups; these are separated “into two major groups: those that have *N*-acetyl glucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) and those which have galactose (Gal) as the first sugar in the O unit” (Liu, et al., 2014). The eight Gal-initiated O antigens are serogroups O2 (A), O4 (B), O8 (C2-C3), O9 (D1), O9,46 (D2), O9,46,27 (D3), O3,10 (E1-E3) and O1,3,19 (E4). The remaining serogroups are in the GlcNAc and GalNAc initiated antigen group. Even though there are fewer associated serogroups with the Gal-initiated O antigens, they represented 84.2% of the human-sourced isolates between 1999 and 2009 (Centers for Disease Control, 2009). Variability with the O-antigens allows for immune system evasion, more effective adhesion, and survival in a variety of environments and selection pressures (Butela & Lawrence, 2010). The O antigens are stable and cannot be altered throughout an infection, unlike the H-antigens.

Salmonella H-antigens are slender structures found on the flagella (Centers for Disease Control, 2020) and are also commonly referred to as the flagellin protein. They are encoded primarily by one of two genes, *fliC* and *fliB*; these express the phase 1 H antigen and the phase 2 H antigen, respectively (Masten & Joys, 1993). The *fliC* gene is present in all *Salmonella*, and the *fliB* gene is only present in four of the *S. enterica* subspecies (subspecies I, II, IIIb, and VI). Serotypes that express one flagellin type are called monophasic, serotypes that express two flagellin types are called diphasic, and the rare isolates that express three flagellin types are referred to as triphasic (Smith & Selander, 1991)The Kauffman-White scheme currently

recognizes 114 H antigen types; sixty-nine are characterized by a single epitope that show little to no immunologic relatedness with other H antigens (e.g., H:a, -b, -c), and the remaining 45 have multiple epitopes and are grouped into complexes that are related antigenically (e.g., the 1 complex, G complex, Z4 complex) (McQuiston, et al., 2011). Multiple methods are available to accurately serotype both the O and H antigens for *Salmonella* and other Gram-negative bacteria.

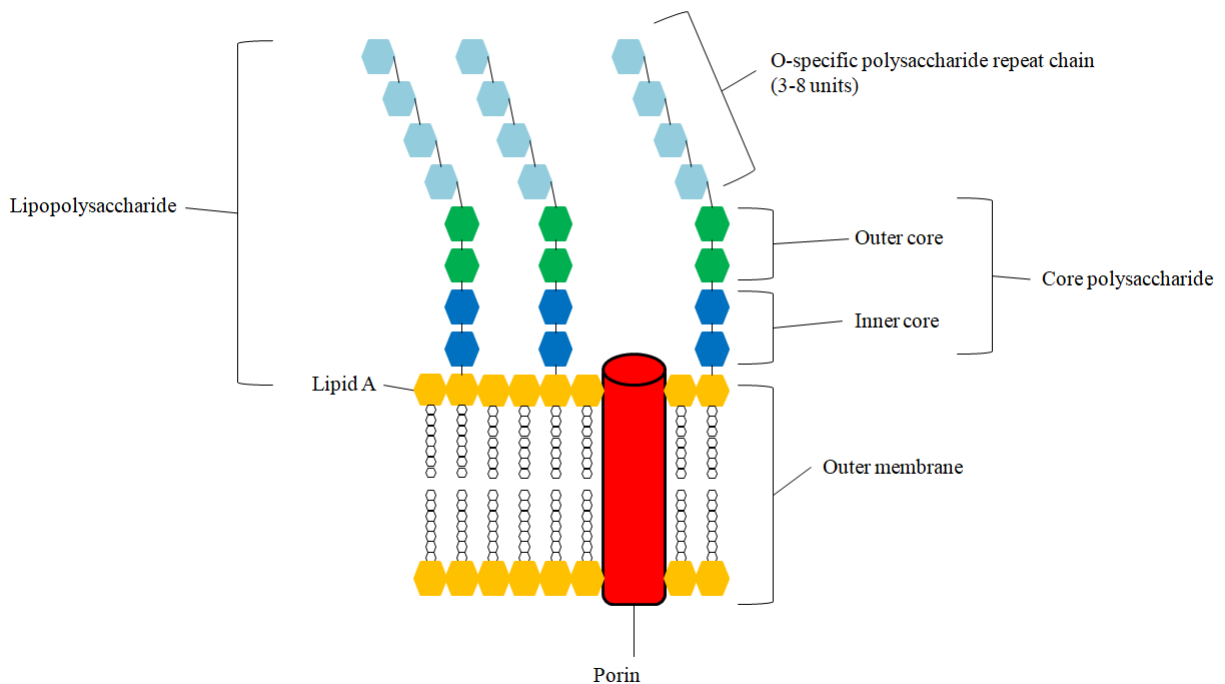


Figure 1.1 Molecular representation of the envelope of a Gram-negative bacterium

White-Kauffmann-Le Minor Serotyping Scheme

The “Kauffmann-White” classification scheme was originally named after British microbiologist Philip Bruce White and bacteriologist Fritz Kauffmann. Philip B. White (1891-1949) published a *Salmonella* classification scheme in 1926 that was later expanded by Fritz Kauffman (1899-1978) (Ewing, 1979). Kauffmann was the head of the World Health Organization Collaborating Centre for Reference and Research *Salmonella* (WHOCC-Salm) lab at the Statens Serum Institut in Copenhagen, Denmark, during the mid-1930s, where he named new serotypes based on their geographical location. After his retirement in 1965, the French microbiologist, Leon Le Minor continued this naming scheme. Originally, when Kauffmann was

naming each serotype, he considered each as a different species, italicizing the serotype in older references. In 2005, the Judicial Commission of the International Committee for Systematics of Prokaryotes recognized the current nomenclature, where only the genus and species (*Salmonella enterica* or *Salmonella bongorii*) are italicized and each serotype is capitalized but not italicized (ex. *Salmonella* Typhimurium, *Salmonella* Enteritidis) (De Vos, et al., 2005). The classification is currently referred to as the White-Kauffmann-Le Minor (WKL) scheme and was last revised in January 2007. There have been two supplements published: 2010 and 2014, which in total recognized 1,585 *Salmonella enterica* subsp. *enterica* serotypes. Each serotype is validated at WHOCC-Salm (Institut Pasteur) as well as laboratories in Hamburg (Institut für Hygiene und Umwelt) and Atlanta (Centers for Disease Control); the serotypes are homologated when all three laboratories agree (Grimont & Weill, 2007).

Each serotype is determined based on the use of specific antisera to distinguish the various antigen combinations. A 20 µL volume of previously prepared antisera is placed on a slide, then a bacterial sample is mixed on the slide with the antisera. Within 5-10 seconds, a visible agglutination should be noticeable to the naked eye as seen in Figure 1.2a. Figure 1.2b shows a negative reaction. Once *Salmonella* is identified using a selective agar medium or biochemical test, the O antigen is determined by first using an O group pool antiserum to help narrow down the selection of O antiserum, and then testing the individual O antiserum covered in each pool to identify the exact O determination. After the O group antisera are tested and the serogroup is identified, the H phase antisera within that serogroup are tested to determine the specific serotype, starting with the Phase 1 H antigens and then the Phase 2 H antigens. Since only one H antigen is present on each individual bacterium at a time, the Phase 2 H antigens may need to be forced via a phage inversion to identify the additional H antigens. Figure 1.3 is an aid to identifying *Salmonella* serotypes using antisera. Once the antisera are tested, the individual formulation determines the specific serotype. An example of popular serotypes and their corresponding antisera formulation is provided in Table 1.2.

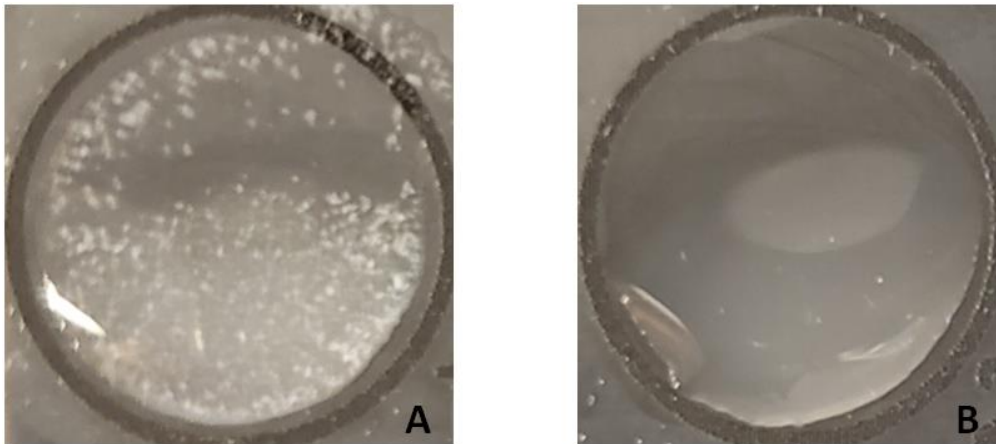


Figure 1.2 Slide agglutination of *Salmonella* reactions

Sample A shows a positive reaction and Sample B shows a negative reaction

Table 1.2 Example of *Salmonella* serotypes and their antisera reactions

<i>Salmonella</i> Serotype	O-Antigen Antisera	H Phase 1 Antisera	H Phase 2 Antisera	Associated Serogroup
<i>S. Typhimurium</i>	<u>1</u> , 4, [5], 12	i	1,2	Group O:4 (B)
<i>S. Enteritidis</i>	<u>1</u> , 9, 12	g,m	--	Group O:9 (D1)
<i>S. Infantis</i>	6, 7, <u>14</u>	r	1,5	Group O:7 (C1)
<i>S. Heidelberg</i>	<u>1</u> , 4, [5], 12	r	1,2	Group O:4 (B)
<i>S. Kentucky</i>	8, <u>20</u>	i	Z6	Group O:8 (C3)
<i>S. Anatum</i>	3, {10}{15}{ <u>15</u> , <u>34</u> }	e,h	1,6 [Z ₆₄]	Group O:3,10 (E1)

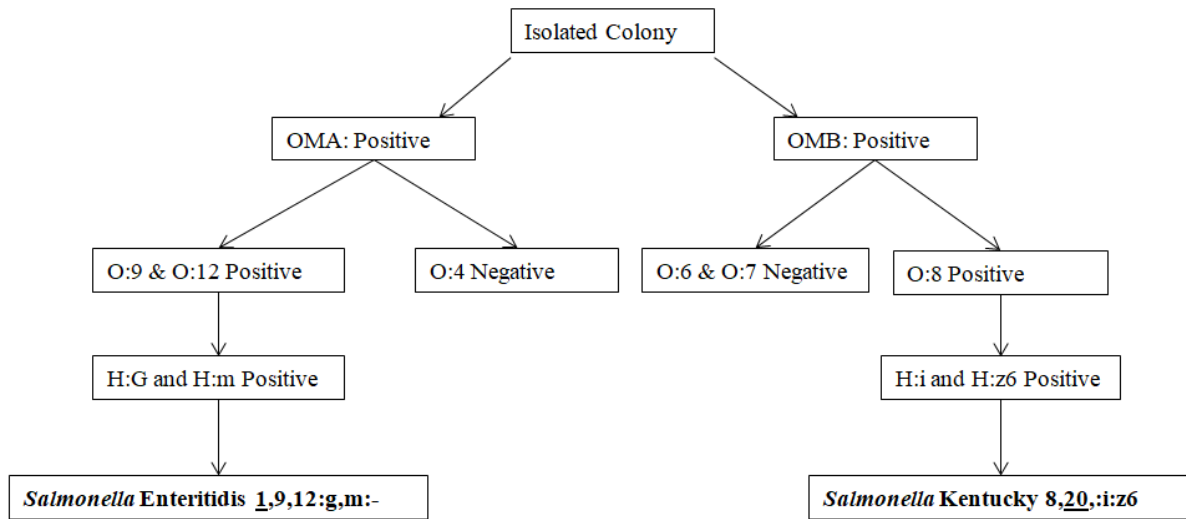


Figure 1.3 Full serotyping of *S. Enteritidis* and *S. Kentucky*

Intergenic Sequence Ribotyping

In addition to serotyping using antisera, there are multiple DNA-based serotyping methods currently available. One such method is Intergenic Sequence Ribotyping (ISR), which evaluates the single nucleotide polymorphisms (SNPs) that occur in the 5S ribosomal gene region along with the flanking sequences that border the gene *dkgB* (2,5-didehydrogluconate reductase B) which is involved in the biosynthetic process of L-ascorbic acid and enables oxidoreductase activity (BioCyc, 2021). When using a DNA serotyping approach, the results are not impacted by the variable expression found on the cell-surface antigens such as seen in using the WKL method (Guard, et al., 2012). With access to a high-throughput sequencing lab, the overall time commitment and cost is decreased per sample when compared to the cost and time associated with performing full WKL for each individual isolate. In *Salmonella enterica* species, a region spanning the end of a 23S ribosomal gene, across a 5S gene and the last base pair preceding a tRNA *aspU* ribosomal gene neighboring *dkgB* has been shown to differentiate closely related serotypes (Guard, et al., 2012); (Morales, et al., 2006). ISR presents a more sensitive assessment of each serotype when compared to DNAhyb, a technique that measures the similarity between DNA sequences. When compared, two types of *Salmonella* Typhimurium

were identified through ISR, Typhimurium and Typhimurium 5-, whereas DNAhyb was unable to identify the 5- variant (Guard, et al., 2012). Currently, ISR can be used to identify over 240 different *Salmonella enterica* subspecies *enterica* sequences for common serotypes with more sequences added each year.

Serotype Prevalence

Even though there have been 2,579 *Salmonella* serotypes identified as of 2007, approximately 98% of all human cases are caused by fewer than 100 serotypes. In 2016, 69.2% of all culture-confirmed human *Salmonella* infections in the United States were caused by only 20 serotypes (National Center for Emergine and Zoonotic Infectious Diseases: Division of Foodborne, Waterborne and Environmental Diseases, 2018). Table 1.3 lists the culture-confirmed human *Salmonella* infections reported to The Laboratory-based Enteric Disease Surveillance (LEDS), with the top 20 most frequently reported serotypes from the United States in 2016.

After *Salmonella* Pullorum and *Salmonella* Gallinarum were eradicated from commercial flocks in the mid-1960s, *S. Enteritidis* emerged as a primary serotype in both birds and eggs. Previously only associated with rodents (Callaway, et al., 2008), *Salmonella* Enteritidis quickly filled the empty niche. Since *S. Gallinarum* and *S. Enteritidis* both express the immunodominant O9 lipopolysaccharide antigen on their cell surface, it is estimated that *S. Gallinarum* competitively excluded *S. Enteritidis* (Rabsch, et al., 2000) since it had an increased ability to colonize and adapt within the poultry industry (Velge, et al., 2005). Production practices, such as bird density, vaccination status, and biosecurity measures can directly affect the prevalence of *S. Enteritidis* on each individual farm. From the mid-1960s until 1997, *S. Enteritidis* was the most prevalent serotype found in commercial poultry, but it was surpassed by *S. Heidelberg* in 1997 (Foley, et al., 2008). Since the mid-1990s, *S. Enteritidis* has been on the decline due to current surveillance programs and an increase in flock immunity through exposure and vaccination. *S. Enteritidis* and *S. Heidelberg* both colonize the reproductive tracts and enter the eggs, passing on the bacteria to the next generation (Gast, et al., 2004).

Salmonella Heidelberg passed *S. Enteritidis* in 1997 to become the most prevalent serotype found in chickens until it was surpassed by *S. Kentucky* in 2007 (Foley, et al., 2008). Similar to *S. Enteritidis*, it colonizes the reproductive tract and infects the eggs. *S. Heidelberg*

made headlines in March 2013 when a multistate outbreak, originating with Foster Farms Chicken, infected 634 individuals. Seven outbreak strains of multidrug-resistant *S. Heidelberg* were reported in 29 states and Puerto Rico with 77% of the ill persons coming from California. No deaths were associated with the outbreak, but 38% of the ill persons were hospitalized before the end of the outbreak in July 2014 (Centers for Disease Control, 2014). The outbreak resulted in severe backlash from the country, and Foster Farms invested \$75 million into new equipment and improvements to limit the prevalence of *Salmonella* within their company (Food Business News, 2018).

In 2007, *S. Kentucky* became the most prevalent serotype in chickens; however, it is infrequently isolated from humans. In 2016, an outbreak linked to alfalfa sprouts resulted in twenty-five individuals infected with the outbreak strain of *S. Muenchen* and one *S. Kentucky* infected individual (Centers for Disease Control, 2016). *S. Kentucky* is a concern due to being highly resistant to various antibiotics and that it is widespread throughout the poultry industry, and now, the dairy industry (Haley, et al., 2016). There are multiple distantly related sequence types (ST) of *S. Kentucky*, but two that have been regularly isolated at a global rate: ST152 and ST198. Clinical cases of ST152 in humans are rare; however, ST198 infections have been associated with Africa, Southeast Asia, and the Middle East travelers (Le Hello, et al., 2011). *S. Kentucky* is a polyphyletic serovar determined in a whole-genome phylogenetic analysis, and it has been associated with lateral gene transfer of the antigenic coding regions (Haley, et al., 2016). The two sequence types are more similar to other serotypes than to each other due to differences in sialic acid transport regions, inositol catabolism, amino acid sequence of virulence-associated proteins and a homolog of the Typhi colonization factor (tcf). These differences account for the various interactions in the bovine, human and poultry gastrointestinal tracts.

Salmonella Typhimurium is the serotype that is commonly isolated from human cases of Salmonellosis. In 2016, it was the third most isolated serovar in the United States (Table 1.3). In addition to poultry, *S. Typhimurium* can be isolated from other small pets, like hedgehogs and turtles and other food items, such as coconuts (Centers for Disease Control, 2021); (Centers for Disease Control, 2019); (Centers for Disease Control, 2018). *S. Typhimurium* presents as a gastrointestinal infection but may spread to other organs such as the liver and spleen. In 2018, *S. Typhimurium* was proven to colonize mouse brains by crossing the blood-brain barrier, an area

where antibiotics cannot fully treat infections (Chaudhuri, et al., 2018). A major virulence factor is the bacterium's flagella which aids in the initial colonization of the host (Das, et al., 2018) A monophasic serotype of *S. Typhimurium*, called 4,5,12:i:-, lacks the second-phase flagellar antigens and has emerged as a public health threat throughout Europe and the United States (Arruda, et al., 2019). It has been reported throughout the world since the 1990s, but it has increased at a dramatic rate. In 2016, it was the fifth most isolated serotype in human cases within the United States as reported by the CDC (Table 1.3). The monoflagellated *Typhimurium* has an advantage in that the loss of the second flagella allows it to hide from the immune system (Switt, et al., 2009).

Table 1.3 Culture-confirmed human *Salmonella* infections report to LEDS, with the 20 most frequently reported serotypes listed individually, United States, 2016

Rank	Serotype	Number reported	Percent	Incidence (per 100,000)
1	Enteritidis	7,830	16.8	2.44
2	Newport	4,728	10.1	1.47
3	Typhimurium	4,581	9.8	1.43
4	Javiana	2,719	5.8	0.85
5	I 4,[5],12:i:-	2,179	4.7	0.68
6	Infantis	1,281	2.7	0.4
7	Muenchen	1,216	2.6	0.38
8	Montevideo	1,018	2.2	0.32
9	Braenderup	1,001	2.1	0.31
10	Thompson	792	1.7	0.25
11	Saintpaul	778	1.7	0.24
12	Heidelberg	754	1.6	0.23
13	Oranienburg	692	1.5	0.22
14	Mississippi	536	1.1	0.17
15	Typhi	423	0.9	0.13
16	Bareilly	412	0.9	0.13
17	Berta	369	0.8	0.11
18	Agona	362	0.8	0.11
19	Paratyphi B var. L(+) tartrate+	343	0.7	0.11
20	Anatum	257	0.6	0.08
	Subtotal	32,271	69.2	
	Other serotyped*	7,709	19.3	2.4
	Unknown serotype	5,625	12.1	1.75
	Partially serotyped	801	1.7	0.25
	Rough, mucoid, and/or nonmotile	217	0.5	0.07
	Subtotal	14,352	30.8	
	Total	46,623	100	14.51

(National Center for Emerging and Zoonotic Infectious Diseases: Division of Foodborne, Waterborne and Environmental Diseases, 2018)

“Reference to specific commercial products, manufacturers, companies, or trademarks does not constitute its endorsement or recommendation by the U.S. Government, Department of Health and Human Services, or Centers for Disease Control and Prevention”

Salmonella Surveillance

With *Salmonella* ubiquitous worldwide and over 2500 different serotypes currently identified, surveillance around the world is of utmost importance. The globalization of food production, marketing, manufacturing, and international travel has caused the risk of transferring infectious agents, such as *Salmonella*, to increase as well; therefore, surveillance around the world is important. Surveillance helps to identify which serotypes are present, monitor how they change over time, alert to possible outbreaks, link outbreaks to their source, and determine if existing programs need to be modified to ensure an adequate and relevant food safety program (Kaferstein, et al., 1997). With the increased production of food and international travel, the outbreaks can quickly progress from mild to severe, affecting hundreds or thousands of people worldwide. It is important for the programs implemented within each country to collaborate with each other so that they can determine whether the outbreak is confined to one country or multiple countries. A network, such as Salm-Net, can be a useful tool to aid in the interactive international collaboration (Kaferstein, et al., 1997). Collaboration between the various programs can also help maintain international food safety standards and aid in the spread of new information and research.

It is important that each surveillance system monitors the epidemiologic data, populations at risk, types of food, locations of each outbreak, practices that lead to the contamination and which causative agents are implicated. The systems should also have guidelines and resources in place to inform people on how to properly prepare foods, especially if the food has been imported from another country or if it is food that is culturally different from what they are used to preparing (Kaferstein, et al., 1997). These systems can help ensure proper responses and information when dealing with outbreaks of *Salmonella* and other various bacterial based foodborne illnesses.

In the United States, multiple departments and programs manage and monitor *Salmonella* infections; these will be described in more detail below. As explained, the National Poultry Improvement Plan aids in new diagnostic technology that can be applied to the improvement of poultry and poultry products. (NPPI). The Center for Disease Control monitors various outbreaks, their causes, and reports to other government programs worldwide (Centers for Disease Control, 2021). The United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) works to prevent foodborne illness and protect public health by

creating and upholding regulations that ensure proper food production (United States Department of Agriculture (USDA): Food Safety and Inspection Service (FSIS), 2018). The USDA also investigates outbreaks, attempts to control them, and then, works to prevent similar outbreaks from occurring in the future (Food and Drug Administration, 2021).

National Poultry Improvement Plan

Established in the 1930s to eliminate pullorum disease (PD) caused by *Salmonella* Pullorum, the National Poultry Improvement Plan (NPIP) provides “a cooperative industry, state, and federal program through which new diagnostic technology can be effectively applied to the improvement of poultry and poultry products throughout the country” (National Poultry Improvement Plan (NPIP)). The program was eventually expanded to include monitoring and testing for *Salmonella* Typhi, *Salmonella* Enteritidis, *Mycoplasma synoviae*, *Mycoplasma meleagridis*, *Mycoplasma gallisepticum* and avian influenza. While initially only testing chickens, the program now includes commercial poultry, turkeys, waterfowl, exhibition poultry, game birds and backyard poultry. Criteria were developed with the aid and expertise of state and federal officials and industry professionals. The criteria developed by the program and the subsequent standards have eliminated PD from the United States commercial poultry industry and decreased the presence of many other infectious agents (National Poultry Improvement Plan (NPIP), n.d.)). The NPIP provides workshops for laboratory professionals to train and inform them in the best practices for multiple infectious agents including *Salmonella*. Their Program Standards describe the sampling and testing protocols for all agents listed above as well as proper animal care and maintenance for production facilities and were used to isolate the *Salmonella* isolates included in this thesis project.

Center for Disease Control

The Center for Disease Control (CDC) is the United States’ health protection agency. It provides health information and conducts research to protect and respond against health threats that may become dangerous and expensive (Centers for Disease Control, 2019). The CDC utilizes multiple surveillance systems to monitor the prevalence of *Salmonella* throughout the country.

- The National Notifiable Diseases Surveillance System (NNDSS) is utilized to conduct and monitor culture-confirmed cases from public health laboratories across the United States.
- The Foodborne Disease Outbreak Surveillance System (FDOSS) “captures data on pathogenic agents, foods, and settings responsible for outbreaks” (Centers for Disease Control, 2019).
- The Foodborne Disease Active Surveillance Network (FoodNet) tracks the impact of food safety policies, reports the trends in foodborne infections and manages the CDC’s Emerging Infections Program.
- The Laboratory-based Enteric Disease Surveillance (LEDS) collects a variety of information regarding the *Salmonella* isolates affecting humans. This information includes serotyping of the isolates to aid in assessing geographic differences and the trends over time.
- The National Antimicrobial Resistance Monitoring System - Enteric Bacteria (NARMS) specifically monitors the antimicrobial resistance in various enteric bacteria, including *Salmonella* from sources such as animals, retail meats and humans.
- The National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet) identifies and connects nationwide cases of illness, including some that may otherwise go undetected or unidentified (Centers for Disease Control, 2019).

These resources allow the CDC to release various annual reports as well as identify and monitor outbreaks throughout the nation.

United States Department of Agriculture

The United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) ensures that poultry, meat, catfish, and eggs are safe and properly packaged and labeled through inspections and compliance regulations (United States Department of Agriculture (USDA): Food Safety and Inspection Service (FSIS), 2020). Regarding the inspection of meat products, FSIS is responsible for ensuring that the commercial supply of meat is safe, wholesome, and correctly labeled. They inspect all parts of the carcass of any sheep, swine, goat, and cattle per the Federal Meat Inspection Act. For poultry products, FSIS ensures

that the poultry is wholesome, safe, and correctly labeled per the Poultry Products Inspection Act (PPIA). For egg products, FSIS inspects activities at the egg production plants and ensures that they are wholesome, not adulterated and properly labeled, and packaged per the Egg Products Inspection Act (EPIA). For siluriformes, like catfish, FSIS ensures that the products are not adulterated and are properly packaged and labeled (United States Department of Agriculture (USDA): Food Safety and Inspection Service (FSIS), 2020).

Food and Drug Administration

The Food and Drug Administration (FDA) investigates various outbreaks with the intention of controlling and preventing them. Outbreak investigations are managed by the FDA's Coordinated Outbreak Response and Evaluation (CORE) team. The CORE team also conducts surveillance and post-response activities (Food and Drug Administration, 2021). Since their establishment in 2011, CORE has identified over 950 potential outbreaks, identified specific foods in 100 outbreaks, and responded to over 230 outbreaks. In order to properly detect, respond and prevent outbreaks, the CORE team includes multiple smaller teams.

- The CORE Signals and Surveillance Team collaborates with the CDC, FDA, and state agencies to monitor emerging outbreaks and the surveillance trends by reviewing past inspections, product distribution, sampling results, and source information (Food and Drug Administration, 2021).

The CORE Communications Team is responsible for issuing the public warnings regarding an outbreak as well as preparing responses to questions from the media and FDA Stakeholders (Food and Drug Administration, 2021).

Conclusion

Typhoid and non-typhoidal *Salmonella* serotypes play important roles in animal and human health throughout the world. From the ancient Athenians' encounter with *S. Typhi* as an agent of bioterrorism, to today's battle against *Salmonella*-induced food poisoning, *Salmonella* has impacted health and military regulations, meat production, food handling and animal husbandry on a global basis. Mary Mallon showed how one person can impact an epidemic through her spread of typhoid fever in New York. With typhoid fever, pullorum disease and fowl

typhoid no longer endemic in developed countries, *Salmonella*'s primary impact is currently in foodborne illnesses. Globalization of the food industry has created a situation where serotypes that were previously only found in a specific geographic location have now traveled around the world as the food products are shipped to new locations.

The need for early identification, detection and collaboration of international government agencies is paramount to the goal of managing *Salmonella*'s presence in the food industry. The United States NPIP, CDC, USDA FSIS and FDA agencies all work together and with other government agencies around the world to track, monitor and endeavor to reduce the impact of outbreaks on a global scale. Antibiotic resistance is becoming an increasingly difficult problem when it comes to treating infections in critically ill human patients; therefore, antibiotic use in the food industry to treat the infections has decreased. The goal now lies in preventing *Salmonella* infections in the beginning stages of animal production to keep it from infecting the end consumers. Vaccines, such as custom autogenous vaccines, may play an important role in managing these outbreaks before they have a chance to occur.

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Chapter 2 - *Salmonella* Autogenous Vaccines and the Phenotypic Characteristics that Aid in the Selection of a Formulation

Autogenous Vaccines – Background Information

Autogenous veterinary vaccines are defined as “any immunological veterinary medicinal products manufactured for the purpose of producing active immunity from pathogenic organisms obtained from an animal or animals from the same herd that have been inactivated and used for” the prevention of microbials in the same animals or herd (Ceva Sante Animale, 2021). They are prepared from bacterial or viral cultures from an individual or farm and then used to immunize the same individual or farm to protect against further spread and infection. The vaccines are produced in a licensed facility according to an approved Outline of Production and are approved for use under the direction of a veterinarian or approved non-veterinarian specialist (United States Department of Agriculture, 2016). They are considered for use when there are no commercially licensed products available or when the commercial products do not provide adequate protection. Autogenous biologics can only be used in the herd of origin or an approved adjacent herd; for any non-adjacent herd use, approval must be received prior to shipment from the appropriate State Veterinarians and notification made to the Center for Veterinary Biologics (CVB) of the USDA. Since their introduction in 1903 by Sir Almroth Edward Wright (Britannica, 2021) (PharmProm.ru, 2021), autogenous vaccines have developed into a large global impact valued at over \$400 million in 2018 with an estimated Compound Annual Growth Rate (CAGR) of 5.5% by 2031 (Persistence Market Research, 2021).

There are multiple types of autogenous licenses, including killed virus autogenous vaccine, autogenous bacterin, killed virus + autogenous bacterin vaccine, and killed fungus autogenous vaccine (United States Department of Agriculture, 2016). They are regulated and guided by the following documents: 9 CFR 113.113 Autogenous Biologics, 9 CFR 112.7 Labeling Requirements, Veterinary Services Memorandum (VSM)800.85 Avian Influenza Vaccines, VSM 800.69 Guidelines for Autogenous Biologics, VSM 800.103 Guidelines Concerning Restrictions on the Production and Use of Veterinary Biologics, CVB Notice 02-04 Autogenous Vaccine Use in Turkeys, and CVB Notice 09-15 Autogenous Vaccines Containing Porcine Circovirus (United States Department of Agriculture, 2016). The Outlines of Production

(OPs) for autogenous products follow the same format used for all vaccines, toxoids and bacterins as outlined in 9 CFR 114.9 and are generally worded to allow for production flexibility. The adjuvant selected must be approved for use in each specific species (per VSM 800.51). For viral autogenous products, only cell lines that are approved by the USDA and satisfy the requirements put forth in 9 CFR 113.52 – identified in Section II.C.1 of the OP and must be approved for use in each species the vaccine will be used in – or primary cells that satisfy requirements in 9CFR 113.51 and VSM 800.65 may be used. They must also be produced in a licensed facility and only approved for use by, or under the immediate direction of, a veterinarian or non-veterinarian approved specialist (United States Department of Agriculture, 2016). They are tested in mice, poultry, fish, or guinea pigs for safety and must not contain bacterial or fungal contamination, but they do not have to demonstrate efficacy or host animal safety (Erdman, et al., 2020).

Autogenous isolates may not be used indefinitely. Their use is restricted to 12 months from harvest of the first serial, or 15 months from the date of isolation, whichever comes first (United States Department of Agriculture, 2016). If the company has the appropriate information, as described in VSM 800.69 and 9 CFR 113.113, then the use of these isolates may be extended to 24 months. Other restrictions for autogenous products are listed in VSM 800.103 and state that the “license does not authorize production, distribution, or shipment of autogenous vaccine/bacterin for foot-and-mouth disease, rinderpest, any H5 or H7 subtype of avian influenza, any subtype of avian influenza in chickens, swine vesicular disease, Newcastle disease, African swine fever, classical swine fever, Brucella abortus, vesicular stomatitis, and rabbit hemorrhagic disease or any other disease that the Administrator determines may pose a risk to animal or public health” (United States Department of Agriculture, 2018). These restrictions ensure that the biologics do not interfere with any eradication programs, surveillance programs, does not pose any additional health risks, and restricts the use of certain microorganisms.

There are many benefits to implementing an autogenous vaccination program. Preventing infections with a custom vaccine can limit disease occurrence, especially those involving multi-drug resistant bacteria. Increased antibiotic resistance in bacteria has limited the available treatment options for humans and animals alike. By preventing disease outbreaks with a vaccine, less pressure is put on antibiotics for the control of the associated pathogen. This also helps contribute to public health since so many of these bacterial pathogens are zoonotic and can lead

to human infections (Ceva Sante Animale, 2021). Autogenous vaccines can provide solutions where no currently licensed vaccine exists, such as instances involving a “minor” disease or a “secondary” species (von Hanko, 2009). It can also be created and developed in an urgent manner if there is a sudden onset of an emerging disease. Autogenous vaccines obtain specific immunity to fight against antigenic variability within bacterial species that may not be available in commercial vaccines. Finally, they cost less to produce than a commercial vaccine, since multiple pathogens can be combined into a single vaccine. Therefore, this limits the number of vaccines used on a property while still ensuring appropriate vaccine coverage and decreasing the animal’s overall stress associated with the act of vaccination (Ceva Sante Animale, 2021). Ceva Sante Animale currently prepares autogenous vaccines for swine, avian, fish, equine and rabbit animal species throughout the world, while other animal biologics companies provide autogenous vaccines for cattle and other ruminant species.

Autogenous Vaccines – Poultry

Poultry-based autogenous vaccines are currently used for bacterial species, such as *Escherichia coli*, *Avibacterium* spp., *Pasteurella* spp., *Gallibacterium*, *Enterococcus* spp., *Erysipelothrix rhusiopathiae*, *Mycoplasma* spp., *Ornithobacterium rhinotrachealis*, *Salmonella enterica*, *Riemerella* spp., and *Clostridium* spp., as well as for the following viruses: avian reoviruses, avian adenoviruses, infectious bronchitis, duck hepatitis and low pathogen avian influenza viruses (von Hanko, 2009). For bacteria, such as *Escherichia coli* and *Salmonella enterica*, cross-protection may not occur between serotypes, which means that multiple vaccines would be needed to adequately protect a flock. With an autogenous vaccine implemented on a poultry farm, the specific serotypes found on the farm can be included in one autogenous product, ensuring that the birds are not excessively stressed with additional handling and receiving the most relevant vaccines based on potential exposure.

Injection site injuries in poultry are not uncommon and may result in focal inflammatory myositis, cellulitis, and myositis of the neck region, which may in turn cause postural and/or neurologic signs. The affected birds can show leg paralysis, recumbency, twisting of the neck and ataxia along with subcutaneous and muscle swelling (Van Wettere, 2020). Intramuscular or subcutaneous injections are the most common route of vaccination in poultry and the risk for developing injection site injuries is present for each injection. Decreasing the overall number of

injections and times in which the birds are handled can decrease the chance of developing these injuries. There are other vaccination routes, such as application through drinking water, spray application, eye drops, cutaneous through wing webs or feet or *in-ovo*. Each route also comes with its own set of risk for injury (Cargill & Johnston, 2007).

Autogenous vaccines have been proven to be efficacious in poultry throughout the past few decades based on recent studies. For protection against avian pathogenic *Escherichia coli* (APEC), birds vaccinated with a vaccine combination including an O78 commercial vaccine and an O78 autogenous vaccine, or with an O78, O18, O111 three-valence autogenous vaccine were better protected against the challenge of O78 and O111 APEC strains when compared to the unvaccinated groups and live commercial O78 *E. coli* vaccinated groups. In the same study, when challenged with an O18 APEC strain, the group of birds vaccinated with a vaccine combination including the O78 commercial vaccine and the O78, O18, O111 three-valence autogenous vaccine presented milder clinical signs, a lower re-isolation rate, and a lower mean lesion score. This may be due to each vaccine eliciting a separate immune response, thus triggering a wider immunization spectrum (Koutsianos, et al., 2020). Birds vaccinated with two separate doses of a homologous autogenous vaccine had an increase in their IgY antibody response, a slower clinical onset of infection, and a lower mortality when compared to birds vaccinated with a single dose of the same vaccine and birds without an autogenous vaccination, even when all birds had previously been vaccinated with a commercial *E. coli* vaccine (Li, et al., 2017). Similar results were seen with other bacteria, such as *Avibacterium paragallinarum* (Wambura, 2010), *Riemerella anatipestifer* in Muscovy ducks (Thibault, Lesceau, Gantelet, & Castagnos, 2017), and *Salmonella enterica* (Abd El-Ghany, et al., 2012).

Autogenous Vaccines – Salmonella

Bacterial load, such as that with *Salmonella*, can be reduced in all stages of poultry production, and most infections are via vertical transmission from the broiler breeder and from horizontal transmission in the broiler houses (Heyndrickx, et al., 2002). *Salmonella* vaccination of the layers and the broiler breeders can reduce the rate of vertical transmission from the layers to their progeny, while also providing maternal antibodies to the chicks that may help minimize the horizontal spread throughout the grow-out houses and the amount of *Salmonella* making its

way into the processing plant. While the vaccination of the breeder flocks may not reduce the prevalence or load of *Salmonella* in those specific birds, it increases their antibody titers and results in a significant decrease in the prevalence of *Salmonella* in their progeny (Berghaus, et al., 2011) due to the protection from maternal antibodies (Young, et al., 2007).

Autogenous *Salmonella* vaccines have also been effectively used in other food-producing animals. In feedlot cattle, *Salmonella* autogenous vaccines can reduce the horizontal transfer of bacteria between vaccinated and non-vaccinated cattle housed in adjacent pens (Horton, et al., 2021). In dairy cattle, the effect of any vaccine – live attenuated, killed, or autogenous – has had varied results for a long-term preventive effect but may be beneficial for curbing the initial outbreaks depending on the serotype involved in the outbreak (Holschbach & Peek, 2018).

In swine, autogenous vaccination against the *S. Typhimurium* monophasic variant 1,4,[5],12:i- is effective in controlling the prevalence of fecal shedding, infection, and colonization in the piglets of the vaccinated sows. In the study conducted by Ruggeri et al. 2015, twenty pregnant sows were vaccinated with an autogenous vaccine and twenty pregnant sows were not vaccinated. After farrowing, three piglets from each sow were included in the secondary portion of the study. The sixty piglets from the vaccinated sows were separated into two groups, thirty with a booster vaccination and thirty without a booster. The same was done with the piglets from the non-vaccinated sows. The results indicated that piglets born from vaccinated sows grew more than the non-vaccinated sows' piglets. The sows did not show any changes in their fecal shedding, but the piglets from the vaccinated sows did show decreased fecal shedding. The piglets from the non-vaccinated sows that were in turn vaccinated also did not show decreased fecal shedding. The most protected group was the thirty piglets that were born of vaccinated sows, and then, were vaccinated; they had a lower percentage of colonized animals as well as a lower *Salmonella* prevalence in their organs and their feces. They also had a heavier mean weight during the growing phase and at slaughter (Ruggeri, et al., 2015).

While there are many positives to the implementation of an autogenous vaccine into any livestock farm, when it comes to *Salmonella*, there are too many different serotypes and variabilities between the isolates to ensure a perfect response. When challenged with the same strain included in the vaccine, there are positive responses in poultry, cattle, and swine, but their ability to cross-protect is not guaranteed. Also, with so many different *Salmonella* serotypes present, it can be difficult to select the appropriate formulation.

Autogenous Vaccines – Formulations

Autogenous vaccines exist in many different formulations, and each company may create their vaccine differently. Some companies, such as Vaxxinova, manufacture siderophore receptors and porins (SRP®) autogenous vaccines for cattle that only contain a trace amount of outer cell wall lipopolysaccharide (LPS) and no whole bacterial cells (Vaxxinova, n.d.). Other companies, such as Ceva Biovac, use the whole bacterial cell in their vaccine (Ceva Sante Animale, 2021).

In addition to an appropriate bacterial candidate, the adjuvant is equally important to the formulation of the vaccine. Adjuvants are substances that enhance an immune system's response to antigenic presence. There are two broad groups of adjuvants: immunostimulatory adjuvants that directly activate cells through specific receptors, such as toll-like receptors (TLRs), and adjuvants that deliver the antigen to antigen presenting cells (APCs), such as dendritic cells. Both result in inflammatory responses to increase the immunologic response, which creates a more rapid response and a stronger adaptive immune response with increased antigen specificity (Helson, n.d.). In veterinary autogenous vaccines, adjuvants, such as aluminum hydroxide, water-oil, and water-oil-water emulsions, are common choices, but synthetic adjuvants, such as MVP Adjuvants®, have become increasingly popular due to their user-friendly preparation methods and their ability to be modified for specific vaccine needs (Phibro Animal Health Corporation, 2021).

For the selection of an appropriate bacterial isolate to be used in the autogenous vaccine formulation intended for a specific farm or hatchery, it is important to know and understand the specific bacterial colonies that are prevalent on the location. There are many different serotypes of *Salmonella*, and even the colonies belonging to the same serotype may exhibit variations amongst them, making the selection of an appropriate isolate difficult. Performing a *Salmonella* surveillance study prior to selection can help determine which serotypes are most prevalent in the hatchery as well as in the processing plant and determine which serotypes and isolates should be focused on. Further identification of the isolates could include phenotyping and/or genotyping characterization. In this study, phenotypic characterizations were utilized with various biochemical tests analyzed to establish a clear understanding of bacterial properties and which

ones are most likely to survive from the processing plant to the plates of the end consumers. *Salmonella* isolates can be characterized by the following tests: citrate utilization, acetoin production, d-sorbitol fermentation, arginine dihydrolase, inositol utilization and biofilm formation. A more detailed description of these tests is provided below.

Laboratory Characterization Methods of *Salmonella* Isolates

There are many different phenotypes associated with *Salmonella* spp., and the creation of an autogenous formulation requires selecting a subset of isolates for inclusion in the vaccine. After a surveillance study, there are many different isolates to choose from and a selection based on the serotype alone may not be the most useful. Phenotyping of the samples can further differentiate these isolates and aid in the selection of isolates that represent the bacterial population. Tests such as citrate utilization, acetoin production, d-sorbitol fermentation, arginine dihydrolase, inositol utilization, and biofilm formation can identify isolates that are most likely to survive through the production cycle and potentially create outbreaks in the end consumers.

Citrate Utilization

Citrate is an important organic acid that is used as a carbon and energy source through the Krebs cycle. The transport of citrate into the bacterial cell requires citrate transport proteins, called permeases, to transfer the citrate. Once it enters the cell, it is cleaved by citrate lyase into acetate and oxaloacetate, which is in turn broken down into pyruvate and CO₂. Further breakdown of the pyruvate depends on whether the environment is under aerobic or anaerobic conditions (Tankeshwar, 2021). Under aerobic conditions, it enters the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle or citric acid cycle, to produce carbon dioxide, flavin adenine dinucleotide H₂ (FADH₂), adenosine triphosphate (ATP) or guanosine-5'-triphosphate (GTP) and nicotinamide adenine dinucleotide + H (NADH). Under anaerobic conditions it generates heterolactic fermentation products, such as acetate, ethanol, and L-lactate (Figure 2.1) (Kreth, et al., 2013) (Quintans, et al., 2008).

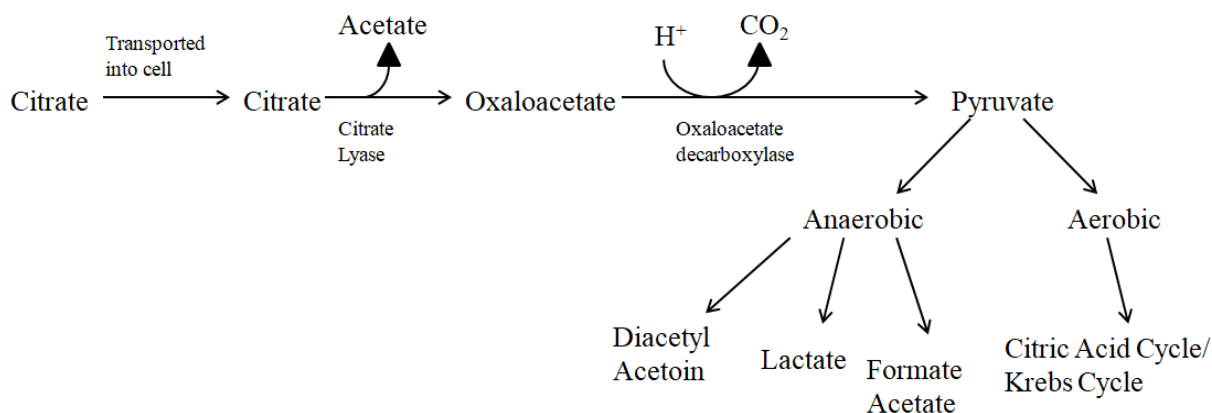


Figure 2.1 The metabolic pathway from citrate to its anaerobic and aerobic derivatives

Some citrate utilization tests use a tube slant of citrate agar, where the agar contains citrate as the only carbon source and inorganic ammonium salts ($\text{NH}_4\text{H}_2\text{PO}_4$) as the only source of nitrogen. Any bacteria that grow on this agar have the ability to produce the enzyme citrate-permease, which converts citrate to pyruvate. The ammonium salts are broken down into ammonia as the bacteria metabolize the citrate that increases the alkalinity. An increase in the pH to anything above 7.6 results in the bromthymol blue indicator in the agar to turn from green to blue (Aryal, 2019).

Other tests, such as the bioMerieux® API 20E identification system for *Enterobacteriaceae* and other non-fastidious Gram-negative rods, involves mixing up 5mL of 0.85% NaCl with an isolated bacterial colony. The inoculum is then added to a cupule on the test strip that contains 0.756 mg of trisodium citrate as the only available carbon source, and the test strip is incubated at 37°C for 24 hours. Figure 2.2 identifies the test results (bioMerieux, 2002).

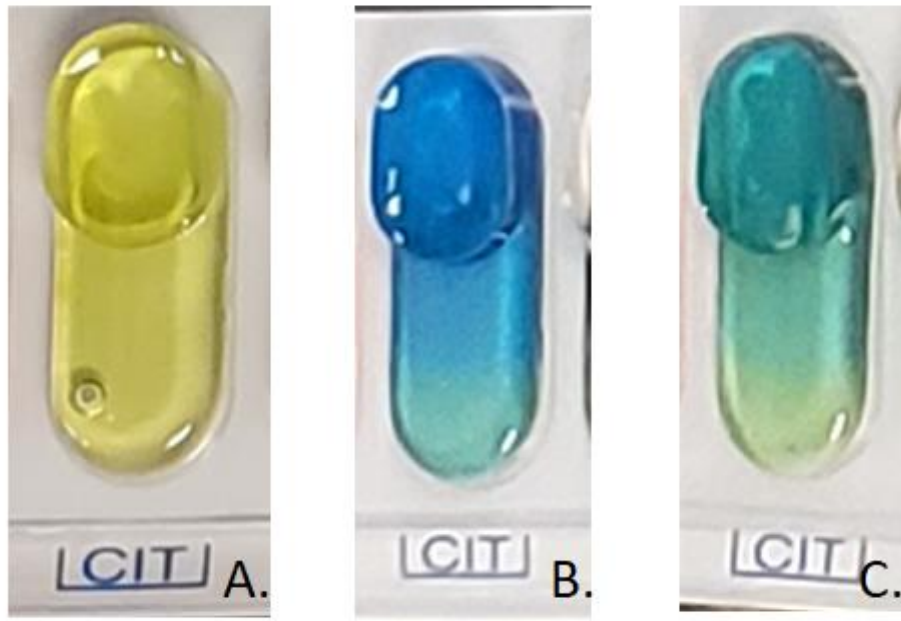


Figure 2.2 Citrate Utilization Reactions

The light green/yellow color in A indicates a negative test reaction.

The green blue/blue colors in B and C both indicate positive test reactions

Salmonella colonies that can utilize citrate as a primary carbon source also have an additional advantage over other colonies due to the important role citrate plays in the host's immune response. Activated immune cells require energy to function properly so they use a modified Krebs cycle that releases citrate as a waste product. Citrate regulates carbohydrate and lipid metabolism, aids in the production of prostaglandins and nitric oxide, impacts cytokine production and can generate aconitate. Aconitate is generated from itaconate and promotes an anti-inflammatory response (Zotta, et al., 2020). During infection, the host macrophages and activated dendritic cells reprogram their metabolic pathways, which results in the accumulation of both succinate and citrate; these waste products have antibacterial effects, and they act as signaling molecules that affect inflammation pathways (Williams & O'Neill, 2018). *Salmonella* that can utilize the citrate will not be affected by these antibacterial effects and can slow the host's inflammatory responses.

Acetoin Production

Acetylmethylcarbinol (Acetoin) is a metabolite that is excreted by many microorganisms including bacteria. It serves as a microbial classification marker, aids in the regulation of the NAD/NADH ratio, the storing of carbon and helps microbes avoid acidification (Xiao & Xu, 2007). Acetoin is produced from glucose under aerobic conditions in the 2,3-butanediol fermentation pathway in which two molecules of pyruvate are converted to α -acetolactate by acetolactate synthase which is decarboxylated to form acetoin as seen in Figure 2.3 (Romick & Fleming, 1998). While the pathway is most notably associated with *Bacillus* spp. and *Serratia*, it has also been associated with *Escherichia coli* (Vivijis, et al., 2014). When stressed for a prolonged period of time, *Salmonella* will initiate the pathway (Abdallah, et al., 2007). *Salmonella* was historically considered negative for acetoin production; however, acetoin production has been noted in *Salmonella* throughout the years, prompting a closer look at the conditions surrounding the acetoin production pathway. When cultured on a minimal medium containing glucose, *Salmonella* has been shown to produce acetoin (Garibaldi & Bayne, 1970).

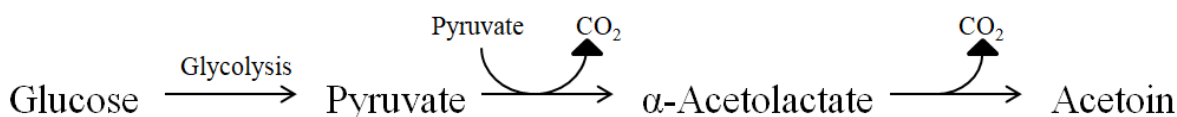


Figure 2.3 The metabolic pathway from glucose to the production of acetoin

The Voges-Proskauer (VP) test has been used to detect acetoin production since 1898. The premise of the test is that glucose is broken down into acetylmethylcarbinol, which when in the presence of oxygen and a strong base, is oxidized to diacetyl. Alpha-naphthol and potassium hydroxide are each added to the tube of inoculum after incubation in methyl red broth as seen in Figure 2.4. Once added, the tube is shaken vigorously and set at room temperature for an hour. If positive, the culture will turn brownish red to pink and a negative culture will turn a brownish green to yellow (University of Wyoming, n.d.). BioMerieux® also includes a VP reaction on their API 20E test strip. To perform this test on the strip, the tube and cupule, containing 1.9 mg of sodium pyruvate, is filled with an inoculum of the selected culture and 0.85% NaCl solution,

and the strip is incubated aerobically at 37°C for 24 hours. Prior to reading the test result, the test kit reagents VP1 and VP2 are added, in order, to the test strip and allowed to sit at room temperature for 10 minutes (bioMerieux, 2002). The test results are identified in Figure 2.5. The variable shades of pink can make reading the test difficult if the result is not a strong positive resulting in some associated observational bias when reading the color strips.

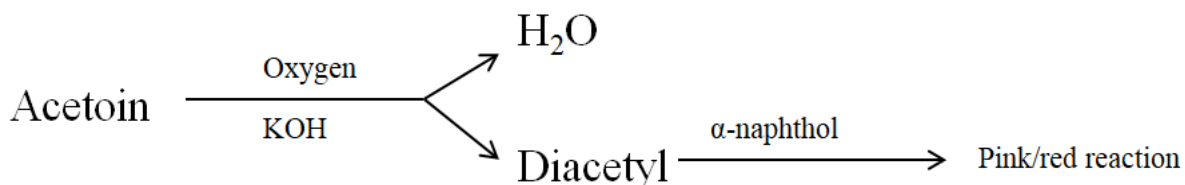


Figure 2.4 The VP chemical reactions that indicate whether acetoin was produced

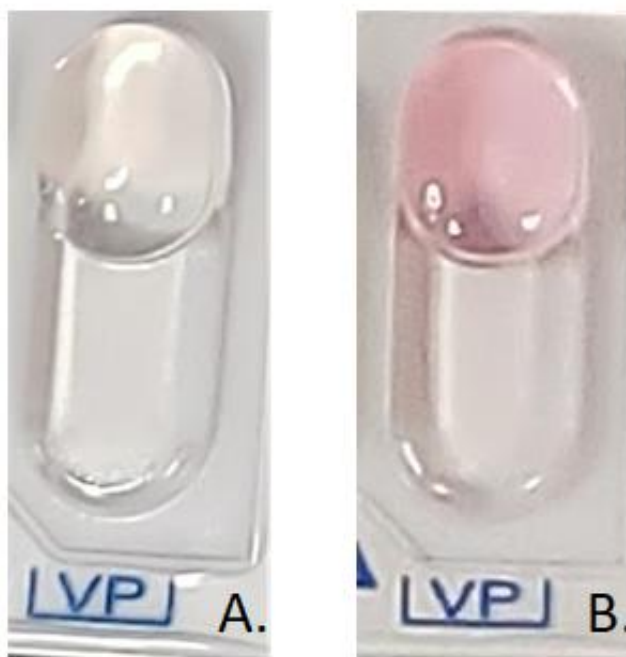


Figure 2.5 Acetoin Production Reactions

The clear/colorless tube in A indicates a negative reaction
 The pink color in B indicates a positive reaction.

Note: A slight pink reaction forming more than 10 minutes after the application of the last VP reagent is still considered a negative reaction.

D-sorbitol Fermentation

Sorbitol is known by many names, including D-sorbitol, glucitol and D-glucitol, and its chemical formula is $C_6H_{14}O_6$. It is naturally occurring in fruits and plants that have diuretic or laxative properties, and it is an *Escherichia coli* metabolite (National Center for Biotechnology Information, 2021). *Salmonella* is typically capable of fermenting sorbitol as well as other carbohydrates, such as L-arabinose, maltose, D-mannitol, D-mannose, L-rhaminose and D-xylose (Holt, et al., 1994). Sorbitol is reduced from glucose via the aldose reductase enzyme with the transference of an H^+ molecule from NADPH. The sorbitol then transfers an H^+ molecule to NAD with the enzyme sorbitol dehydrogenase, which breaks it down into fructose (Figure 2.6).

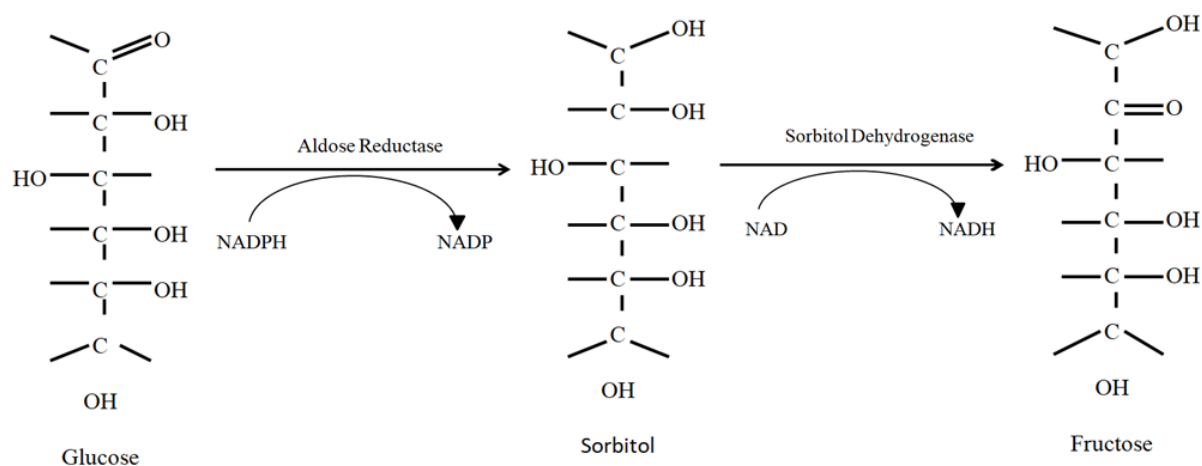


Figure 2.6 The metabolic pathway from glucose to sorbitol to fructose

Sorbitol testing using the bioMerieux® API 20E test strip involves inoculating 5 mL of 0.85% NaCl with the selected *Salmonella* colony, and the sorbitol test tube is filled to the top of the tube, not the cupule. After the strip is incubated aerobically for 24 hours at $37^{\circ}C$, the tube is read as either positive or negative (bioMerieux, 2002). The positive and negative test reactions are indicated in Figure 2.7.

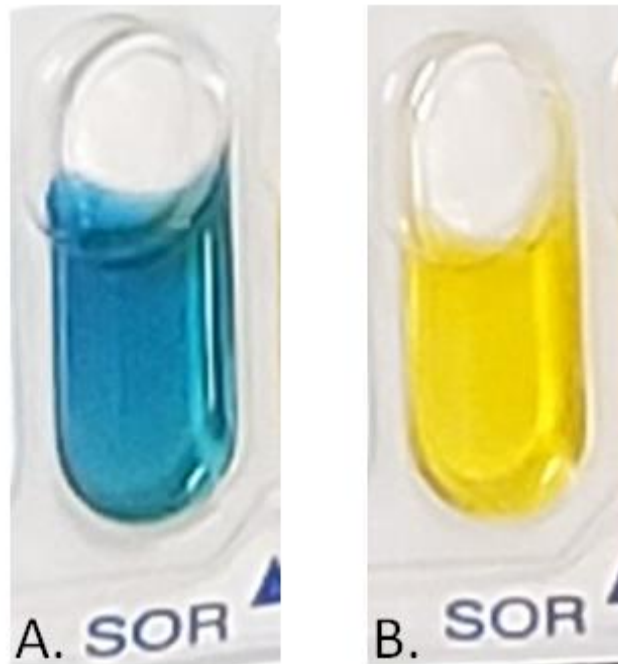


Figure 2.7 d-Sorbitol Fermentation Reactions

The blue color in A indicates a negative reaction

The yellow color in B indicates a positive reaction

Arginine Dihydrolase

Arginine metabolism is important for pathogens, such as *Salmonella*, *Helicobacter pylori* and *Mycobacterium tuberculosis*, for efficient pathogenesis and as a source of energy. Host macrophages transport arginine via cationic amino acid transporter (CAT2) proteins where it is utilized in iNOS and arginase pathways. When infected with a pathogen, iNOS is upregulated and converts arginine to nitric oxide (NO) and citrulline. The enzyme arginase breaks down arginine into urea and ornithine. Nitric oxide has antimicrobial properties, and ornithine aids in polyamine synthesis, which serves as an energy source for parasites, modulates inflammation, and is essential in tissue repair. *Salmonella* infects the host's macrophage and recruits the host's arginine transporter CAT1 to the *Salmonella*-containing vacuole (SCV). This allows the *Salmonella* to access the host's arginine pool. The bacterial arginine transporter (ArgT) imports arginine from the SCV lumen into the bacterial cytosol (Gogoi, et al., 2017). *Salmonella* has effectively adapted a pathway to transfer the host's arginine into its own vacuole and uses it as an energy source to perpetuate infection within the host.

The arginine dihydrolase (ADH) pathway is a major source of energy and nitrogen for bacteria. Within this pathway, arginine is catalyzed “to ornithine, ammonium and carbon dioxide, to generate ATP from ADP and phosphate” (Ropolo, et al., 2019). The arginine is converted to citrulline and NH_4^+ by the enzyme arginine deiminase. Citrulline is then catalyzed by ornithine transcarbamylase into carbamoyl-phosphate and ornithine. The carbamoyl phosphate phosphorylates ADP by carbamate kinase to produce one ATP molecule (Figure 2.8) (Ropolo, et al., 2019).

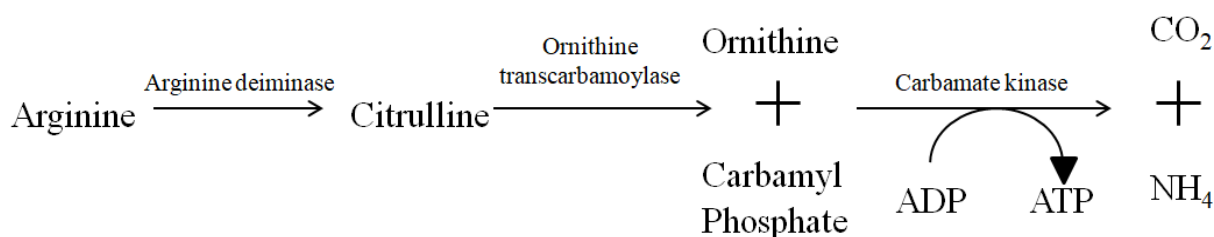


Figure 2.8 The metabolic pathway from arginine to CO_2 and NH_4

The bioMerieux® API 20E test strip includes a test for arginine dihydrolase. The selected bacterial colony is mixed with 5 mL of 0.85% NaCl and inoculated into the test tube. Once the tube is filled, not the cupule, a few drops of mineral oil is applied into the cupule to create an anaerobic environment. After the test strip is incubated for 24 hours at 37°C , the test tube is read and interpreted based on the color change (bioMerieux, 2002) as indicated in Figure 2.9.



Figure 2.9 Arginine Dihydrolase Reactions

The yellow color in A indicates a negative test reaction
 The red color in B indicates a positive test reaction

Inositol Utilization

Inositol is a natural sugar found in cell membrane phospholipids, nucleus, and plasma lipoproteins. It is an intracellular phosphate compound that is involved in cell signaling and may play a role in stimulating tumor cell differentiation. While technically a collection of nine different stereoisomers, the name inositol is generally used to describe myo-inositol. This is the most common type and is structurally similar to glucose. It is considered a pseudovitamin since it plays a vital role in the body but does not result in disease conditions when a deficiency is noted. Myo-inositol is a human metabolite, a mouse metabolite, and an *Escherichia coli* metabolite (National Center for Biotechnology Information, 2021). It is ubiquitous in plants and soil in both a free form and a phospholipid derivative (Kroger & Fuchs, 2009) and can be utilized by livestock when in the presence of phytases (Kroger, et al., 2010).

In *Salmonella*, myo-Inositol (MI) utilization depends on gene *iol* characterization and regulation within the bacterium. The *iol* divergon includes the operons *iolABCDEFGHJI* and *iolRS*, and the gene *iolT* is responsible for the breakdown of MI. These genes are found on a 22.6

kb genomic island located between STM4417 and STM4436. This genomic island is not only present in *Salmonella* Typhimurium strains, but also in other *Salmonella* serovars, such as Saintpaul strain SARA23, Weltevreden strain HI_N05-537, and Paratyphi B strain SPB7. It is notably absent in *Escherichia coli*, *Shigella dysenteriae*, *Salmonella bongori*, *Vibrio cholera* and in *Salmonella enterica* serovars Typhi CT18/Ty2, Paratyphi A/C, Enteritidis, Dublin, Gallinarum and Diarizonae (Kroger & Fuchs, 2009). The genomic island GEI4417/4436 contains two MI transporters, IolT1 and IolT2, to transport the Inositol into the bacteria (Kroger, et al., 2010), where it is degraded into dihydroxyacetone phosphate, acetyl coenzyme A and CO₂ (Kroger & Fuchs, 2009). The degradation pathway starts with myo-inositol being transported into the bacteria, where it interacts with the enzyme *myo*-inositol dehydrogenase which breaks it down into 2-keto-*myo*-inositol which is broken down with the enzyme inosose dehydratase by dehydrating it into D-2,3-diketo-4-deoxy-*epi*-inositol (also referred to as 3D-3,5/4-Trihydroxycyclohexane-1,2-dione) (Klegg, n.d.). It is then rehydrated to 2-deoxy-5-keto-D-gluconic acid, which is phosphorylated to 2-deoxy-5-keto-D-gluconic acid 6-phosphate, and then, cleaved by the IolJ protein into dihydroxyacetone phosphate and malonic semialdehyde. The malonic semialdehyde is converted to acetyl coenzyme A and CO₂ by the *iolA* product (Yoshida, Aoyama, Ishio, Shibayama, & Fujita, 1997). Figure 2.10 includes the enzymes and compounds associated as well as the associated *iol* gene (Krings, et al., 2006).

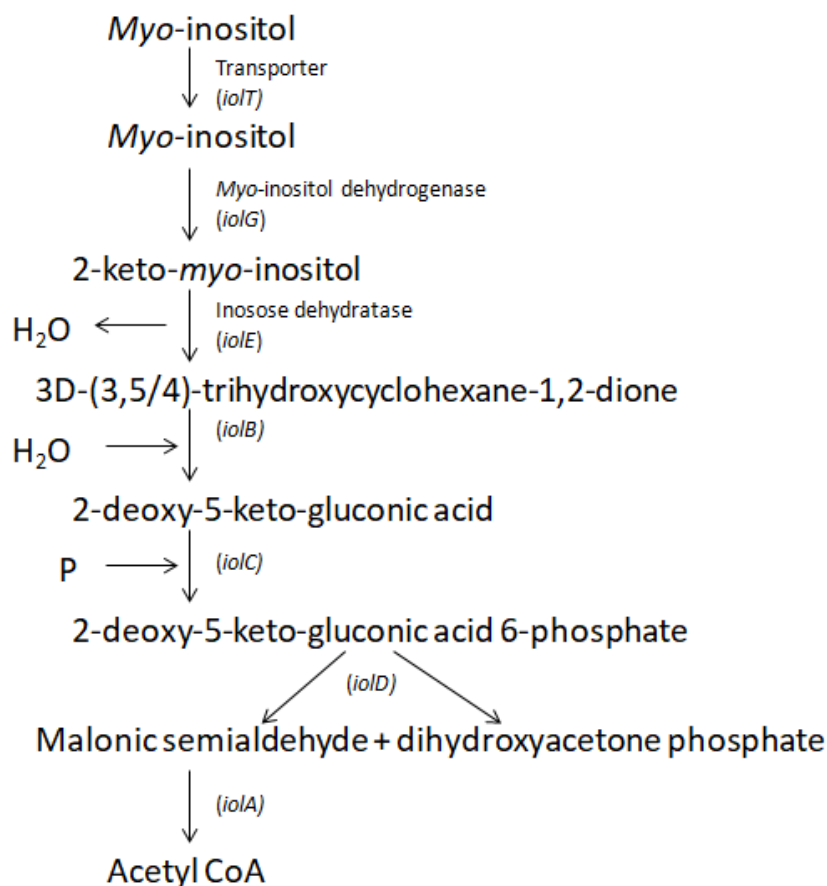


Figure 2.10 Proposed pathway of *myo*-inositol with associated genes

The bioMerieux® test for inositol fermentation/oxidation includes mixing up an inoculation of 0.85% NaCl with an isolated colony and filling up the API 20E test strip tube containing 1.9 mg of inositol. After incubating for 24 hours at 37°C, the inositol tube reaction is read and marked as a positive or negative. Fermentation begins in the lower part of the tube and oxidation begins in the cupule of the tube. If the bacteria have the capability to ferment or oxidize Inositol, it becomes more acidic resulting in the color change seen in Figure 2.11.

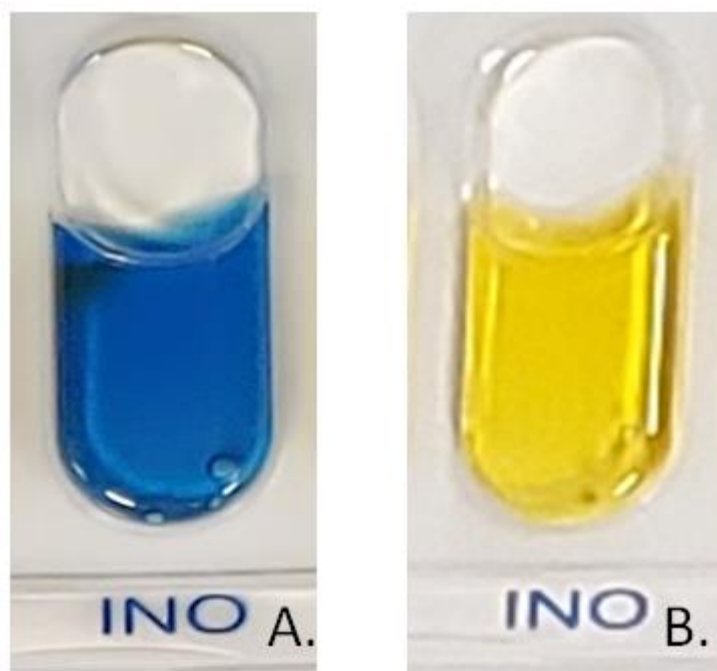


Figure 2.11 Inositol Reactions

The blue color in A indicates a negative test reaction.

The yellow color in B indicates a positive test reaction.

Biofilm Production

A biofilm is a microbiome that includes various bacterial colonies or multiple colonies of a single type of bacteria attached to a surface. The bacterial cells are embedded in a matrix that generally is composed of environmental DNA, polysaccharides, and proteins (Sharma, et al., 2019). The three most important factors that play a role in inducing biofilm formation by bacteria are bis-(3'-5')-cyclic diguanosine monophosphate (c-di-GMP), quorum sensing, and small RNAs (Yan & Wu, 2019). Quorum sensing is important in the production of biofilms because it is a cell-to-cell communication tool to help bacteria communicate for activities, such as biofilm production, luminescence, sporulation, enhancing access to nutrients, defenses, and virulence patterns. It also helps the bacteria communicate with the host (Sholpan, et al., 2021), monitor their surroundings, and quickly adapt to environmental stressors (Williams, et al., 2007).

The formation of biofilm involves multiple steps. First, the bacteria must attach to the selected surface, and then, it forms an irreversible attachment with adhesions or

exopolysaccharides. Next, they develop mushroom shaped microcolonies and begin to create the biofilm structure. If the conditions are favorable, they will stop synthesizing matrix compounds and will enzymatically cleave the matrix compounds to disperse the biofilm. *Salmonella* can form biofilms on a multitude of surfaces, such as plants, metal, glass, plastic, meat, meat-processing equipment, gallstones, and chicken intestinal epithelium (Peng, 2016). The biofilm communities alter their metabolic activity, including altering genes associated with biofilm formation, decreasing growth rates, and increasing exopolysaccharide production. The creation of a biofilm benefits the bacteria by protecting it from antibiotics, immune cells, and altered pH and/or nutrient deficient environments (Sharma, et al., 2019).

The quorum sensing molecules are released during bacterial growth and accumulate in the surrounding environment, allowing bacteria to sense when the population becomes too dense. When the quorum level is reached, transcription factors change the gene expressions in receptor cells. There are four categories of quorum signaling compounds: N-acyl homoserine lactones (AHLs) are fatty acid derivatives commonly called autoinducer-1 (AI-1), and they are used by Gram-negative bacteria for intraspecies communication; furanosyl borate diesters are formed during the recycling of S-adenosyl-homocysteine to homocysteine, are commonly called autoinducer-2 (AI-2), and are produced by both Gram-positive and Gram-negative bacteria as universal interspecies and intraspecies communication signals; autoinducer-3 (AI-3) which communicates between mammalian epinephrine host cell signals; and finally, small auto inducing peptides allow Gram-positive bacteria to communicate between cells and are characterized by their small size of 6-25 amino acids, specificity, diversity and stability (Sholpan, et al., 2021).

In *Salmonella*, the gene *sdiA* encodes for an AI-1 receptor but does not synthesize AHLs. It is also used for both interspecies and intraspecies communications, may aid in the colonization of the host's gut, and can detect many AHLs released by other species. Throughout the many different *Salmonella* serotypes, the *sdiA* gene may regulate different responses and may not always interact in the same way (Sholpan, et al., 2021). In *Salmonella* Typhimurium, AI-2 production is associated with the exponential growth phase. The AI-2 signaling requires low pH, high osmolarity, and a series of enzymatic reactions. S-adenosyl methionine is converted by methyl transferases to S-adenosylhomocysteine, which is then converted by methylthioadenosine/S-adenosylhomocysteine nucleosidase (pfs) and S-ribosylhomocysteinase

to 4,5-dihydroxy-2,3-pentanedione, and then, is finally cyclized into (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetra-hydrofuran, the AI-2. After AI-2 is synthesized, it is transported extracellularly by the YdgG transport protein, where it binds to auto inducer-binding protein LsrB and is then transported back into the cell by a *lsr* operon encoded ATP transporter, where it is phosphorylated by LsrK and inactivates via a dose-dependent manner the LsrR transcription repressor protein. The LsrR inhibits gene expression for oxidative stress responses, flagellar expression, and virulence, and so, the inactivation by AI-2 of LsrR is necessary for flagellar expression and *Salmonella* pathogenicity island 1 (SPI-1) transcription (Taga & Bassler, 2003). The synthetic pathway and chemical formula of AI-3 is currently unknown, but the two component regulators are associated with the recognition of norepinephrine and epinephrine, which are both catecholamines produced by the host (Sholpan, et al., 2021).

Small RNA (sRNA) molecules are abundant, stable, and short (50-600 nucleotide) transcripts that act as post-translational regulators. They carry out their repression or activation activity in two ways: direct sRNA-mRNA pairing and sRNA-protein interaction. They are either trans-encoded sRNAs or cis-encoded sRNAs. The trans-encoded sRNAs are mostly studied in bacteria and base-pair with target transcripts with limited and imperfect complementarities resulting in an altered translation and stability, which indicates that they may control many genes and processes, such as stress resistance and metabolic regulation (Sholpan, et al., 2021). Due to the imperfect pairing, they require the aid of the Hfq RNA chaperone to facilitate the binding (Papenfert & Vogel, 2010). The cis-encoded sRNAs pair perfectly complementary to the target transcript. They are generally associated with regulating mobile elements like phages, transposons, and plasmids. Their role in *Salmonella* quorum sensing is still relatively unknown, but in *Vibrio cholerae*, the Hfq RNA chaperone aids interactions between sRNAs and the mRNA of the *V. cholerae* quorum sensing regulator HapR, which is helpful in both biofilm formation and virulence patterns (Sholpan, et al., 2021).

The bis-(3'-5')-cyclic diguanosine monophosphate (c-di-GMP) is important for biofilm formation, because it is a complex signaling network that is responsible for deciding whether the bacteria will develop a biofilm or remain free-living (planktonic). High levels of cyclic-AMP (cAMP) can lead to decreased c-di-GMP and inhibit biofilm formation in *Pseudomonas aeruginosa*. The cAMP levels can be increased by the expression of acute virulence factors since the initial infection requires the virulence factors, which can only occur with the up-regulation of

the cAMP-Vfr signaling pathway (Almblad, et al., 2019). *BpeGReg*, a globin-coupled diguanylate cyclase, enhances biofilm formation in *Salmonella Typhimurium*. Globin-coupled sensors are signal transducers that bind heme when in the presence of oxygen. In motile bacteria, high levels of c-di-GMP will suppress motility to increase exopolysaccharide production and biofilm formation (Wan, et al., 2009).

When it comes to antibiotics, bacteria that develop biofilms are 10-1000 times more capable of resisting the effects of antibiotics and can eventually lead to multi drug resistant bacteria. The antibiotic resistance in biofilm communities occurs due to multiple methods including slow or inadequate diffusion of the antibiotics into the biofilm, multiple populations within the biofilm, which may be able to sporulate, and a changed chemical environment (Sharma, et al., 2019). The exopolysaccharides in the biofilm matrix can induce resistance to aminoglycosides by dismantling the effect of the antibiotics through diffusion-reaction inhibition, which chelates the antibiotics or enzymatically breaks down the antibiotics (Khan, et al., 2010). Also, many antibiotics work by affecting the growth of the bacteria, but since most bacteria in a biofilm are in a stationary or lag growth phase, they are not as affected by the antibiotics. Another antibiotic resistance mechanism involves horizontal gene transfer between the bacteria in the biofilm. Their high density, accumulation of genetic elements, and increased genetic competency allows for increased conjugation between bacterial cells to transfer the resistance genes (Sharma, et al., 2019).

There are multiple methods to test for biofilm production, including tube method, Congo Red Agar method, tissue culture plate method, bioluminescent assay, piezoelectric sensors, and fluorescent microscope examination (Hassan, et al., 2011). Three of the most common tests include the tissue culture plate method, tube method and Congo Red Agar method.

The tissue culture plate method involves inoculating a fresh colony with 10 mL of trypticase soy broth with 1% glucose followed by dilution. Two hundred microliters of the diluted cultures are added to a 96 well-flat bottom tissue culture plate and incubated for 24 hours at 37°C. The contents are removed and washed with 0.2mL of phosphate buffer saline four times to remove the remaining planktonic bacteria. The bacteria that form biofilm adhered to the walls of the plate and are fixed with 2% sodium acetate, stained, and an optical density is performed with a micro-ELISA auto reader (Abdel Halim, et al., 2018).

The tube method involves inoculating 10 mL of trypticase soy broth + 1% glucose with a selected colony and incubating for 24 hours at 37°C. The tubes are decanted and washed with PBS and then dried and stained. The test is considered positive when there is a clear film on the bottom of the tube, and the amount is scored as 1-weak/none, 2-moderate, and 3-high/strong (Hassan, et al., 2011).

The Congo Red Agar method involves preparing a Congo Red Agar medium then inoculating it with a colony of bacteria and incubating it for 24 hours at 37°C. The formation of black colonies with a dry crystalline structure indicates biofilm production. Overall, the tissue culture plate method is the most sensitive in identifying bacteria that can produce biofilm (Abdel Halim, et al., 2018).

Conclusion

Autogenous vaccines play an important role in mitigating the presence of *Salmonella enterica* on poultry farms throughout the United States. Selecting the specific isolates to be included in the vaccine formulation can be difficult when a surveillance study can yield hundreds of isolates. In addition to identifying the serotypes of the isolates, the phenotypic characteristics can further distinguish the isolates and provide more information on differences between them. Bacteria isolated from the processing equipment are most likely to make their way to the end consumer and cause outbreaks; therefore, it is important to restrict their ability to make it into the processing plant with the use of preventive measures, such as vaccines. Isolated *Salmonella* serotypes used for autogenous vaccine production, including their phenotypic characteristics, can change over time, impacting the overall effectiveness of the vaccine. The selection of isolates to be included in an autogenous vaccine formulation can be difficult since there are many different serotypes and characteristics of the *Salmonella* found on poultry farms. There have not been any studies to show how the selection of an autogenous product effects the prevalence and phenotypic changes in a *Salmonella* population after vaccination. Therefore, the hypothesis of this thesis project is that the implementation of a selected autogenous vaccine onto a poultry farm will affect the overall prevalence and the individual phenotypic characteristics of the *Salmonella* recovered after vaccination. The focus of this thesis project was to measure differences in the serotypes and phenotypes of *Salmonella* isolated from hatcheries and processing plants of four different study sites over a four-year period in which broiler chickens

were routinely vaccinated with an autogenous *Salmonella* vaccine. The details and findings of this study are reported in Chapter 3.

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Chapter 3 - Effect of Autogenous *Salmonella* Vaccination on the Overall Prevalence, Recovery and Phenotypic Characteristics of *Salmonella* in Poultry

Introduction

The bacteria *Salmonella* belongs to the family Enterobacteriaceae and is found in a variety of environments throughout the world. *Salmonella* contamination can cause food poisoning from many different food sources with poultry representing many of these illnesses globally. Nearly all poultry farms contain *Salmonella*, and the bacteria can be transferred through the poultry production process to the end consumer. The poultry farms implement multiple procedures to aid in the prevention and spread of *Salmonella* between farms and to humans.

Salmonella is a Gram-negative, motile, zoonotic, facultative anaerobic bacteria. It is hardy and thrives in a variety of environments, such as soil, litter, dry and wet conditions, internal organs, and some can thrive in the harsh chemical conditions of the poultry processing plants. There are two species of the bacteria: *Salmonella enterica* and *Salmonella bongori* (V). *Salmonella enterica* is further classified into six subspecies (Brenner, et al., 2000). All serotypes associated with poultry are classified as *S. enterica* subsp. *enterica*.

Poultry meat is the second most consumed meat globally with a 2019 global market worth \$231.5 billion. The United States is the second largest consumer of poultry products with 19 million tons (38 billion pounds) consumed annually (Poultry World, 2020). The United States poultry industry includes 9.22 billion chickens, 224 million turkeys, 185 million chickens (excluding broilers) and 112 billion eggs and was valued at over 40 billion dollars in 2019 (United States Department of Agriculture. National Agricultural Statistics Service, 2021). With an industry this large, involving so many birds, the spread of diseases such as pullorum disease, infectious coryza, fowl typhoid and necrotic enteritis is inevitable.

The Centers for Disease Control (CDC) reports that non-typhoidal *Salmonella* bacterial infections cause approximately 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year (Centers for Disease Control, 2021). The World Health Organization (WHO) reports that approximately 550 million people fall ill to foodborne diseases and that “*Salmonella* is 1 of 4 global causes of diarrheal diseases” (World Health Organization,

2018). Salmonellosis is characterized by abdominal pain, diarrhea, acute onset of fever, nausea and vomiting that can occur 6-72 hours post ingestion with an illness that lasts 2-7 days. Most cases are mild and are managed by replacing electrolytes, but others become life-threatening and require hospitalization. Antibiotics are rarely used due to the high prevalence of antibiotic resistance (World Health Organization, 2018). *Salmonella* can also be found in other food sources, such as vegetables, due to contamination of soil, contamination during harvest and preparing food near uncooked meat. Precautions include proper handwashing, separating raw meat from other foods, storing raw meats at appropriate temperatures, washing utensils, washing fruits and vegetables, and avoiding unpasteurized milk and raw or undercooked eggs (Minnesota Department of Health, 2019).

The globalization of food has allowed for an increase in disease outbreaks and an increased rate of antibiotic resistance. Food items can move around the world faster than ever before and the cuisines found in other cultures are able to be brought into new countries as delicacies. Some of these locations may allow the use of certain antibiotics in their livestock industries, and these uses can directly increase the *Salmonella* resistance to human antibiotic treatments (Aarestrup, 2015). The current recommendations for severely sick individuals infected with *Salmonella*, are the use of antibiotics, such as fluoroquinolones and extended spectrum cephalosporins and carbapenems, as a last resort (World Health Organization, 2012). Therefore, other prevention methods are preferred to prevent the outbreaks of *Salmonella* from occurring.

With more 2500 serotypes of *Salmonella* currently identified, removing *Salmonella* completely can prove difficult. Vaccines offer an aid in managing the prevalence of *Salmonella* on the poultry farms. Commercial vaccines are implemented on farms to provide a base level of protection against common serotypes that can affect the health and production of the birds and create dangerous outbreaks in the consumer. *Salmonella enterica* subsp. *enterica* serotype Typhimurium (*Salmonella* Typhimurium), *S. Enteritidis*, *S. Kentucky* and *S. Heidelberg* are serotypes that are often included in commercial vaccines. Some farms have large populations of other serotypes not commonly covered in the commercial vaccines and need additional vaccine coverage since cross-protection is not always guaranteed between serotypes.

Autogenous vaccines are custom vaccines that can be created quickly in response to an increase in prevalence or outbreak and can be made using any serotype isolated from that

specific company or farm. These allow producers to implement vaccine protocols that target their individual bacterial populations. The selection of an autogenous vaccine formulation can be difficult since the bacterial load of the property should be known to ensure that the isolates selected represent the farm's resident bacteria. Using a *Salmonella* surveillance study to first analyze the bacterial load and movement throughout the company or farm allows for an informed formulation decision. The bacteria surviving through the harsh chemical environment of the processing plant are most likely to create an outbreak in the consumer, so preventing the colonization of the bacteria prior to their arrival to the processing plant decreases the chance of an outbreak.

The selection of an autogenous vaccine formulation involves narrowing down the isolate list to isolates that represent the bacterial load on the farm. In addition to knowing the most prevalent serotypes, knowing the phenotypic characteristics of the isolates can help select isolates most likely to survive additional antimicrobial efforts. The following tests may differ between isolates; arginine dihydrolase, citrate utilization, acetoin production, d-sorbitol fermentation, inositol utilization and biofilm formation. Once a vaccine formulation has been selected and the vaccine implemented on the farms, it is important to continue monitoring the prevalence of *Salmonella* so that the vaccine can be updated as the bacterial load changes.

The purpose of this study was to determine whether there were any changes in serotype prevalence and phenotypic characteristics after the utilization of an autogenous vaccine formulation by analyzing the changes in four different study sites over a four-year period. It was hypothesized that a farm utilizing an effective autogenous vaccine formulation would have a decreased *Salmonella* prevalence and demonstrate alterations in their phenotypic characteristics.

Methods and Materials

Autogenous Vaccines

All study sites in this study implemented an autogenous vaccine protocol throughout the entire period of the study. The autogenous vaccines were prepared using five different *Salmonella* isolates that were isolated throughout the poultry company's multiple farms (Table 3.1). The vaccines were prepared using approved production outlines to kill and prepare the vaccines. The formulations were altered as necessary due to changes in serotype prevalence or

isolate expiration. The adjuvant was a ready-to-use polymer adjuvant proven to induce an appropriate immune response in chickens. The vaccines were given to the broiler breeders at 12 weeks of age via an intramuscular injection. Commercial vaccines were administered to all locations in addition to the autogenous vaccines.

Table 3.1 Autogenous Vaccine Formulations

Vaccine Formulation	Year Produced	Serotype	Arginine Dihydrolyase	Citrate Utilization	Acetoin Production	d-Sorbitol Fermentation	Inositol Utilization	Biofilm Formation
Formulation #1	2017	S. Enteritidis	Positive	Positive	Negative	Positive	Negative	2
		S. Enteritidis	Positive	Negative	Positive	Positive	Negative	2
		S. Enteritidis	Positive	Negative	Negative	Positive	Negative	2
		S. Typhimurium*	Positive	Negative	Positive	Positive	Positive	3
		S. Heidelberg	Positive	Positive	Negative	Positive	Negative	1
Formulation #2	2018	S. Enteritidis	Positive	Positive	Positive	Positive	Negative	3
		S. Enteritidis	Positive	Negative	Positive	Positive	Negative	2
		S. Infantis	Positive	Positive	Positive	Positive	Positive	2
		S. Typhimurium*	Positive	Negative	Positive	Positive	Positive	3
		S. Heidelberg	Positive	Positive	Negative	Positive	Negative	1
Formulation #3	2020	S. Enteritidis	Positive	Positive	Negative	Positive	Negative	3
		S. Enteritidis	Positive	Negative	Negative	Positive	Negative	2
		S. Infantis	Positive	Positive	Positive	Positive	Positive	2
		S. Infantis	Positive	Positive	Negative	Positive	Positive	2
		S. Typhimurium	Positive	Positive	Positive	Positive	Positive	3

*Monoflagellated

Study Design and Site Selection

This study focused on a top ten United States broiler chicken company with more than 5 different locations throughout the southern states. Four locations were selected for analyses over a four-year period from January 2017 through March 2021. The locations were eligible if they: 1) were consistently sampled over a four-year period from both the hatcheries and the processing plants, 2) contained a complete dataset with demographic and vaccination histories, and 3) consistently utilized a *Salmonella* autogenous vaccine protocol. The four study sites accounted for a total of 682 samples producing 572 different isolates. Some samples had no *Salmonella* recovered or contained more than one serotype resulting in multiple isolates from one sample. The samples submitted included chick papers from the hatcheries, carcass rinses and swabs from the processing plants, ceca and necropsy swabs from the broiler houses, and environmental swabs from the breeder houses.

Study site #1 was in the state of Arkansas and accounts for four (4) environmental swabs from the breeder houses, ninety-two (92) chick paper samples from the hatchery, and sixty-eight (68) isolates from the processing plant for a total of 164 samples and 108 total isolates. An autogenous vaccine containing three *S. Enteritidis* isolates, one *S. Heidelberg* and one *S. Typhimurium* (monoflagellated) isolates was introduced to the breeder flocks at 12 weeks of age during the 2017 Q2 sampling period (Table 3.1). This vaccine was combined with a protein particle vaccine given between 18-21 weeks of age and commercial *Salmonella* vaccines given between one and six weeks of age. In 2018 Q3, the autogenous vaccine formulation was changed due to the increasing prevalence of *S. Infantis*. The updated formulation included two *S. Enteritidis* isolates, one *S. Infantis* isolate, one *S. Typhimurium* isolate and one *S. Heidelberg* isolate. Two years later in 2020, the expired autogenous vaccine was updated to replace the *S. Heidelberg* with a second *S. Infantis* isolate and the *S. Typhimurium* (monoflagellated) isolate was replaced with a multi-flagellated *S. Typhimurium*. The protein particle vaccine was replaced in 2020 with a second autogenous vaccine booster given at 18 weeks of age (Table 3.2).

Table 3.2 Study Site #1 Vaccine Program and Sampling Schedule

	2017				2018			2019				2020			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q1	Q2	Q3	Q4	Q1	Q3	Q4	Total
1 & 14 Days	Commercial Vaccine: <i>S. Enteritidis</i> , <i>S. Typhimurium</i> , <i>S. Heidelberg</i>														
6 Weeks	Commercial Vaccine: <i>S. Enteritidis</i>														
12 Weeks		Autogenous Vaccine: Formulation #1					Autogenous Vaccine: Formulation #2					Autogenous Vaccine: Formulation #3			
18-21 Weeks	Particle Vaccine												Autogenous Vaccine: Formulation #3		
Chick Papers*	12 58.3%	12 33.3%	12 41.7%	8 25.0%	12 16.7%	0	4 0.0%	4 50.0%	4 25.0%	4 0.0%	4 50.0%	4 25.0%	4 0.0%	4 100.0%	88
Processing	1	0	1	0	2	0	3	0	26	18	8	8	2	0	69

*Percent refers to the percent of positive samples in each sampling. Processing isolates were confirmed positive prior to arrival to lab.

Study site #2 was in the state of North Carolina and accounted for twenty-five (25) cecal samples from the broiler houses, twelve (12) necropsy swabs from the broiler houses, seventy-five (75) chick paper samples from the hatchery and sixty-seven (67) isolates from the processing plant for a total of 179 samples and 139 total isolates. An autogenous vaccine containing three *S. Enteritidis* isolates, one *S. Heidelberg* and one *S. Typhimurium* (monoflagellated) isolates was introduced to the breeder flocks at 12 weeks of age during the 2017 Q2 sampling period. This vaccine was combined with a protein particle vaccine given between 18-21 weeks of age and a commercial *Salmonella* vaccine given between one and six weeks of age. In 2018 Q1, the autogenous vaccine formulation was changed for this specific site due to the increasing prevalence of *S. Infantis*. The updated formulation included two *S. Enteritidis* isolates, one *S. Infantis* isolate, one *S. Typhimurium* isolate and one *S. Heidelberg* isolate. Two years later in 2020, the expired autogenous vaccine was updated to replace the *S. Heidelberg* with a second *S. Infantis* isolate and the *S. Typhimurium* (monoflagellated) isolate was replaced with a multi-flagellated *S. Typhimurium* (Table 3.3).

Table 3.3 Study Site #2 Vaccine Program and Sampling Schedule

	2017				2018			2019				2020			Total
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q1	Q2	Q3	Q4	Q1	Q3	Q4	
1 & 14 Days	Commercial Vaccine: <i>S. Enteritidis</i> , <i>S. Typhimurium</i> , <i>S. Heidelberg</i>														
6 Weeks	Commercial Vaccine: <i>S. Enteritidis</i>														
12 Weeks		Autogenous Vaccine: Formulation #1			Autogenous Vaccine: Formulation #2								Autogenous Vaccine: Formulation #3		
18-21 Weeks	Particle Vaccine														
Chick Papers	9 100%	6 55.6%	9 33.3%	6 55.6%	6 44.4%	6 0.0%	6 100%	3 100%	3 33.3%	3 33.3%	3 100%	3 100%	3 100%	3 100%	69
Processing	3	0	0	0	3	0	15	0	17	2	9	3	7	0	59
Broilers -Ceca	0	0	0	0	0	0	0	25 16.0%	0	0	0	0	0	0	25

*Percent refers to the percent of positive samples in each sampling. Processing isolates were confirmed positive prior to arrival to lab.

Study Site #3 was in the state of Alabama and accounts for one hundred and four (104) chick paper samples from the hatchery and twenty-nine (29) isolates from the processing plant for a total of 133 samples and 124 total isolates. An autogenous vaccine containing three *S. Enteritidis* isolates, one *S. Heidelberg* and one *S. Typhimurium* (monoflagellated) isolates was introduced to the breeder flocks at 12 weeks of age during the 2017 Q2 sampling period. This vaccine was combined with a protein particle vaccine given between 18-21 weeks of age and a commercial *Salmonella* vaccine given between one and six weeks of age. In 2018 Q3, the autogenous vaccine formulation was changed due to the increasing prevalence of *S. Infantis*. The updated formulation included two *S. Enteritidis* isolates, one *S. Infantis* isolate, one *S. Typhimurium* isolate and one *S. Heidelberg* isolate. Two years later in 2020, the expired autogenous vaccine was updated to replace the *S. Heidelberg* with a second *S. Infantis* isolate and the *S. Typhimurium* (monoflagellated) isolate was replaced with a multi-flagellated *S. Typhimurium* (Table 3.4).

Table 3.4 Study Site #3 Vaccine Program and Sampling Schedule

	2017				2018			2019				2020			Total
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q1	Q2	Q3	Q4	Q1	Q3	Q4	
1 & 14 Days	Commercial Vaccine: <i>S. Enteritidis</i> , <i>S. Typhimurium</i> , <i>S. Heidelberg</i>														
6 Weeks	Commercial Vaccine: <i>S. Enteritidis</i>														
12 Weeks		Autogenous Vaccine: Formulation #1					Autogenous Vaccine: Formulation #2					Autogenous Vaccine: Formulation #3			
18-21 Weeks	Particle Vaccine														
Chick Papers*	12 100%	12 83.3%	12 58.3%	12 83.3%	12 66.7%	4 75.0%	4 100%	4 100%	4 100%	4 100%	4 25.0%	4 75.0%	8 62.5%	4 100%	100
Processing	2	0	2	0	5	0	5	0	4	0	4	0	5	1	28

*Percent refers to the percent of positive samples in each sampling. Processing isolates were confirmed positive prior to arrival to lab.

Study site #4 was in the state of Alabama and accounts for one hundred and sixty (160) chick paper samples from the hatchery and forty-six (46) isolates from the processing plant for a total of 206 samples and 201 total isolates. An autogenous vaccine containing three *S. Enteritidis* isolates, one *S. Heidelberg* and one *S. Typhimurium* (monoflagellated) isolates was introduced to the breeder flocks at 12 weeks of age during the 2017 Q2 sampling period. This vaccine was combined with a protein particle vaccine given between 18-21 weeks of age and a commercial *Salmonella* vaccine given between one and six weeks of age. In 2018 Q3, the autogenous vaccine formulation was changed due to the increasing prevalence of *S. Infantis*. The updated formulation included two *S. Enteritidis* isolates, one *S. Infantis* isolate, one *S. Typhimurium* isolate and one *S. Heidelberg* isolate. Two years later in 2020, the expired autogenous vaccine was updated to replace the *S. Heidelberg* with a second *S. Infantis* isolate and the *S. Typhimurium* (monoflagellated) isolate was replaced with a multi-flagellated *S. Typhimurium* (Table 3.5).

Table 3.5 Study Site #4 Vaccine Program and Sampling Schedule

	2017				2018			2019				2020			Total
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q1	Q2	Q3	Q4	Q1	Q3	Q4	
1 & 14 Days	Commercial Vaccine: <i>S. Enteritidis</i> , <i>S. Typhimurium</i> , <i>S. Heidelberg</i>														
6 Weeks	Commercial Vaccine: <i>S. Enteritidis</i>														
12 Weeks		Autogenous Vaccine: Formulation #1					Autogenous Vaccine: Formulation #2					Autogenous Vaccine: Formulation #3			
18-21 Weeks	Particle Vaccine														
Chick Papers*	19 89.5%	18 77.8%	18 83.3%	20 70.0%	18 77.8%	6 100%	7 100%	8 100%	8 100%	7 100%	7 71.4%	6 50.0%	6 83.3%	6 100%	154
Processing	3	0	3	0	1	0	2	0	14	1	3	8	8	0	43

*Percent refers to the percent of positive samples in each sampling. Processing isolates were confirmed positive prior to arrival to lab.

Samples were submitted on a quarterly basis from both the hatchery and the processing plant with additional samples submitted based on individual farm needs. Quarter 1 indicates sampling occurred during January, February, or March of that specific year, Quarter 2 during April, May, or June, Quarter 3 during July, August, or September, and Quarter 4 during October, November, or December. Each sample was processed based on specific laboratory protocols with the *Salmonella* positive samples undergoing serotyping via the White-Kauffmann-Le Minor (WKL) scheme and Intergenic Sequence Ribotyping (ISR) protocols. Priority serotypes were determined based on the most prevalent and those that present the most danger to both the birds and the human end-consumers. Priority serotypes for this study are *Salmonella enterica* serotype Enteritidis, *Salmonella Typhimurium*, *Salmonella Infantis*, *Salmonella Heidelberg* and *Salmonella Kentucky*. All isolates with the serotypes *S. Enteritidis*, *S. Typhimurium*, *S. Infantis* and *S. Heidelberg* underwent phenotypic analysis after the initial isolations due to their possible inclusion in an autogenous vaccine formulation.

Chick Paper Processing

Study sites #1 and #3 selected four (4) flocks, site #2 selected three (3) flocks and site #4 selected six (6) flocks per sampling period to represent the various sizes of their farms. Each flock had three separate boxes with a chick paper placed inside. One hundred chicks were placed

into each box (300 chicks per flock) for a minimum of four (4) hours. The three chick papers per flock were placed into a single Ziploc® bag, labeled with identifying information, placed in a cooler with ice packs and mailed overnight to the lab where they were logged into the sample submission database and given a case number.

Tetrathionate broth base was prepared by adding 46g of dehydrated tetrathionate base (BD Difco™ Dehydrated Culture Media: Fluid Tetrathionate) to 1L of deionized water and heating to a boil for one hour while stirring on a stir plate. Iodine solution was prepared by adding 300g of iodine crystals (Iodine Resublimed Crystals/USP, Fisher Chemical) and 250g of potassium iodide (Potassium Iodide, 1.0N, Fisher Chemical) to 1L of deionized water. The 0.1% Brilliant Green solution was prepared by dissolving 1.0g of brilliant green to 1000mL of deionized water. The sulfathiazole solution was prepared by dissolving 0.125g of sulfathiazole Sodium salt into 100mL of deionized water. The solution was filter sterilized using a 0.22µm filter and stored in the fridge. The tetrathionate complete broth was prepared by aseptically mixing 1L of prepared tetrathionate broth base with 20mL of prepared Iodine solution, 10mL of prepared brilliant green solution and 1.0mL of prepared sulfathiazole solution.

After the samples were processed into the submission database, 150mL of the prepared tetrathionate brilliant green complete broth (TTBG) was added to a sampling swab bag containing buffered peptone water (Labplas KSS-67310-BPW Sani-Stick). The three chick papers were individually swabbed with the sampling swab until all chick material and meconium was accounted for, and the swab was released into the sampling bag. The sampling bag was closed and incubated overnight at 37°C aerobically. After 18-24 hours of incubation, the bags were agitated and swabbed onto Xylose-Lysine-Tergitol 4 (XLT4) plates (Hardy Diagnostics™) and Brilliant Green agar (BG) plates (Hardy Diagnostics™) and incubated overnight at 37°C aerobically. The sampling bags were then placed at room temperature. The plates were read after 18-24 hours of incubation. Suspected *Salmonella* colonies were re-inoculated to fresh XLT4 and BG plates to further purify the sample. Any plates that did not indicate *Salmonella* growth after the initial overnight incubation were left to incubate at room temperature for an additional 24 hours. These plates were then read to identify any delayed *Salmonella* colony formations. Isolated colonies were transferred to Nutrient agar (NA) slants (Hardy Diagnostics™) and 1mL of Tryptic Soy Broth (TSB) (Remel, 22092) and incubated overnight at 37°C. Delayed Secondary Enrichments (DSE) were performed on the original sampling bags 5-7 days after being placed at

room temperature. DSEs were performed by adding 1mL of the original sampling enrichment to 9mL of fresh TTBG complete broth and incubating overnight at 37°C. These samples were then plated and isolated as previously discussed. The NA slants were used for WKL antisera testing and the 1mL TSB tubes were used for ISR testing (discussed below). All isolates were inoculated into 2- 2mL cryovials containing 1.8mL each of an 80:20 TSB:Sterile Glycerin (Fisher) solution and stored in an upright -70°C freezer (Eppendorf).

Processing Plant Isolates

Carcass rinses and processing swabs were received as isolates, plated directly from the shipped NA slant to fresh XLT4 and BG plates, and then incubated overnight at 37°C aerobically. The plates were read after 18-24 hours of incubation, and further passaged to XLT4 and BG plates if further isolation was needed. Isolated colonies were transferred to fresh NA slants and 1mL TSB for WKL and ISR testing, respectively and then stored at -70°C.

Necropsy and Environmental Swabs

All necropsy swabs and environmental swabs were plated directly to XLT4 and BG plates and incubated aerobically overnight at 37°C. The reading and passaging of the plates were performed as described above.

Cecal Tissue Samples

The cecal tissue samples were processed by adding approximately 25mL of TTBG Complete broth to the sample bag, mashing the sample into the media and incubating overnight at 41°C. After the incubation, samples were then plated onto XLT4 and BG plates and incubated overnight at 37°C and the original sample bags were placed at room temperature. Colonies were passaged as necessary until isolated. The isolates were then transferred to NA slants and TSB broth for WKL and ISR serotyping. The original sample bags were left at room temperature for 5-7 days and then a delayed secondary enrichment was performed by adding 1mL of the original sample material to 9mL of TTBG Complete broth and incubated at 41°C overnight. They were

then plated to XLT4 and BG plates, passaged until isolated, submitted for WKL and ISR serotyping, and frozen down to -70°C for storage.

White-Kauffmann-Le Minor Serotyping Scheme

The NA slants were used for the WKL serotyping scheme. Approximately 20µL of a selected antiserum (Serum Staten Institut) was placed on a glass agglutination slide. A 1µL loop was used to place a small loopful of bacteria from the NA slant and mix it into the drop of antiserum. The slide was rocked back and forth and monitored for a positive agglutination reaction within 10-20 seconds. A positive or negative result was recorded, and the process was repeated on the next antiserum or sample. The antisera were tested by first identifying the poly-O group (OMA, OMB, or OMC) and then testing the individual O-antigen antisera within the group (O:4, O:7, O:8, O:9, etc.) to determine the serogroup. Once the serogroup was identified, the H-group antisera within that serogroup were tested to determine the phases (H:i, H:r, H:d, H:g,m, etc.). When necessary, H phases were forced by taking a motility medium (Hardy Diagnostics™), heating it to a liquid and mixing it with the first H phase antisera in a clean petri dish. Once the medium re-solidified, a 1µL loopful of bacteria is stabbed into one side of the plate and the plate was incubated overnight at 37°C. After incubation, a loopful of the bacterial growth furthest from where the bacteria stabbed into the media was used to test the secondary H phase antisera. The combination of positive results was utilized to identify the specific serotype of each individual isolate. Table 3.6 identifies the WKL scheme for the priority serotypes in this study.

Table 3.6 WKL Scheme for Priority Serotypes

Serotype	Poly O Group	O-Antigen	H-Antigen		Serogroup
			Phase 1	Phase 2	
<i>Salmonella</i> Enteritidis	OMA	O: <u>1</u> ,9,12	H:g,m	--	D
<i>Salmonella</i> Typhimurium	OMA	O: <u>1</u> ,4,[5],12	H:i	H:1,2	B
<i>Salmonella</i> Infantis	OMB	O:6,7, <u>14</u>	H:r	H:1,5	C1
<i>Salmonella</i> Heidelberg	OMA	O: <u>1</u> ,4,[5],12	H:r	H:1,2	B
<i>Salmonella</i> Kentucky	OMB	O:8, <u>20</u>	H:i	H:z ₆	C3

Intergenic Sequence Ribotyping (ISR)

The inoculated TSB tubes were used for ISR polymerase chain reaction (PCR) serotyping after incubating overnight in TSB broth. The samples were added to a 96-well PCR plate and DNA was extracted using the MagMax™ Core Nucleic Acid Purification kit (Applied Biosystems). The extraction method was performed according to the manufacturer instructions. The PCR master mix was prepared by adding the following amounts per sample: 12.5µL of Promega 2x Master Mix (Promega M7505), 1µL of forward primer (ISR PCR 10µM), 1µL of reverse primer (ISR PCR 10µM), 8.5µL of nuclease free water and 2µL of the extracted DNA samples. The plate was placed in a thermocycler and cycled under the following parameters: initial denaturation at 95.0°C for 2 minutes x 35 cycles, denaturation at 95.0°C for 30 seconds, annealing at 60.0°C for 30 seconds, extension at 72.0°C for 2 minutes, final extension at 72.0°C for 5 minutes and holding at 4.0°C.

Purification of the PCR occurred by pipetting 2µL of Exo-SAP-IT® (Applied Biosystems, Carlsbad, CA) into each well of an additional 96-well plate and adding 8µL of the PCR product. The plate was sealed and placed back in the thermocycler and cycled at the following parameters: incubation at 37.0°C for 15 minutes, incubation at 80.0°C for 15 minutes and holding at 4.0°C. Two plates (one forward and one reverse) were labeled and prepared for sequencing in the following manner. For each sample, 18µL of nuclease free water was mixed with 2µL of the corresponding sequencing primer, either ISR Hfs Forward or ISR Hrs Reverse, and 4µL of the Exo-SAP-IT® purified PCR product was added to the corresponding well of each of the forward and reverse plates. The samples were then submitted to Eurofins Genomics for sequencing.

After the samples were sequenced at Eurofins, the results were sent via the Eurofins website and processed through Geneious Prime® (2021 2.2, Biomatters LTD) bioinformatics software using the following procedure. The compressed (zipped) file was exported from Eurofins to the local server. The .ab1 files (files that contain the raw chromatograms data from the sequencing run) were moved into the Geneious Prime® software for the analysis. The sequences were trimmed to remove the poor-quality sequences at the ends and the trimmed regions were annotated. Heterozygotes were found by identifying and annotating bases where two different nucleotides have been called in the same position, then the read directions were determined based on whether they were the forward or reverse sequences. The batches were renamed to

remove unnecessary characters from the file name and the sequences were assembled using the De Novo Assemble (Geneious 2021 2.2) with the following parameters: first part of name separated by space, highest sensitivity/slow, save assembly report, save list of unused reads, save in sub-folder, save contigs, save consensus sequences and choose to use the existing trim regions. The consensus sequences were then BLAST (Basic Local Alignment Search Tool) against the ISR database supplied by Dr. Jean Guard from the USDA. The assembled sequences were saved as a .csv file in the local server.

API® 20E Test Strip

The API® 20E (Biomérieux™) test strip allows for the identification of Enterobacteriaceae and other non-fastidious Gram-negative bacteria in 18-24 hours. The bacteria for the test were first prepared by inoculating a fresh NA plate (Hardy Diagnostics™) and incubating overnight at 37°C. The test strip was prepared by adding 5-10mL of deionized water to the bottom of the plastic tray and the strip placed on top. One colony of bacteria from the NA plate is added to 5mL NaCl 0.85% medium (Biomérieux™) and mixed thoroughly to homogenize the suspension. The test strip was inoculated with this inoculum so that the tube and cupule of the tests citrate utilization (CIT), Voges-Proskauer (VP) and gelatin (GEL) were filled and only the tubes were filled in all other tests. Mineral oil (Biomérieux™) was added to the cupules in tests arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), hydrogen sulfide (H₂S), and urease (URE) to create an anaerobic environment. The plastic cover was placed on the test strip and the whole tray was incubated at 37°C for 18-24 hours.

After incubation, the strip was read. Some tests required additional reagents to be added to the test wells prior to reading. For the tryptophane deaminase (TDA) test, 1 drop of TDA reagent (Biomérieux™) was added to the test cupule. For the VP test, 1 drop of VP 1 and then 1 drop of VP 2 reagents (Biomérieux™) were added to the test cupule. After waiting for 10 minutes, the test cupule was interpreted. For the indole (IND) test, 1 drop of JAMES reagent (Biomérieux™) was added to the test cupule. The indole test was performed last since the reactions create gases that can interfere with other tests on the strip. All test reactions were recorded as either positive or negative based on the color changes identified and the reactions

were entered into the Biomerieux APIWEB™ database. The database analyzed the reactions and released a bacterial identification.

Motility

The motility tests were performed by taking a 1µL loop of bacteria from the NA slant and stabbing it into a tube of motility agar with tetrazolium growth indicator (TTC) and incubating the tubes at 37°C overnight. The movement of the bacteria away from the initial stab point indicates a positive reaction. The TTC allows for the easy visualization of this movement.

Biofilm

The biofilm was determined using a modified plating method for identifying biofilm production (Guard, et al., 2011). A 1µL loop of isolated bacteria from the NA slant was spot transferred to a single nutrient agar plate in ten places resulting in ten areas of individual growth on a single plate with maximum space allowed between each area. The plate was incubated for 18-24 hours at 37°C aerobically and then stored in a moist container for 120 hours at room temperature and evaluated daily. After 120 hours, the biofilm formations were scored as follows: 0- no biofilm formation, 1-weak and slow biofilm formation, 2-moderate biofilm formation, 3-strong and rapid biofilm formation. A score of 0 appeared as a smooth colony with no biofilm formation, a score of 1 appeared with a small crater in the center of the colony and very little formation towards the outer edges. A score of 2 took longer than 48 hours to organize and develop a spider web-like appearance going towards the outer edges. A score of 3 indicated a well-organized matrix that covered the entire colony that organized quickly within 48-hours of incubation (Guard, et al., 2011). See Figure 3.1 for identification of the various biofilm scores.

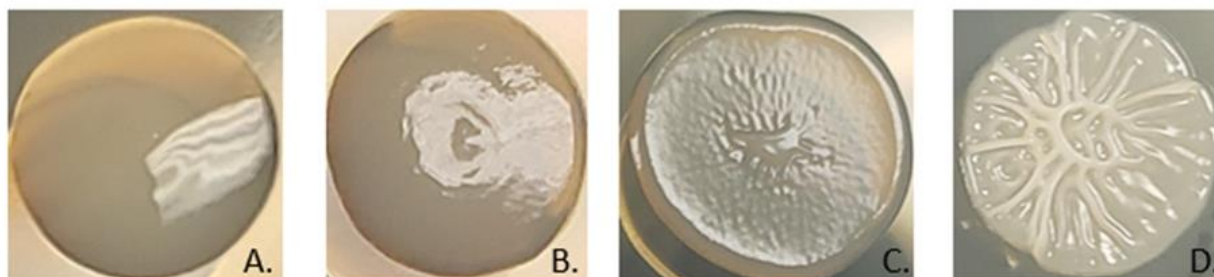


Figure 3.1 Biofilm Formation

The smooth colony with no indentations or formations (A) represents a biofilm score of 0. The smooth colony with the crater in the center and minimal formations (B) represents a biofilm score of 1. A colony with formations in the center and middle of the colony but that does not extend to the outer edges (C) represents a score of 2 and a colony showing a thorough and fast formation (D) indicate a score of 3. All images were taken on the 5th day of incubation.

Data Analyses

The overall *Salmonella* prevalence was presented as a percentage of all samples. The serotype prevalence was presented as a percentage of all positive isolates. The percent differences were determined by finding the difference between the 2017 and 2020 sampling periods. The *Salmonella* database was maintained and managed as an Excel spreadsheet and PivotTables were used to create and manipulate the graphs and tables.

Results

Overall Survey Results

Between January 1, 2017 and December 7, 2020, six hundred and thirty-eight (638) samples were analyzed from four different study sites throughout the southern United States. Of these 638 samples, 153 (22.4%) were negative for *Salmonella* on both primary and DSE enrichments. Of the 485 positive samples, 54 (11.1%) had more than one serotype present in the sample. The chick papers from the hatcheries represented 414 of the 638 samples with 130 (31.4%) samples negative for *Salmonella* on both primary and DSE enrichments. The isolates from the processing plants represented 195 of the 638 samples with two (1.0%) of the samples ending up negative for *Salmonella*. This may be due to an incorrect isolation at the independent lab or the bacteria not surviving through the transit from the lab. Therefore, 193 processing plant isolates were included in this analysis. Figure 3.2 shows the composition of the sample types from 2017-2021.

Independent of study site or isolate origin, the most isolated serotype overall was *Salmonella* Kentucky with 220 out of the 539 individual isolates (40.8%), followed by *Salmonella* Enteritidis with 168/539 (31.2%), and then, *Salmonella* Infantis with 59/539 (11.0%).

In 2017, *Salmonella* Enteritidis was the most isolated serotype with 49.1% of the isolates (Figure 3.3) followed by *Salmonella* Kentucky with 28.9%. In 2020, the prevalence of *S. Enteritidis* dropped to 21.65%, a decrease of 27.5% when compared to the 2017 prevalence data, and the prevalence of *S. Kentucky* increased by 14.4%, when compared to the 2020 data (Table 3.8). Regarding the hatchery samples, the most isolated serotype in 2017 was *Salmonella* Enteritidis at 47.5% but dropped to 33.9% by 2020 (Figure 3.4). The *Salmonella* Kentucky prevalence increased from 31.0% in 2017 to 46.4%, in 2020. Regarding the processing isolates, the most isolated serotype in 2017 was *Salmonella* Enteritidis at 66.7%, and by 2020, it had decreased to 5.4%, and *S. Kentucky* had increased from 6.7% in 2017 to 43.2%, in 2020 (Figure 3.5).

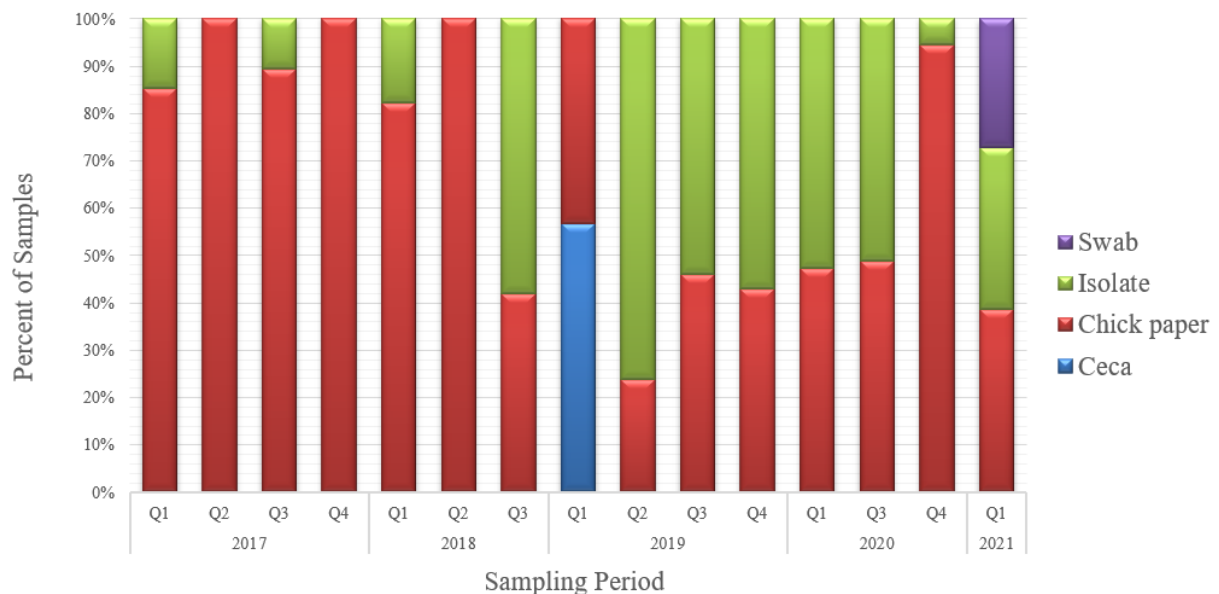


Figure 3.2 Sample Types Submitted Between 2017 and 2020

Table 3.7 Sample Prevalence of all Study Sites

		S. Enteritidis	S. Kentucky	S. Typhimurium	S. Infantis	S. Heidelberg	S. Schwarzengrund	S. Anatum
Hatchery	2017	47.5%	31.0%	7.6%	0.0%	1.9%	0.6%	0.0%
	2018	55.4%	26.8%	3.6%	0.0%	0.0%	0.0%	0.0%
	2019	23.5%	51.5%	0.0%	0.0%	0.0%	0.0%	4.4%
	2020	33.9%	46.4%	1.8%	0.0%	0.0%	3.6%	5.4%
	% Differences*	-13.5%	+15.4%	-5.8%	0.0%	-1.9%	+2.9%	+5.4%
Processing	2017	66.7%	6.7%	13.3%	6.7%	6.7%	0.0%	0.0%
	2018	13.9%	30.6%	0.0%	44.4%	0.0%	0.0%	5.6%
	2019	9.5%	60.0%	0.0%	28.6%	0.0%	1.0%	0.0%
	2020	5.4%	43.2%	0.0%	21.6%	0.0%	10.8%	2.7%
	% Differences*	-61.3%	+36.6%	-13.3%	+14.9%	-6.7%	+10.8%	+2.7%
Overall**	2017	49.1%	28.9%	8.1%	0.6%	2.3%	0.6%	0.0%
	2018	39.1%	28.3%	2.2%	17.4%	0.0%	0.0%	2.2%
	2019	14.7%	57.6%	0.0%	17.0%	0.0%	0.6%	1.7%
	2020	21.7%	43.3%	1.0%	12.4%	0.0%	6.2%	4.1%
	% Differences*	-27.5%	+14.4%	-7.1%	+11.8%	-2.3%	+5.6%	+4.1%

*Percent differences are the difference between the 2017 and 2020 sampling period prevalence

** Overall includes the hatchery, processing plant, broiler, and breeder samples

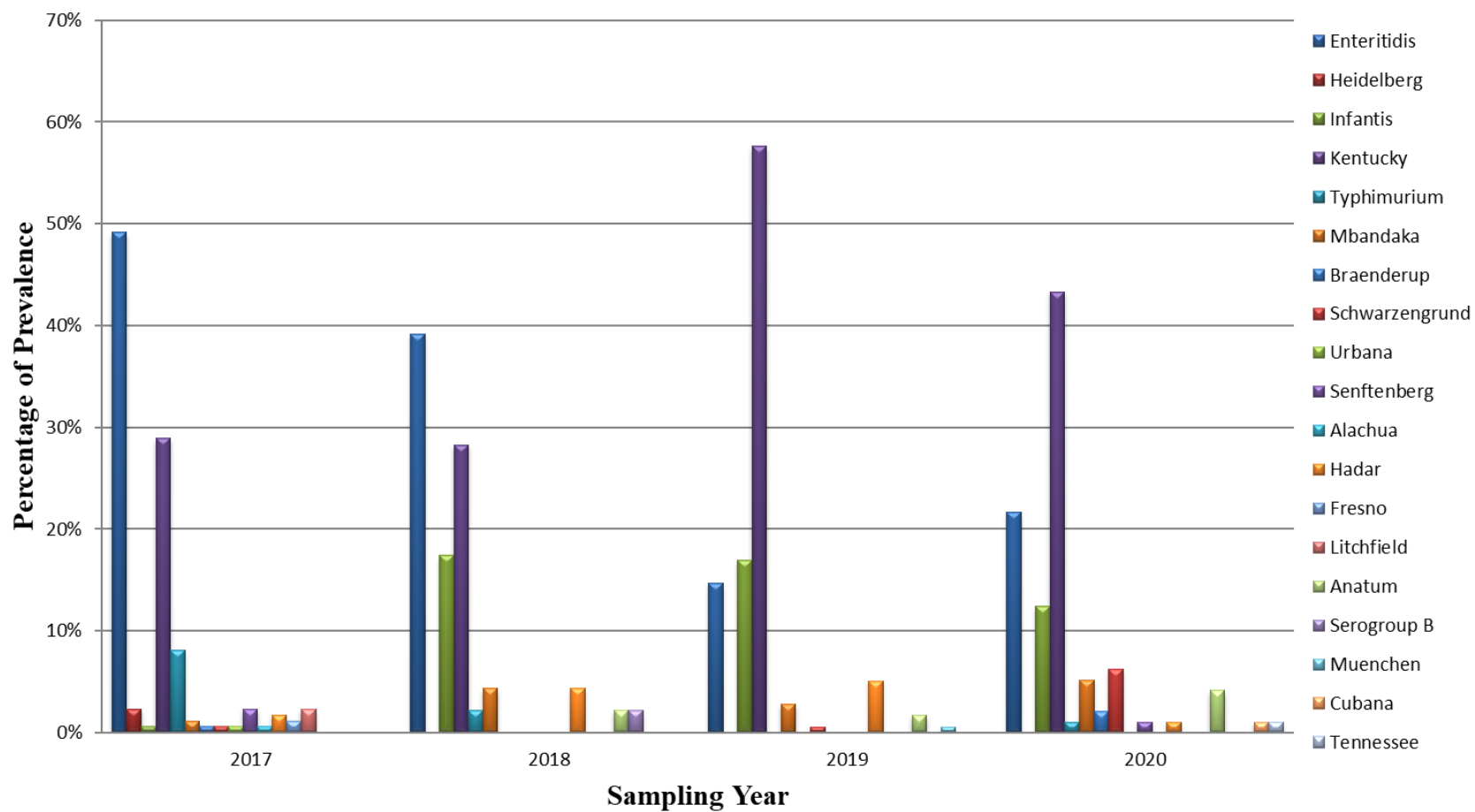


Figure 3.3 Overall *Salmonella* Prevalence throughout all Study Sites 2017-2020

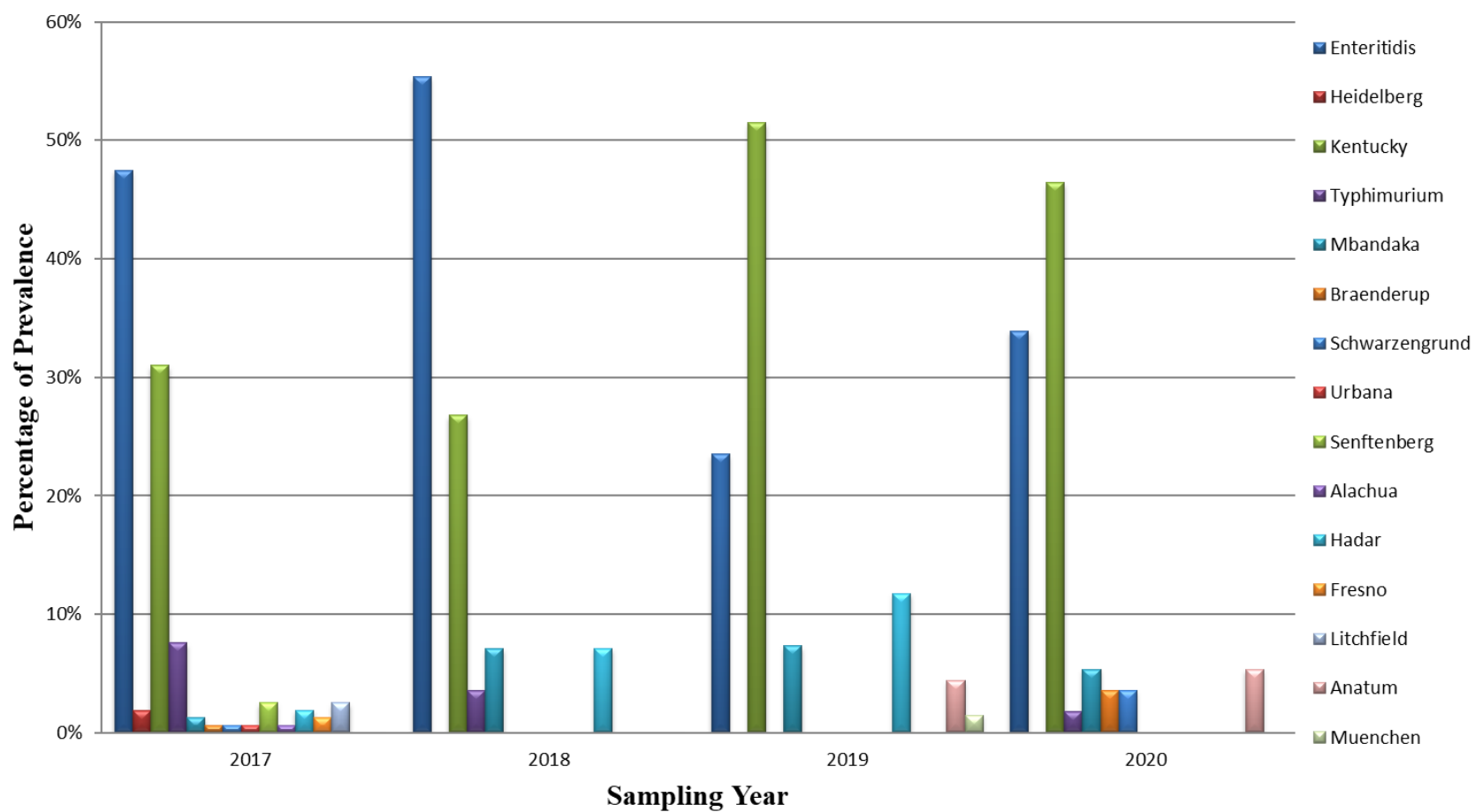


Figure 3.4 *Salmonella* Prevalence of the Hatcheries in all Study Sites Between 2017-2020

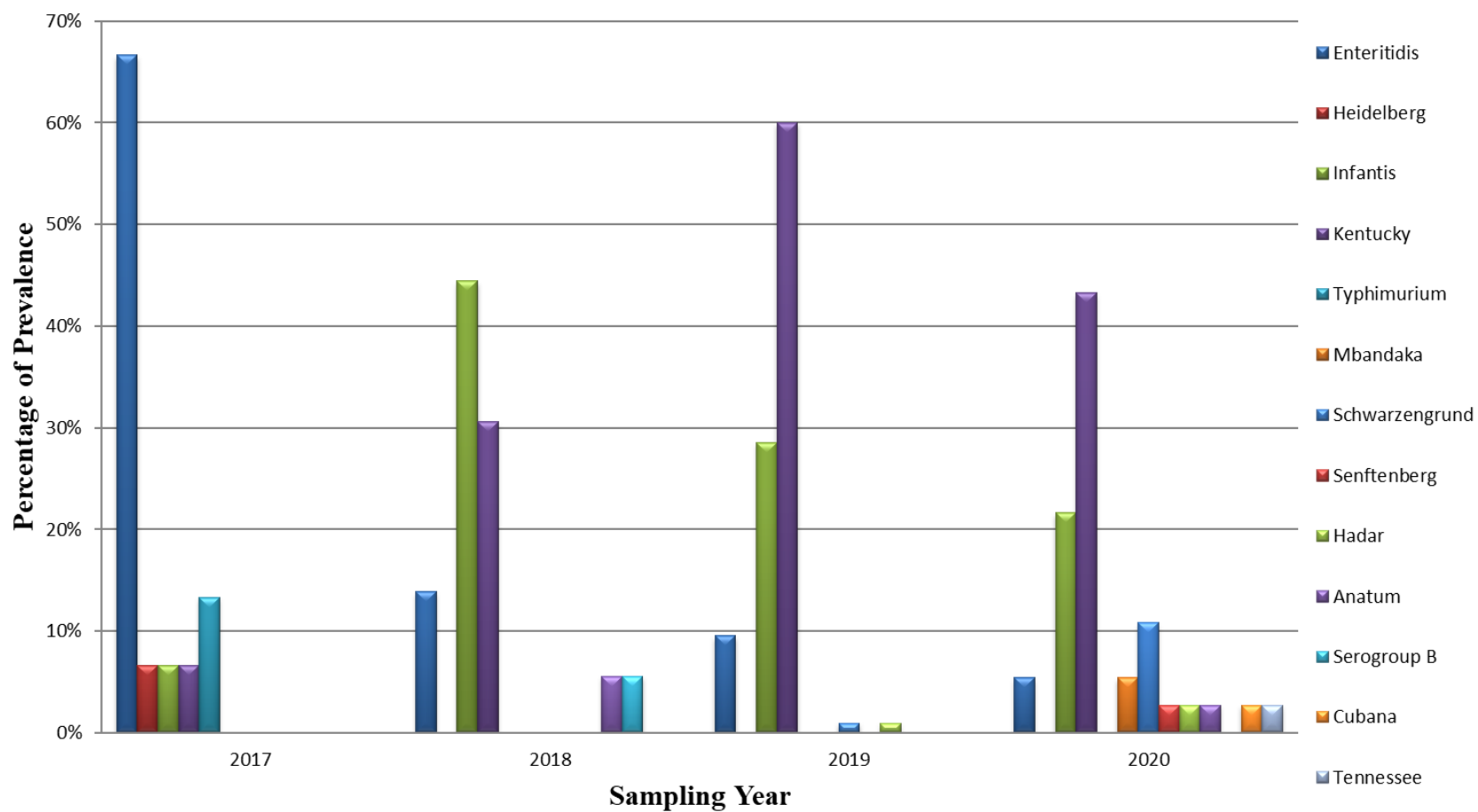


Figure 3.5 *Salmonella* Prevalence of the Processing Plants Throughout all Study Sites 2017-2020

Overall, the prevalence of *Salmonella* Enteritidis and *Salmonella* Typhimurium decreased in both the hatcheries and the processing plants, whereas the prevalence of *Salmonella* Kentucky and *Salmonella* Infantis increased. *Salmonella* Heidelberg has not been isolated since 2017 in any study site, and *Salmonella* Infantis was only isolated from the processing plants.

Survey Results by Study Site

Study Site #1 Results

Study site #1 was in the state of Arkansas. In 2017, the most isolated serotype was *Salmonella* Enteritidis with 43.5%, but by 2020, the prevalence had decreased to 25.0% (Figure 3.6). In the hatcheries in 2017, the most isolated serotype was *Salmonella* Enteritidis with 47.6% followed by *Salmonella* Kentucky and *Salmonella* Senftenberg with 19.1% each. Interestingly, in 2018 the only serotype isolated was *Salmonella* Typhimurium, and in 2019, the only serotype isolated was *Salmonella* Kentucky. In 2020, the most isolated serotype was *Salmonella* Enteritidis at 66.7%, and then, *Salmonella* Mbandaka and *Salmonella* Kentucky, each at 16.7% (Table 3.9). In the processing plant in 2017, the two serotypes isolated were *Salmonella* Heidelberg and *Salmonella* Infantis, each at 50.0% prevalence. In 2020, the most isolated serotype was *Salmonella* Kentucky at 33.3% followed by *Salmonella* Mbandaka, Schwarzengrund, Cubana and Tennessee each at 16.7% prevalence.

In 2017, *Salmonella* Enteritidis represented 47.6% of the serotypes recovered in the hatcheries, but it was not isolated in the processing plants that same year. The same trend was noted in 2020, where *Salmonella* Enteritidis represented 66.7% of the isolates recovered but was not represented in the processing plant isolates (Figure 3.7). *Salmonella* Typhimurium was only found in the hatchery samples but not in the processing plant samples. *Salmonella* Infantis was found in the processing plant and the breeders (Figure 3.8), but not in the hatchery. After the implementation of the second autogenous vaccine formulation, the prevalence of *S. Infantis* decreased further. Even though it was found in the breeders in 2020, it was not found in the hatchery or processing plant.

Table 3.8 Sample Prevalence of Study Site #1 2017-2020

		S. Enteritidis	S. Kentucky	S. Typhimurium	S. Infantis	S. Heidelberg	S. Schwarzengrund
Hatchery	2017	47.6%	19.1%	9.5%	0.0%	4.8%	0.0%
	2018	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%
	2019	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%
	2020	66.7%	16.7%	0.0%	0.0%	0.0%	0.0%
	% Differences*	+19.1%	-2.4%	-9.5%	0.0%	-4.8%	0.0%
Processing	2017	0.0%	0.0%	0.0%	50.0%	50.0%	0.0%
	2018	0.0%	40.0%	0.0%	0.0%	0.0%	0.0%
	2019	3.9%	80.4%	0.0%	13.7%	0.0%	2.0%
	2020	0.0%	33.3%	0.0%	0.0%	0.0%	16.7%
	% Differences*	0.0%	+33.3%	0.0%	-50.0%	-50.0%	+16.7%
Overall**	2017	43.5%	17.4%	8.7%	4.4%	8.7%	0.0%
	2018	0.0%	28.6%	28.6%	0.0%	0.0%	0.0%
	2019	3.6%	82.1%	0.0%	12.5%	0.0%	1.8%
	2020	25.0%	18.8%	0.0%	25.0%	0.0%	6.3%
	% Differences*	-18.5%	+1.4%	-8.7%	+20.7%	-8.7%	+6.3%

*Percent differences are the difference between the 2017 and 2020 sampling period prevalence

** Overall includes the hatchery, processing plant, broiler, and breeder samples

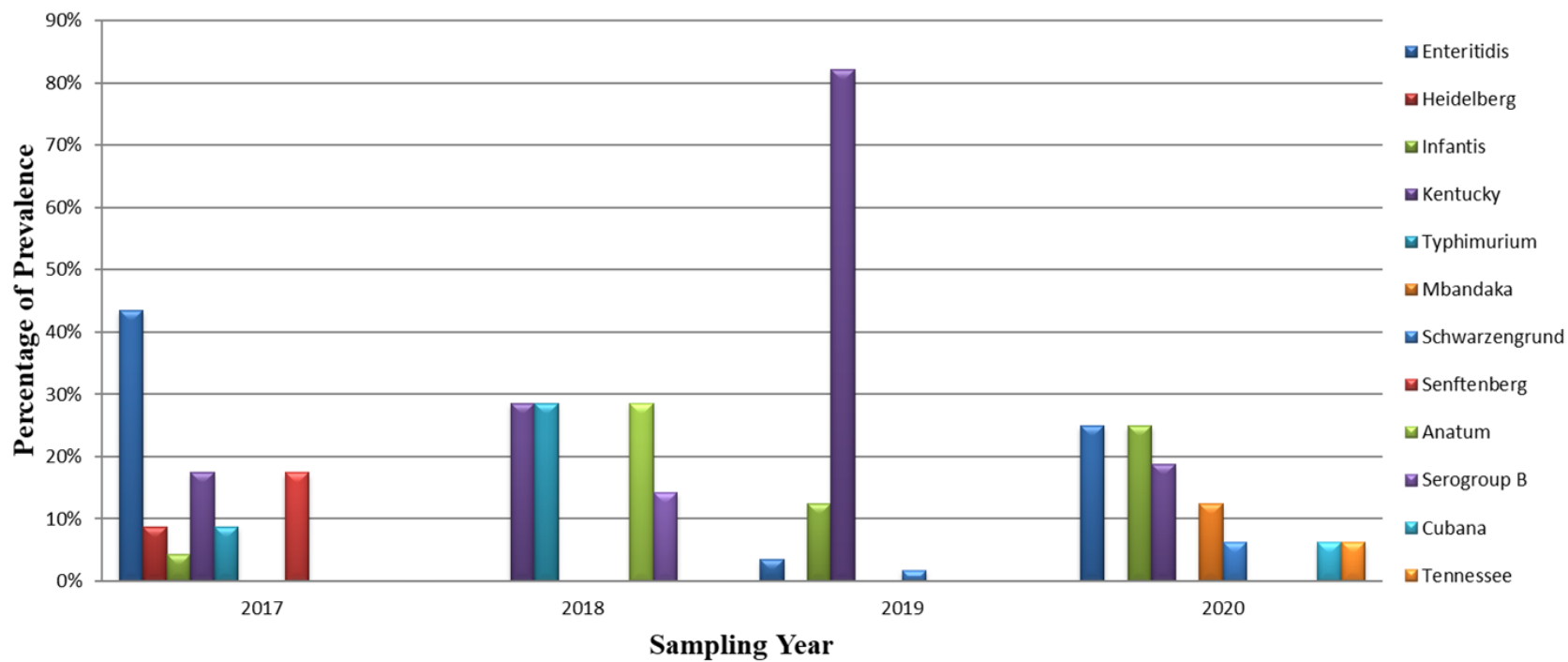


Figure 3.6 Overall *Salmonella* Prevalence throughout Study Site #1 2017-2020

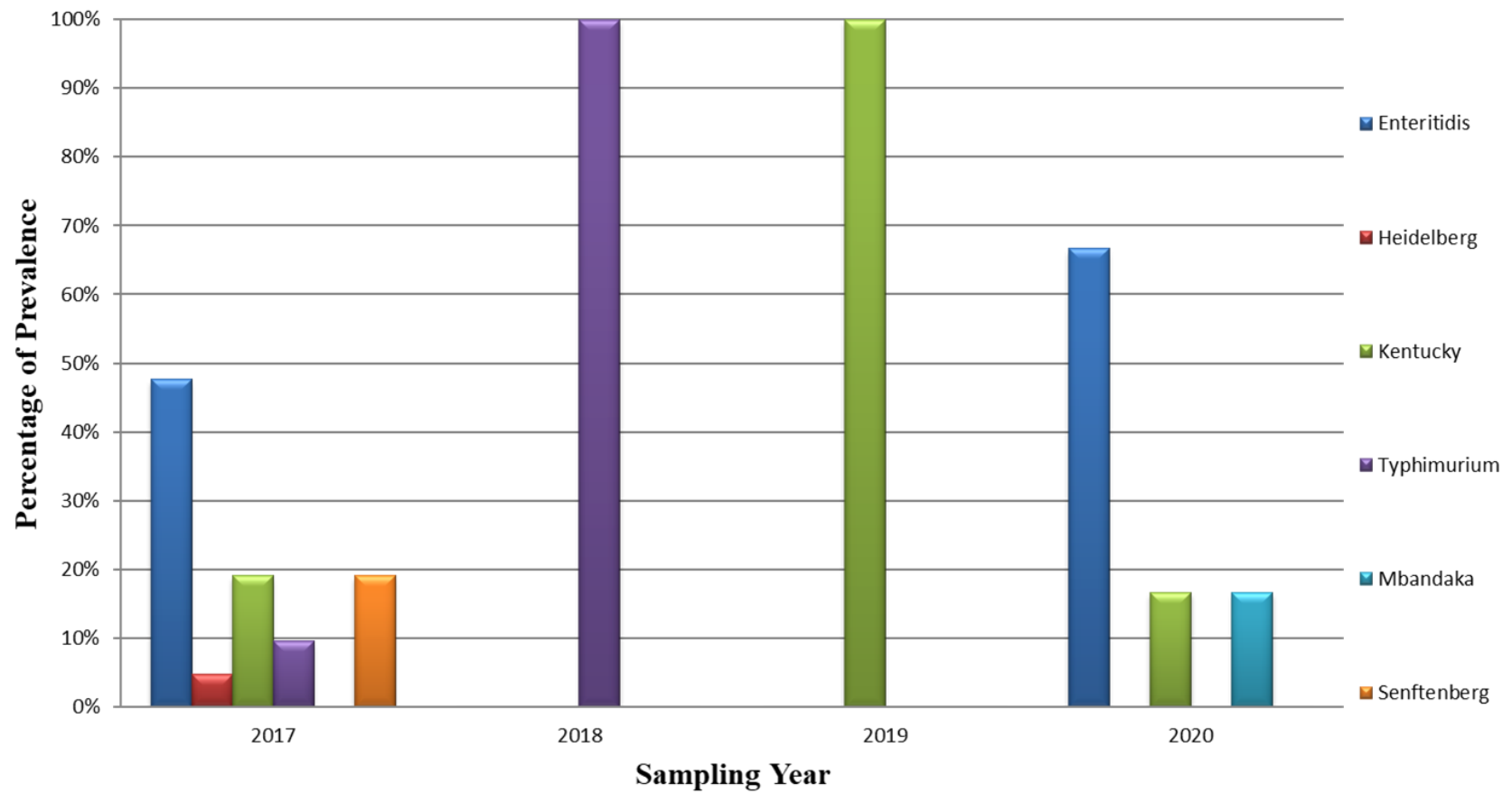


Figure 3.7 *Salmonella* Prevalence in the Hatchery of Study Site #1 2017-2020

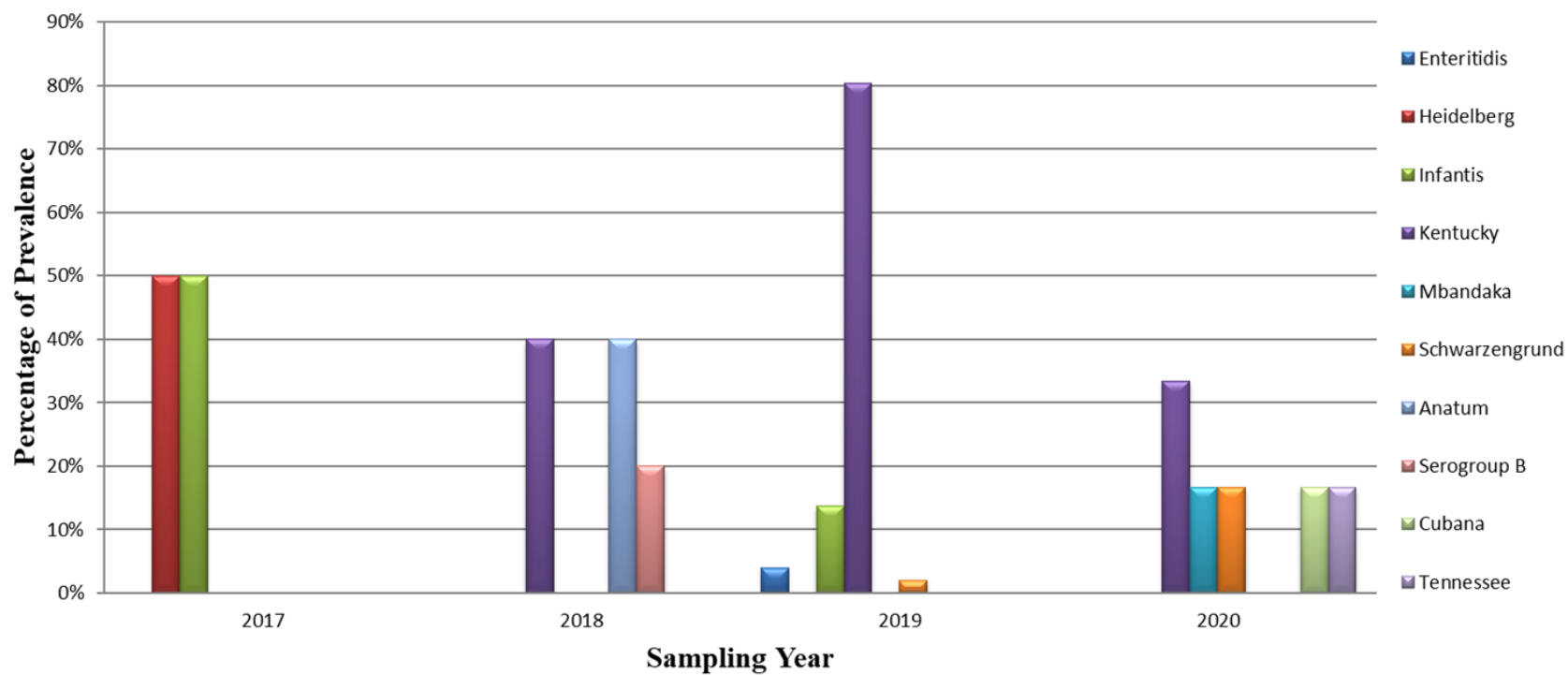


Figure 3.8 *Salmonella* Prevalence in the Processing Plant at Study Site #1 2017-2020

The autogenous vaccine formulation #1 was introduced in 2017 Q2 and contained three *S. Enteritidis* isolates. One isolate was positive for citrate utilization, the other two were negative. Two were negative for acetoin production, one was positive. All three isolates were negative for inositol utilization and positive for sorbitol fermentation. When the vaccine was implemented, the prevalence was 37.5% for that quarter and 57.6% for the following quarter. By 2018 Q3, the prevalence was down to 12.1%. The prevalence of *S. Enteritidis* in 2017 Q1 was 54.6%, with 66.7% of them being negative for citrate utilization (Figure 3.9). Since 2017 Q3, all *S. Enteritidis* isolates recovered from this study site have all been negative for citrate utilization regardless of sample type. At the implementation of the vaccine, 83.3% of the isolates were positive for acetoin production and the prevalence of positive isolates decreased throughout 2018 and 2019. By the time the updated vaccine was implemented in 2020, the prevalence has evened out in the hatchery. In the processing plant, the prevalence of positive and negative isolates wavered from 2017 through 2019. Since 2020 Q1, all processing plant isolates have tested positive for acetoin production (Figure 3.10).

The 2017 autogenous vaccine contained one *S. Heidelberg* isolate that was positive for citrate utilization and d-sorbitol fermentation, and negative for acetoin production and inositol utilization. When the vaccine was implemented during the 2017 Q2, the *S. Heidelberg* was found in 8.7% of the isolates and has not been isolated since 2017 Q3 in any samples. All the *S. Heidelberg* isolates from 2017 were citrate and d-sorbitol positive, inositol utilization negative and variations were found in their ability to produce acetoin. All hatchery isolates were negative in their acetoin production and all processing plant isolates were positive in their acetoin production. The vaccine matched the hatchery isolates in all tests so determining whether one specific test played a larger role in the decreased prevalence is not possible. *S. Heidelberg* was removed from the 2020 autogenous vaccine formulation and no new isolates have been recovered.

The 2017 autogenous vaccine contained one *S. Typhimurium* (monoflagellated) isolate that was positive for acetoin production, inositol utilization and d-sorbitol fermentation, and was negative for citrate utilization. When the vaccine was implemented during the 2017 Q2, the *S. Typhimurium* prevalence was at 8.7% with all the isolates coming from the hatchery. No *S. Typhimurium* isolates from this study site were monoflagellated. All were negative for arginine dihydrolase, citrate utilization, and d-sorbitol fermentation and 50.0% of the isolates were

positive for acetoin production. The samples ranged from mild to moderate biofilm producers with all isolates from 2017 Q4 ranked a score of 2 and all isolates from 2018 Q1 ranked a score of 1. In 2020, the isolate was replaced with a multi-flagellated isolate and *S. Typhimurium* has yet to be recovered from this study site since 2018.

S. Infantis was not included in the 2017 autogenous vaccine formulation for this company but was added to the 2018 formulation due to an increase in prevalence throughout 2018 in other company locations. All isolates were positive for arginine dihydrolase, d-sorbitol fermentation, inositol utilization. In 2017, 7.7% were positive for acetoin production and when the isolate resurfaced in 2019, an average of 26.9% were positive (Figure 3.10). The two isolates included in the 2020 autogenous vaccine encompassed all variations seen in these isolates. The biofilm formation capabilities of *S. Enteritidis* from 2017 to 2020 ranged from moderate to strong biofilm producers, even after the implementation of various autogenous vaccines. *S. Infantis* isolates ranged from weak to moderate biofilm producers regardless of the vaccine implemented (Figure 3.11).

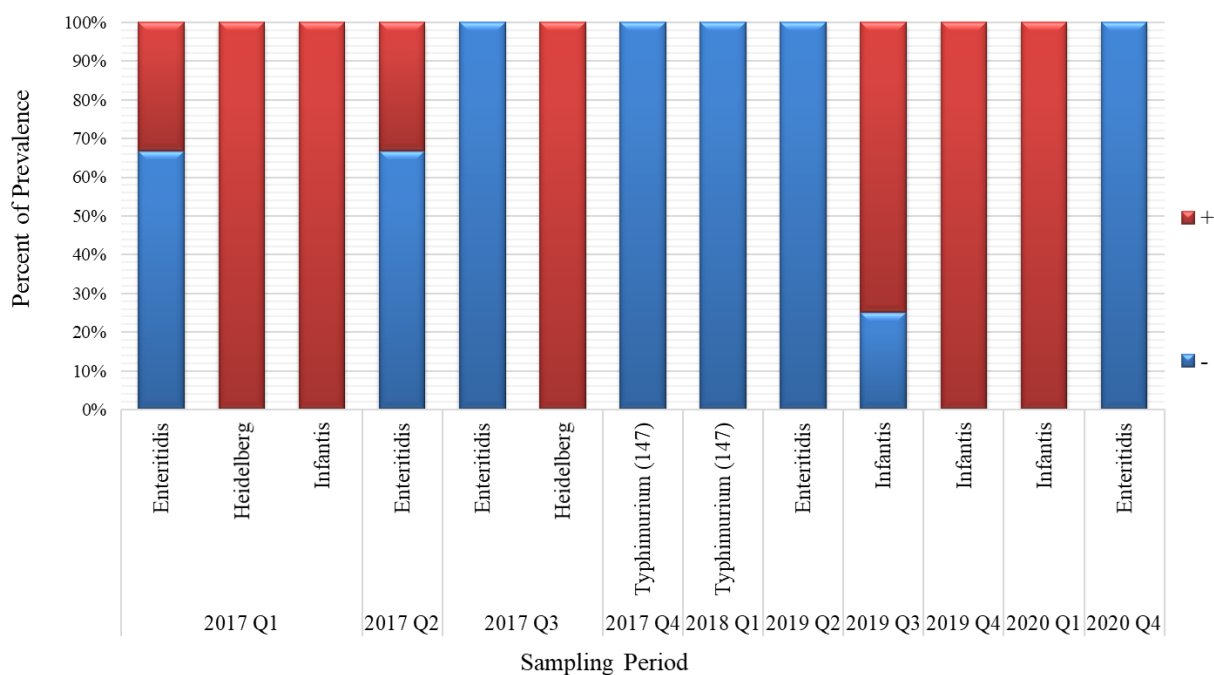


Figure 3.9 Prevalence of Citrate Utilization in Isolates throughout Study Site #1 2017-2020

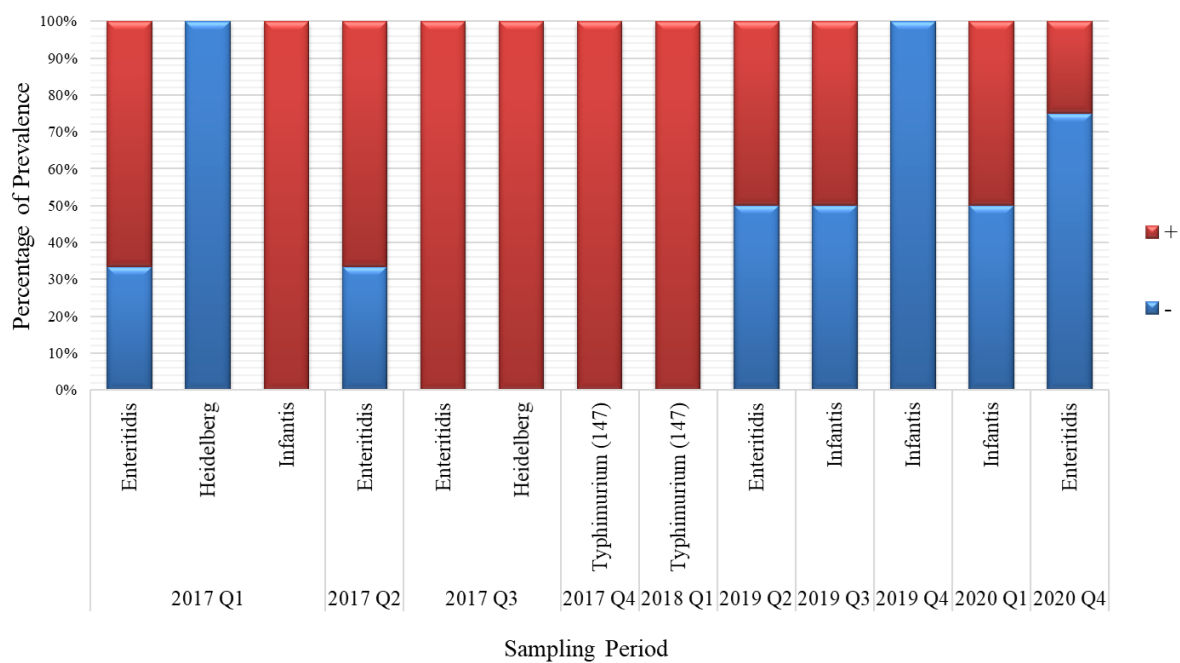


Figure 3.10 Prevalence of Acetoin Production in Isolates throughout Study Site #1 2017-2020

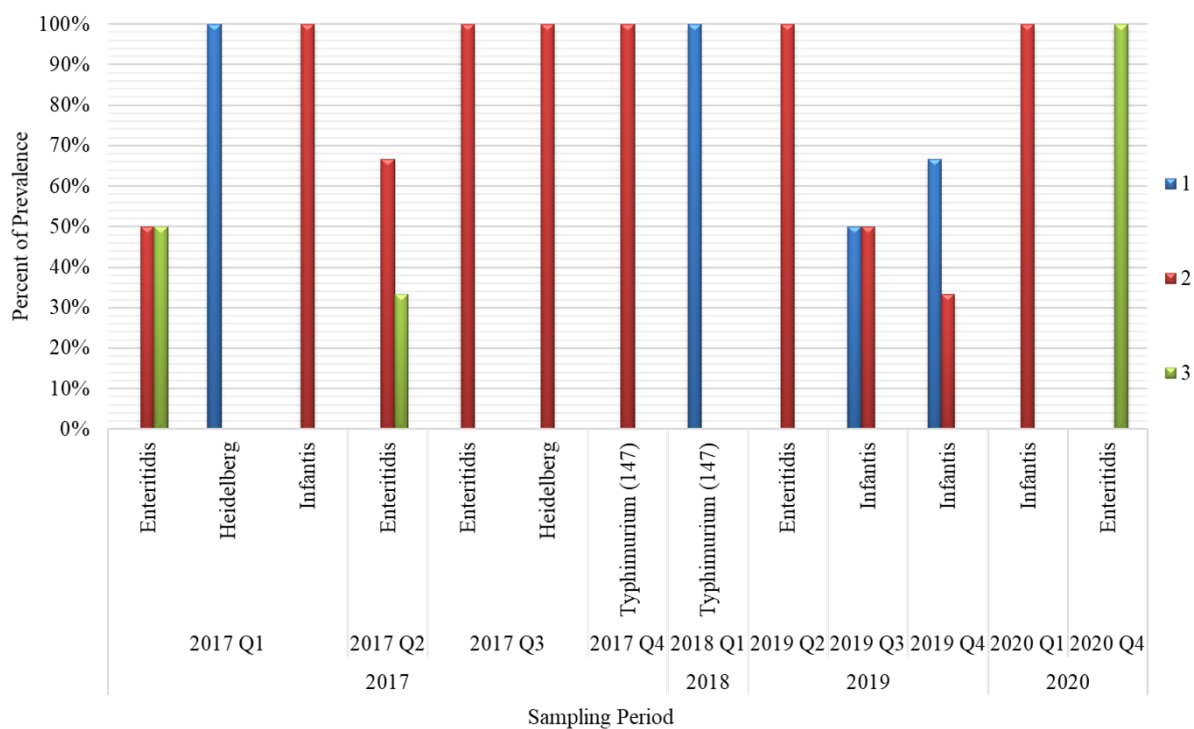


Figure 3.11 Biofilm Score Prevalence in Isolates throughout Study Site #1 2017-2020

Study site #1 showed differences in the recovery and prevalence of the various *Salmonella* serotypes isolated from 2017 through 2020. The implementation of the autogenous vaccines resulted in the reduction of *S. Heidelberg* and *S. Typhimurium* in the hatcheries and processing plant. While the *S. Enteritidis* prevalence increased in the hatchery, it was not recovered from the processing plant. The *S. Kentucky* serotype prevalence increased in the processing plant, but not in the hatchery. Since they have not been included in a vaccine formulation, it is anticipated that the *S. Kentucky* prevalence will continue to increase as the prevalence of the other serotypes decreases. After the implementation of the second autogenous vaccine formulation in 2018, the prevalence of *S. Infantis* decreased in the processing plant and was not isolated in 2020.

Differences were noted in various phenotypic characteristics in relation to the autogenous vaccine implementation. *S. Enteritidis* saw changes in the prevalence of citrate utilization, acetoin production and biofilm formations. No differences were seen in the *S. Typhimurium* isolates' phenotypic characteristics that were isolated in 2017 and 2018 in response to the vaccine, but since no new isolates have been recovered since 2018, the vaccines affected the overall prevalence.

Study Site #2 Survey Results

Study site #2 was in the state of North Carolina. In 2017, the most isolated serotype was *Salmonella* Kentucky at 53.6% prevalence followed by *Salmonella* Enteritidis with 35.7% then *Salmonella* Typhimurium at 7.1% (Figure 3.12). In 2020, the most isolated serotype was *Salmonella* Enteritidis with 37.5% prevalence followed by *Salmonella* Infantis with 33.3% and then *Salmonella* Kentucky with 29.17%. In the hatcheries in 2017, the most prevalent serotype was *Salmonella* Kentucky with 56.0% which dropped to 42.9% in 2020. In the hatcheries in 2020, *Salmonella* Enteritidis was the most isolated serotype with 57.1% prevalence, a 21.1% increase when compared to 2017. In the 2017 processing plants, serotypes *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Kentucky all represented 33.3% of the recovered isolates. In 2020 *Salmonella* Infantis represented 80.0% of the isolates recovered with *Salmonella* Enteritidis and *Salmonella* Kentucky each representing 10.0% (Table 3.10).

In 2017, *S. Enteritidis* represented 36.0% of the serotypes recovered in the hatcheries (Figure 3.13) and 33.3% in the processing plants for the same year. In 2020, *S. Enteritidis*

represented 57.1% of the serotypes recovered from the hatcheries but only represented 10.0% of the serotypes recovered from the processing plant. *S. Kentucky* represented 56.0% of the isolates recovered from the hatchery in 2017 but only represented 33.3% of the isolates in the processing plant for the same year. In 2020, it represented 42.7% of the hatchery isolates but only 10.0% of the processing plant isolates. *S. Typhimurium* was found in the hatchery and processing plant in 2017, but not in 2020. *S. Infantis* was not found anywhere in 2017 and in 2020, it represented 80.0% of the processing plant isolates (Figure 3.14). It was not found in the hatcheries or the breeder and broiler houses, but was a major serotype in the processing plant indicating that it has been introduced to the processing plant through an additional source such as the transportation trucks, employees, etc.

Table 3.9 Sample Prevalence of Study Site #2 2017-2020

		<i>S. Enteritidis</i>	<i>S. Infantis</i>	<i>S. Kentucky</i>	<i>S. Typhimurium</i>	<i>S. Mbandaka</i>
Hatchery	2017	36.0%	0.0%	56.0%	4.0%	4.0%
	2018	50.0%	0.0%	37.5%	0.0%	12.5%
	2019	40.0%	0.0%	50.0%	0.0%	0.0%
	2020	57.1%	0.0%	42.9%	0.0%	0.0%
	% Differences*	+21.1	0.0%	-13.1%	-4.0%	-4.0%
Processing	2017	33.3%	0.0%	33.3%	33.3%	0.0%
	2018	0.0%	88.9%	11.1%	0.0%	0.0%
	2019	0.0%	82.1%	17.9%	0.0%	0.0%
	2020	10.0%	80.0%	10.0%	0.0%	0.0%
	% Differences*	-23.3%	+80.0%	-23.3%	-33.3%	0.0%
Overall**	2017	35.7%	0.0%	53.6%	7.1%	3.6%
	2018	15.4%	61.5%	19.2%	0.0%	3.9%
	2019	9.5%	54.8%	33.3%	0.0%	0.0%
	2020	37.5%	33.3%	29.2%	0.0%	0.0%
	% Differences*	+1.8	+33.3%	-24.4%	-7.1%	-3.6%

*Percent differences are the difference between the 2017 and 2020 sampling period prevalence

** Overall includes the hatchery, processing plant, broiler, and breeder samples

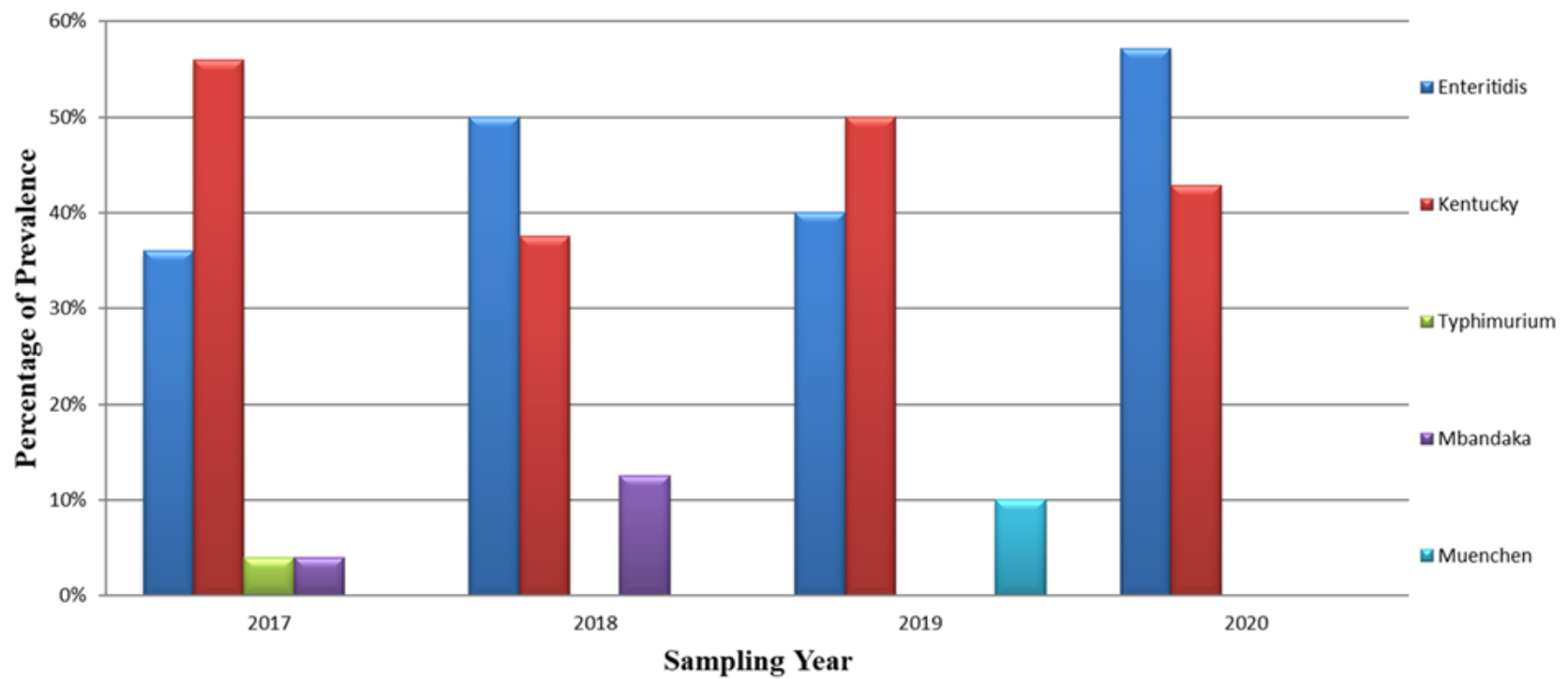


Figure 3.12 Overall Prevalence of *Salmonella* throughout Study Site #2 2017-2020

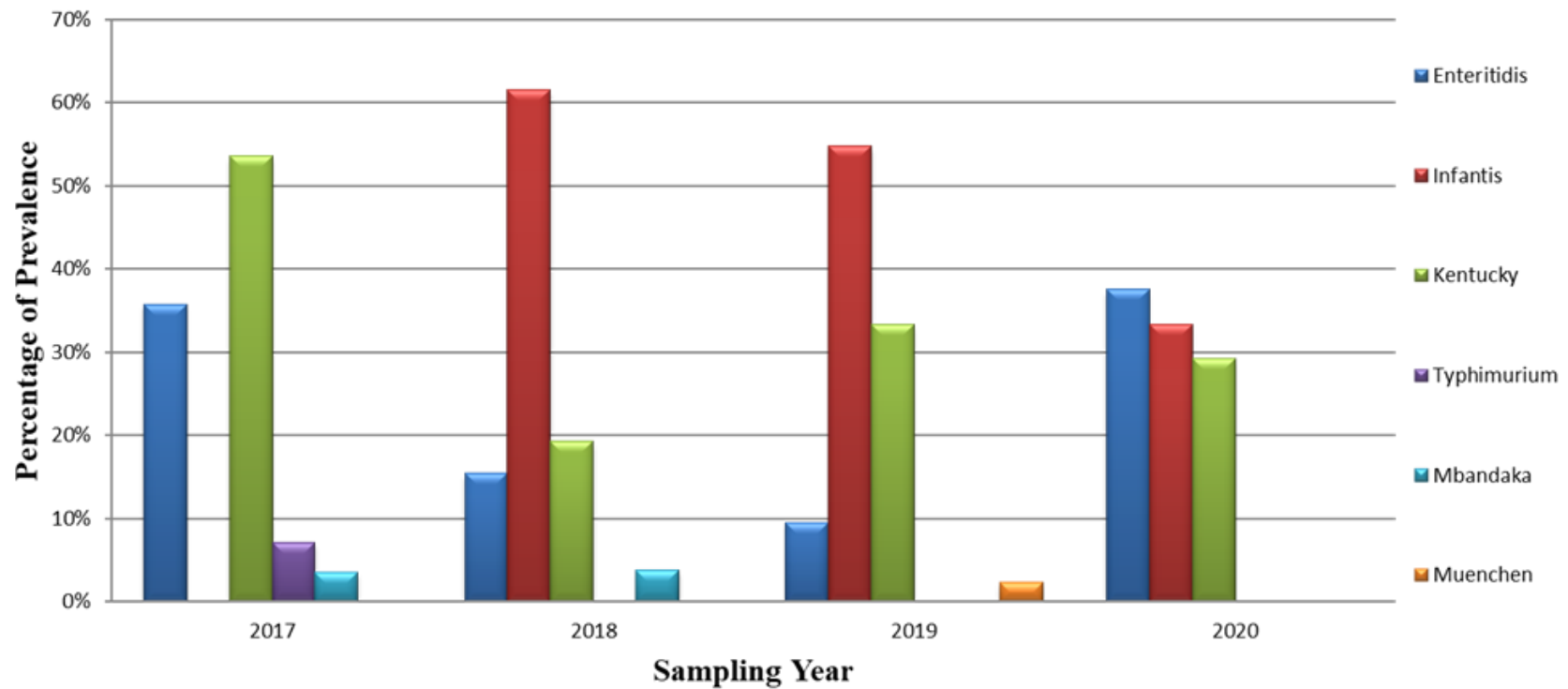


Figure 3.13 *Salmonella* Prevalence in the Hatchery of Study Site #2 2017-2020

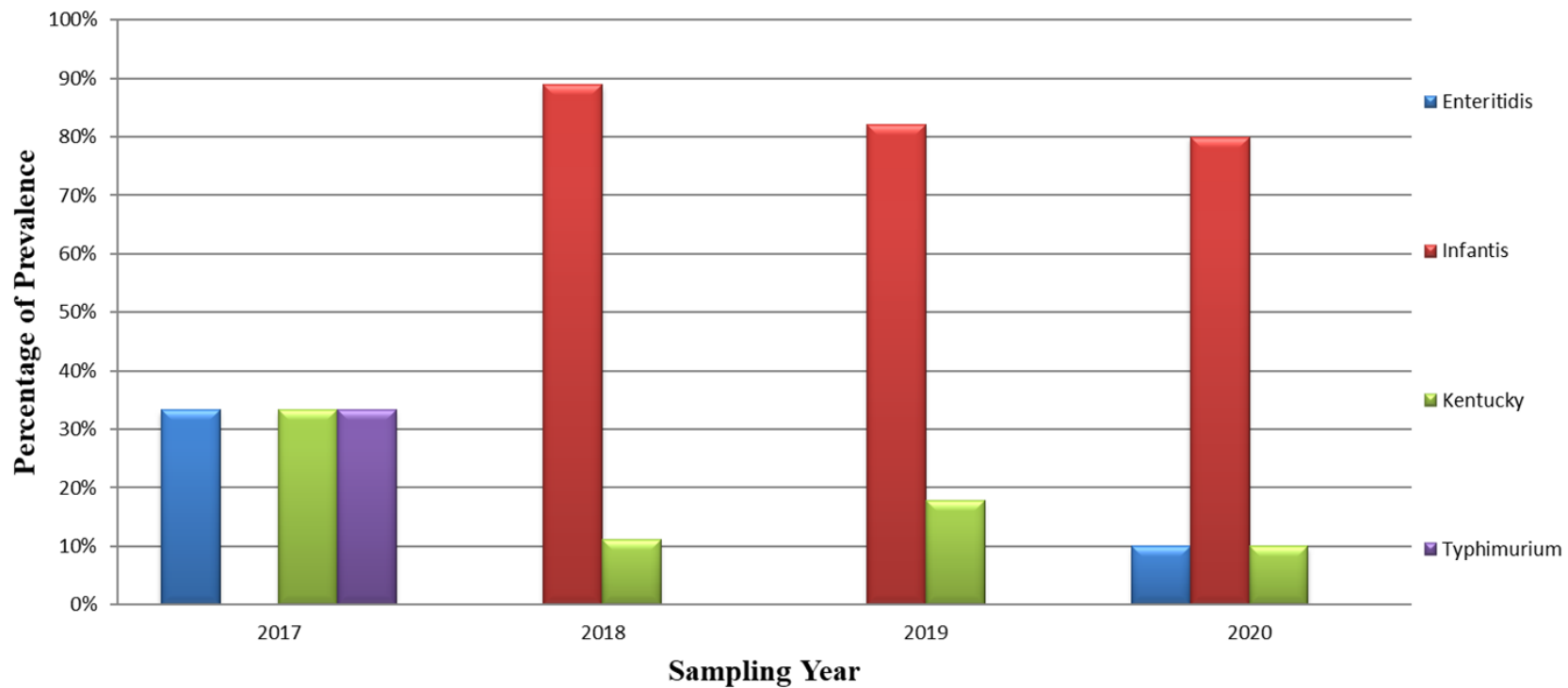


Figure 3.14 *Salmonella* Prevalence in the Processing Plant of Study Site #2 2017-2020

In 2017, an autogenous vaccine formula with three *S. Enteritidis* isolates, one *S. Typhimurium* and one *S. Heidelberg* isolate was introduced to the site. The prevalence of *S. Enteritidis* was at 33.3% when the vaccine was first introduced and quickly rose to 80.0% of the prevalence. In 2018, it dropped to 57.1% in Q1 and was not recovered from 2018 Q3-2019 Q2. Two of the three vaccine isolates were negative for citrate utilization, two were negative for acetoin production and all three were negative for inositol utilization and positive for d-sorbitol fermentation. All recovered isolates from 2017 through 2018 were positive for arginine dihydrolase, negative for inositol utilization, 18.3% were positive for citrate utilization (Figure 3.15), 95.0% were positive for acetoin production (Figure 3.16) and 100.0% were positive for d-sorbitol fermentation. Most of the isolates were moderate to strong biofilm producers with 51.0% receiving a score of 2 and 41.7% receiving a score of 3 (Figure 3.17).

In 2018, the autogenous vaccine was altered to account for the increase in *S. Infantis* isolates and one *S. Enteritidis* isolate was dropped to add a *S. Infantis* isolate. Between 2019 and 2020 Q3, the prevalence of *S. Enteritidis* decreased to an average of 29.9% prevalence per quarter. One of the *S. Enteritidis* isolates was citrate positive, one was negative, both were positive for acetoin production and d-sorbitol fermentation and negative for inositol utilization. The *S. Enteritidis* isolates recovered between 2019 and 2020 Q3 were all negative for inositol utilization, 35.4% were positive for citrate utilization, all 2019 isolates were negative for acetoin production and 55.6% of the 2020 isolates were positive for acetoin production. All the 2019 isolates were positive for d-sorbitol fermentation, 33.3% of the 2020 Q1 isolates were positive and none of the 2020 Q3 isolates were positive for d-sorbitol fermentation. All isolates were moderate to strong biofilm producers with 95.5% receiving a score of 3. In *S. Enteritidis*, the majority of the 2017 isolates were negative for citrate, but the trend evened out throughout 2019 and 2020 to about 65% negative, with most of the positive isolates originating in the processing plant. In 2018, all *S. Enteritidis* isolates were positive for acetoin production, but in 2019, all isolates recovered were negative for acetoin production. The prevalence of positive isolates increased throughout 2020, but the prevalence of the negative isolates remained between 30% and 65%. A similar trend was noted in *S. Infantis*, indicating that the vaccine formulation affects the acetoin production characteristic.

In 2020 Q3, the autogenous vaccine was updated due to expired isolates. The new vaccine formulation included two *S. Enteritidis* isolates, *S. Infantis* isolates, and one *S.*

Typhimurium (multi-flagellated) isolate. The two *S. Enteritidis* isolates were negative for acetoin production and inositol utilization, positive for d-sorbitol fermentation and one isolate was positive for citrate utilization. Since the implementation of this vaccine, the prevalence of *S. Enteritidis* has averaged 56.3%. 16.67% of the isolates tested positive for citrate utilization, most of these were from the processing plant isolates, 54.2% were positive for acetoin production, 50.0% were positive for d-sorbitol fermentation, all were negative for inositol utilization and all were strong biofilm producers with a score of 3.

In the 2017 vaccine formulation, the *S. Typhimurium* (monoflagellated) isolate was citrate negative, and positive for acetoin production, inositol utilization and d-sorbitol fermentation. The prevalence of *S. Typhimurium* in this study site was 6.7% in 2017 Q1 and 20.0% in 2017 Q2. It has not been recovered since 2017 Q2. The isolates that were recovered were positive for arginine dihydrolase, the processing isolates were positive for citrate utilization and the hatchery isolates were negative, the hatchery isolates were positive for acetoin production and the processing isolates were negative. All isolates were positive for d-sorbitol fermentation, and inositol utilization. The vaccine isolate had a biofilm score of 3 as well as the processing plant isolates from 2017. The most recent 2020 autogenous vaccine formulation included a multi-flagellated *S. Typhimurium*, but no new isolates have been recovered from this study site

The 2017 vaccine formulation also included a *S. Heidelberg* isolate that was citrate and sorbitol positive, and acetoin and inositol negative. No *S. Heidelberg* isolates have been recovered from this site since prior to 2017.

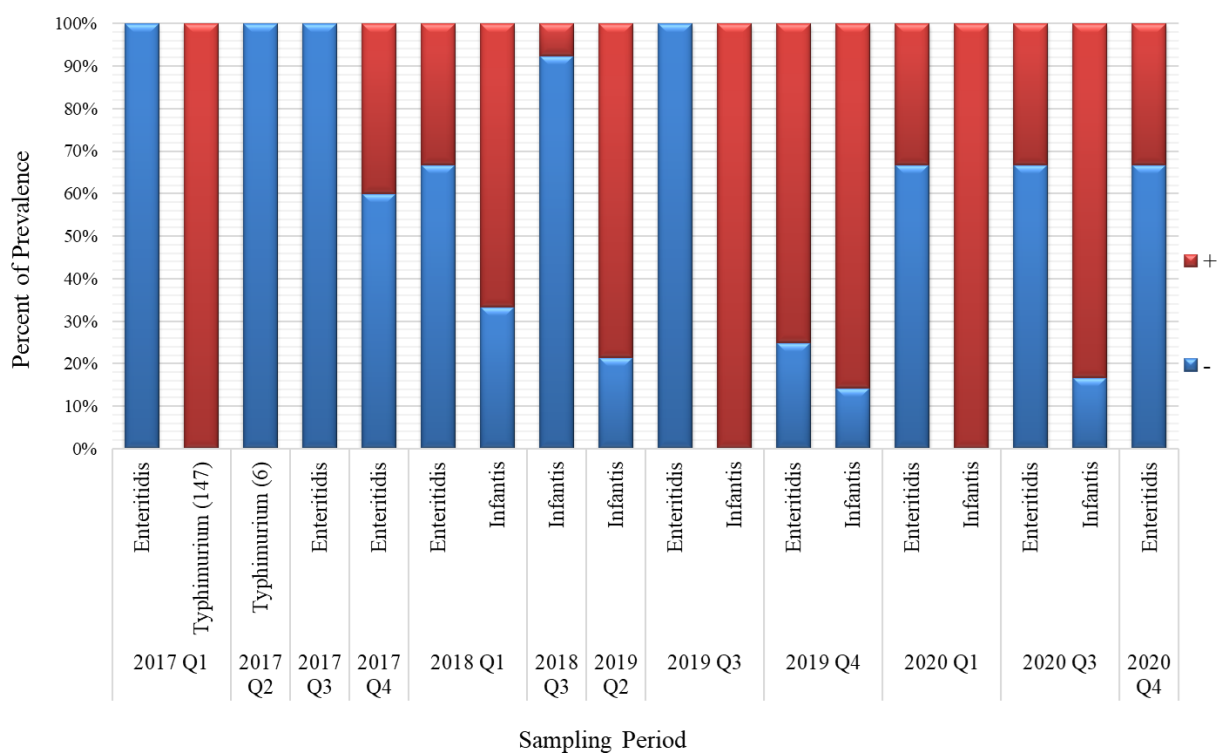


Figure 3.15 Prevalence of Citrate Utilization in Isolates throughout Study Site #2 2017-2020

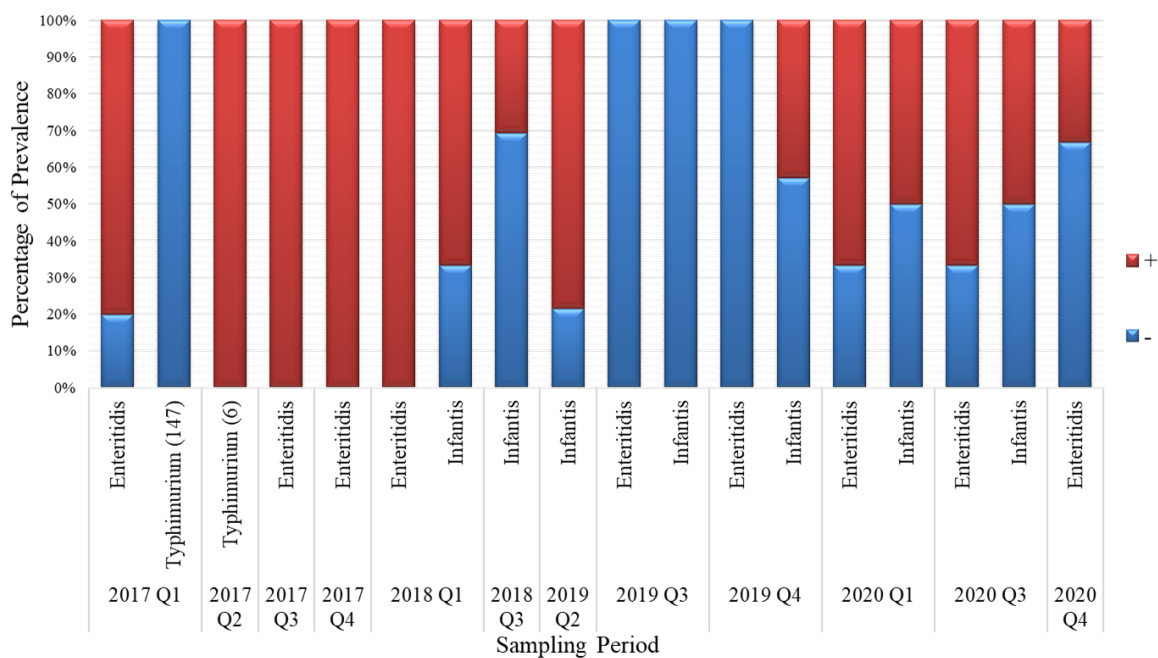


Figure 3.16 Prevalence of Acetoin Production in Isolates throughout Study Site #2 2017-2020

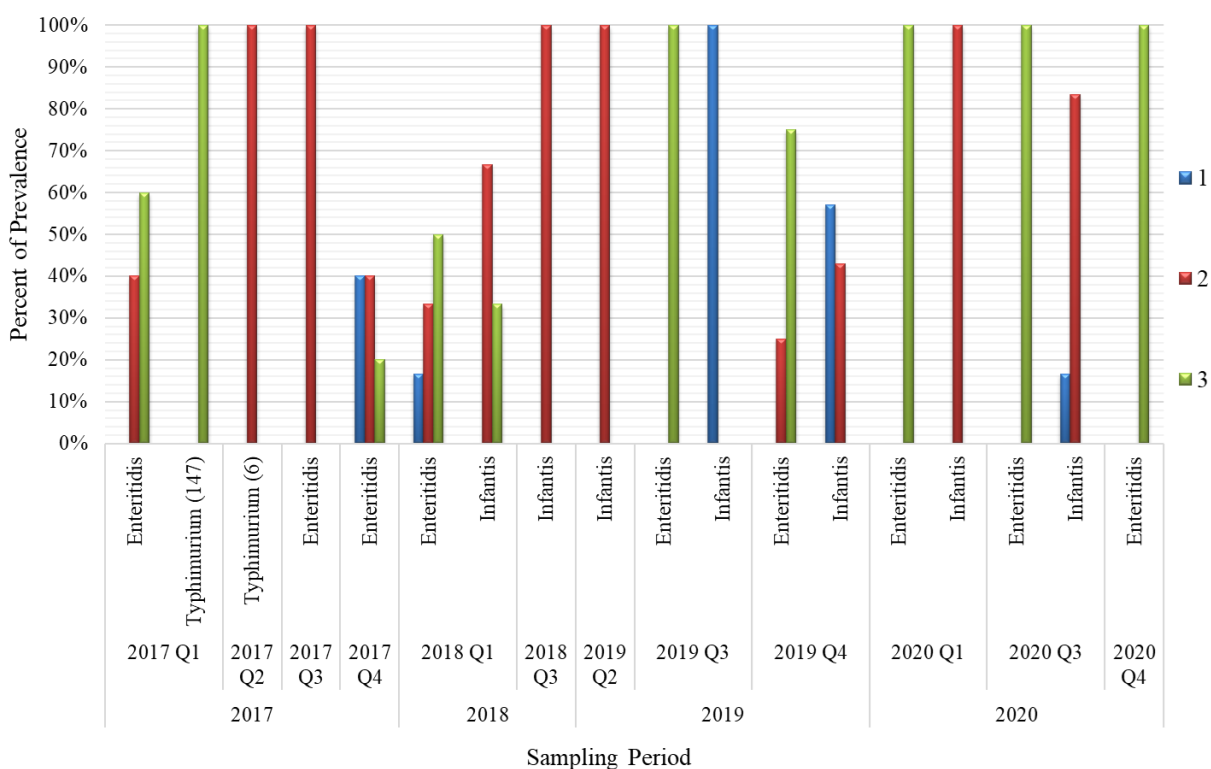


Figure 3.17 Biofilm Score Prevalence in Isolates throughout Study Site #2 2017-2020

Study site #2 had changes in the overall *Salmonella* prevalence due to the implementation of the autogenous vaccine protocols. In 2017, 36.0% of the hatchery samples were *S. Enteritidis* along with 33.3% of the processing plant isolates. In 2020, the prevalence of *S. Enteritidis* in the hatchery increased to 57.1%, but the prevalence of *S. Enteritidis* in the processing plant decreased to 10.0%. *Salmonella* Infantis isolates were not recovered in 2017, but in 2018, they were responsible for 88.9% of the positive processing plant isolates. After the implementation of autogenous vaccine formulation #2, there was no statistical difference in prevalence between 2018 and 2020 (Table 3.10). *Salmonella* Kentucky isolates were not included in the autogenous vaccine formulations, but their prevalence dropped 24.4% between 2017 and 2020. This may be due to environmental changes on the farm that affected the prevalence of *Salmonella* or due to cross-protection with another serotype present in the vaccine. *Salmonella* Typhimurium was recovered in both the processing plant and the hatchery in 2017 but was not recovered in any samples from 2018-2020, after the implementation of the monoflagellated vaccine isolate.

Differences were noted in various phenotypic characteristics after the implementation of the autogenous vaccines. Characteristics such as citrate utilization, acetoin production and biofilm formation altered throughout the sampling periods. In *S. Infantis*, the use of a citrate positive isolate in the autogenous vaccine formulation did not appear to have any effect on the prevalence of citrate positive isolates recovered in the processing plant.

Study Site #3 Survey Results

Study site #3 was in the state of Alabama. In 2017, the most isolated serotype was *Salmonella* Enteritidis with 48.0%, followed by *Salmonella* Typhimurium with 18.0% and then *Salmonella* Kentucky with 16.0% (Figure 3.18). In 2020, the most isolated serotype was *Salmonella* Kentucky with 59.1%, a 43.09% increase, followed by *Salmonella* Enteritidis and *Salmonella* Schwarzengrund with 18.2% each and then *Salmonella* Senftenberg with 4.6%. In the hatcheries in 2017, the most isolated serotypes were *Salmonella* Enteritidis with 43.5% followed by *Salmonella* Typhimurium with 19.6% and then *Salmonella* Kentucky with 17.4% prevalence. In 2020, the most isolated serotypes were *Salmonella* Kentucky with 62.5%, a 45.1% increase, followed by *Salmonella* Enteritidis with 25.0%, an 18.5% decrease (Figure 3.19). In the processing plant in 2017, *Salmonella* Enteritidis was the only serotype isolated (Figure 3.20). In 2020, the most isolated serotype was *Salmonella* Kentucky with 50.0%, followed by *Salmonella* Schwarzengrund with 33.3% and then *Salmonella* Senftenberg with 16.7% (Table 3.6). Overall, *S. Kentucky* increased by 43.1% between 2017 and 2020.

In 2017, *Salmonella* Enteritidis represented 43.5% of the hatchery isolates and 100.0% of the processing plant isolates. By 2020, it represented 25.0% of the hatchery isolates and was not found in the processing plant. *Salmonella* Typhimurium represented 19.6% of the hatchery samples in 2017 but was not found in the processing plant. *Salmonella* Infantis was not found at this site during any point between 2017-2020. *Salmonella* Heidelberg was found in the hatchery in 2017, but not in the processing plant. It has not been isolated since 2017 at this site.

Table 3.10 Sample Prevalence of Study Site #3 2017-2020

		S. Enteritidis	S. Heidelberg	S. Kentucky	S. Typhimurium	S. Mbandaka	S. Schwarzengrund	S. Senftenberg
Hatchery	2017	43.5%	2.2%	17.4%	19.6%	2.2%	0.0%	0.0%
	2018	70.0%	0.0%	25.0%	0.0%	0.0%	0.0%	0.0%
	2019	31.3%	0.0%	56.3%	0.0%	12.5%	0.0%	0.0%
	2020	25.0%	0.0%	62.5%	0.0%	0.0%	12.5%	0.0%
	% Differences*	-18.5%	-2.2%	+45.1	-19.6%	-2.2%	+12.5%	0.0%
Processing	2017	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	2018	40.0%	0.0%	60.0%	0.0%	0.0%	0.0%	0.0%
	2019	25.0%	0.0%	75.0%	0.0%	0.0%	0.0%	0.0%
	2020	0.0%	0.0%	50.0%	0.0%	0.0%	33.3%	16.7%
	% Differences*	-100.0%	0.0%	+50.0%	0.0%	0.0%	+33.3%	+16.7%
Overall**	2017	48.0%	2.0%	16.0%	18.0%	2.0%	0.0%	0.0%
	2018	61.5%	0.0%	38.5%	0.0%	0.0%	0.0%	0.0%
	2019	29.2%	0.0%	62.5%	0.0%	8.3%	0.0%	0.0%
	2020	18.2%	0.0%	59.1%	0.0%	0.0%	18.2%	4.6%
	% Differences*	29.8%	-2.0%	+43.1	-18.0%	-2.0%	+18.2%	+4.6%

*Percent differences are the difference between the 2017 and 2020 sampling period prevalence

** Overall includes the hatchery, processing plant, broiler, and breeder samples

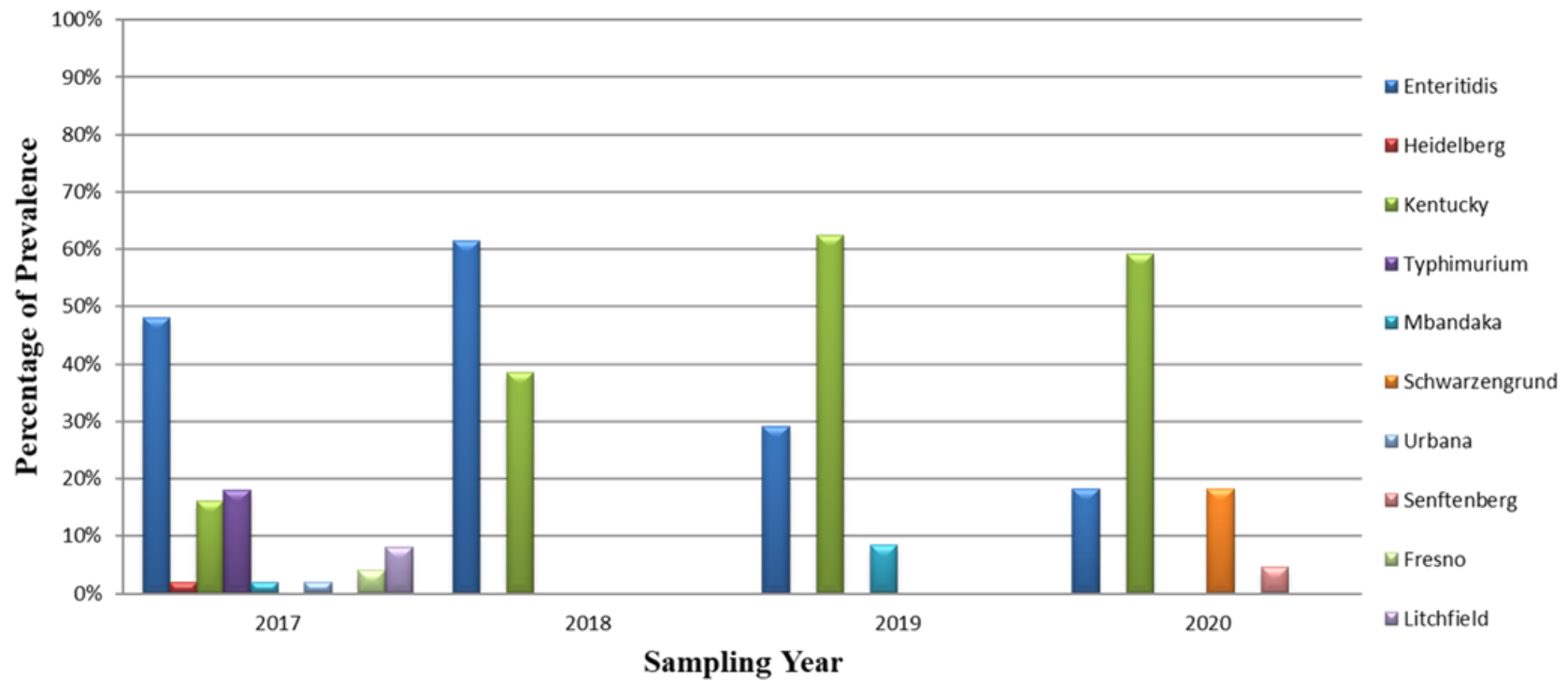


Figure 3.18 Overall Prevalence of *Salmonella* throughout Study Site #3 2017-2020

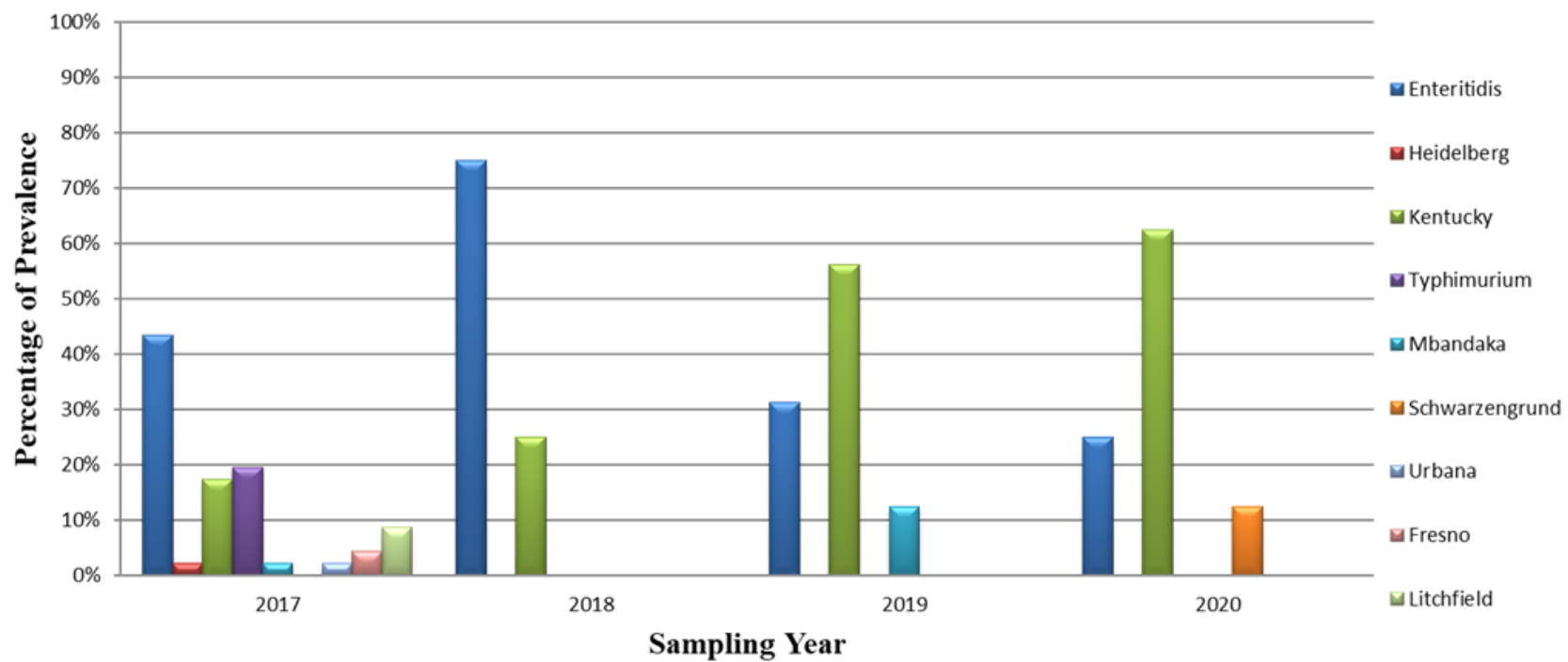


Figure 3.19 *Salmonella* Prevalence in the Hatchery of Study Site #3 2017-2020

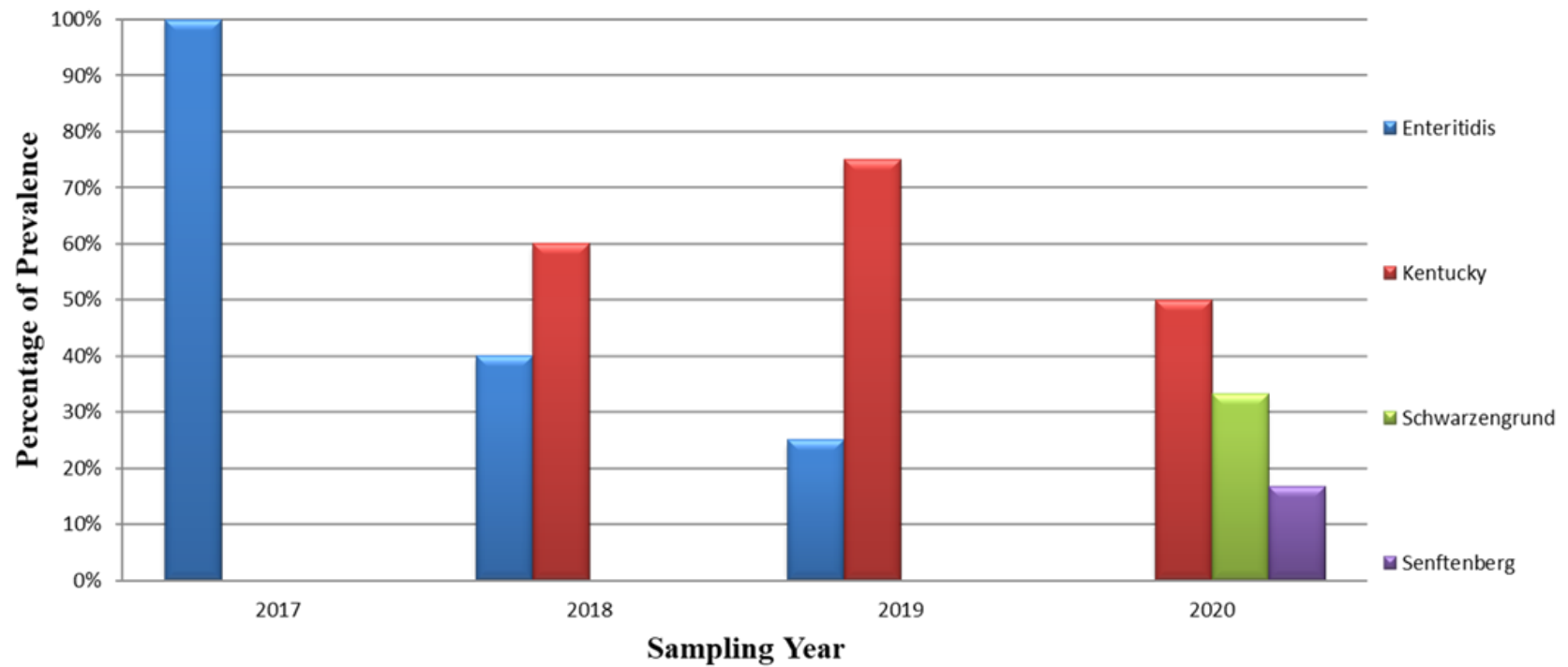


Figure 3.20 *Salmonella* Prevalence in the Processing Plant of Study Site #3 2017-2020

The 2017 autogenous vaccine formulation was utilized in this study site from 2017 through 2018 Q2 and the 2018 autogenous vaccine formulation was utilized from 2018 Q3 to 2021 Q1. The *S. Enteritidis* isolates recovered during this time were 99.2% positive for arginine dihydrolase, 37.5% positive for the utilization of citrate (Figure 3.21), 57.9% were positive for acetoin production (Figure 3.22), all isolates were positive for d-sorbitol fermentation and 97.5% were negative for inositol utilization (Figure 3.23). For the biofilm formations, 66.5% of the isolates were strong biofilm producers and 31.1% were moderate biofilm producers (Figure 3.24). There were no apparent changes in the prevalence of citrate utilization in response to either vaccine formulation. Throughout the entire time of the study, the prevalence of citrate utilization remained mostly negative in the processing plant and hatchery. Some sampling periods included only positive citrate isolates, but it is unclear whether they are due to the vaccine formulation or external influences on the individual farm. After the implementation of the vaccine formulation #2 in 2018, the prevalence of *S. Enteritidis* isolates negative for acetoin production increased throughout 2019 and 2020.

The 2020 autogenous vaccine formulation was implemented during the 2020 Q3 sampling period. The *S. Enteritidis* isolates recovered during this period were all positive for arginine dihydrolase and inositol utilization, 33.3% were positive for citrate utilization, 66.7% were positive for acetoin production and d-sorbitol fermentation. All the samples were negative for inositol utilization and were strong biofilm producers.

The 2017 autogenous vaccine formulation included a *S. Typhimurium* (monoflagellated) isolate. All recovered isolates were from the hatchery with 77.8% of the isolates belonging to the monoflagellated serotype throughout 2017. All monoflagellated *S. Typhimurium* isolates were positive for arginine dihydrolase, 50.0% were positive for citrate utilization, 75.0% were positive for acetoin production, 100.0% were positive for d-sorbitol fermentation and inositol utilization. All were moderate to strong biofilm producers. All multi-flagellated *S. Typhimurium* isolates were positive for arginine dihydrolase, negative for the utilization of citrate, positive for acetoin production and d-sorbitol fermentation and 50.0% were positive for the utilization of inositol. All were moderate to strong biofilm producers. The 2020 autogenous vaccine includes a multi-flagellated *S. Typhimurium* isolate, but *S. Typhimurium* has not been isolated from this site since 2017.

The *S. Heidelberg* isolated in 2017 from this site was positive for arginine dihydrolase, citrate utilization, d-sorbitol fermentation and were negative for acetoin production and inositol utilization. The isolates were weak biofilm producers. The implementation of the vaccine directly affected the recovery of *S. Heidelberg*. The most recent autogenous vaccine did not have a *S. Heidelberg* since no isolates had been recovered since 2017.

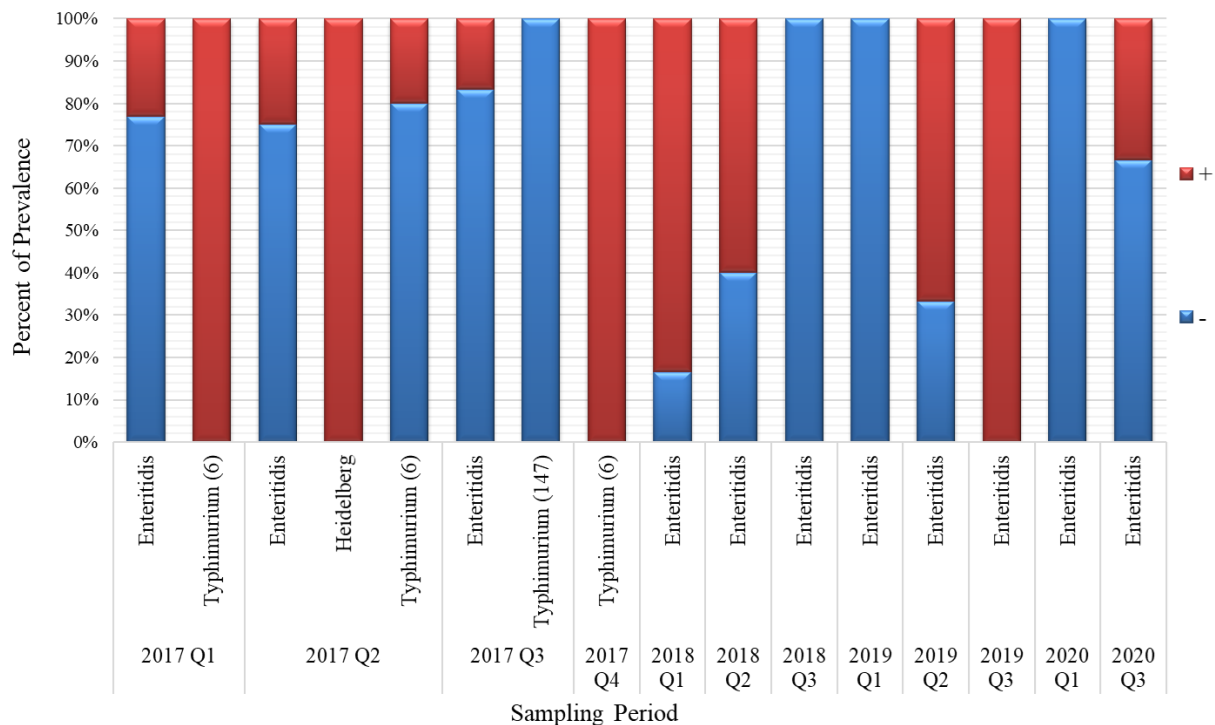


Figure 3.21 Prevalence of Citrate Utilization in Isolates throughout Study Site #3 2017-2020

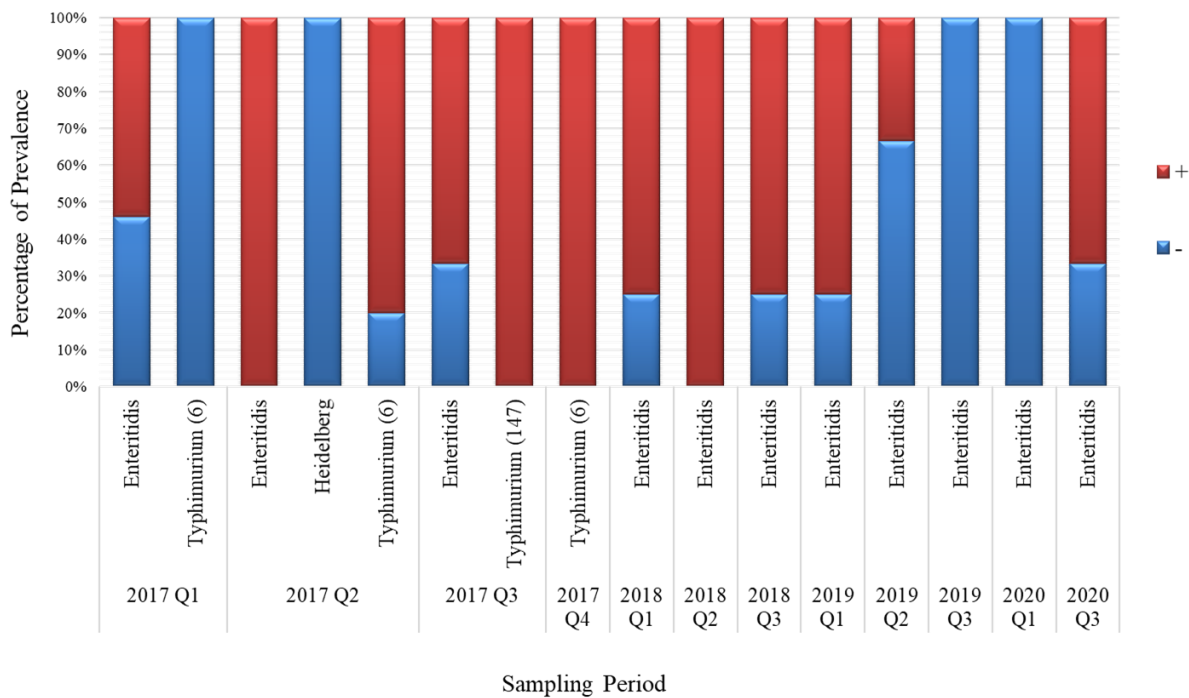


Figure 3.22 Prevalence of Acetoin Production in Isolates throughout Study Site #3 2017-2020

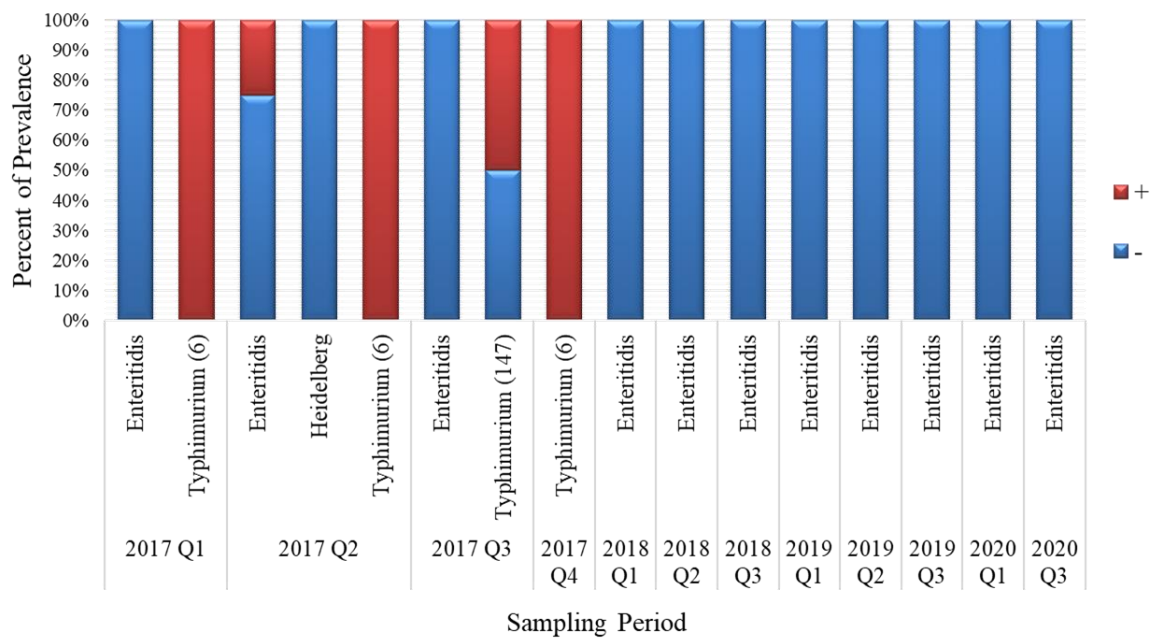


Figure 3.23 Prevalence of Inositol Utilization in Isolates throughout Study Site #3 2017-2020

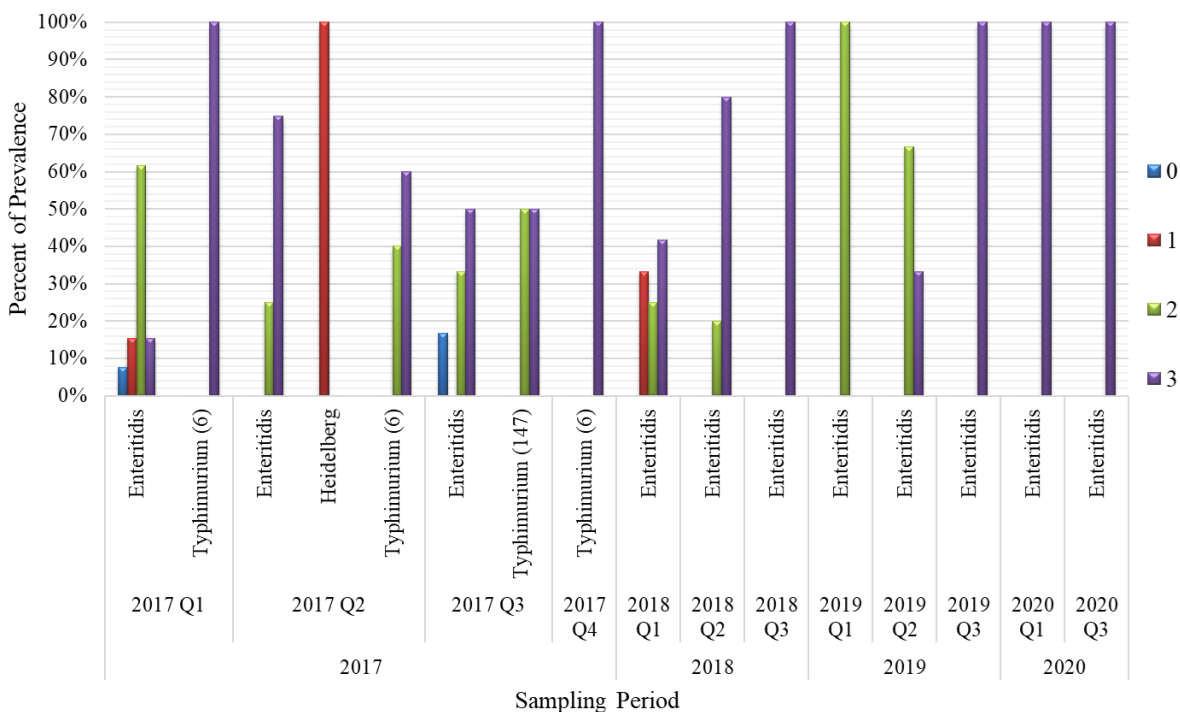


Figure 3.24 Biofilm Score Prevalence in Isolates throughout Study Site #3 2017-2020

Study site #3 showed differences in the recovery and prevalence of the various *Salmonella* serotypes isolated from 2017 through 2020. The implementation of the vaccines resulted in the reduction of *S. Typhimurium*, *S. Enteritidis* and *S. Heidelberg* isolates in the hatcheries and processing plant. *Salmonella Typhimurium* and *S. Heidelberg* have not been isolated at this site since 2017. *Salmonella Enteritidis* represented 43.5% of the isolates recovered in the hatchery in 2017 and 100.0% of the processing isolates, however by 2020, there was a decrease of 18.5% in the hatchery and was not isolated in the processing plant. As *S. Enteritidis*, *S. Typhimurium* and *S. Heidelberg* decreased in response to the vaccine, other isolates such as *S. Kentucky* and *S. Schwarzengrund* increased to fill the niche. Between 2017 and 2020, the prevalence of *S. Kentucky* and *S. Schwarzengrund* increased by 43.1% and 18.2%, respectively, in both the hatchery and the processing plant.

Citrate utilization fluctuated in *S. Enteritidis* throughout the study period with some sampling periods consisting of all negative isolates and others consisting of all positive isolates. The fluctuations do not appear to be in relation to any specific vaccine formulation. The prevalence of acetoin negative isolates increased throughout 2019 and 2020 after the

implementation of the vaccine formulation #2 which included two *S. Enteritidis* isolates that were positive for acetoin production, indicating that the shift is due to the effect of the vaccine. The inositol utilization did not change in response to the vaccine since most of the *S. Enteritidis* isolates were negative prior to the implementation of the vaccine and remained so after the implementation. The biofilm formations for *S. Enteritidis* shifted slightly throughout the course of this study. In 2017 and 2018, the biofilm scores ranged from 0 to 3, but by the end of 2018, the scores were in the 2-3 range and by 2020, all recovered isolates developed strong biofilm formations and were scored a 3. This may be because as the overall prevalence of *S. Enteritidis* decreases on the farms, the remaining isolates are going to be the hardiest and most capable of surviving the various environments. Since biofilm formations aid the bacteria in this survival, it is common for these remaining isolates to produce strong biofilms.

Study Site #4 Survey Results

Study Site #4 was in the state of Alabama. The most isolated serotype in 2017 was *Salmonella* Enteritidis with 56.9% followed by *Salmonella* Kentucky with 31.9% and then *Salmonella* Hadar with 4.2% (Figure 3.25). In 2020, the most isolated serotype was *Salmonella* Kentucky with 54.3%, a 22.4% increase, followed by *Salmonella* Enteritidis with 11.4%, a 45.5% decrease. In the hatchery in 2017, the most isolated serotype was *Salmonella* Enteritidis with 54.6% , which dropped to 15.0% in 2020. In the processing plant in 2017, the most isolated serotypes were *Salmonella* Enteritidis with 83.3% and then *Salmonella* Typhimurium with 16.7%. In 2020, the most isolated serotypes were *Salmonella* Kentucky with 66.7% followed by *Salmonella* Enteritidis, Mbandaka, Schwarzengrund, Hadar and Anatum all with 6.7% (Table 3.7).

In 2017, *Salmonella* Enteritidis represented 54.6% of the hatchery isolates (Figure 3.26) and 83.3% of the processing plant isolates (Figure 3.27). By 2020, it dropped to 11.4% of the hatchery isolates and 6.7% of the processing plant isolates. *Salmonella* Typhimurium represented none of the hatchery isolates in 2017 but represented 16.7% of the processing plant isolates, indicating that it was coming into the processing plant from another source such as the broiler houses. In 2020, it represented 5.0% of the hatchery isolates but was not found in the processing plant. *Salmonella* Kentucky represented 34.9% of the hatchery isolates in 2017 but was not found in the processing plant. In 2020, it represented 45.0% of the hatchery isolates and 66.7%

of the processing plant isolates. *Salmonella* Infantis was not found at this site at any point from 2017-2020.

Table 3.11 Sample Prevalence of Study Site #3 2017-2020

		S. Enteritidis	S. Heidelberg	S. Kentucky	S. Typhimurium	S. Mbandaka	S. Anatum	S. Hadar
Hatchery	2017	54.6%	1.5%	34.9%	0.0%	0.0%	0.0%	4.6%
	2018	50.0%	0.0%	26.7%	0.0%	10.0%	0.0%	13.3%
	2019	18.9%	0.0%	43.2%	0.0%	8.1%	8.1%	21.6%
	2020	15.0%	0.0%	45.0%	5.0%	10.0%	15.0%	0.0%
	% Differences*	-39.6%	-1.5%	+10.2%	+5.0%	+10.0%	+15.0%	-4.6%
Processing	2017	83.3%	0.0%	0.0%	16.7%	0.0%	0.0%	0.0%
	2018	33.3%	0.0%	33.3%	0.0%	0.0%	0.0%	0.0%
	2019	33.3%	0.0%	61.1%	0.0%	0.0%	0.0%	5.6%
	2020	6.7%	0.0%	66.7%	0.0%	6.7%	6.7%	6.7%
	% Differences*	-76.7%	0.0%	+66.7%	-16.7%	+6.7%	+6.7%	+6.7%
Overall**	2017	56.9%	1.4%	31.9%	1.4%	0.0%	0.0%	4.2%
	2018	48.5%	0.0%	27.3%	0.0%	9.1%	0.0%	12.1%
	2019	23.6%	0.0%	49.1%	0.0%	5.5%	5.5%	16.4%
	2020	11.4%	0.0%	54.3%	2.9%	8.6%	11.4%	2.9%
	% Differences*	-45.5%	-1.4%	+22.4%	+1.47%	+8.6%	+11.4%	-1.3%

*Percent differences are the difference between the 2017 and 2020 sampling period prevalence

** Overall includes the hatchery, processing plant, broiler, and breeder samples

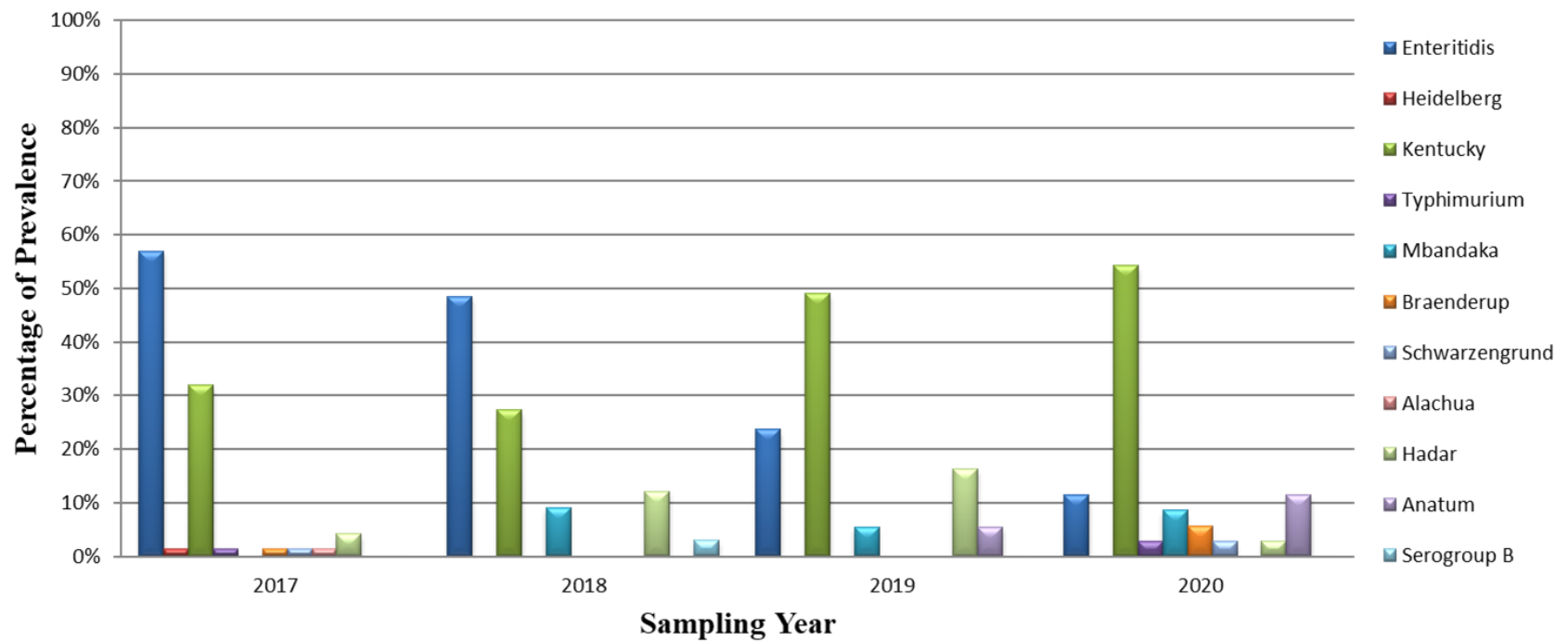


Figure 3.25 Overall Prevalence of *Salmonella* throughout Study Site #4 2017-2020

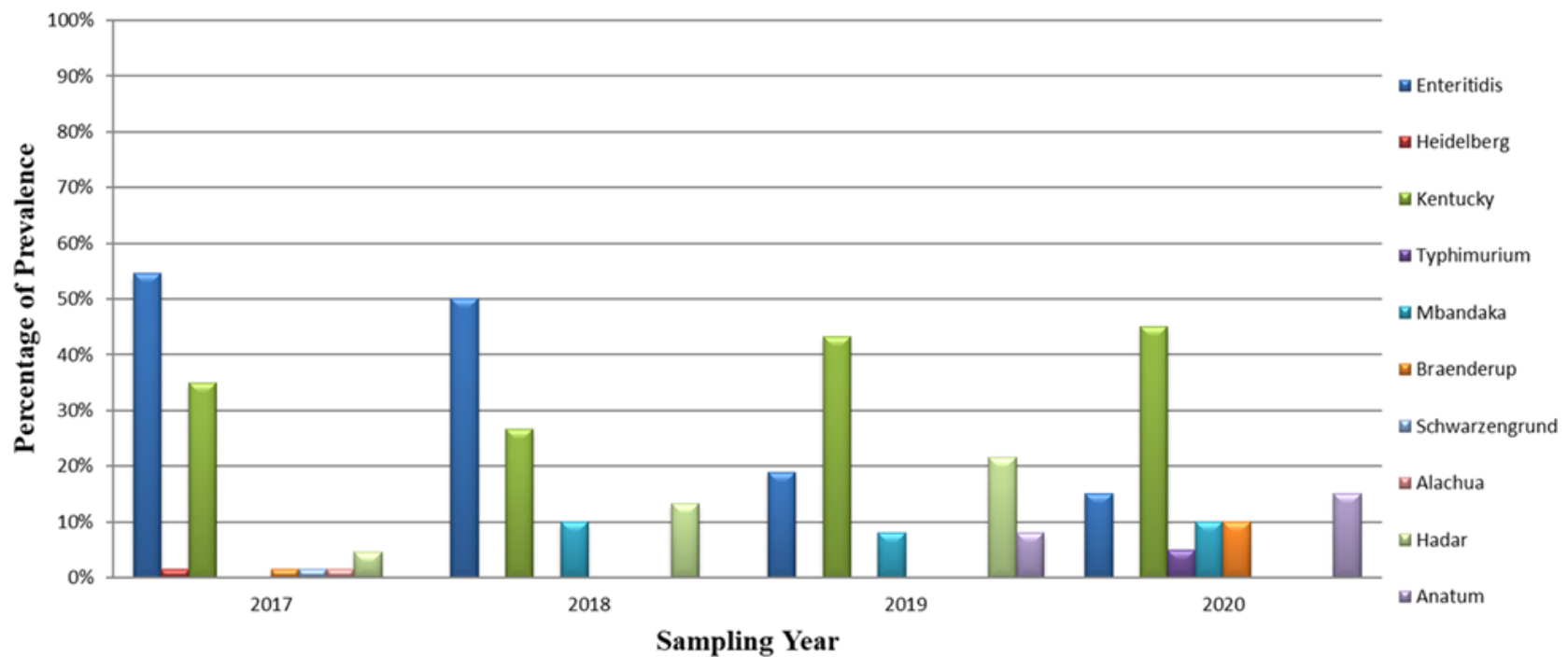


Figure 3.26 *Salmonella* Prevalence in the Hatchery of Study Site #4 2017-2020

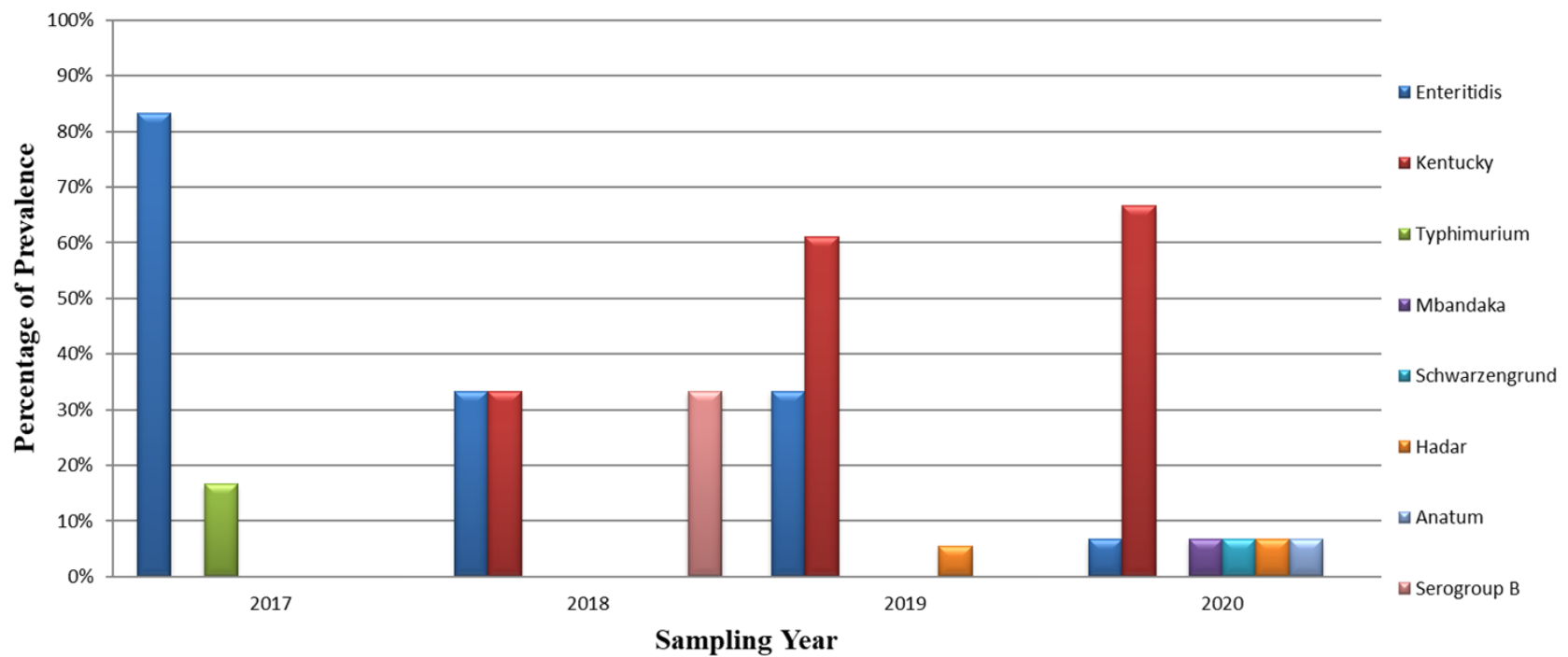


Figure 3.27 *Salmonella* Prevalence in the Processing Plant of Study Site #4 2017-2020

The 2017 vaccine was utilized in this study site from 2017 through 2018 Q2, with the 2018 vaccine being utilized between 2018 Q3 and 2020 Q1. The *S. Enteritidis* recovered from this site during that time were positive for arginine dihydrolase, d-sorbitol fermentation, 96.3% were negative for inositol utilization, 76.4% were positive for acetoin production (Figure 3.29), and 39.5% were positive for citrate utilization (Figure 3.28). For biofilm production, 44.8% were strong biofilm producers, 53.6% were moderate biofilm producers and 1.6% were weak producers (Figure 3.30). The 2020 autogenous vaccine formulation was implemented during the 2020 Q3 sampling period. The *S. Enteritidis* isolates recovered during this time were all positive for arginine dihydrolase, d-sorbitol fermentation, negative for citrate utilization, inositol utilization, and 50.0% were positive for acetoin production. All isolates were strong biofilm producers.

S. Typhimurium (monoflagellated) was isolated from this site in 2017 Q3 and 2020 Q4. The 2017 isolates were from the processing plant and the 2020 isolates were from the hatchery. All isolates from both sampling periods and vaccine formulations were positive for arginine dihydrolase, d-sorbitol fermentation, inositol utilization and were strong biofilm producers. The 2017 isolates were all negative for citrate utilization and positive for acetoin production which matched the vaccine isolate for that period. The 2020 isolates were all positive for citrate utilization and negative for acetoin production.

The *S. Heidelberg* isolates from 2017 were all positive for arginine dihydrolase, citrate utilization, d-sorbitol fermentation, negative for acetoin production, inositol utilization and were all weak biofilm producers. No isolates were recovered after 2017 Q2.

No *S. Infantis* isolates were recovered from this site at any time between 2017 and 2021.

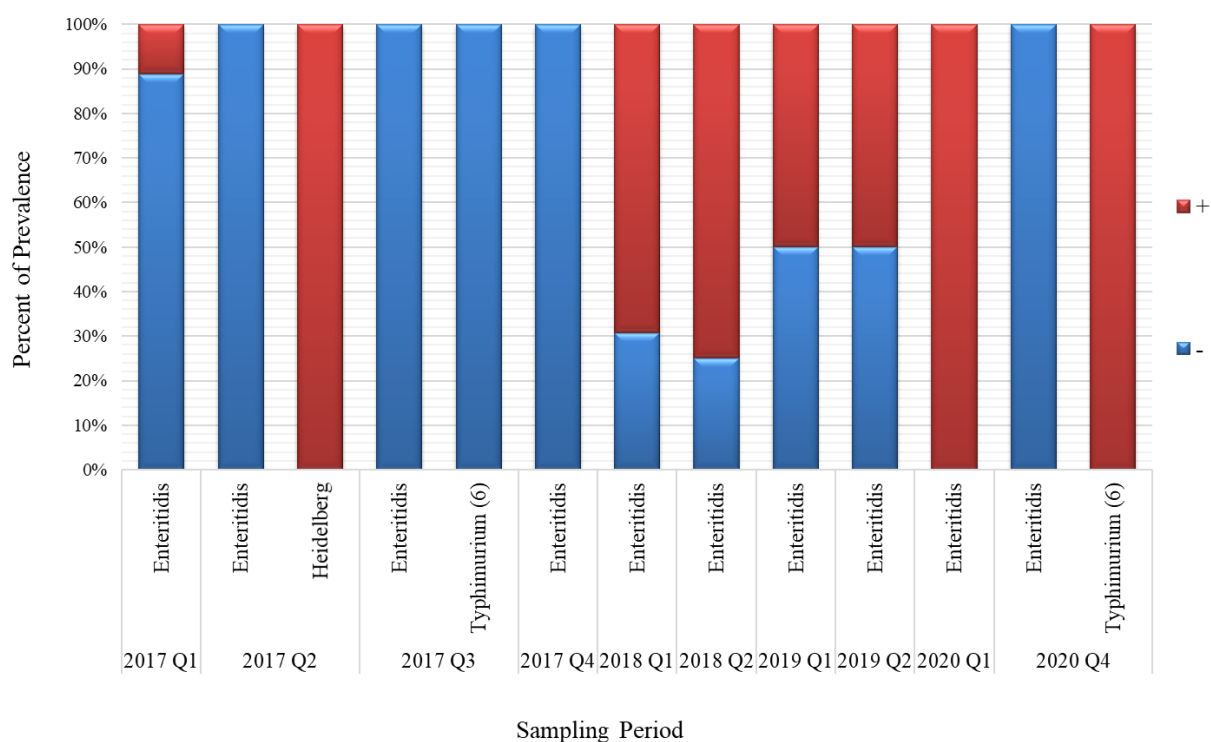


Figure 3.28 Prevalence of Citrate Utilization in Isolates throughout Study Site #4 2017-2020

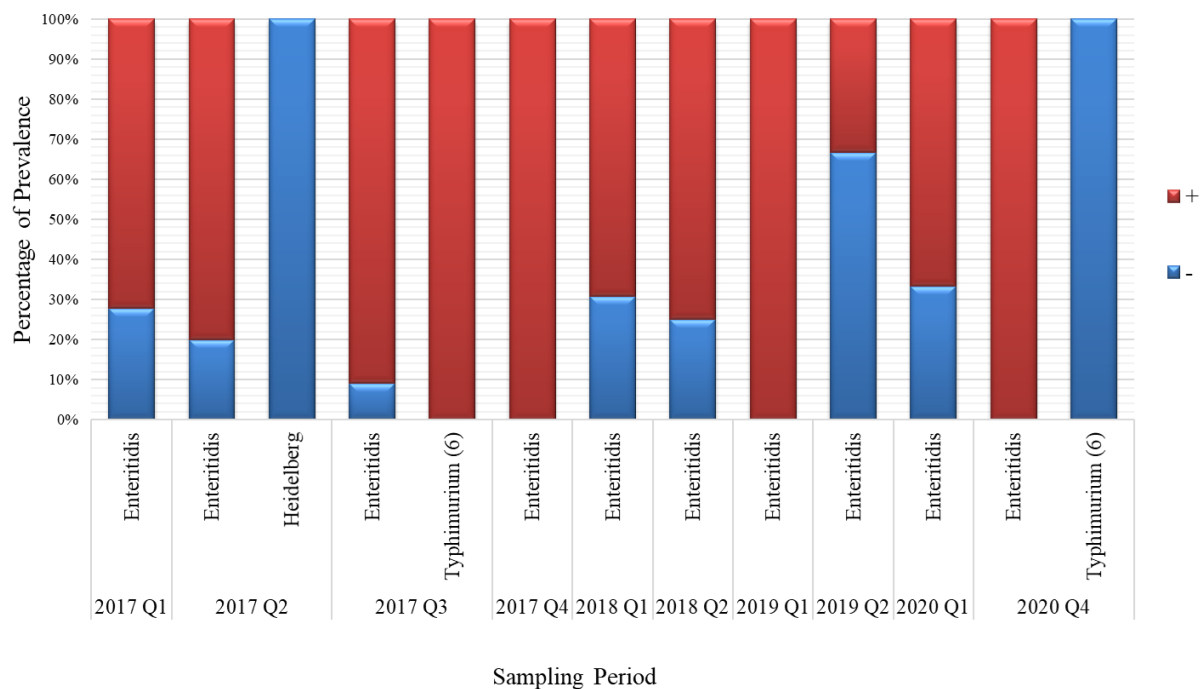


Figure 3.29 Prevalence of Acetoin Production in Isolates throughout Study Site #4 2017-2020

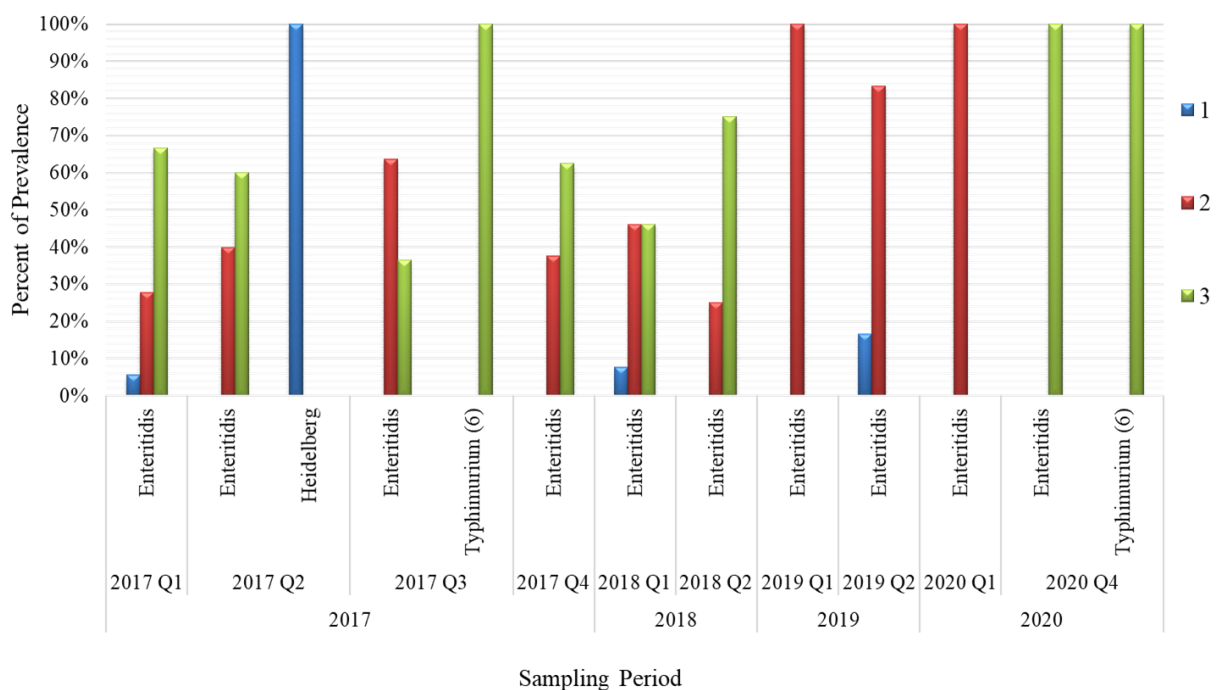


Figure 3.30 Biofilm Score Prevalence in Isolates throughout Study Site #4 2017-2020

Study site #4 showed differences in the recovery and prevalence of serotypes *S. Enteritidis*, *S. Heidelberg*, *S. Kentucky*, and *S. Typhimurium* in response to the vaccine formulations. *Salmonella Enteritidis* represented 54.6% of the hatchery isolates and 83.3% of the processing plant isolates in 2017, but by 2020, they had decreased to 15.0% and 6.7%, respectively. *Salmonella Heidelberg* was not isolated on this site after 2017. In 2017, *S. Typhimurium* was isolated from the processing plant and then again in the hatchery in 2020. *Salmonella Kentucky* increased by 10.2% in the hatchery between 2017 and 2020, and by 66.7% in the processing plant during the same timeframe. *Salmonella Mbandaka* and *S. Anatum* also increased in both the hatchery and processing plant between 2017 and 2020. The vaccines implemented resulted in a decreased prevalence of the isolates included in the vaccine formulation and an increase in isolates not included.

Fluctuations were noted in the prevalence of citrate utilizing isolates throughout the study timeframe. In 2017, most *S. Enteritidis* and *S. Typhimurium* isolates were negative for the utilization of citrate, but between 2018 and 2020, the prevalence leveled out with more positive isolates recovered. The *S. Typhimurium* isolates recovered in 2020 were positive for citrate

utilization, which differed from the previous vaccine formulations, indicating that the vaccine had an impact on citrate utilization for the *S. Typhimurium* isolates. The vaccine also impacted the prevalence of acetoin producing *S. Typhimurium* isolates. The 2017 and 2018 vaccines included an acetoin positive isolate and the isolates recovered in 2020 were negative. In *S. Enteritidis*, acetoin production fluctuated throughout the study timeframe, but remained mostly positive for acetoin production regardless of the vaccine formulation. With regards to biofilm formation, both the 2017 and 2020 *S. Typhimurium* isolates formed strong biofilms. The *S. Enteritidis* isolates showed some variability with their biofilm formations, from 1 to 3, in 2017, but by 2020, all isolates were moderate to strong biofilm producers.

Biochemical Test Results

In addition to the serotype prevalence changes, some overall changes have occurred in the biochemical test reactions between 2017 and 2020. All priority isolates have been positive for arginine dihydrolase since 2018 Q1 regardless of serotype. Citrate utilization varied throughout the years in *S. Enteritidis* isolates, with some sampling periods having all positive reactions and others having all negative with no definitive pattern. Each formulation used since 2017 has included citrate positive and citrate negative *S. Enteritidis* isolates. *S. Infantis* isolates had a spike in citrate utilization negative samples in 2018 Q3, then decreased over the next two years. This initial spike in negative samples was seen in site #2 within a year of the implementation of the 2018 vaccine formulation in which a citrate positive *S. Infantis* isolate was included.

Salmonella *Enteritidis* isolates have ranged over the years in their ability to produce acetoin, with some quarters having isolates with all positive isolates and others with all negative isolates. The *S. Infantis* isolates showed no increase or decrease in the overall acetoin production between 2017 and 2020 in these study sites.

All *S. Enteritidis* isolates from 2017 through 2019 were positive for d-sorbitol fermentation with some negative isolates appearing in 2020. The negative isolates were seen in both the hatchery and all 2020 Q3 processing plant isolates. The vaccine formulations between 2017 and 2020 have all included d-sorbitol fermentation positive isolates, so the recovery of the negative d-sorbitol isolates may be due to the vaccine. The *S. Infantis* isolates have all been positive for d-sorbitol fermentation between 2017 and 2020, along with all *S. Typhimurium*

(monoflagellated) isolates. The *S. Typhimurium* multi-flagellated isolates had some variations in the hatchery samples, but all processing isolates were positive.

All *S. Enteritidis* processing plant isolates were negative for inositol utilization, but some positive isolates were found in the hatchery in 2017 Q2 and 2020 Q1. All *S. Infantis* isolates have been positive except for 2019 Q2 which had 7.1% of isolates test negative. All *S. Typhimurium* monoflagellated isolates were positive, but differences were noted in the multi-flagellated isolates. In 2017, the multi-flagellated *S. Typhimurium* isolates switched from all positive reactions to all negative reactions by 2018. The vaccine implemented at this time consisted of a positive inositol *S. Typhimurium* which may have played a role in the changes seen in this characteristic.

The biofilm formation trends changed in *S. Enteritidis* isolates between 2017 and 2020. Throughout 2017 and 2018 Q1, biofilms ranged from a score of 0 to 3, but by the end of 2018, majority of the isolates were moderate to strong biofilm producers. In *S. Infantis* isolates, all isolates were moderate to strong biofilm producers until 2019 Q3. Since then, all isolates have been weak to moderate biofilm producers. The 2018 autogenous vaccine included an isolate that was a moderate biofilm producer and by 2019 Q3 the isolates included weak biofilm producers instead of strong biofilm producers. Most *S. Typhimurium* isolates from 2017 through 2020 were strong biofilm producers, but the isolates ranged from weak to strong producers. The most recently isolated *S. Typhimurium* isolates were strong biofilm producers.

Overall, the utilization of the autogenous vaccines affected the prevalence of multiple phenotypic characteristics in all four study sites. Some characteristics such as citrate utilization, acetoin production and biofilm formation were more likely affected by the vaccine formulation than characteristics such as the arginine dihydrolase. The utilization of phenotypic characteristics to determine an autogenous vaccine formulation results in changes in the phenotypic characteristics after the implementation of the vaccine.

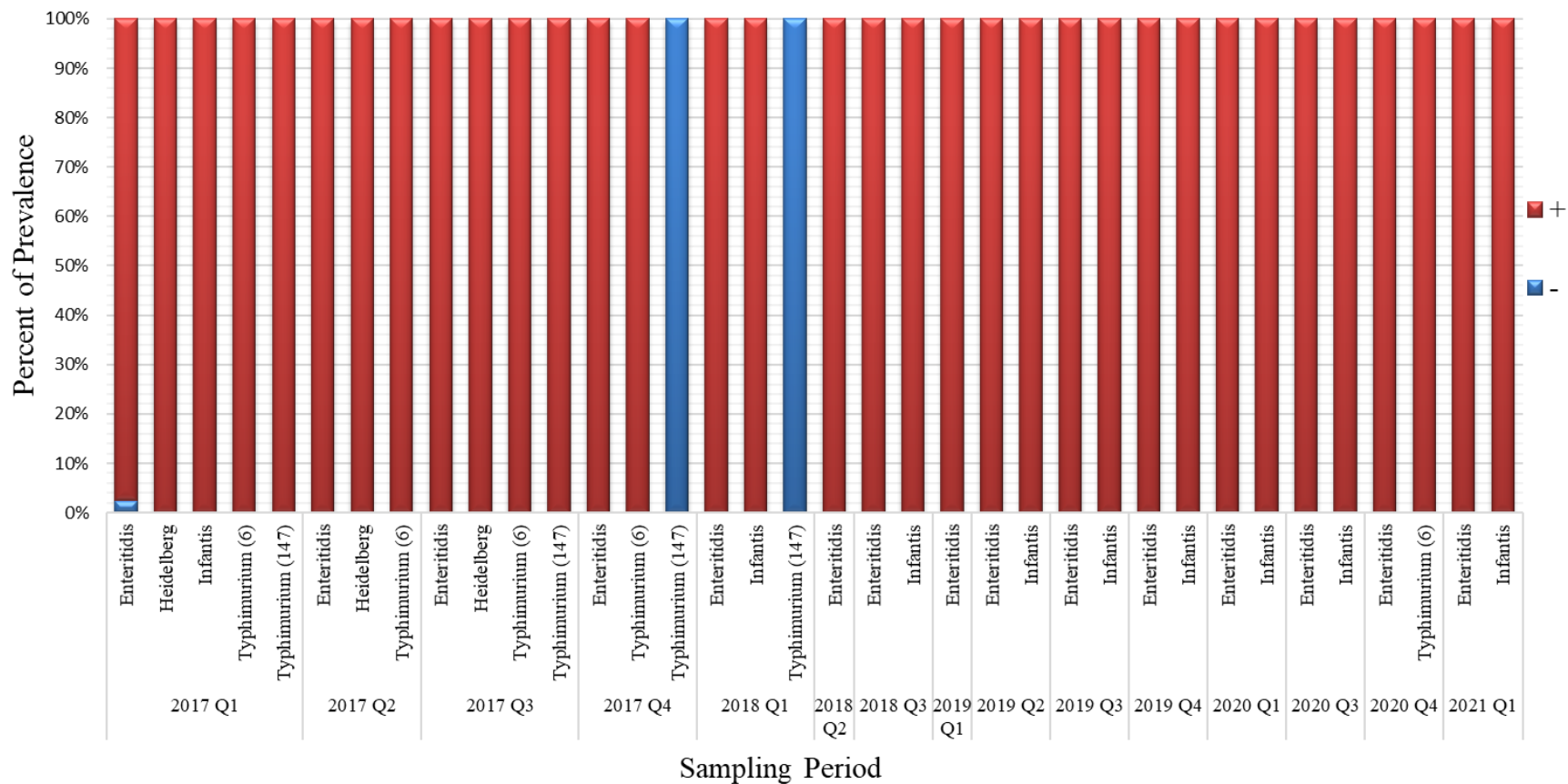


Figure 3.31 Prevalence of Arginine Dihydrolase in Isolates throughout All Study Sites 2017-2020

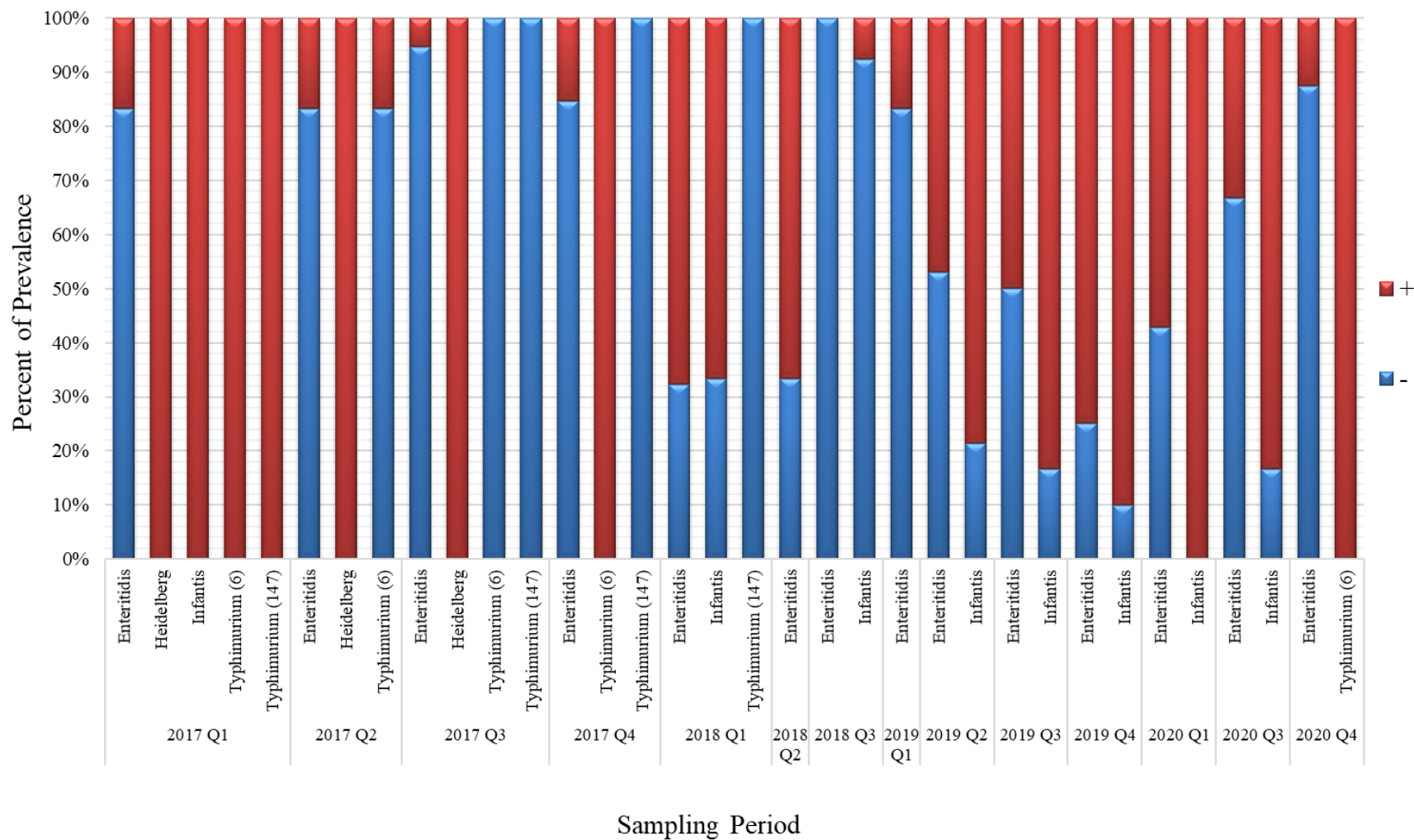


Figure 3.32 Prevalence of Citrate Utilization in Isolates throughout All Study Sites 2017-2020

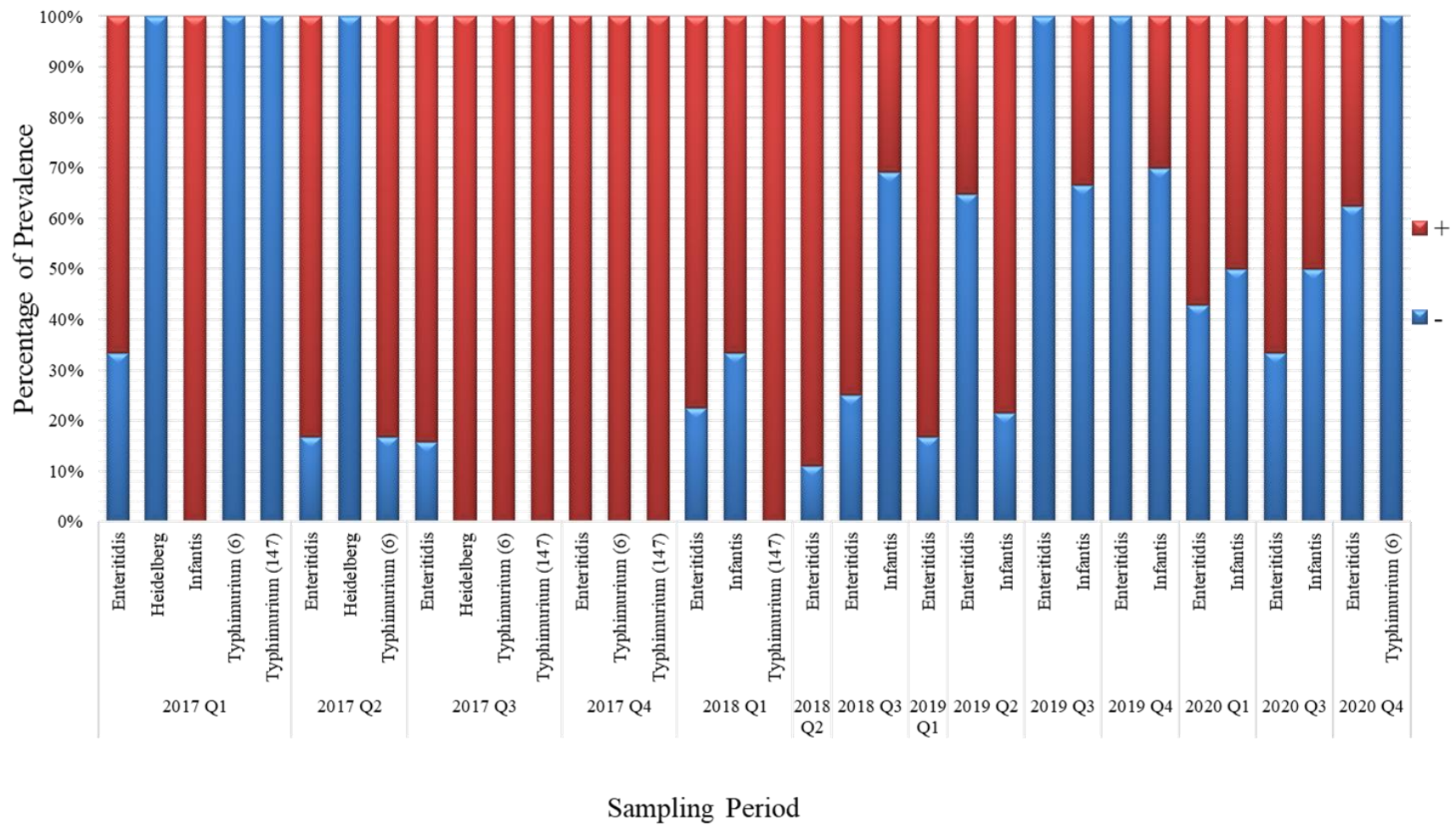


Figure 3.33 Prevalence of Acetoin Production in Isolates throughout All Study Sites 2017-2020

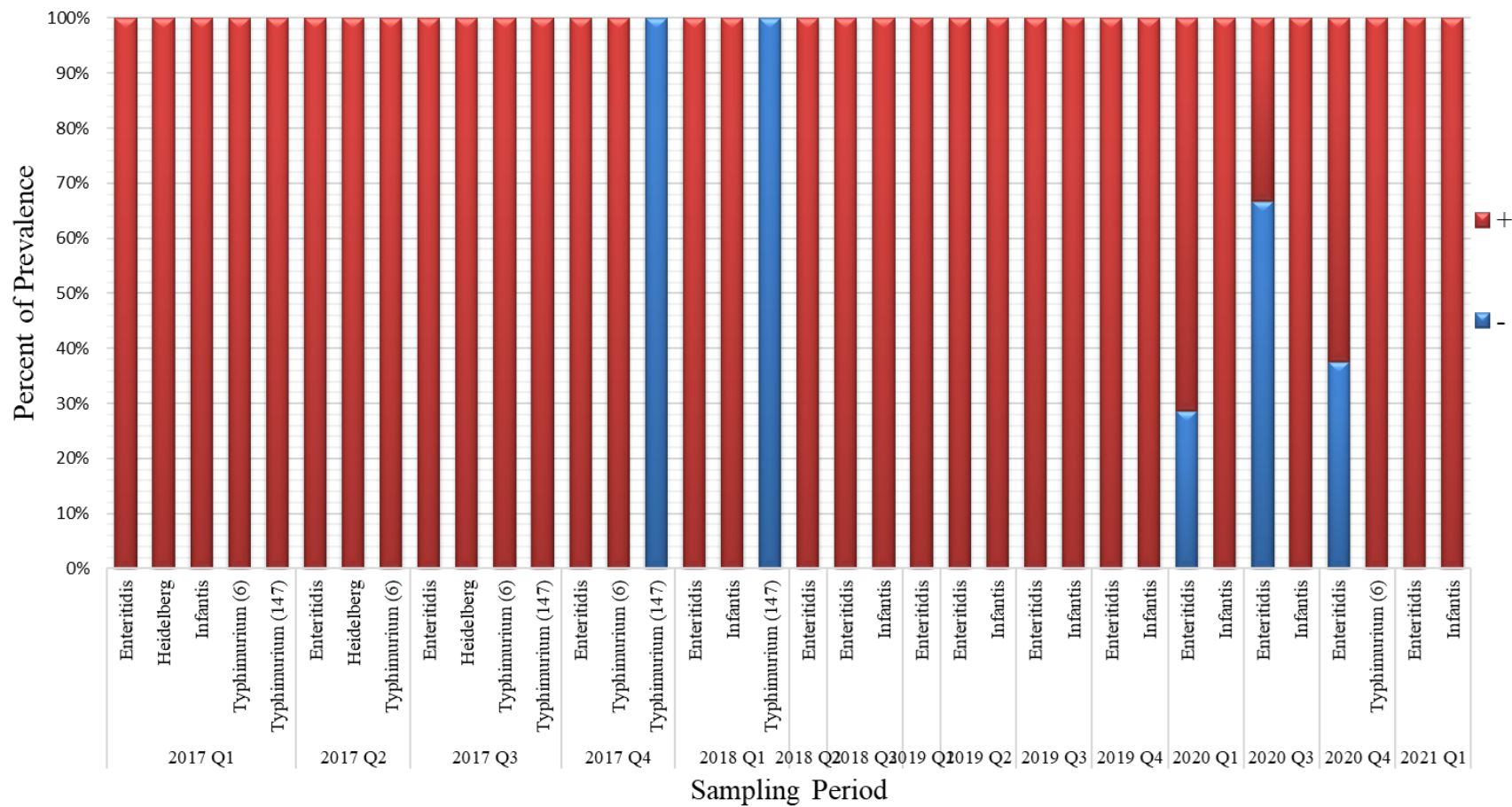


Figure 3.34 Prevalence of d-Sorbitol Fermentation in Isolates throughout All Study Sites 2017-2020

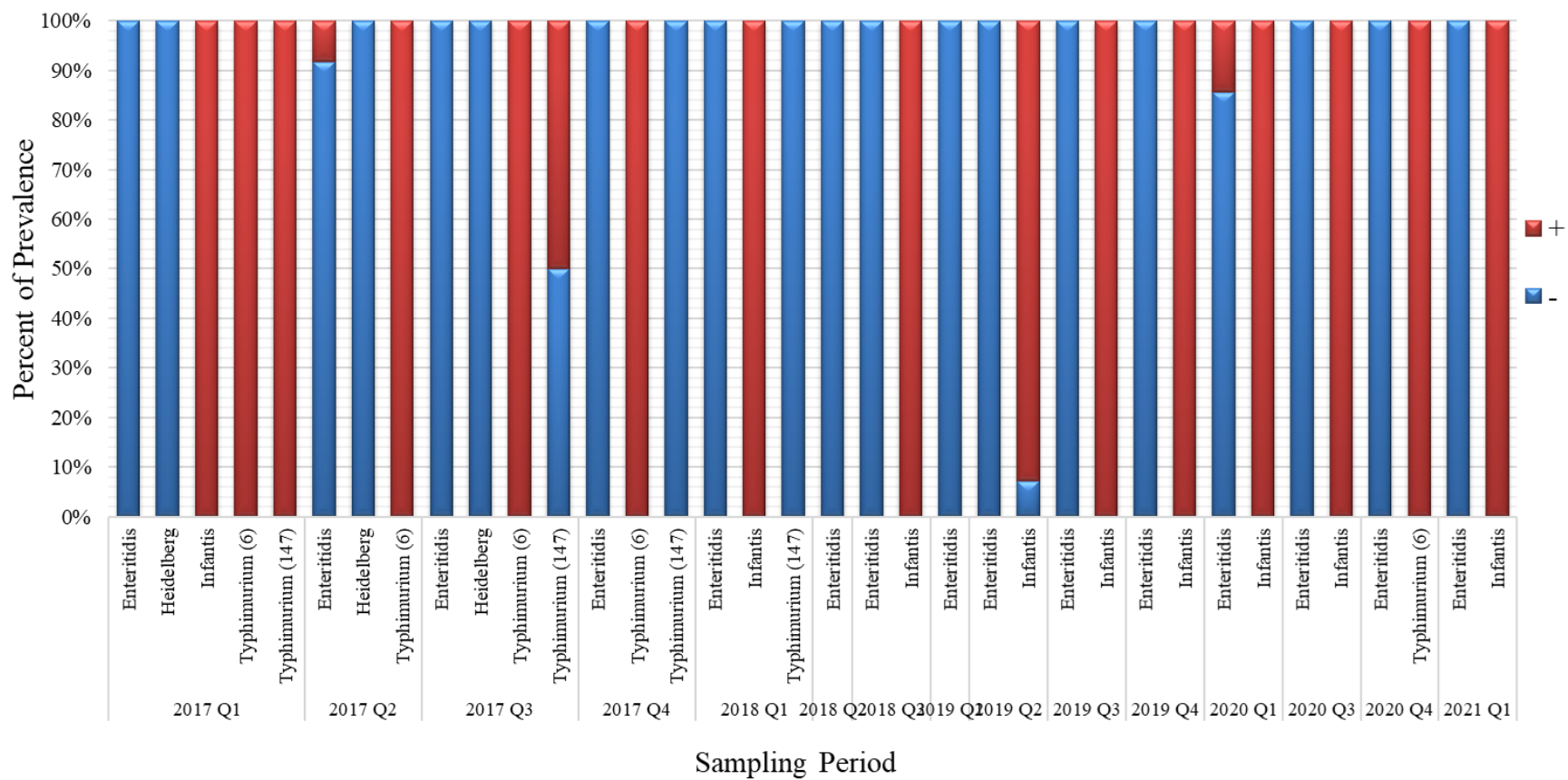


Figure 3.35 Prevalence of Inositol Utilization in Isolates throughout All Study Sites 2017-2020

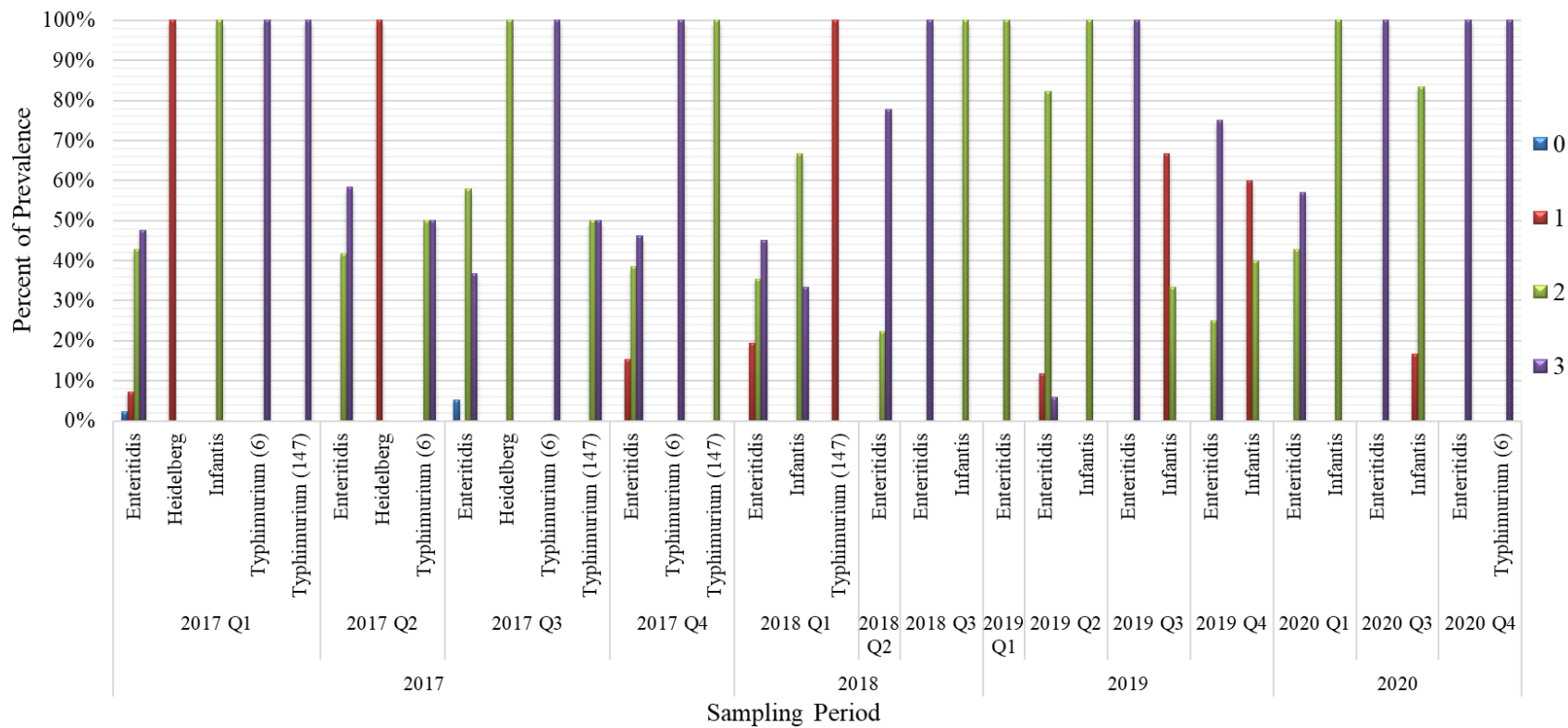


Figure 3.36 Biofilm Score Prevalence in Isolates throughout All Study Sites 2017-2020

Discussion

The hypothesis that the use of an autogenous vaccine protocol would result in a decreased *Salmonella* prevalence and recovery as well as demonstrate alterations in their phenotypic characteristics was supported. Overall, the prevalence of the priority serotypes *Salmonella* Enteritidis, Typhimurium, and Heidelberg decreased in both the hatcheries and the processing plants between 2017 and 2020 following the implementation of autogenous vaccines in 2017. *Salmonella* Kentucky had not been included in the autogenous vaccine formulation since prior to 2017, and so, it was not surprising that its prevalence increased by 15.4% in the hatcheries and 36.5% in the processing plants between 2017 and 2020. *Salmonella* Infantis increased by 14.9% in the processing plant from 2017 to 2020; however, it not been isolated in the hatcheries in these sites, indicating that the source of infection is located elsewhere in the production process. In study site #2, it was added to the autogenous vaccine formulation in Quarter 2 of 2018, which resulted in the overall prevalence decreasing from 17.4% to 12.4% in 2020. In 2020, a second *S. Infantis* isolate was added to the vaccine formulation, and the prevalence dropped to 6.1% in the first quarter of 2021 for the same site. *Salmonella* Heidelberg has not been isolated in either the hatchery or the processing plant since Quarter 3 of 2017 in any site. It was removed from the autogenous vaccine formulation in 2020 and has yet to be isolated. *Salmonella* Typhimurium has been included in the autogenous vaccine formulation since 2017 and has had a decrease of 7.1% overall from 2017 to 2020. *Salmonella* Enteritidis has decreased by 27.5% between 2017 and 2020.

The biochemical test reactions varied throughout the years in response to the vaccine formulation for citrate utilization, d-sorbitol fermentation, inositol utilization and biofilm formation. No differences were seen in arginine dihydrolase after the implementation of a vaccine; therefore, it is not a phenotypic characteristic that should be highly valued when selecting an autogenous vaccine. While differences were noted after the use of the vaccines, it was unclear and beyond the scope of this study to determine whether each individual characteristic directly affected the outcome of vaccine implementation. Additional studies should be performed to determine whether each phenotypic characteristic is directly linked to the impact of the vaccine. Overall, the prevalence of weak and slow biofilm producers decreased and most of the *S. Enteritidis* and *S. Typhimurium* isolates recovered throughout 2019 and 2020 were

moderate to strong biofilm producers. The biofilm formations help the bacteria survive their respective environments, so the hardest to kill bacteria are more likely to form strong biofilms in a quick manner. Therefore, the use of the vaccines resulted in the survival of strong biofilm producers and the reduction of weak biofilm producers.

Based on the findings of this study, the implementation of an autogenous *Salmonella* vaccine directly affected the overall prevalence of *Salmonella* within a poultry company and altered the phenotypic characteristics seen within the priority serotypes. Individual phenotypic characteristics should be studied further in the future to determine whether there is a direct impact relationship to the effectiveness of the vaccine. There may be other bacterial characteristics that could impact the overall effectiveness of autogenous vaccination, such as serotype cross-protection, cellular signaling proteins, and external changes in the environment, that could also be explored in the future.

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Chapter 4 - Conclusion and Discussion

The bacteria *Salmonella* is found throughout the world in a variety of different hosts and environments. With over 2500 serotypes, it is very difficult to maintain control over these hardy bacteria. Throughout the ages, this bacterium has proven to cause trouble for humans and animals alike. From its role in the siege of Athens during the Peloponnesian War (430-426 BC) (Papagrigrakis, et al., 2013), its decimation of the assembly camps during the Spanish-American War in 1898 (Cirillo, 2000), to its rampant spread throughout New York City in the early 1900s, *Salmonella* has impacted the outcomes of wars and civilizations, created opportunities for the advancement of science, changes in military regulations, and the understanding of how epidemics spread throughout a population. After the introduction of the typhoid vaccine in 1911 (Fanning, 1997), current *Salmonella* outbreaks typically involve the non-typhoidal *Salmonella* serotypes that manifest as food-borne illnesses.

The second most consumed meat in the world is poultry. With a global market worth over \$231 billion (Poultry World, 2020), its impact on the global economy and the health of the global population is vast. The United States alone produced 20,296 metric tons of chicken meat in July 2021. With a poultry industry containing over 9.22 billion chickens, 224 million turkeys and 112 billion eggs, (United States Department of Agriculture. National Agricultural Statistics Service, 2021) the United States poultry industry is constantly at risk for the spread various infections. Diseases such as the *Salmonella*-induced pullorum disease and fowl typhoid have been nearly eradicated in the United States, (Shivaprasad, 2000) but the presence of the non-typhoidal *Salmonella* serotypes still present an issue when it comes to the overall health of the birds and the end consumers of the poultry products.

The poultry industry utilizes many different tools to monitor and control the prevalence of *Salmonella* on the individual farms. The use of commercial vaccines to mitigate the presence of *Salmonella* has been an important part of controlling the bacteria for many years. With so many different serotypes, it is impossible to cover all the serotypes that may be found on an individual farm with just commercial vaccines. Autogenous veterinary killed bacterin vaccines are used to fill the gaps when different serotypes are isolated within the companies. The selection of an autogenous vaccine formulation for a company can be difficult since there are many different characteristics that the formulation could be based on. In addition to the serotype,

characterizing the bacteria by its phenotypic characteristics may aid in the selection of an appropriate vaccine. After the vaccine is implemented, continued surveillance of the farm will indicate whether the vaccine had a direct result on the *Salmonella* prevalence and recovery. A study was conducted over a four-year timeframe, in which all samples submitted for a *Salmonella* surveillance study from four locations of a large United States broiler chicken producer were documented. Isolates that were serotyped as *S. Enteritidis*, *S. Typhimurium*, *S. Heidelberg*, and *S. Infantis*, were further characterized for the study. The objective of the study was to determine whether the implementation of an autogenous vaccine protocol, formulated based on phenotypic characteristics, would result in a decreased *Salmonella* prevalence, and changes to their respective phenotypic characteristics.

The implementation of the autogenous vaccine protocol resulted in a decrease in the overall *Salmonella* prevalence of all serotypes included in the vaccine formulation. *Salmonella* Heidelberg has not been detected in any study site since the implementation of formulation #1 in 2017. *Salmonella* Typhimurium decreased to the point in which it was not detected in three of the four sites after 2017. In study site #3, *S. Typhimurium* was recovered from the hatchery in 2020, but the isolate showed different phenotypic characteristics when compared to the original vaccine formulation. As the prevalence of the serotypes included in the vaccine formulations decreased, the prevalence of other serotypes such as *S. Kentucky* increased. This is because as the prevalence change, there are fewer competitors for these other serotypes. *Salmonella* Kentucky was not included in the vaccine formulation, even after it became the most prevalent serotype, because it does not pose a strong risk to the chickens or to the end consumers (Centers for Disease Control, 2016). If the prevalence of *S. Kentucky* were to decrease, it can create an opening that allows another, possibly more dangerous, serotype to fill that niche.

The use of the autogenous vaccine formulations also affected the phenotypic characteristics of the isolates. Characteristics such as citrate utilization, acetoin production, inositol fermentation, d-sorbitol utilization and biofilm formation appeared to be altered in some capacity due to the vaccine. For citrate utilization and acetoin production, it was not uncommon for one vaccine formulation to include both positive and negative isolates. Therefore, additional studies should be performed in which only positive and only negative isolates are included in a specific formulation, to determine the direct effect these characteristics may have on the outcome of the vaccine. It was not within the scope of this study to determine the direct link between the

vaccine and the isolates. The phenotypic characteristic arginine dihydrolase was not influenced by the vaccine formulation. Over the course of the study, the biofilm formations tended to become stronger in the *S. Enteritidis*, *S. Infantis* and *S. Typhimurium* isolates. This is most likely because as the overall prevalence of these serotypes decrease, the remaining bacteria are hardier and most likely to continue surviving their environments. The formation of biofilms allows these bacteria to thrive as their environments, internal organs, and processing plants, become harsher. Therefore, the bacteria capable of forming strong biofilms quickly, are most likely to continue to thrive. Additional studies should be conducted to look at each phenotypic characteristic individually to identify whether it directly impacts the effects of the autogenous vaccine.

The use of phenotypic characteristics to create an autogenous vaccine is a proven method to reducing the prevalence of *Salmonella* on poultry farms. As technology continues to advance, other methods, such as Whole Genome Sequencing, can further classify the isolates and provide an additional approach to formulating a vaccine. Additional studies should be conducted to look at a genotypic approach, compare it to the phenotypic approach used in this study, and determine whether a formulation based on both approaches results in the further reduction of *Salmonella* on poultry farms.

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