# Biofilm Development and Comparative Genomic Analysis on Biofilm Formation in *Salmonella* Enteritidis and *Salmonella* Kentucky strains isolated from poultry

By

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## Abstract

*Salmonella* Enteritidis has been a leading cause of foodborne illnesses worldwide, and food contamination continue to occur despite implemented foodborne pathogen reduction interventions. A key factor that might contribute to *Salmonella* persistence is the formation of biofilms, which enhance *Salmonella's* resilience. This study aims to evaluate the biofilm formation abilities of *Salmonella* Enteritidis (SE) and *Salmonella* Kentucky (SK) at 20-22°C, linking phenotypic date with genomic insights through comparative analysis of marker genes associated with biofilm formation and strain resilience. Fifteen SE and 24 SK strains isolated from poultry processing facilities were compared to assess their biofilm-forming abilities. Biofilm were formed for 5 d at 20-22°C and assessed using the crystal violet method (CV). Genomes were sequenced using Illumina, assembled and annotated using the BV-BRC pipeline. A comparative systems analysis was conducted to screen for genes associated with biofilm formation. A protein-coded gene comparison was conducted to compare the only SE intermediate bovine strain to a strong SE poultry strain. Single Nucleotide Polymorphism (SNP) variation analysis was used to screen for genetic mutations. There were 11 strong and 4 intermediate SE strains, and 4 strong, 5 weak, and 15 non-biofilm SK strains. For curli and cellulose production, 11/15 SE and 4/24 SK strains were positive for both. A pan-genome of 39 *Salmonella* strains generated 5,396 genes and 122 of them were associated with biofilm formation, curli, cellulose, fimbria, flagellum, pilus, and type IV secretion system (T4SS) that might impact biofilm formation, and 107 of them were common to all strains. Strains resulted in almost the same genomic profiles except for curli genes *csgDEF* and T4SS genes *virB1*, *virB3*, *virB4*, *virB6*, *virB10*, *virB11,* and *virD4*. Twenty-four genes with mutations could possibly influence the biofilm formation, including *bcsFGQ*, *flgAB*, *motAB*, and *tolABR*. The nonsynonymous mutations of the *bcs* and *tol* genes might play important roles in biofilm forming capacity, explaining the weaker biofilm within some *Salmonella* strains. Protein-coded gene comparison revealed the absence of some T4SS proteins, including VirB1, VirB3, VirB4, VirB5, VirB6, VirB9, VirB10, VirB11, TraR (VirD2 homolog), and VirD4 in the intermediate strain while present in the strong biofilm strain.

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## Thesis Organization

The following thesis is divided into four chapters.

The first chapter introduces *Salmonella* Enteritidis and *Salmonella* Kentucky, causing biofilm formation through poultry contamination, and genomic information associated with biofilm formations found in those *Salmonella* species. The research hypotheses and objectives are described at the end of the chapter.

The second chapter is the literature review that includes the background information about *Salmonella*, *Salmonella* infection, biofilm formation, and genes that have been researched regarding biofilm formation.

Chapter three is the manuscript that includes the wet lab experiments on the biofilm formation and the production of curli and cellulose of *Salmonella* Kentucky strains, and the dry lab comparative genomic analysis on the gene information acquired from *Salmonella* Enteritidis and *Salmonella* Kentucky strains.

Chapter four is the overall conclusion and prospect for future work.

Table of Contents

## List of Tables

## List of Figures

# 1. Chapter 1: Introduction

## 1.1. Introduction

*Salmonella* is a genus of Gram-negative bacteria belonging to the *Enterobacteriaceae family*. Within the *Salmonella* genus, *Salmonella enterica* subsp. *enterica* is the major cause of the common foodborne disease, salmonellosis, worldwide. It causes approximately 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States annually (CDC, 2023a), and contributes to an estimated 87,500 cases of salmonellosis and 17 deaths in humans every year in Canada (Fonseca et al., 2024). S. *enterica* comprises 60% of total serotypes and accounts for 99% of *Salmonella* infections in humans and warm-blooded animals (Chan et al., 2003). Within S. *enterica*, *Salmonella enterica* subsp. *enterica* Enteritidis (for simplicity, the term *Salmonella* in this thesis will be used as a short-hand for the members of the *enterica* subspecies of *Salmonella enterica*) is the second most common serovar or serotype in *Salmonella enterica* subsp. *enterica* causing illness in humans in both North America and Oceania, followed by *Salmonella* Typhimurium, the most common serotype in Europe (Ferrari et al., 2019). Even though salmonellosis can be self-limiting, it can be severe in vulnerable populations such as children, the elderly, and immune-compromised people (Stanaway et al., 2019). Poultry, eggs, and egg-associated products are major sources of protein, but they are often associated with *Salmonella* outbreaks, especially the serovar *Salmonella* Enteritidis. Despite all the measures taken by the poultry industry to reduce salmonellosis incidences, outbreaks caused by the consumption of contaminated poultry and chicken products still need more investigation (CDC, 2012, 2018a, 2018b, 2019, 2024b). *Salmonella*-related food contamination and outbreaks have led to considerable economic losses and a heavy societal burden. The USDA estimated that the annual economic loss of *Salmonella* infections from all sources is $2.7 billion, with an average cost of $1,896 per case (Ricke & Gast, 2014). In Canada, the financial impact of nontyphoidal salmonellosis linked to fresh produce, eggs, and poultry totals $287.78 million for all cases and $166.28 million for reported cases (Jain et al., 2019). *Salmonella* Enteritidis is the major cause (65%) of non-typhoidal salmonellosis in North America (Galanis et al., 2006). Not only Enteritidis, but *Salmonella* Kentucky serovar was also commonly associated with poultry outbreaks. In Canada 2019, *Salmonella* Kentucky was the top serovar identified among broiler chicken farms, whereas, at least in Canada, it is not a pathogen of concern since it does not appear to cause a high burden of human disease, unlike Enteritidis (Canada, 2024). *Salmonella* Kentucky does not cause as many human illnesses as *Salmonella* Enteritidis, the reason could be due to the lack of some virulence genes (Cheng et al., 2015; Dhanani et al., 2015; Tasmin et al., 2017), or different transmission pathways since Kentucky is more associated with poultry but not eggs. Animals are the primary reservoir of non-typhoidal *Salmonella* and people can be infected by consumption of contaminated food or by direct contact with infected animals (WHO, 2018). Multiple factors have influenced the ability of *Salmonella* serovars to cause infections and illnesses, including biofilm formation (Römling et al., 2003), virulence factors (Dhanani et al., 2015), antibiotic resistance (Obe et al., 2021), and environmental factors (Roy et al., 2021).

Many studies have made connections between *Salmonella* Enteritis and the formation of biofilms (Latasa et al., 2005; Shivaning Karabasanavar et al., 2020; Solano et al., 2002; Yang et al., 2016). Bacteria are found in two forms in nature: a planktonic form (free-floating bacteria in water) and a sessile form (attached to a surface). A biofilm is a group of microbes in a sessile form when the bacteria cells congregate in large numbers and are embedded in a matrix of extracellular polymeric substances (EPS) (Fratamico et al., 2009). EPS serves an important role in biofilm formation (Kumar et al., 2011), cell aggregation (Burdman et al., 2000), and cell adhesion (Frank & Belfort, 2003). Due to the lack of sufficient sanitation and disinfection procedures in food processing facilities, food-contacting surfaces with bacteria and organic matter present are more susceptible to becoming the ideal reservoir for biofilms. After the maturation stage of the biofilm formation, the biofilm can act as a shield and protect the bacteria inside, thus, making the bacteria more resistant to antimicrobials (Singh et al., 2021) and sanitations (Obe et al., 2021). When the biofilms are developed, it is harder for the bacteria underneath to be removed by conventional practices and more likely for survival, therefore, posing a threat to a higher possibility of contamination on equipment surfaces in the future.

Genetic factors play an important role in biofilm formation (Donlan, 2002; Jenal & Malone, 2006; Römling & Galperin, 2015; Steenackers et al., 2012). In order to figure out the relationship between genetic factors and biofilm formation, methods such as whole-genome sequencing followed by genome assembly (Ryan et al., 2017), genome annotation (Brettin et al., 2015), comprehensive genome analysis (Davis et al., 2020), bacterial genome phylogenetic tree (Stamatakis, 2014), heatmap (Dhanani et al., 2015) and variation analysis (H Li, 2013) have been used in the past for biofilm formation analysis. Numerous studies have revealed the functions of various genes in controlling the development of biofilm through biofilm regulation, the synthesis of curli and cellulose (two major components found in the biofilms), and the movement of fimbriae, pilus, and flagella on the bacterial cells (Barnhart & Chapman, 2006; Domka et al., 2006; U. Römling, Z. Bian, et al., 1998; Solano et al., 2002; Steenackers et al., 2012; Wang et al., 2020). Such research papers studied the role of individual genes; however, few have integrated the mechanisms altogether in review. Additionally, connections between the phenotypic and the genomic results of *Salmonella* Enteritidis and Kentucky strains with different biofilm-forming abilities are still opaque.

### 1.2. Research Hypothesis

Hypothesis 1: *Salmonella* Enteritidis and *Salmonella* Kentucky strains with different biofilm formation abilities would show different genomic results accordingly such as the presence or absence of certain genes that are related to biofilm formation.

Hypothesis 2: Genetic mutations found in those *Salmonella* strains may play a role in explaining the contradictory phenotypic and genomic results of Hypothesis 1.

### 1.3. Research Objectives

The main objectives of this study are to 1) evaluate the biofilm formation of *Salmonella* Enteritidis and *Salmonella* Kentucky strains isolated from poultry facilities; 2) to conduct comparative genomic analysis on *Salmonella* Enteritidis and *Salmonella* Kentucky strains on genes associated with biofilm formation as well as to determine potential mutations found in those *Salmonella* strains using SNP variation analysis. 3). to perform a BLASTp comparison between the SE bovine strain 107, which has intermediate biofilm-forming ability, and the SE poultry strain 53932, a strong biofilm former (as reference), in order to identify which essential genes are required for the production of a strong biofilm that are absent in a strain which only produced an intermediate/weak/non biofilm.

# 2. Chapter 2: Literature Review

## 2.1. *Salmonella* Overview

To date, *Salmonella* genus has two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* can then be further divided into six different subspecies: *Salmonella enterica* subsp. *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI) (Lamas et al., 2018), while *Salmonella bongori* was formerly known as subspecies V (Crosa et al., 1973) (Figure 2-1). More than 2600 serovars in *Salmonella enterica* have been discovered and almost all are able to cause illness in humans and animals (Banerji et al., 2020; Guibourdenche et al., 2010; Mezal et al., 2014).

Different *Salmonella enterica* subspecies have different biochemical characteristics resulting in different outcomes in dulcitol, gelatinase, sorbital, lactose, growth with KCN, L(+)-tartato, malonate, galacturonate, and lysed by phage O1 (Lamas et al., 2018). Distinguishing serovars or serotypes within *Salmonella* genus is by serotyping, which is based on the detect of lipopolysaccharide O antigen and flagellar H antigen by reaction with specific antisera (Samuel & Reeves, 2003).

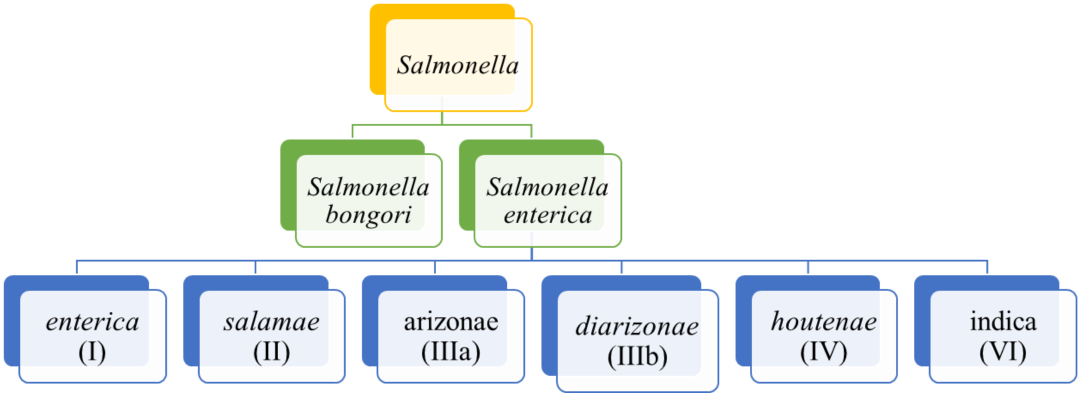

Figure 2-1: *Salmonella* nomenclature

### 2.1.1. *Salmonella* epidemiology

*Salmonella enterica* subsp. *enterica* is one group of the leading food-borne pathogens associated with human diseases. It contributes to an estimated 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year (CDC, 2023a). In Canada, an estimated 87,500 cases of salmonellosis and 17 deaths in humans take place every year (Fonseca et al., 2024). According to a CDC report in 2011 (Scallan et al., 2011), in the United States *Salmonella* (non-typhoidal) was the primary cause of deaths and hospitalizations and second cause of the number of illnesses when compared to other foodborne pathogens, accounting for 378 counts, 28% deaths; 19,336 counts, 35% hospitalizations; and 1,027,561 counts, 11%

illnesses. In the U.S. in 2022, *Salmonella* contamination caused 28 recalls, which is 21% of all the recalls of all food products (FDA, 2022). USDA estimated that the economic cost associated with *Salmonella* infections for all sources was $2.7 billion annually, with an approximate cost of $1,896 per case (Ricke & Gast, 2014). In Canada, the monetary cost associated with nontyphoidal Salmonellosis from fresh produce, eggs, and poultry, accounts for $287.78 million for total cases and $166.28 million for reported cases (Jain et al., 2019).

*Salmonella* has long been a significant public health concern. Although gastrointestinal (non-invasive) salmonellosis typically has a low fatality rate due to its self-limiting nature, invasive *Salmonella* infections can be severe, especially in vulnerable populations. In complicated cases, antibiotic treatment may be necessary. High fatality rates are observed in children under five (13.5%), elderly individuals over seventy years old (51.2%), people with HIV infection (41.8%), and in low-development countries (15.8%) (Stanaway et al., 2019). These fatality rates highlight the ongoing need for effective public health strategies to address *Salmonella* infections and protect public health.

### 2.1.2. Reservoirs and transmission pathways of *Salmonella*

Salmonellosis affects the gastrointestinal tract of humans and animals such as chickens, turkeys, pigs, and cows (Giannella, 1996; Popa & Papa, 2021) primarily, and it is ranked as the third most common cause of death among illnesses that are transmitted through food (Ferrari et al., 2019). Animals are the primary reservoir of non-typhoidal *Salmonella*, and transmission can be directly from contact with infected animals or through consumption of contaminated food or water (WHO, 2018). There are a variety of food sources that can potentially expose humans to *Salmonella*, including contaminated meat and animal products (chicken, turkey, beef, pork, eggs, chicken nuggets, stuffed chicken entrees, raw tuna), fruits and vegetables (sprouts, mushrooms, onions, peaches, papayas), and frozen products (CDC, 2023b; Ian Plumb, 2023). The majority of human salmonellosis is caused by *Salmonella enterica* subsp. *enterica*. Within the *Salmonella* enterica subspecies *enterica*, *Salmonella* Gallinarum, is more restricted in poultry, with very few human infection cases (Zhou et al., 2022). However, there are several zoonotic serotypes can infect not only animals but also transmit to humans including *Salmonella* Typhimurium, Enteritidis, Heidelberg, and Minnesota. (Kipper et al., 2022). All of them have the ability to infect poultry, cattle, and humans, while only the first three serotypes are associated with swine (Kipper et al., 2022). In poultry facilities in Brazil, several other serotypes (Infantis, Newport, Hadar, Senftenberg, Schawarzengrund, and Mbandaka) are also detected in poultry production but the cases are relatively less frequent (Kipper et al., 2022). In the U.S., *Salmonella* Kentucky is considered the most prevalent serovar isolated during poultry production, especially found in young chicken carcasses, even though it is not tightly associated with human illness

(Siceloff et al., 2022; USDA, 2015).

The majority of non-typhoidal salmonellosis cases are caused by *Salmonella* Enteritidis (65%), Typhimurium (12%), and Newport (4%) worldwide, however, *Salmonella* Typhimurium (29%) is more common than Enteritidis (21%) in North America (Galanis et al., 2006). According to a meta-analysis report, *Salmonella* Enteritidis is considered the most frequently reported case of human salmonellosis in Europe, followed by the U.S. (Ferrari et al., 2019). Even though non-typhoidal *Salmonella* infection is the leading cause of food poisoning worldwide, the actual numbers are likely much higher since people with milder symptoms often underreport or fail to self-diagnose the illness (Health, 2023).

### 2.1.3. *Salmonella* associated foodborne outbreaks

The rates and morbidity of *Salmonella* infection differ according to the *Salmonella* serotypes involved. According to the Centers for Disease Control and Prevention (CDC), since 2007, food contamination sources that resulted in more than 500 people include contaminated raw produce with *Salmonella* Saintpaul (1,442 cases); peanut butter with *Salmonella* Typhimurium (714 cases); shell eggs with *Salmonella* Enteritidis (1,939 cases); chicken meat with *Salmonella* Heidelberg (634 cases); cucumbers with *Salmonella* Poona (907 cases); onions with *Salmonella* Newport (1,127 cases); onions with *Salmonella* Oranienburg (1,040 cases) (CDC, 2024a). Live poultry and backyard poultry are another important source of *Salmonella* infection. From 2012 to 2023, there were an estimated 10,105 illnesses, 2,153 hospitalizations, and 14 deaths (CDC, 2024a).

## 2.2. *Salmonella* Enteritidis overview

*Salmonella* species *enterica* have more than 2,600 serotypes. One of the most found serovars to form biofilms in *Salmonella enterica* subsp. *enterica* is *Salmonella* Enteritidis. *Salmonella* Enteritidis is a rod-shaped, gram-negative, non-spore-forming, facultatively anaerobic with a cell size of approximately 0.7–1.5 x 2.0–5.0 µm and the presence of peritrichous flagella (Ricke & Gast, 2014). *Salmonella* Enteritidis is the primary cause of foodborne salmonellosis in humans (Shah, Elder, et al., 2017). It is a zoonotic pathogen that can be transmitted between animals and humans. Human infection typically occurs through the consumption of raw or undercooked contaminated animal products (chicken, beef, pork, and eggs) or through the ingestion of fresh produce contaminated by the feces of infected animals.

Many environmental factors such as temperatures, pH, and water activity can influence the growth of *Salmonella*, however, it can easily adapt to environmental conditions that are not ideal for its growth (Podolak et al., 2010). *Salmonella* Enteritidis is a mesophilic and neutrophile bacteria, but it can also grow in a wide range of temperatures (5°C-45°C, typically 37°C), pH (4.0-9.0, typically at 7.0), and water activity

(>0.93) (Ricke & Gast, 2014).

### 2.2.1. *Salmonella* Enteritidis prevalence

In 2009, the FDA issued a final regulation to egg producers to prevent egg contamination with *Salmonella* Enteritidis on the farm, during storage, and transportation. This regulation is expected to prevent approximately 79,000 cases of foodborne illnesses and 30 deaths due to *Salmonella* Enteritidis egg contamination and to reduce the infection rate from eggs by nearly 60% (FDA, 2009). Illnesses and outbreaks with confirmed cases of human *Salmonella* infection by *Salmonella* Enteritidis still have been linked to the consumption of contaminated eggs, egg products, chicken meat, and chicken products (CDC, 2012, 2018a, 2018b, 2019, 2023a, 2023b, 2024b). According to a United States Department of Agriculture report in 2014, *Salmonella* Enteritidis was the second most isolated reservoir associated with young chicken carcasses in the U.S., with *Salmonella* Kentucky being the most common serotype (USDA, 2015).

Overall, the occurrence of salmonellosis varies from serotypes and locations. In Taiwan, the prevalence of all *Salmonella* Enteritidis isolates has increased by 19.7% between 2004 to 2022 (Liao et al., 2024). In Japan, the *Salmonella* Enteritidis contamination rate in commercial eggs has decreased by tenfold compared to the mid-1990s as of the year of 2013 (Esaki et al., 2013).

Although the number of *Salmonella* contamination cases has generally been declining worldwide in recent years, *Salmonella* infections continue to pose a significant threat to public health. Despite the efforts to decrease their incidences, the persistence of these infections remains because of various factors such as the emergence of new strains, insufficient food safety procedure supervision, and the widespread nature of *Salmonella* in both humans and animals. As a result, *Salmonella* still remains as major concern, thus, leading to substantial illnesses, economic costs, and public health burdens across different regions.

### 2.2.2. *Salmonella* Enteritidis in poultry and eggs

*Salmonella* Enteritidis outbreaks have been a recurring issue within the poultry and egg industries, with illness, hospitalizations, death, and recalls that happens globally in recent years. According to CDC, in 2018, 44 cases of illness and 12 hospitalizations were reported. During the epidemiological investigation, it was found that people had eaten at restaurants serving dishes containing eggs supplied by Gravel Ridge Farm, whose eggs were recalled shortly after being linked to *Salmonella* Enteritidis (CDC, 2018b). In 2018, Harvest Creek Chicken Nuggets were recalled in Canada after being linked to a *Salmonella* Enteritidis outbreak, which resulted in 33 reported cases of illness and 4 hospitalizations across 6 provinces (Canada, 2018). In the year of 2019, a total of 98 cases of illness were reported across10 provinces and 1 territory, linked to 4 different brands of uncooked frozen chicken nuggets which were shortly recalled after contaminated with *Salmonella* Enteritidis (Canada, 2019). In 2021, 36 illnesses and 12 hospitalizations were reported from people consuming frozen chicken products and after linked to *Salmonella* Enteritidis. Upon investigation, five brands of raw frozen breaded stuffed chicken products were recalled (CDC, 2012). In 2023, two sub-clusters of *Salmonella* Enteritidis ST11 has infected at least 134 people, resulting in one death across 11 EU/EEA countries. The outbreak was later linked to the consumption of dishes made with chicken meat including chicken burrito, chicken schnitzel, kebab, and pizza (ECDC, 2023). More recently, on October 17, 2024, Milo's Poultry Farms recalled their eggs after making 93 people ill and 34 hospitalizations due to *Salmonella* Enteritidis infections in the U.S. (CDC, 2024b).

Eggs are known for their valuable nutrients, ie, higher protein digestibility-corrected amino acid score (PDCAAS) in children (Tome, 2012) and low cost compared to other proteins such as beef and fish (Drewnowski, 2010). This nature has made them more accessible for the population of every economic household to purchase. According to the USDA, The production of eggs in the United States decreased from 9.4 billion dozen in 2019 to 9.1 billion dozen in 2022 due to highly pathogenic avian influenza (HPAI), however, the number of egg production would recover in 2024, and reach 10.8 billion dozen by 2033 (Miller. M. et al., 2024). Apart from the unavoidable cause of egg reduction from influenza, eggs and egg products are also commonly associated with *Salmonella* Enteritidis contamination and foodborne outbreaks, which is another leading cause of egg reduction. In the U.S. from 1985 to 2022, 81% of *Salmonella* Enteritidis outbreaks were associated with the consumption of eggs (Ricke & Gast, 2014). Products such as homemade mayonnaise, hollandaise sauce, custards, raw eggs, and poached eggs have been implicated as the sources of the outbreaks due to inadequate cooking (Ricke & Gast, 2014). Consumption of contaminated egg and egg products is especially dangerous and happens mostly in daycare centers, nursing homes, and hospitals where people who are more susceptible to the infection of foodborne decrease live (Ricke & Gast, 2014).

### 2.2.3. Horizontal/Vertical transmission of *Salmonella* from hens to eggs

There are two ways for unhatched eggs to be infected with *Salmonella*, horizontally and vertically. Horizontal transmission involves the penetration of *Salmonella* through the eggshell after fecal contact in a relatively moist cool condition or from the colonized cloaca (Berrang et al., 1999). For vertical transmission, the hen's ovary carries the bacteria that can pass them down and cause transovarian infection before the eggs have formed the shell components (Miyamoto et al., 1998). The bacteria can be deposited in not only the eggshell membranes and the eggshells but also in the yolk and albumen (Gantois et al., 2008). Non-host-specific serovars such as *Salmonella enterica* subsp. *enterica* Enteritidis can be transmitted to the eggs via vertical transmission from infected laying hens. After the infected eggs are hatched, the chicks are able to transmit the bacteria via a horizontal transmission (Wray & Wray, 2000).

Of all *Salmonella enterica* subsp. *enterica* serovars, *Salmonella* Enteritidis is particularly problematic to the egg industry because of vertical transmission i.e., ovo-transmission while birds remain outwardly healthy since the adult hens can adapt *Salmonella* in their gut microflora. One of the routine egg-processing steps involves washing the eggs, however, the conditions need to be controlled critically since the outermost layer of the egg (the cuticle) could be removed upon washing. If the processing environment is unstable, the unequal pressure between the outside and the inner shell creates a pressure gradient, which accelerates the movement of bacteria into the eggshells (Ricke & Gast, 2014). According to Ricke & Gast, only 0.005% of eggs from commercial flocks in the United States experienced an incidence of *Salmonella* Enteritidis contamination (Ricke & Gast, 2014). However, considering the colossal daily consumption of eggs, even a small percentage is still a giant number.

### 2.2.4. Other *Salmonella* serotypes besides Enteritidis in poultry and eggs

*Salmonella* Pullorum and *Salmonella* Gallinarum (two distinct biovars in *Salmonella enterica* subsp. *enterica* serovar Gallinarum) can cause diseases in poultry and aquatic birds called Pollorum disease and fowl typhoid, respectively (Batista et al., 2015). These two diseases have been eliminated in developed countries such as the US, Canada, and Western Europe, but are still present in developing countries (Andino & Hanning, 2015). After the eradication of *Salmonella* Pullorum and *Salmonella* Gallinarum, the rise of *Salmonella* Enteritidis brought more problems to poultry farms. *Salmonella* Enteritidis is not only found in poultry, such as chickens and turkeys but also uses rodents and flies as additional reservoirs. This makes it more challenging to control the spread of the infection in poultry farms (Davies & Wray, 1995; Holt et al., 2007).

Between 2018 and 2019, a total of 129 people were infected with *Salmonella* Infantis in 32 states in the U.S., resulting in 25 hospitalizations and 1 death, and the implicated food was contaminated raw chicken or chicken products from different sources, brands, and locations (CDC, 2019). Between 2017 and 2018, 45 cases of *Salmonella* Braenderup infections, including 11 hospitalizations, were reported following the consumption of restaurant egg dishes made with eggs supplied by Rose Acre Farms (CDC, 2018a). In 2014, an outbreak of *Salmonella* Heidelberg contamination was detected in Tyson-Brand mechanically-separated chicken, resulting in 9 reported illnesses in Tennessee, leading to the recall of 33,840 pounds of affected chicken products (CDC, 2014). In Australia in 2020, an outbreak of 38 *Salmonella* Typhimurium infections was linked to backyard poultry (Laidlow et al., 2022). Interestingly, it is reported that between 2001 and 2011 in Australia, *Salmonella* Typhimurium dominated foodborne *Salmonella* outbreaks that are linked to eggs (90% of all egg outbreaks) (Moffatt et al., 2016) and Pande et al. have suggested that the main route of egg contamination with Typhimurium in laying hens is through horizontal infection (Pande et al., 2016).

### 2.3. *Salmonella* virulence factors

Many *Salmonella* serotypes can cause gastroenteritis (non-typhoidal salmonellosis) such as *Salmonella* Enteritidis, Typhimurium, Heidelberg etc., and typhoid fever such as *Salmonella* Typhi and Paratyphi. Gastroenteritis is usually self-limiting and is caused by mucosal edema and inflammation in the large intestine, resulting in diarrhea, vomiting, nausea, fever, and abdominal pain (Santos et al., 2001). The most common host-to-host transmission of *Salmonella* is via the fecal-oral route (Gopinath et al., 2012). Humans or animals that carry *Salmonella* bacteria would excrete them in their feces and transmit the pathogen to contaminate water, soil, and foods that would later be consumed or contacted by another human or animal through the oral route.

For *Salmonella* to infect host, it uses a large variety of genes encoding a large group of virulence factors (Valdez et al., 2009). Different strains of *Salmonella* vary in their ability to cause infection, with some strains being more virulent and capable of causing severe illnesses or even death. The severity of outbreaks is influenced by various factors, and the virulence factors of *Salmonella* must be considered when assessing the potential impact. *Salmonella enterica* can carry numerous virulence factors such as Type III Secretion System (T3SS), flagella, fimbriae, *Salmonella* enterotoxins, adhesins, invasins etc. (Jajere, 2019).

*Salmonella* pathogenicity islands (SPIs) are located in certain areas of the chromosomes or plasmids in the bacterial cells, clustering genes that encodes the various virulence factors such as adhesion, invasion, toxin genes, etc. (Foley et al., 2008). To date, a total of 5 *Salmonella* pathogenicity islands (SPI-1 to -5) have been reported (Foley et al., 2008) and different pathogenicity islands play different roles in *Salmonella* virulence and pathogenesis. Two most SPIs are SPI-1 and -2. Pathogenesis of *Salmonella* is assisted by Type III Secretion System (T3SS) encoded by genes of *Salmonella* pathogenicity islands 1 & 2 (SPI-1 and SPI-2) (Figueira & Holden, 2012; Waterman & Holden, 2003). SPI-1, found in both species (*S. bongori* and *S. enterica*), is essential for the invasion of host epithelial cells by injecting effector-proteins into the host cells to facilitate bacterial entry (Lou et al., 2019). SPI-2 is exclusive to *Salmonella enterica* subspecies, and it can translocate effectors across *Salmonella*-containing vacuole (SCV) membrane in infected host epithelial cells and macrophages (Figueira & Holden, 2012). T3SS is found in *Salmonella* enterica (Ellermeier & Slauch, 2007) and it is essential for the invasion of the epithelial cells and allows bacteria to survive within the host. It contributes to the development of gastrointestinal disease and it plays a major role in causing both acute and chronic infections by the bacteria. T3SS is embedded in the bacterial cells, both the inner and outer membrane, to inject effector proteins into the host cell membrane via a needle-like structure (Mueller et al., 2008). Before it injects the effector proteins, *Salmonella* cells need to first adhere to the epithelial cells of the gut by using adhesins and fimbriae to facilitate the attachment, and then multiply in the host cells (Wiedemann et al., 2015).

## 2.4. Microbial biofilm

### 2.4.1. Biofilm overview

Biofilms refer to a group of surface-associated microbial communities that are enclosed in an amorphous and hydrated extracellular polymeric substance (EPS) matrix that has the ability to attach to either biotic or abiotic surfaces (Donlan, 2002), showing different forms morphologically and physiologically. EPS is a complex mixture of polysaccharides, proteins, lipids, and nucleic acids that provide structural support and protection to the microbial community (Flemming, 2016).

Planktonic cells and sessile cells refer to two distinct forms of cellular organizations. Planktonic cells are free-floating cells suspended in a fluid medium, where bacteria can move and disperse independently. They can move using flagella or other appendages; thus, they have greater mobility and can colonize new habitats. In contrast, sessile cells are stationary and attached to a substrate or surface. They are unable to move independently once they have attached themselves. Sessile cells can be found in various organisms, such as plants, fungi, and bacteria. They typically exhibit specialized structures or mechanisms for attachment, such as adhesion proteins or biofilm matrix components (Rollet et al., 2009).

The structural components of the extracellular polymeric substances (EPS) vary based on the environmental conditions in which biofilms are formed, but they predominantly consist of polysaccharides, proteins, phospholipids, and other biomolecules (Steenackers et al., 2012). EPS serves as a structure for the biofilm’s three-dimensional layout, allowing the cells to adhere to surfaces and stick together within the biofilm (Flemming & Wingender, 2010), and it serves as an essential adaptation for microorganisms residing within it. Bacteria within the biofilms have strong capabilities to survive in hostile environments, because the EPS not only shows tolerance to antimicrobial compounds and persistence against host defence, but also can adapt and survive under a wide range of temperatures, pH, and moisture levels (Morita et al., 2011; Webber et al., 2019; Xylia et al., 2022). Biofilms can be composed of single species or multispecies, with either single or multi-layers (Schulze et al., 2021). They can be developed on numerous biotic and abiotic surfaces in the environment from biotic surfaces in nature (hot springs, rocks, roots of a plant) and humans and animals (intestinal tract, tooth, lungs, epithelial cells) to abiotic surfaces like man-made materials (stainless steel, plastic, rubber, glass) (Steenackers et al., 2012).

According to methods outlined in several research papers (Adator et al., 2018; Stepanović, Cirković, et al., 2004), biofilm biomass formation can be classified into four categories: strong, intermediate/medium, weak, and non-biofilm formers or producers. Crystal violet will stain the biofilm in the wells and the dye binds to the biomass of the biofilm including the EPS. After adding ethanol to dissolve the dye, the bond between biofilm and the crystal violet is broken, and thus releases the dye into the solution. The amount of dye is proportional to the amount of biofilm formed. The density of the biomass is calculated using an optical density (OD) value at 630mm wavelength, which will absorb the light remaining. The higher OD value is another indicator of a stronger biofilm former.

### 2.4.2. Biofilm formation stages

The formation of biofilm consists of four stages.

The first step is the initial attachment of the bacteria cells in the planktonic stage to a surface, which is a reversible attachment (weak interactions between the bacterial cells and the substratum). Biofilms can grow under the van der Walls attraction forces, electrostatic forces and hydrophobic interactions in any environment on almost any surface (Kumar & Anand, 1998). The nutrient availability is also important in that an increased level of nutrients from the food processing surface can act as a conditional film, which is the buildup of molecules on food-contact surfaces at the interface between solid and liquid, and attract microorganisms to that surface (Hood & Zottola, 1997). Different surfaces exhibit different roughness; for example, nylon and Teflon have smooth surfaces, while stainless steel and aluminum are rougher (Kumar & Anand, 1998). A rough surface can facilitate the trapping of bacteria, making it more difficult for shear forces from the liquid or routine cleaning efforts to dislodge them. These rougher surfaces can hinder cleaning processes, along bacteria to remain embedded (Kumar & Anand, 1998).

The second step is the irreversible attachment. Various short-range forces such as dipole-dipole interactions, hydrogen bonding, ionic and covalent bonding, and hydrophobic interactions occur (Kumar & Anand, 1998). During this step, motile planktonic cells become sessile and once attached, bacteria begin to secrete EPS (Rutherford & Bassler, 2012). Much strong forces such as scrubbing or scrapping are required to remove the bacterial cells compared to the initial attachment step (Marshall et al., 1971).

In the third step is biofilm development and maturation. The biofilm keeps maturing, and nutrients and signalling molecules are passing through in the biofilm structure (Preda & Săndulescu, 2019). The bacterial cells form microcolonies by growing and dividing while using the nutrients available (Kumar & Anand, 1998). Then, the biofilm is formed as a result of the continuous attachment of the cells to the substratum and the production of EPS. The size and the number of layers of biofilms depend on the species of microorganisms within the biofilm (Kumar & Anand, 1998).

Finally, the detachment and dispersion of some of the bacterial cells as planktonic cells into the environment start a new cycle of attachment to a new surface (Aleksandrowicz et al., 2023). This step is detrimental to the food processing environments since the dispersion indicate a new set of bacteria that can act as a source of cross-contamination.

### 2.4.3. *Salmonella* and biofilm formation

*Salmonella* was the first foodborne bacterium ever reported to have the ability to adhere to food surfaces and form biofilm (Duguid et al., 1966). Biofilms developed in food processing areas are especially significant and worth studying because they can serve as a persistent source of microbial contamination, which would result in food spoilage or cross-contamination. The attachment of *Salmonella* biofilms to different surfaces is closely associated with several bacterial cell surface components for example, flagella, fimbriae, and cellulose (components that will be explained in the later sections) (Kroupitski et al., 2009). According to Marin et al., 50% of *Salmonella* isolated from poultry farms, regardless of serotypes, were able to form biofilms (Marin et al., 2009). Another study done by Dantas et al., even suggested that 77.5% of *Salmonella* strains isolated from poultry slaughterhouse mats were able to produce biofilms on polystyrene (Dantas et al., 2020).

Many environmental factors can influence the biofilm formation, such as temperature, pH, and water activity. According to Webber et al., *Salmonella* Enteritidis biofilms can grow in a wide range of temperatures (42±1 °C, 36±1 °C, 25±1 °C, 9±1 °C, and 3±1 °C) on all three surface materials (polyethylene, stainless steel, and polyurethane) (Webber et al., 2019). USDA (USDA, 2017) and Tortora et al. (Tortora, 2012) suggested that the lowest temperature for *Salmonella* to grow is 5°C, however, the study by Morey and Singh (Morey & Singh, 2012) demonstrated that *Salmonella* is not growing between 4°C and 8°C.

Different values of pH also influence *Salmonella* Enteritidis growth. A higher optical density (OD) was observed in lettuce grown in Brain heart infusion (BHI) broth with descending pH values (8, 7, 6, and then 5) and a higher OD value was observed in the same pH level under growth temperature of 37 °C than 21°C (Xylia et al., 2022). *Salmonella* Typhimurium shares many bacterial properties with *Salmonella* Enteritidis. The rate of biofilm formation increased with increasing temperature (28°C, 37°C, and 42°C) and pH (6 and 7) on stainless steel and acrylic material surfaces, however, there was no difference between pH 6 and 7 after 240h (Nguyen et al., 2014). The lowest *Salmonella* growth temperature is believed to be 3.8°C (Jay, 2003).

*Salmonella* can also survive on dry surfaces for a long period of time (Podolak et al., 2010) and the EPS material in the biofilm has the ability to protect *Salmonella* bacterial cells from dryness (Iibuchi et al., 2010). *Salmonella* Enteritidis can be recovered from stainless steel surfaces transferred from kitchen sponges for at least 4 days when the contamination level is high ($10^5$ CFU/$cm^2$). When the contamination level was moderate (10 CFU/$cm^2$), the surviving numbers decreased below the detection limit (4 CFU/100 $cm^2$) within 24h (Kusumaningrum et al., 2003). *Salmonella* Enteritidis strains with high biofilm-forming ability can survive under dry conditions (2-15% humidity) for approximately a year on stainless steel bolt threads, while only 20% of weak biofilm formers survived (Morita et al., 2011).

Cleaning should be the first step for removing the biofilms. Without the cleaning step, disinfectants would only remove the first layer and not be able to penetrate the matrix, therefore, biofilms would still remain on the surface (Simões et al., 2006). Common disinfectants that are used involve chemical compounds such as biocides, chlorine, hydrogen peroxide, peracetic acid, and acidic compounds (Bremer et al., 2006; Simões et al., 2006). Once the biofilm is formed over some residual microflora that has not been completely sanitized, it is significantly difficult and challenging to remove compared to planktonic cells of the same bacteria since with the protection of the EPS structure, they are more resistant to conventional cleaning and sanitation measures (Joseph et al., 2001). Therefore, because of *Salmonella* biofilms' strong resistance to disinfection and sanitizers, another effective way to eradicate the biofilms is to prevent the formation in the first place such as using well-designed equipment (Coughlan et al., 2016).

## 2.5. Genes and biofilm formation

The genetics of *Salmonella* plays a crucial role in biofilm formation. Specific genes involved in biofilm formation enhance the bacterium's resistance to environmental stress, antimicrobial agents, and the host immune system, making infections more difficult to control and treat. In poultry processing facilities, specific genes enable *Salmonella* to produce biofilms that can protect it from hostile environments and sanitation, increasing the risk of persistent contamination and making it even harder to eliminate from these critical food production environments.

The research is focused on finding a relationship and linkage between the phenotypes (different biofilm-forming abilities, curli and cellulose productions) and genomic information (the present or absence of some of the important protein coding genes associated with curli, cellulose, fimbria, flagellum, pilus and others that might impact biofilm formation as well as the SNPs variants that might play a role in mutations leading to misfunctioned genes.

### 2.5.1. Overview of genes associated with bacteria biofilm formation

Approximately 80% of the bacteria and archaea on Earth can form biofilms (Flemming & Wuertz, 2019). Under the large family of Enterobacteriaceae in the gram-negative bacteria, *Escherichia* and *Salmonella* spp. often share a similar pathway to form biofilms, which includes the production of type 1 fimbriae, flagella, curli, cellulose, LPS synthesis, fimbriae regulation, stress response regulation, and transmembrane transport (Aleksandrowicz et al., 2023) (Holden et al., 2022).

During the development of biofilms, different gene expression of various pathways plays an essential and beneficial role in different stages. For *Salmonella* Typhimurium, genes responsible for adhesion, fimbriae expression, cellulose and amino acid biosynthesis are crucial after 12 hours of growth. After 24 hours of growth, genes involved in flagella, cAMP, purine, LPS biosynthesis, respiration, and stress-response transcriptional regulators determine the fitness of biofilm. Finally, after 24 hours of growth, genes responsible for biofilm matrix, fimbriae, flagella, lipopolysaccharide (LPS) biosynthesis and transmembrane transport are important for the last stage of biofilm formation (Aleksandrowicz et al., 2023).

### 2.5.2. Flagella

Flagella are whip-like appendages found on the surface of many bacteria, including most of the *Salmonella* serovars and *Escherichia coli,* enabling them to swim in liquid environments (Neidhardt, 1996). Flagella are the primary organelle for pelagic motility, however they possess other critical functions such as biofilm formation, protein export, and adhesion (Haiko & Westerlund-Wikström, 2013). The synthesis of the flagellum is regulated. It is involved in both motility and chemotaxis. The flagellum itself consists of several part: a basal body with rotary motor, a hook and a flagellar filament extruding in the medium (Haiko & Westerlund-Wikström, 2013). It is important for the attachment to epithelial cells and intestinal colonization (Haiko & Westerlund-Wikström, 2013).

The master regulator FlhDC encodes a transcriptional regulator that controls the expression of flagellar genes and it also plays an important role in the regulation of biofilm formation in *Salmonella* Enteritidis. FlhA and FlhB are flagellar biosynthesis proteins and FlhC and FlhD are flagellar transcriptional activators. FlhE belongs to the flagellar regulon and is responsible for the swarming motility of motile bacteria, thus increasing the lubrication of the movement and affecting the composition of the extracellular matrix (Stafford & Hughes, 2007).

### 2.5.3. Fimbriae

Fimbriae are hair-like appendages found on the surface of many bacteria. Type I fimbriae (T1F) are one of the most common bacterial adhesins found in *Salmonella* spp. and it is an essential virulence factor since adhesion to host tissues plays an essential role in *Salmonella* pathogenesis. Fimbriae are involved in adhesion (Tan et al., 2016), motility, biofilm formation, and surface sensing conjugation. It was shown that the structure of *Salmonella* Typhimurium is similar to that of the *E. coli* fimbriae, which is comprised of a major fimbrial subunit and other minor fimbrial proteins (Clegg & Swenson, 2020). The fimbrial clusters contain seven groups of genes (*fim, bcf, stb, sth, std, saf,* and *sti*) (Yue et al., 2012). Several of those genes were found in our *Salmonella* samples including *stiC, stdB, stbC, sthB, fimI, fimW, fimZ, fimY* etc. FimWYZ are the three major regulatory proteins that control the expression of the *fim* operon (Yue et al., 2012).

### 2.5.4. Curli

Curli are a type of fimbriae with an amyloid structure produced by certain bacteria. Curli are amyloid fibers which are not only responsible for biofilm formation but also for surface adhesion, cell aggregation and environmental persistence (Jonas et al., 2007). Within *Salmonella enterica* and other species from the *Enterobacteriaceae* family, curli production is one of the major extracellular matrix components found. Phenotypically speaking, *Salmonella* biofilms can form four different morphotypes that can be seen on Congo Red agar plates: red, dry, and rough; pink, dry and rough; brown, dry and rough; and smooth and white. The red, dry, and rough (RDAR) morphotype appears when both curli and cellulose are present, while the presence of pink, dry and rough (PDAR), brown, dry, and rough (BDAR), and smooth and white (SAW) represents the absence of curli, cellulose, and both, respectively (Jonas et al., 2007).

Curli is especially important during the first attachment stage of biofilm formation. There are two operons that are directly related to the curli production in *Salmonella* spp.: *csgBAC* and *csgDEFG*. The operon *csgBAC* encodes the proteins CsgB, CsgA, and CsgC. The operon *csgDEFG* consists of a transcriptional activation gene *csgD* and three accessory genes *csgE*, *csgF*, and *csgG*, that encode for putative curli assembly factor (Hammar et al., 1995).

#### 2.5.4.1. Curli subunit genes Csg family: *csgA, csgB, csgC, csgD, csgE, csgF,* and *csgG*

The gene *csg*A from a primary curli structural protein, encodes the major curlin subunit protein and it is transcribed as an operon from *csgB* (Arnqvist et al., 1994). *agfA* gene codes the AgfA fimbrin, which makes up the thin, aggregative fimbriae produced by *Salmonella* Enteritidis, is 74% identical and 86% similar to protein CsgA found in E. coli (Arnqvist et al., 1994). Therefore, the two different nomenclatures lead to a homologous gene (U. Römling, Z. Bian, et al., 1998). Several studies have shown that almost all (over 90%) *Salmonella* Enteritidis and *Salmonella* Typhimurium strains express the red, dry, and rough morphotype at 28°C instead of 37°C. (Römling et al., 2003). Römling et el. also showed that a CsgA signal was detected in *Salmonella* Typhimurium samples at 28°C but not at 37°C, which corresponded with his result of a curli production at 28°C and no curli production at 37°C (U. Römling, Z. Bian, et al., 1998). The deletion of the *csgA* gene resulted in a morphology that is pink, dry, and rough (PDAR), which indicates the absence of curli and the presence of cellulose only (U. Römling, Z. Bian, et al., 1998).

CsgB protein is homologous to CsgA sequence. The presence of CsgB protein helps the stability of the expression of CsgA and it has shown that insertion with a *csgB*- *csgA*+ mutant does not result in curli production (Hammar et al., 1995).

The role of CsgC is not completely understood in terms of curli biosynthesis. CsgC is a small beta-sheet-rich periplasmic chaperone-like protein (Evans et al., 2015). During the secretion of curli fiber, CsgC inhibits the premature folding and polymerization of CsgA in the periplasm by acting as a safeguard against runaway polymerization (Bhoite et al., 2019) and some suggested that CsgC can inhibit CsgA amyloid formation and keep CsgA in a non-beta-sheet-rich conformation (Evans et al., 2015).

CsgD protein is a putative transcription regulator and activator that initiates the curli fimbriae production by activating the *csgBAC* operon that encodes the curli fimbriae structural subunits and facilitates the biofilm formation in Enterobacteria such as *Escherichia coli* and other strains as well (Brombacher et al., 2006). Additionally, CsgD facilitates the upregulation of the biosynthesis of cellulose (Gerstel & Römling, 2003). Environmental factors such as nutrients, oxygen tension, temperature, pH and ethanol play an important role in the expression and transcription level of CsgD (Gerstel & Römling, 2003). The study has shown that the transcription of *csgD* is stimulated when the nutrients are scarce and cell density is high. Additionally, the transcription is lower in the log phase and increases substantially in the stationary phase (Gerstel & Römling, 2001). *Salmonella* Typhimurium with a *csgD* mutation was deficient in forming a biofilm, and by overexpression of this gene, a much thicker and more rapidly growing biofilm was observed (Jonas et al., 2007). The regulation of gene expression by the curli activator CsgD protein has been well studied in *Salmonella* Typhimurium and *Escherichia coli* (MacKenzie Keith et al., 2015; Ute Römling et al., 1998), but study was conducted on *Salmonella* Enteritidis strain. CsgD can also indirectly activate *adrA* at a transcriptional level, and therefore, regulates the cellulose production in *Salmonella* spp. (Hammar et al., 1995).

CsgE, a periplasmic protein, often interacts with CsgG at the outer membrane and helps to facilitate the curli assembly. csgE mutant strains were unable to bind the congo red dye and showed smooth and white morphology (saw) (Barnhart & Chapman, 2006). CsgF, like CsgE, is a periplasmic protein that can interact with CsgG at the outer membrane. However, unlike *csgE* mutant, *csgF* mutant behaves like *csgB* mutant and secretes unpolymerized and soluble CsgA (Chapman et al., 2002). CsgG**,** a putative curli assembly factor, is an outer membrane lipoprotein that has shown huge resistance to protease digestion, therefore, it is used to protect the stability and to help the secretion of CsgA and CsgA (Barnhart & Chapman, 2006).

#### 2.5.4.2. Curli gene *bapA*

BapA (biofilm-associated protein), a large cell-surface protein is also required for the formation of biofilm in *Salmonella* Enteritidis (Latasa et al., 2005). BapA is secreted through the type I secretion system. The deletion of the *bapA* gene can cause the loss of capacity in biofilm formation, when overexpressing this gene, the biofilm biomass increased. The expression of *bapA* was coordinated with the expression of both curli and cellulose through the action of CsgD (Latasa et al., 2005), which affects the biofilm formation process. It has also been shown that animals inoculated with a *bapA*-deficient *Salmonella* Enteritidis strain lived longer compared to those inoculated with wild-type strains since there is less invasion to the epithelial cells from *Salmonella* Enteritidis penetrating the intestinal barriers (Latasa et al., 2005).

### 2.5.5. Cellulose genes: *adrA* and *the bcs* family

Cellulose is a major water-insoluble component found in the EPS matrix, and it is one of the homopolysaccharides participating in biofilm formation (Flemming & Wingender, 2010). Cellulose contributes to long-range cell-cell interactions and offers mechanical, chemical, and biological protections to the bacteria (Solano et al., 2002). Additionally, cellulose production is associated with the chlorine resistance, therefore, promote biofilm formation.

AdrA (*agfD* regulated gene) is a putative transmembrane protein sound in many *Salmonella* serovars (Römling et al., 2000). Encodes a diguanylate cyclase protein, the activation of the transcription of the *adrA* gene can stimulate the cellulose production by *csgD* and the expression of *adrA* gene requires RpoS, a global regulator (Robbe-Saule et al., 2006). Both *crl* and *rpoS* mutant could substantially reduce the expression of *adrA* gene (Robbe-Saule et al., 2006), however, the expression of the *bcs* genes were independence on AdrA (Zogaj et al., 2001).

Research has identified essential genes for cellulose biosynthesis within the *bcs* (bacterial cellulose synthesis) operons. The *yhjR-bcsQABZC* operon encodes *yhjR, bcsQ, bcsA, bcsB, bcsZ,* and *bcsC*, while the *bcsEFG* operon encodes *bcsE, bcsF,* and *bcsG* (Serra et al., 2013; Shi et al., 2019; Solano et al., 2002; Zogaj et al., 2001). The expression of the *bcs* genes are independent on CsgD, AdrA and RpoS, and they are considered to be constitutively transcribed (Zogaj et al., 2001). Gene *bcsC* is required for maximal for the production of cellulose (Solano et al., 2002), however all of the *bcs* genes are involved in the synthesis of cellulose.

### 2.5.6. A global regulator RpoS

Rpos, a stationary-phase sigma factor, regulates many genes in *Escherichia coli* and *Salmonella* Typhimurium (Ute Römling et al., 1998). It is one of the stress response genes that influence biofilm formation (Corona-Izquierdo & Membrillo-Hernández, 2002). In *Escherichia coli*, a mutation in *rpoS* can promote biofilm formation during the exponential phase of bacterial growth (Corona-Izquierdo & Membrillo-Hernández, 2002). It is responsible for the development of red, dry, and rough (RDAR) morphotype, virulence, and biofilm formation in *Salmonella* Typhimurium and *Escherichia coli* strains and its interaction with the *crl* protein can activate the expression of associated genes for example the *csgBAC* operon encoding the curli proteins subunits (Robbe-Saule et al., 2006). Genes *rpoS* and *ompR* are necessary for the transcriptional activation of the *csgD* promoter (U. Römling, Z. Bian, et al., 1998). Römling et al. have shown that the RDAR morphotype expression with *csgD* promotor is partially dependent on *rpoS* in *Salmonella* Enteritidis, however, *rpoS* is required in that of *Salmonella* Typhimurium (Ute Römling et al., 1998).

### 2.5.7. Other important genes

#### 2.5.7.1. Gene *invA*

*InvA* is a *Salmonella* invasive and virulence gene that encodes proteins in the membranes of the bacterial cells that are necessary for the invasion of the host epithelial cells (Yanestria SM, 2019), in addition, it can be involved in the process of translocation of the InvE protein. It plays an essential role in the *Salmonella* type III secretion system (T3SS) apparatus (Worrall et al., 2010). It was shown that *invA* at 285 bp is only exclusively possessed by *Salmonella* bacteria (Rahn et al., 1992). In this study, all the isolates from *Salmonella* Enteritidis and *Salmonella* Typhimurium contained 284 bp invA (100%) and *stn* gene (100%); 77.27% contained 310 bp *spvR* gene; 22.72% contained 571 bp *spvC* gene (Shivaning Karabasanavar et al., 2020). However, not all types of *Salmonella* serotypes contain the *invA* gene, which could be a better way to differentiate. *Salmonella* (serotype not specified) isolated from milkfish in Indonesia and 4 positive *Salmonella* samples contained 284 bp *invA* gene (Yanestria SM, 2019). *Salmonella* Litchfield and *Salmonella* Senftenberg were not detected as having a 284 bp *invA* gene (Rahn et al., 1992).

#### 2.5.7.2. Quorum sensing

Quorum sensing refers to a type of cellular signaling, which is a cell-to-cell communication of the bacteria community based on chemical signals that regulate their gene expression accordingly based on sensing the fluctuation of the cell population density (Rutherford & Bassler, 2012). Quorum sensing involves the production, secretion, group-wide detection, and responding to autoinducers, the small extracellular signaling molecules (Papenfort & Bassler, 2016). The concentration of autoinducers increase as the bacterial population density grows. The changes in cell numbers lead to changes in the concentration of autoinducer, and therefore, alter the gene expression (Papenfort & Bassler, 2016). The purpose of this cell-to-cell communication includes getting nutrients, passing on genetic materials between bacterial cells, adjusting cellular functions, motility, and population density-based pathogenesis (Preda & Săndulescu, 2019), as well as altering microbiota composition, virulence factors secretion, and enhancing biofilm formation and bacterial dispersion in eukaryotic hosts (Gill et al., 2015). Many gram-negative bacteria use N-acyl-L-homoserine lactones (AI-1) and furanose borate diester (AI-2) for quorum sensing signaling to regulate gene transcription (Smith et al., 2011). The synthesis of AI-1 is dependent on the luxLM locus (Bassler et al., 1993). AI-2, which is to date the most common bacterial autoinducer, is produced by the enzyme LuxS (encoded by *luxS*), which is also involved in cellular metabolism (Papenfort & Bassler, 2016; Smith et al., 2011).

### 2.6. Conclusion

In conclusion, this literature review has shed light on the prevalence of *Salmonella enterica* serovar Enteritidis in the poultry and egg industry and the necessity of proper cleaning and sanitizing to effectively decrease and prevent the formation of biofilms in the food processing environments which could lead to potential public health issues. The mechanism of biofilm formation in *Salmonella* spp. was mentioned. Those protein coding genes associated with biofilm formation, curli, cellulose, fimbria, flagellum, and pilus were discussed, underlying their importance in the formation of biofilms. In addition, the literature review emphasized the potential genetic mutations and their results in the protein coding genes for biofilm formation.

# 3. Chapter 3: Biofilm formation and comparative genomic analysis on *Salmonella* Enteritidis and Kentucky strains in poultry sources

## 3.1. Abstract

This study aimed to investigate the biofilm-forming ability and genomic differences between *Salmonella* Enteritidis (SE) and *Salmonella* Kentucky (SK), focusing on genes associated with biofilm formation. The goal was to identify genetic factors that contribute to the resilience of strong biofilm-forming strains compared to moderate, weak or non-biofilm-forming strains. Fifteen SE and 24 SK strains isolated from various poultry products and processing facilities were analyzed. Biofilm formation was assessed using the crystal violet method (CV) on 96-well microplates at 20-22°C for 5 days. The curli and cellulose production was examined using Congo red plates and Calcofluor dye respectively. The comparative system analysis was conducted using the BV-BRC pipeline to screen for genes associated with biofilm formation. A comparison of the protein-coding DNA was performed to check the DNA for the cell's biofilm production potential between genomes with different biofilm-forming abilities to determine which essential genes were present in the strong strain but absent in the intermediate strain. SNP variation analysis was performed to screen for genetic mutations in the genes of interest. Of the 15 SE strains, 11 were strong, 4 were intermediate biofilm formers, and 14 were positive for both curli and cellulose. Of the 24 SK strains, 4 were strong, 5 weak, and 15 non-biofilm formers, and 4 strong biofilm formers were positive for both curli and cellulose. For genome comparisons, 122 protein-coding genes were found to be associated with biofilm formation, fimbriae, flagella, pilus, curli, cellulose, and type IV secretion system and 107 of them were present in all 15 SE strains and all 24 SK strains. Variation analysis revealed that 24 protein-coding genes with mutations have potential connections to biofilm formation including but not limited to BcsG, BcsF, BcsQ, FlgA, FlgB, MotA, MotB, TolA, TolB, TolR. Synonymous variants and stop-gained variants in certain cellulose genes, such as those in the *bcs* group, may contribute to weaker biofilms in *Salmonella* strains. Additionally, nonsynonymous missense variants in the *tol* family found in both the SE and SK strains could result in amino acid changes that impair biofilm formation and flagellar biosynthesis.

## 3.2. Introduction

Despite numerous efforts to implement food safety measures to reduce salmonellosis infections, outbreaks still occur and impact food safety and human health worldwide. *Salmonella* Enteritidis has been reported to be linked to numerous poultry, eggs, and egg product-associated outbreaks (CDC, 2012, 2018a, 2018b, 2019, 2024b) and its persistence in food processing facilities is still very problematic. One reason for its persistence is the formation of biofilms (Steenackers et al., 2012; Vestby et al., 2009).

Biofilm refers to a group of surface-associated microorganisms that have the ability to attach to a surface, produce an amorphous extracellular polymeric substance (EPS) matrix and that enclosed the bacteria within. EPS is composed of polysaccharides, nucleic acids, lipids, and proteins (Donlan, 2002; Flemming, 2016). In 2006, the National Institute of Health estimated that over 80% of human bacterial infections could be associated with biofilm formation, which includes but is not limited to dental plaques caused by *Streptococcus* spp. and *Actinomyces viscosus*; chronic rhinosinusitis caused by *Staphylococcus aureus*; and catheter-associated urinary tract infections caused by *Escherichia. coli*, *Enterococcus* spp, *Pseudomonas aeruginosa*, and *Klebsiella* spp. (Hung & Henderson, 2009). *Salmonella* can form biofilms and persists on food contact surfaces such as plastic, glass, and stainless steel (Giaouris & Nychas, 2006; Joseph et al., 2001; Solano et al., 2002). The density and amount of biofilm produced can be classified as strong, intermediate/moderate, weak, and non (Stepanović, Cirković, et al., 2004). By forming biofilms, bacteria can survive in harsh environments, such as food processing plants and slaughterhouses. (Steenackers et al., 2012).

Similar to *Salmonella* Enteritidis, *Salmonella enterica* serovar Kentucky is another serovar at which poultry is the primary carrier, transmitting the bacteria to humans through the consumption of contaminated foods (Ferrari et al., 2019). *Salmonella* Kentucky is a polyphyletic non-typhoidal *Salmonella* serovar that includes two major sequence types (STs), ST152 and ST198, which represent two distinct genetic lineages (Soltys et al., 2021). ST 152 is more prevalent in the U.S. poultry and poultry products and while it is sporadically linked with human illness, it has not been associated with major foodborne *Salmonella* outbreaks in the U.S. (Shah, Paul, et al., 2017). In contrast, ST 198 is more prevalent in international poultry (poultry outside the U.S.) and is more frequently associated with human disease (Soltys et al., 2021). Fluoroquinolone-resistant *Salmonella* Kentucky ST198 is commonly isolated from poultry and poultry products, and frequently associated with human illnesses in Africa, Southeast Asia, the Middle East, and some European countries (Le Hello et al., 2013; Le Hello et al., 2011). However, more fluoroquinolone-resistant *Salmonella* Kentucky ST 198 strains were detected in international travelers returning to the United States and Canada from those areas mentioned above.

*Salmonella* Kentucky is the most prevalent serovar associated with poultry in Canada (Canada, 2024) and North America (Ferrari et al., 2019), while *Salmonella* Enteritidis dominates in Asia, Latin America, Europe, and Africa (Ferrari et al., 2019). In the United States, *Salmonella* Kentucky accounts for 0.2% (1,026 out of 477,861 cases) of all confirmed human *Salmonella* infections (CDC, 2018c). An analysis of 5,151 *Salmonella* Kentucky genomes collected from 2017 to 2021 revealed that 90.3% of isolates came from chicken broilers, 5.9% from humans, and 3% from cattle (Richards et al., 2023). Although *Salmonella*

Kentucky is not considered a significant public health threat in the United States or Canada (Canada, 2024; Khan et al., 2023), it remains the most commonly isolated serovar from broiler chickens in the U.S. and is among the top 10 serovars linked to human salmonellosis in Europe (Richards et al., 2023). This difference could be due to mutations in sequence types and therefore genomic differences across regions.

In the EU, the number of confirmed and reported human-associated salmonellosis cases from *Salmonella* Kentucky between year 2010 and 2016 was 0.4%, 0.3%, 0.3%, 0.4%, 0.3%, 0.3%, and 0.4%, respectively. It was significantly less prevalent when compared to that of *Salmonella* Enteritidis, which was 51.6%, 52.7%, 50.9%, 48.0%, 54.6%, 54.7%, and 57.1%, respectively (Koutsoumanis et al., 2019). Like salmonellosis caused by *Salmonella* Enteritidis, it is likely that human illness cases associated with *Salmonella* Kentucky are underreported as well due to its self-limiting nature. However, it is still considered one of the new five target serovars for breeding hens in the EU after 2014, and it had the highest proportion of travel-associated cases (53.2%) in the EU in 2016 (Koutsoumanis et al., 2019). Research has suggested that *S.* Kentucky is not associated with many human salmonellosis might be related to the absence of some virulence genes such as *sopD2*, *pipB2*, *sspH2*, and *srfH* (Dhanani et al., 2015) or *grvA*, *sseI*, *sopE*, and *sodC1* (Cheng et al., 2015), or *seeI*, *sseK2*, and *sspH2* (Tasmin et al., 2017).

The RDAR morphotype is linked to forming a robust biofilm matrix, and it shows how *Salmonella* established persistent infections in food processing environments. The RDAR morphotype has already been well-studied in terms of biofilm formations in *Salmonella*. The RDAR phenotype expressing colonies consist of proteinaceous compounds such as adhesive curli fimbriae and exopolysaccharides such as cellulose, and sometimes O-antigenic capsule, capsular polysaccharide, and lipopolysaccharide (Steenackers et al., 2012). Curli are amyloid-like cell-surface proteins that are responsible for host colonization, persistence, motility, and invasion (Steenackers et al., 2012). Curli in *Salmonella* spp. are essential for biofilm formation since they promote cell-surface and cell-to-cell interactions (Steenackers et al., 2012).

Many research papers have investigated the relationship between biofilm formation and the expression of curli fimbriae and cellulose production (Gerstel & Römling, 2003; U. Römling, Z. Bian, et al., 1998). However, few studies have addressed the correlation between biofilm phenotypic results (e.g., biofilm density) and its genomic results. This research aims to explore the genetic differences between strong biofilm-forming *Salmonella* strains and those weak or non-biofilm producers.

## 3.3. Materials and Methods

### 3.3.1. *Salmonella* biofilm formation

#### 3.3.1.1. *Salmonella* bacterial strain selection and culture conditions

Fifteen *Salmonella* Enteritidis (SE) strains (Table 3-1) were included in study from previous work by Bucher et al. (Bucher et al., 2007) to determine biofilm formation capabilities. These SE strains were selected based on the source, specifically the poultry environment, facilities, and processing. Some SE strains were found in the end-product (chicken nuggets, feed, and chicken breast), indicating that these bacteria have survived all interventions applied, and thus it is hypothesized that perhaps those strains have some qualities different from others, such as biofilm formation.

Before selecting these strains for genomic comparisons, biofilm formation ability was assessed. Results showed that of 15 *Salmonella* Enteritidis strains tested, 11 were strong biofilm formers, and 4 were intermediate. There were no weak or non-biofilm former strains, therefore to include all levels of biofilm formation. Twenty-four *Salmonella* Kentucky (SK) strains were included (Table 3-1). SK strains were isolated in Agasiz, Canada from a poultry processing facility. The strains were recovered from six locations: crate, plucking belt, carcass wash/pre-chill, final wash/post chill, plucking water, and chill water. *Salmonella* Kentucky was included for more varieties of the biofilm categories, since *Salmonella* Enteritidis strains in our study.

All bacterial strain cultures used in this research were kept in a -80 °C freezer with Trypticase Soy Broth (TSB; Becton, Dickinson and Company, MB, United States) and 15% glycerol. A loopful of the frozen culture was streaked on Xylose Lysine Deoxycholate (XLD) (Hardy Diagnostics Inc., Santa Maria, CA, United States) agar plates and incubated at 37 °C overnight to contain single colonies. A single colony of each bacterial strain was transferred and inoculated from the overnight agar plate into a 10 ml sterile Lennox Broth no-salt (LN-NS (10g tryptone (VWR Chemials LLC, Solon, USA) +5 g yeast extract (Termo Fisher Scientific, Sasingstoke, UK) in 1L distilled water) and incubated at 37 °C overnight. The overnight culture was adjusted to a concentration of $10^8$ colony-forming units (CFU/ml) by diluting it with LB-NS to read an OD (optical density) between 0.30 nm to 0.35 nm on the spectrometer or using a 0.5 McFarland Standard for culture concentration confirmation.

Table 3-1: *Salmonella enterica* subsp. *enterica* serovar Enteritidis and serovar Kentucky

| Serovar | Strain ID | Source | Serovar | Strain ID | Source |
|---|---|---|---|---|---|
| Enteritidis | 51094 | Chicken nuggets | Kentucky | C45-1 | Crate |
| Enteritidis | 51095 | Chicken nuggets | Kentucky | B38-3 | Plucking belt |
| Enteritidis | 52239 | Chicken nuggets | Kentucky | W36-3 | Carcass wash/Pre-chill |
| Enteritidis | 618901 | Chicken nuggets | Kentucky | W37-2 | Carcass wash/Pre-chill |
| Enteritidis | 619102 | Chicken nuggets | Kentucky | W38-1 | Carcass wash/Pre-chill |
| Enteritidis | 78501 | Chicken nuggets | Kentucky | W39-1 | Carcass wash/Pre-chill |
| Enteritidis | 79801 | Chicken nuggets | Kentucky | W40-3 | Carcass wash/Pre-chill |
| Enteritidis | 79901 | Chicken nuggets | Kentucky | W41-2 | Carcass wash/Pre-chill |
| Enteritidis | 719001 | Chicken nuggets | Kentucky | W42-2 | Carcass wash/Pre-chill |
| Enteritidis | 710901 | Chicken nuggets | Kentucky | W44-3 | Carcass wash/Pre-chill |
| Enteritidis | 53932 | Chicken breast | Kentucky | F36-3 | Final wash/post chill |
| Enteritidis | 53936 | Chicken breast | Kentucky | F37-3 | Final wash/post chill |
| Enteritidis | 61401 | Feed | Kentucky | F38-2 | Final wash/post chill |
| Enteritidis | 715701 | Feed | Kentucky | F39-3 | Final wash/post chill |
| Enteritidis | 107 | Bovine | Kentucky | F40-3 | Final wash/post chill |
| | | | Kentucky | F41-2 | Final wash/post chill |
| | | | Kentucky | F42-3 | Final wash/post chill |
| | | | Kentucky | F43-3 | Final wash/post chill |
| | | | Kentucky | F44-1 | Final wash/post chill |
| | | | Kentucky | F45-2 | Final wash/post chill |
| | | | Kentucky | PW8-3 | Plucking water |
| | | | Kentucky | PW9-3 | Plucking water |
| | | | Kentucky | CW8-3 | Chill water |
| | | | Kentucky | CW9-3 | Chill water |

#### 3.3.1.2. Biofilm assessment using the crystal violet method

For *Salmonella* biofilm formation, the $10^8$ CFU 10ml LB-NS overnight culture tube was vortexed and was diluted to $10^7$ CFU/ml by adding 1ml $10^8$ CFU culture into 9ml LB-NS broth. In a 96-well microplate, 180µL of sterile LB-NS was dispensed into each microplate well. Using a multi-channel pipette, 20µL of the $10^7$ CFU/ml culture was dispensed into the wells to achieve a final population of $10^6$ CFU/ml in the microplates. Each plate included four *Salmonella* Kentucky samples, one positive control (20µL *E. coli* R508 strain+180µL LB-NS), and one sterility control (200µL LB-NS total-no bacteria), and the experiment was repeated four times in two replicates. The microplate was sealed with parafilm, covered with the lid, and incubated at 20-22 °C for 5 days (Adator et al., 2018).

After the 5-day incubation, biofilm development was assessed. For this purpose, unattached cells were removed from microplate wells. The microplates wells were washed with 200µL Butterfield's Phosphate Buffer ($KH_2PO_4$) (BPB) (Hardy Diagnostics Inc., Santa Maria, CA, United States) three times using a microplate washer (405 LS, BioTek, Winooski, VT, United States) and air dried for 10 minutes. Then 200µL of absolute methanol was added to each well, and the contact was allowed for 15 minutes to fix the attached biofilm. After 15 minutes, the methanol was removed and air dried for 15 mins. The biofilm was then stained with 200µL of 0.1% crystal violet (CV) (Sigma Aldrich) (Adator et al., 2018) for 20 minutes. Next, the fixed biofilm was washed three times with 200µL BPB, and air-dried for 15 minutes. Finally, 200µL of 85% ethanol was added to each well to dissolve the stained biofilm and the microplates were agitated. The optical density (OD) was measured at 630nm using a microplate reader (BioTek ELx800; BioTek Instruments Inc., Winooski, VT, United States).

#### 3.3.1.3. Curli and cellulose

*Salmonella* Enteritidis and Kentucky strains (Table 3-1) were assessed on their capacity to produce curli and cellulose at 20-22°C. For curli production, the overnight cultures of *Salmonella* Kentucky strains were steaked on Congo red agar (10g/L casamino acids (VWR, Solon, Ohio, USA), 1g/L yeast extract (Thermo Fisher Scheintific, Basingstoke, UK), and 20g/L agar (Difco, Sparks, USA)), supplemented with 20 µg/mL Coomassie brilliant blue dye (Sigma Aldrich, St. Louis, USA) and 40 µg/mL Congo red dye (Sigma Aldrich, St. Louis, USA) (Adator et al., 2018). For cellulose production, the overnight cultures of *Salmonella* strains were steaked on Luria Bertani (LB) agar (Hardy Diagnostics CulGenex™, Santa, Maria, USA) supplemented with 200 mg/L Calcofluor dye (Sigma Aldrich, St. Louis, USA). Those plates were incubated at 22°C for 1-2 days. A positive result for curli production is shown as red, dry, and rough (RDAR) morphotype, which indicate the presence of curli in the extracellular matrix, other morphotypes indicate negative results. The presence of fluorescence colonies on LB agar with Calcofluor dye at 366-nm UV light indicates a positive result for cellulose, and colonies that don't fluoresce indicate a negative result.

#### 3.3.1.4. Statistical data analysis method

The obtained data from the microplate reader was analyzed using the method described by Stepanović et al. and Adator et al. The biofilm classification cutoff line was based on the value of the negative controls, where ODc (optical density cut-offs) =mean of negative control+3*SD of the negative control. The biofilm-forming ability, OD value, was then fitted into the range. When $OD \leq ODc$, it is a non-biofilm former; when $ODc < OD \leq 2ODc$, it is a weal biofilm former; when $2ODc > OD \leq 4ODc$, it is an intermediate biofilm former; when $4ODc < OD$, it is a strong biofilm former (Adator et al., 2018; Stepanović, Ćirković, et al., 2004).

For assessing the biofilm forming ability of the *Salmonella* strains, interactions between strains and biofilm categories were analyzed using the Chi-Square method. For all statistical analyses, the significance was declared at $p<0.0001$.

### 3.3.2. Genomic comparison of *Salmonella* Enteritidis (SE) and *Salmonella* Kentucky (SK) with regards to their biofilm formation gene information

#### 3.3.2.1. Whole genome sequencing and bioinformatics analysis

Genomic DNA from *Salmonella* strains was extracted using the DNeasy Blood and tissue kit-Gram Negative protocol (Qiagen, Inc., Toronto, Ontario, Canada) following manufacturer recommendations. DNA concentration and quality were tested using NanoDrop (NanoDrop Technologies, Wilmington, United States). The whole genome sequence for the fifteen *Salmonella* Enteritidis (SE) strains and twenty-four *Salmonella* Kentucky (SK) strains described above (Table 3-1) were obtained from the National Microbiological Laboratory Public Health Agency of Canada located in Winnipeg.

Quantification of the DNA was done using the Qubit HS kit. The genomic DNA (gDNA) was first normalized using the Biomek i7 robot. The library samples were prepared using the Illumina DNA prep kit, followed by post-PCR purification with AmpPure XP beads. Size selection was carried out using the Blue Pippin system, and the average base pair size was determined using the Agilent Tapestation automated electrophoresis instrument. Sequencing was performed on the Illimina MiSeq platform with the 600 v3 kit. The National Microbiology Laboratory, Winnipeg, Genomics Core conducted all sequencing and library preparation procedures.

#### 3.3.2.2. Genomic assembly and annotation and comprehensive genomic analysis

Whole genome sequence raw data were assembled (Wick et al., 2017) and annotated (Brettin et al., 2015) using the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) platform SPAdes and RAST/SEED systems, respectively, using the Illumine platform with a unicycler. In total, all 15 SE and 24 SK whole genomes showed good quality (completeness at 100%, contamination at 0%) according to the annotation report in the Comprehensive Genome Analysis (CGA) generated by the BV-BRC (Davis et al.,

2016) (shown in Table 5-1 in the complementary material).

#### 3.3.2.3. Phylogenetic tree and heatmapping

A phylogenetic tree was used after the single reads were paired and fully annotated. Three phylogenetic trees were generated, one for SE strains, one for SK stains, and one for both stains together using 500 single-copied gene parameters in the mafft alignment program and RAxML Fast Bootstrapping (Stamatakis, 2014). The genes in the phylogenetic tree were picked randomly from a defined number of the BV-BRC Global Protein Families to build an alignment and develop a phylogenetic tree based on the sequence differences. The number of genes ranges from 10 to 1000 for selections based your genomes of interest (Olson et al., 2023). Since all strains in this study belong to the same subspecies (*Salmonella enterica* subsp. *enterica*) and the same serotype within the Enteritidis and Kentucky strains, their genomes are closely related.

A comparative system (Davis et al., 2016) in the BV-BRC was used to compare up to 500 genes and protein families using the whole genome sequences of those 15 SE and 24 SK strains. Under the protein family sorter, genes of interest were searched, filtered, and grouped into a heatmap to examine the distribution of the protein families across the genomes. A heatmap that consisted of a pan-genome of 5,396 genes was generated under the comparative system tool with a focus on the intra-genus comparison (PLfam) to examine the presence and absence of genes of interest that are associated with biofilm formation, curli and cellulose production, flagella, fimbriae and pilus expression. Since all the strains belong to the same genus (*Salmonella*), the PLfam uses only proteins within a genus and more stringent criteria (BV-BRC, 2023). More than 100 genes were filtered and selected as they were genes associated with biofilm formation, the production of curli and cellulose, the functions of flagella, fimbriae, and pilus (Brombacher et al., 2006). Filtered genes were examined upon their functions and were illustrated with iTol service (Letunic & Bork, 2021).

#### 3.3.2.4. SNP variation analysis

In addition to the tools above, a variation analysis (Heng Li, 2013) was also implemented to identify and annotate sequence variations and to look for any mutations mentioned above using BWA-mem as the aligner algorithm and FreeBayes of the SNP caller method.

A single nucleotide polymorphism (SNP) analysis was utilized to determine genomic variations that occurs at a specific nucleotide position within the DNA sequence, where a single base pair differs between individuals or populations (NIH, 2024). This variation can potentially influence gene expression, protein functions, or regulatory mechanisms and one of the possible type of genetic differences to environmental factors, and susceptibility to cause diseases (Dar et al., 2023).

The genetic variations of SNPs were examined among the 15 SE strains and the 24 SK strains. SE strain 53932 and SK strain PW9-3 were selected as the reference genome due to their strong biofilm-forming abilities to compare with the other SE and SK strains, respectively. Using a strong biofilm-former strain as a reference could provide more precise insights into genomic mutations regarding their impact on the expression of protein coding genes. This method could help to explain the rationale behind the different phenotypic and genomic results.

#### 3.3.2.5. A BLASTp comparison

A comparison is based on protein sequence similarity and finds the best unique/unidirectional/bidirectional hit when compared to the reference genome (Boratyn et al., 2013). This comparison is a sequence-based comparison that was part of RAST (Overbeek et al., 2014) and it was conducted in the present study to determine whether there are proteins present in the strong biofilm former strains that are missing in the intermediate/weak/non-biofilm former strains. Within the SE strains comparison, a strong biofilm-forming isolate from poultry (strain 53932) was used as a reference strain and BLASTed against the only intermediate SE biofilm-forming strain 107 (isolated from bovine).

#### 3.3.2.6. Statistical data analysis method

Interactions between the phenotypic results (the biofilm categories, and the production of curli and cellulose), and the genomic results (types of SNPs and the SNPs impact) were analyzed using the Chi-Square method. For all statistical analyses, the significance was declared at $p<0.0001$.

## 3.4. Results and Discussion

### 3.4.1. Biofilm formation

Numerous studies have investigated the biofilm-forming abilities of *Salmonella*. In the present study, the results from the 15 SE strains (Table 3-2) showed that at 20ºC, 11 out of 15 strains (73.3%) produced strong biofilms, while 4 out of 15 strains (26.7%) produced intermediate biofilms ($p<0.0001$) (Figure 3-1, Table 3-2). The four intermediate biofilm producers were isolated from chicken nuggets (n=2), feed (n=1), and bovine (n=1). These findings indicate that SE biofilms can grow effectively at 20-22ºC, the typical temperature of chicken barns. The 20-22°C temperature range is critical because it represents the ambient temperature in chicken barns where chickens are raised and eggs are laid. At 20-22ºC, *Salmonella* Enteritidis forms strong biofilms that protect underlying bacteria to survive and contribute to their persistence in the environment. The ideal environmental temperature for laying hens ranges between 18-24 ºC (Holik, 2009). In Canada, the average temperature in commercial laying hen barns was reported to be 21.7°C ±1.44°C (range:19.0–25.0°C) (Van Staaveren et al., 2018). In Manitoba province, the temperature of the chicken barns was between 21-25°C (information provided by local producers and the University of Manitoba layers barn at Gleanlea Research station). Our findings on temperature and biofilm formation results are similar to the study conducted by Giaouris et al., who identified 20°C as the optimum temperature for SE biofilm formation after six days of incubation (Giaouris et al., 2005). Within 15 SE isolated *in vitro* obtained in Serbia from a laboratory, 7 strains were strong, 5 were intermediate, and 2 were weak biofilm producers at 25°C (Ivana et al., 2015). Borges et al. (Borges et al., 2018), found that 85.6% of SE strains produced biofilms at all temperatures tested (37°C, 28°C, 12°C, and 3°C). In addition, Schonewille et al. described SE strain as the strongest biofilm producer at both 20 ± 1 and 37 ± 1 °C (Schonewille et al., 2012).

However, a study conducted by De Oliveira et al. on *Salmonella* biofilm formation from poultry products in Brazil provided contrasting information. After incubating for 96 hours, at 20ºC, non-biofilm producers dominated SE strains grown on stainless steel (120, 69%), glass (135, 77.6%), polyvinyl chloride (105, 60.3%), followed by weak (stainless steel (48, 27.6%), glass (33, 19%), polyvinyl chloride (69, 39.7%)), intermediate (stainless steel (6, 3.4%), glass (6, 3.4%), polyvinyl chloride (0, 0%)), and strong formers (0% on all three surfaces) (De Oliveira et al., 2014). Similarly, another study investigated SE biofilm formation in Brazil and found that strains from foodborne outbreaks (foods and stool samples) and poultry products (n=171) incubated at 25 ºC for 24 hours exhibited varied biofilm formation abilities using the same crystal violet method at 550nm in the biofilm reader. Of the outbreak strains, 51.25% were weak biofilm formers, followed by intermediate (33.75%), non (8.75%), and strong producers (6.25%). Among strains isolated from poultry products, the majority were non-producers (53.33%), followed by weak (38.89%), intermediate (4.44%), and strong producers (3.33%) (Rodrigues et al., 2019). Both studies highlight that strains with strong biofilm-forming capacities were a minority. This discrepancy suggested that SE strains from Brazil possess different biofilm formation abilities compared to those isolated in North America, perhaps associated with different genomic traits. Additionally, it is also possible that slight differences in the method and conditions such as incubation time and culture media used (diluted TSB) could also contribute to the differences in results.

In the present study, the only intermediate biofilm SE strain originated from bovine. There is limited information available on *Salmonella* Enteritidis biofilm formation in bovine since *Salmonella* Enteritidis is less commonly found in cattle. However, in another study, the *Salmonella* strains were incubated in TSB broth at 37°C for 72 hours, the crystal violet method was used, and the microplates were read at 570nm. Of the 5 *Salmonella* Enteritidis strains isolated from beef collected from meat sale centers after incubated, the majority of them (4 out of 5) were intermediate and 1 was a strong biofilm former (Manafi et al., 2020).

In our research, the results from the 24 SK strains (Figure 3-1, Table 3-2) revealed that at 22°C, 4 strains were strong biofilm formers (16.7%), 0 strains were intermediate (0.0%), 5 strains were weak (20.8%), and 15 strains were non-biofilm formers (62.5%) ($p<0.0001$). In contrast, Cufaoğlu et al. have classified the SK serotype as a weak biofilm producer after isolating a strain (n=1) from scalding tank water and incubating it in dilute TSB broth at room temperature (21 ± 1°C) for 48 hours (Cufaoğlu et al., 2021). Obe et al., tested two SK strains and found both to be weak biofilm producers in TSB broth at 15°C on plastic and steel surfaces, while at 25°C, one strain was weak, and the other intermediate (Obe et al., 2022). A research reported SK strains (n=12) isolated from commercial processing equipment ranged from strong (n=3), to moderate (n=2), to weak biofilm producers (n=7) after incubated in TSB broth for 48 hours at 25°C (Obe et al., 2021). Despite the small sample sizes, the findings in the first two studies are valuable as they align with broader trends observed in SK biofilm formation. Additionally, the experimental conditions and methodologies used were similar to the one applied in this study except for the broth used, suggesting that SK strains generally exhibit weak biofilm formation under these conditions. In a food processing environment, various strains with different attachment properties could form a biofilm matrix, which may explain why some weak biofilm-forming strains were recovered from the surface, due to the presence of other strong biofilm-forming strains (Obe et al., 2021). Another research has shown that the biofilm formation of different strains isolated from the same food processing plant could range from strong to weak (Obe et al., 2021). This result aligns with our present study's results, which in addition to the strong to weak categories, the non-biofilm category was introduced. In our study, of the 8 SK strains isolated from the carcass wash/pre-chill step, 7 were non-biofilm formers, while 1 was a strong biofilm former. Similarly, of the 10 SK strains isolated from final wash/post chill, 9 were non-biofilm formers and 1 was a strong former. Two isolated from plucking water resulted in 1 weak and 1 strong former, additionally, two isolated from chill water resulted in 1 weak and 1 non-former. These results suggest that even when strains are isolated from the same location and under similar environmental conditions, can still exhibit significant variability in their biofilm-forming abilities. Poultry processing facilities contain not just single species, but multiple species of a bacteria within the same processing location and the same environment; a single strain may behave differently when it is present alone compared to when it was interacting with other strains (Arnold & Silvers, 2000).

Compared to SE strains, SK strains produced significantly less biofilm at room temperature. The room temperature in the chicken barns typically ranges between 21 and 25°C, with lower temperatures in winter and higher temperatures in summer, depending on external weather conditions. The recommended relative humidity in Canadian poultry barns is 40- 60% (Manitoba Canada). Our findings suggest that SK strains have a lower risk of biofilm formation, potentially reducing their persistence in poultry barns. Similarly, non-biofilm producers may represent a lower risk for food contamination in food processing environments.

The incubation time could also affect biofilm formation, as shown by Stepanović et al. who found that for 30 *Salmonella* strains tested at 22°C, 30°C, and 37°C, biofilm production peaked at 30°C after 24h and was at 22°C after 48h in TSB broth (Stepanović et al., 2003). In a study of 40 *Salmonella* spp. isolates from pig slaughterhouses and their environment, in 1/20 TSB broth at 22°C, 25 isolates (62.5%) were weak, 3 isolates (7.5%) were moderate, and 12 isolates (30%) were non-biofilm producers; while at 35°C, 9 isolates (22.5%) were weak, and 31 (77.5%) were non-producers (Piras et al., 2015). Similarly, in poultry processing plants, 98 *Salmonella* spp. isolated from poultry processing plants cutting rooms showed 1 strong adherent, 28 intermediate adherent, and 69 weak adherent after 96 hours of incubation in LB broth at 35°C (Ziech et al., 2016). These studies demonstrate the wide variability in biofilm formation abilities among *Salmonella* serotypes, highlighting their capacity to grow and form biofilms at a range of temperatures, from strong biofilm formers to non-formers.

The selection of broth could significantly influence *Salmonella* biofilm formation. Lennox Broth no-salt (LB-NS) broth was used in our study, while tryptic soy broth (TSB) was used in most of the literatures. LB-NS broth contains tryptone and yeast extract. While both are considered enriched media, the LB-NS is chosen since it is prone to grow the biofilms over TSB broth. Another study confirmed a greater cell attachment on stainless steel in 1/10 diluted TSB than in full-strength TSB broth at 25°C (Dhakal et al., 2019). Additionally, the production of curli and cellulose contribute more in the biofilm formation in low nutrient conditions (1/20 TSB) than in nutrient-rich conditions (TSB) at 25°C (Castelijn et al., 2012). Since LB-NS used in our study is also a low-nutrient medium similar to the diluted 1/20 TSB, biofilm formation and the curli/cellulose production are expected to align with these findings.

The tested temperature and the result of strong biofilm formation of the SE strains isolated from poultry and poultry-related sources in the present study are important for the poultry industry and further emphasizes the importance of proper cleaning and sanitation procedures such as drubbing the food-contacting surface effectively, the use of detergents with the appropriate concentration, and the contact time of sanitizers to the food-contacting surface to avoid the formation of any biofilms regardless of their intensities.

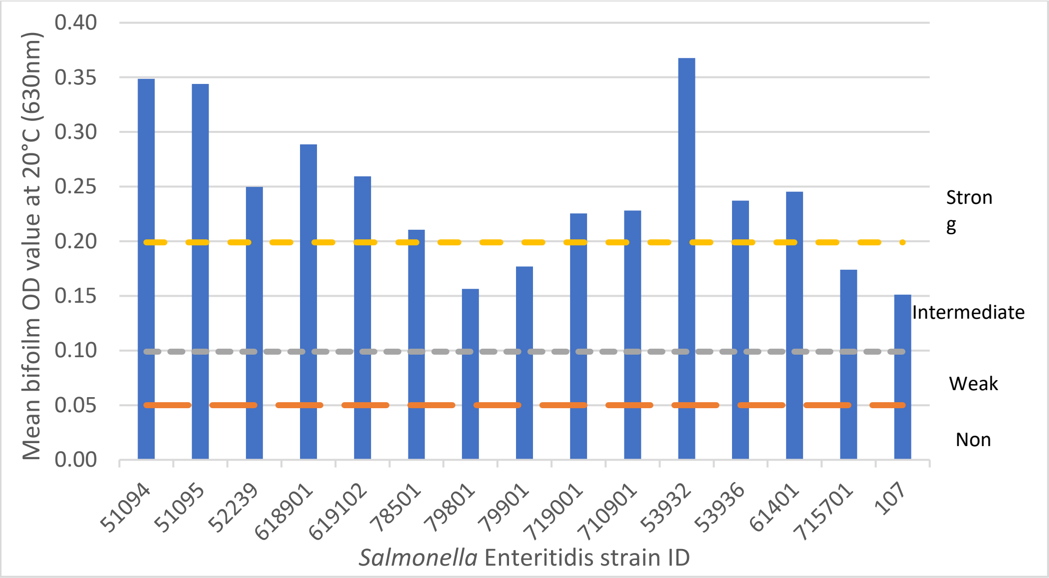

Figure 3-1: *Salmonella* Enteritidis strains biofilm formation at 22°C after 5 days. The biofilm formation ability is categorized by strong (above yellow line), intermediate (above grey line), weak (orange line), and non-biofilm formers (below orange line).

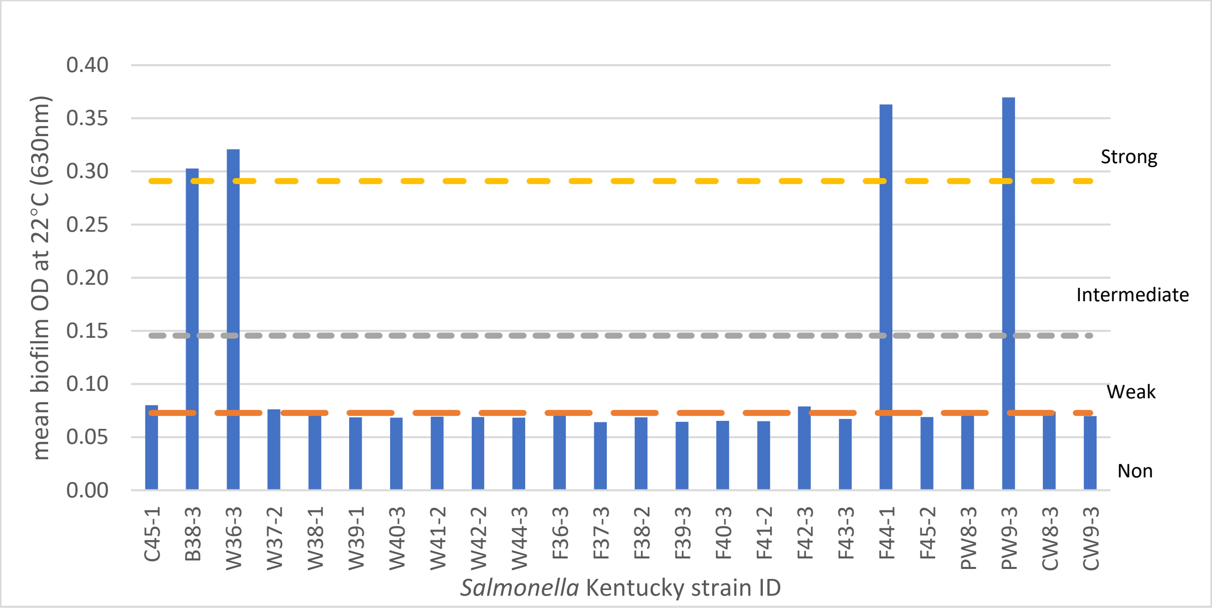

Figure 3-2: *Salmonella* Kentucky strains biofilm formation at 22°C after 5 days. The biofilm formation ability is categorized by strong (above yellow line), intermediate (above grey line), weak (orange line), and non-biofilm formers (below orange line).

### 3.4.2. Curli and cellulose phenotypic results for SE and SK strains

Curli and cellulose are two of the major components in the extracellular matrix, and their production have been reported as an essential component of *Salmonella* spp. biofilms (Gerstel & Römling, 2003; Solano et al., 2002). Curli fibers are involved in surface adhesion, cell aggregation, and biofilm formation, and are also the mediator for host cell adhesion and invasion (Barnhart & Chapman, 2006), and cellulose production helps with bacterial cell-to-cell interactions, biofilm formation, and chlorine resistance to the organism (Römling, 2007). Curli and cellulose contribute to the survival of *Salmonella* during long-term desiccation (White et al., 2006) and *Salmonella* biofilm phenotypes are usually correlated with the production of curli and cellulose (Ivana et al., 2015).

We analyzed the production of curli and cellulose in 15 *Salmonella* Enteritidis strains at 20°C. Results showed that 14/15 SE strains (93.3%) were positive for both curli and cellulose, with one exception, the strain #107. Interestingly, SE 107 was categorized as intermediate biofilm formation, perhaps impacted by the lack of curli and cellulose production. The other 3 intermediate SE strains all had positive results for curli and cellulose, despite their intermediate biofilm categories. Another study suggested that *Salmonella* spp. such as Typhimurium and Enteritidis produce highly regulated curli expression with RDAR morphology, which is only expressed at temperatures below 30°C (Gerstel & Römling, 2001).

For those 24 SK strains, 4/24 (16.7%) showed RDAR morphology and fluoresce for curli and cellulose production, respectively (Table 3-2), corresponding to the only 4 strong biofilm former strains. The rest of the 20 SK strains showed a new type of morphology for curli expression: smooth, red, and dry colonies (SRAD). This new morphotype was also seen in several research studies (Anriany et al., 2006; Turki et al., 2012) and was linked to diminished expression of curli structures caused by two insertion mutations in genes *ddhC* and *waaG* involved in lipopolysaccharide synthesis in *Salmonella* Typhimurium (Anriany et al., 2006). Gene *ddhc* (formerly *rfbH*), codes for CDP-4-keto-6-deoxy-D-glucose-3-dehydrase, is responsible for synthesizing abequose, the last sugar component of the O antigen in LPS. Gene *waaG* (formerly *rfaG*), encodes UDP-glucose-LPS α1,3-glucosyltransferase, an enzyme that adds a glucose molecule to heptose II of the core polysaccharide of LPS (Anriany et al., 2006). Both mutations result in decreased levels of curli while increasing cellulose productions. In the present study, the gene *waaG* was present in all 15 SE and 24 SK strains, while the gene *ddhC* was absent in all strains.

Table 3-2. *Salmonella* Enteritidis and *Salmonella* Kentucky Strains used in testing biofilm formation (at 20-22°C), their sources, and their phenotypic results.

| Strain | Strain ID | Source | Biofilm | OD value | Standard deviation | Curli | Cellulose |
|---|---|---|---|---|---|---|---|
| *Salmonella* Enteritidis | 51094 | Chicken nuggets | Strong | 0.3487 | 0.048 | + | + |
| *Salmonella* Enteritidis | 51095 | Chicken nuggets | Strong | 0.3440 | 0.043 | + | + |
| *Salmonella* Enteritidis | 52239 | Chicken nuggets | Strong | 0.2498 | 0.031 | + | + |
| *Salmonella* Enteritidis | 618901 | Chicken nuggets | Strong | 0.2888 | 0.132 | + | + |
| *Salmonella* Enteritidis | 619102 | Chicken nuggets | Strong | 0.2595 | 0.154 | + | + |
| *Salmonella* Enteritidis | 78501 | Chicken nuggets | Strong | 0.2107 | 0.012 | + | + |
| *Salmonella* Enteritidis | 79801 | Chicken nuggets | Intermediate | 0.1565 | 0.010 | + | + |
| *Salmonella* Enteritidis | 79901 | Chicken nuggets | Intermediate | 0.1768 | 0.016 | + | + |
| *Salmonella* Enteritidis | 719001 | Chicken nuggets | Strong | 0.2255 | 0.123 | + | + |
| *Salmonella* Enteritidis | 710901 | Chicken nuggets | Strong | 0.2282 | 0.059 | + | + |
| *Salmonella* Enteritidis | 53932 | Chicken breast | Strong | 0.3678 | 0.073 | + | + |
| *Salmonella* Enteritidis | 53936 | Chicken breast | Strong | 0.2373 | 0.032 | + | + |
| *Salmonella* Enteritidis | 61401 | Feed | Strong | 0.2453 | 0.026 | + | + |
| *Salmonella* Enteritidis | 715701 | Feed | Intermediate | 0.1740 | 0.004 | + | + |
| *Salmonella* Enteritidis | 107 | Bovine | Intermediate | 0.1512 | 0.013 | - | - |
| *Salmonella* Kentucky | C45-1 | Crate | Weak | 0.0800 | 0.024 | - | - |
| *Salmonella* Kentucky | B38-3 | Plucking belt | Strong | 0.3027 | 0.162 | + | + |
| *Salmonella* Kentucky | W36-3 | Carcass wash/Pre-chill | Strong | 0.3207 | 0.141 | + | + |
| *Salmonella* Kentucky | W37-2 | Carcass wash/Pre-chill | Weak | 0.0762 | 0.023 | - | - |

| | | | | | |
|---|---|---|---|---|---|
| *Salmonella* Kentucky | W38-1 | Carcass wash/Pre-chill | Non | 0.0708 | 0.009 |
| *Salmonella* Kentucky | W39-1 | Carcass wash/Pre-chill | Non | 0.0685 | 0.009 |
| *Salmonella* Kentucky | W40-3 | Carcass wash/Pre-chill | Non | 0.0683 | 0.009 |
| *Salmonella* Kentucky | W41-2 | Carcass wash/Pre-chill | Non | 0.0691 | 0.010 |
| *Salmonella* Kentucky | W42-2 | Carcass wash/Pre-chill | Non | 0.0689 | 0.014 |
| *Salmonella* Kentucky | W44-3 | Carcass wash/Pre-chill | Non | 0.0683 | 0.014 |
| *Salmonella* Kentucky | F36-3 | Final wash/post chill | Non | 0.0712 | 0.016 |
| *Salmonella* Kentucky | F37-3 | Final wash/post chill | Non | 0.0640 | 0.008 |
| *Salmonella* Kentucky | F38-2 | Final wash/post chill | Non | 0.0684 | 0.011 |
| *Salmonella* Kentucky | F39-3 | Final wash/post chill | Non | 0.0643 | 0.009 |
| *Salmonella* Kentucky | F40-3 | Final wash/post chill | Non | 0.0653 | 0.009 |
| *Salmonella* Kentucky | F41-2 | Final wash/post chill | Non | 0.0649 | 0.009 |
| *Salmonella* Kentucky | F42-3 | Final wash/post chill | Weak | 0.0787 | 0.022 |
| *Salmonella* Kentucky | F43-3 | Final wash/post chill | Non | 0.0670 | 0.010 |
| *Salmonella* Kentucky | F44-1 | Final wash/post chill | Strong | 0.3630 | 0.183 |
| *Salmonella* Kentucky | F45-2 | Final wash/post chill | Non | 0.0689 | 0.010 |
| *Salmonella* Kentucky | PW8-3 | Plucking water | Weak | 0.0734 | 0.009 |
| *Salmonella* Kentucky | PW9-3 | Plucking water | Strong | 0.3697 | 0.195 |
| *Salmonella* Kentucky | CW8-3 | Chill water | Weak | 0.0739 | 0.014 |
| *Salmonella* Kentucky | CW9-3 | Chill water | Non | 0.0698 | 0.012 |

### 3.4.3. Phylogenetic tree of the SE and SK strains

#### 3.4.3.1. Phylogenetic tree of the SE strains

The phylogenetic tree analysis of the 15 SE strains revealed two major clades: blue and green clades (Figure 3-3). Strain 107, represented by a red branch, did not belong to either clade due to its distinct genetic makeup, making it an outgroup. An outgroup is any strain that is not assumed to belong to the clade of interest (Abaza, 2020). Strain 107 was more distantly related to the other SE strains, which aligns with its bovine origin rather than poultry. The blue clade consists of strains collected from chicken nuggets and feed, while the green clade consists of strains isolated from chicken nuggets, feed, and chicken breast meat. All the strains in the green clade were strong biofilm-formers with positive curli and cellulose results, while half of the strains (n=3) in the blue clades were intermediate biofilm-formers which also have positive curli and cellulose results.

The bootstrap values, represented as percentages in the tree (Figure 3-3) indicate how many times the same branch is observed when the phylogenetic tree is repeated 100 times (Ojha et al., 2022). A bootstrap value of 100% indicates that this arrangement was observed every time, confirming that the result is reliable and not due to random chance (Ojha et al., 2022). For example, strain 53936 (from chicken breast) and 61401 (from chicken feed) are closely related, with a bootstrap value of 100%. Similarly, the clade containing strain 619102 and strain 618901 (both from chicken nuggets) is also strongly supported with a 100% bootstrap value. Strain 51095 and strain 51094 (both from chicken nuggets) show a high degree of similarity, with a 91% bootstrap value. In contrast, the clades in the green group had relatively low bootstrap value (<85%), indicating a less confident branch grouping compared to those with 99% or 100% values.

The horizontal lines in the tree represent the amount of genetic change and evolutionary lineages change over time, with the branch length representing the number of substitutions per 100 nucleotide sites (Rambaut, 2018). The shorter the horizontal lines indicate fewer genetic base differences along the sequences, suggesting closer relatedness between the strains. Strain 107 (from bovine) has a larger number of base differences (4.7e-5 substitutions per site) compared to the rest of the tree branches, reflecting its more distinct genomic profile compared to the other strains. This difference is consistent with strain 107 being the only one with negative curli and cellulose production results. Furthermore, strains within the smallest clade, which have a branch length of 1.0e-6, show no divergence.

This phylogenetic tree result could help us understand the phenotypic and genomic similarities among the strains. For example, among the 15 SE strains, 4 were intermediate biofilm formers (shown in the grey squares). Apart from strain 107, the other 3 intermediate strains (715701, 79901, and 79801) belonged to the same clade (Figure 3-3).

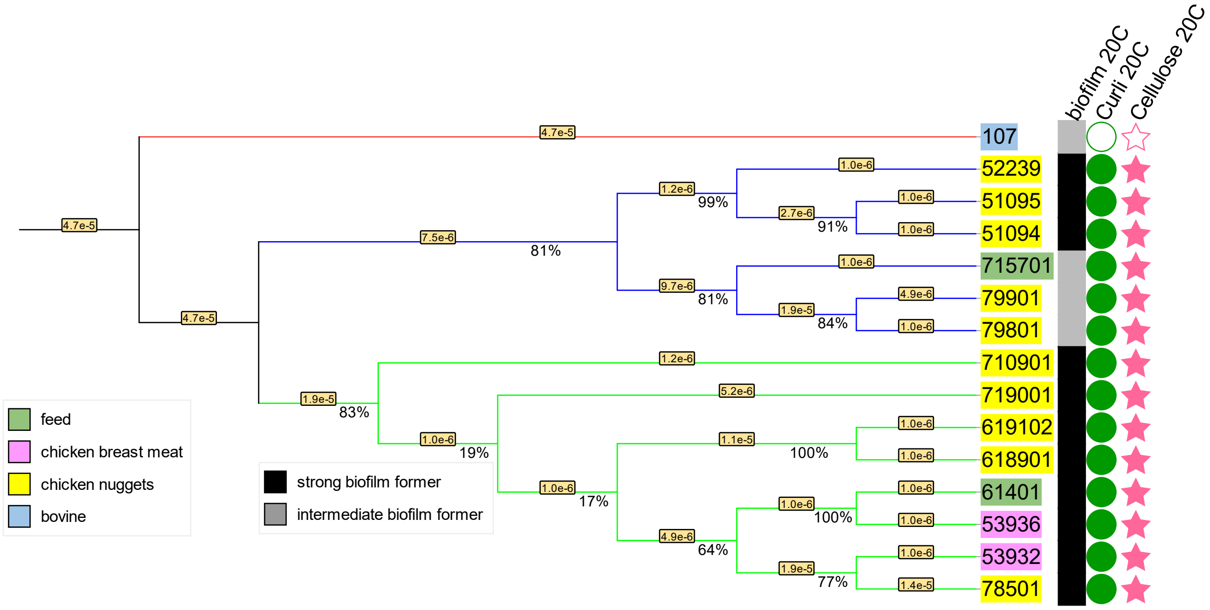

Figure 3-3: Phylogenetic tree of 15 *Salmonella* Enteritidis strains isolated from poultry samples using 500 single-copied genes parameter with the alignment program called mafft and the branch supporting method called RAxML Fast Bootstrapping. The tree was divided into two clades and an outgroup. The numbers in percentages indicate how confident is this way of clustering, and the number in the horizontal line represent the closeness of those strains. The strains ID were color-coded to show the sources of isolation. The black squares indicate strong biofilm formers and grey square indicate intermediate biofilm formers. For the curli and cellulose results, solid-colored shapes represent positive results, while non-colored shapes indicate negative results.

### 3.4.3.2. Phylogenetic tree of the SK strains

The phylogenetic tree of the 24 *Salmonella* Kentucky strains, generated using the BV-BRC codon tree (Figure 3-4), showed that the strains were grouped into three major clades in red, blue and green color. The letters in the strain IDs represent their isolation locations: C=crate; B=Plucking belt; W=Carcass wash/pre-chill; F=Final wash/post chill; PW=Plucking water; CW=Chill water.

Compared to the SE strains (Figure 3-3), the SK strains appear more closely related, as indicated by the horizontal branch distance (the numbers of substitutions). Strains from various isolation sources were clustered in different clades. Four of the 24 SK strains were strong biofilm producers, showing positive curli and cellulose production results. However, despite having similar phenotypic results, these four strains were scattered across different clades. The bootstrap values for the smaller clades are relatively low compared to the major clades, suggesting less confidence in their grouping. The branch length of 1.0e-6 suggested nearly no divergence between the strains, corresponding with the result that some strains are clonal. From the results of the phylogenetic tree, no clear patterns emerge that link between the phylogenetic tree and the phylotypic results. Although *Salmonella* Kentucky strains are considered polyphyletic, it is important to note that the SK strains tested in our study are monophyletic. This means they have many evolutionary origins, making it less likely for them to cluster closely together in a phylogenetic tree (Haley et al., 2016).

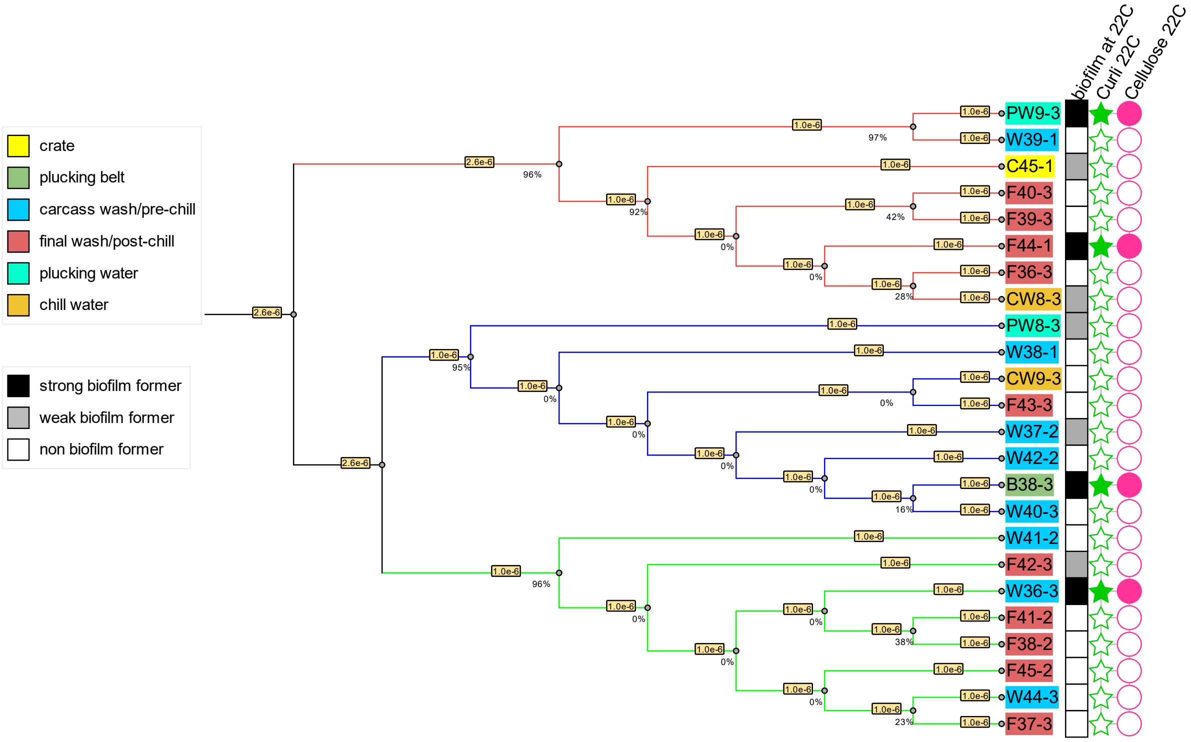

Figure 3-4: Phylogenetic tree of 24 SK strains using 500 single-copied genes parameter with the alignment program called mafft and the branch supporting method called RAxML Fast Bootstrapping. The tree was divided into three clades. The numbers in percentages indicate how confident is this way of clustering, and the number in the horizontal line represent the closeness of those strains. The strains ID were color-coded to show the sources of isolation. The black squares indicate strong biofilm formers; grey squares indicate weak biofilm formers; and white squares mean non-biofilm formers. For the curli and cellulose results,

solid-colored shapes represent positive results, while non-colored shapes indicate negative results.

#### 3.4.3.3. Phylogenetic tree containing both SE and SK strains

The phylogenetic tree with 15 SE and 24 SK strains comparing 500 randomly selected core genes (Figure 3-5), revealed two distinct major clades marked in red and blue. All strains within the red clade were identified as *Salmonella* Enteritidis, while those in blue clade belonged to *Salmonella* Kentucky. The branch length of 5.0e-3 suggested a relatively divergent relationship between *Salmonella* Enteritidis and *Salmonella* Kentucky, indicating that these two serovars share less genetic similarity compared to strains within the serovar itself. This clade pattern is supported by the phylogenetic tree created by Dhanani et al, with *Salmonella* Typhimurium, Enteritidis, Tennessee, Typhi, Kentucky, Hadar, Heidelberg, *Escherichia fergusonii, coli,* and *E. albertii* isolates (Dhanani et al., 2015). In their study, most serovars were strongly clustered together except for Enteritidis, which was scattered across the tree. The tree in their study showed a clear distinction between serotype Kentucky and Enteritidis except for one Enteritidis isolate, and a similar pattern was observed in our phylogenetic tree Figure 3-5. In the present study, our SE and SK strains were also genetically distinct, certain strains within *Salmonella* Kentucky show close genetic relationships, suggesting clonal propagation among those strains.

Although the strains in this study were isolated from different locations, it was unexpected that strains from the same location did not cluster together. It is possible that the strains scattering by locations indicates that these strains could be highly adaptable, and be able to survive in diverse environments within the processing facility. This adaptability could lead to their persistence, which refers to the ability of *Salmonella* strains to survive and remain active in harsh environments after a long time (Podolak et al., 2010).

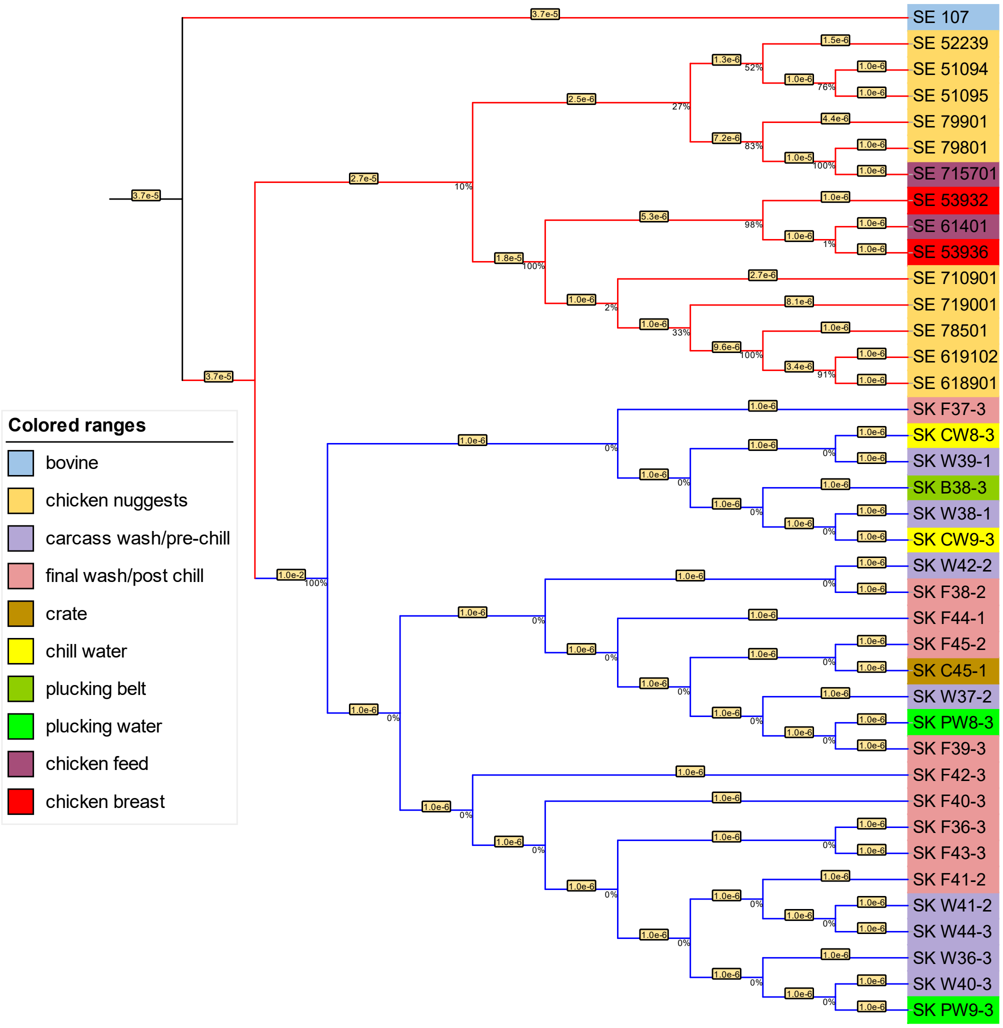

Figure 3-5: Phylogenetic tree of 15 SE strains and 24 SK strains using 500 single-copied genes parameter with the mafft alignment program and RAxML Fast Bootstrapping branch supporting method. The tree was divided into two clades. The red clade has all 15 SE strains clustered together, and the blue clade has all 24 SK strains clustered together. The numbers in percentages indicate how confident is this way of clustering, and the number in the horizontal line as number of nucleotide changes or substitutions per site, represent the closeness of those strains. The strain IDs were color-coded to show their sources of isolation.

### 3.4.4. Genomic comparison between different biofilm formation categories and between serotypes

#### 3.4.4.1. Comparative genomic analysis results with SE and SK strains obtained from BV-

### BRC comparative system

Using the BV-BRC comparative system and heatmapping, a pan-genome comprising 5,396 protein-coding genes was constructed. Of these, 4,043 of them were identified as core genomes and 1,353 of them were accessory genomes. A total of 122 protein coding genes associated with biofilm formation, including those related to curli, cellulose, fimbriae, pilus, flagella, and type IV secretion systems, were examined in these genomes, as listed in Table 3-3. In addition, to see the differences between the core genomes and accessory genomes, a heatmap was generated by filtering protein-coded genes that were in the strong SE and SK strains but absent in the other strains. A total of 67 protein coding genes were generated (Figure 3-6). In which, 12 of those (CspF/H, DNA topoisomerase III, Modification methylase NgoFVII, mRNA interferase RelE, Plasmid encoded restriction endonuclease Per, Resolvase, VirB1, VirB4, VirB10, VirB6, VirB11, VirD4) had specific functions while the rest 55 of those were hypothetical proteins.

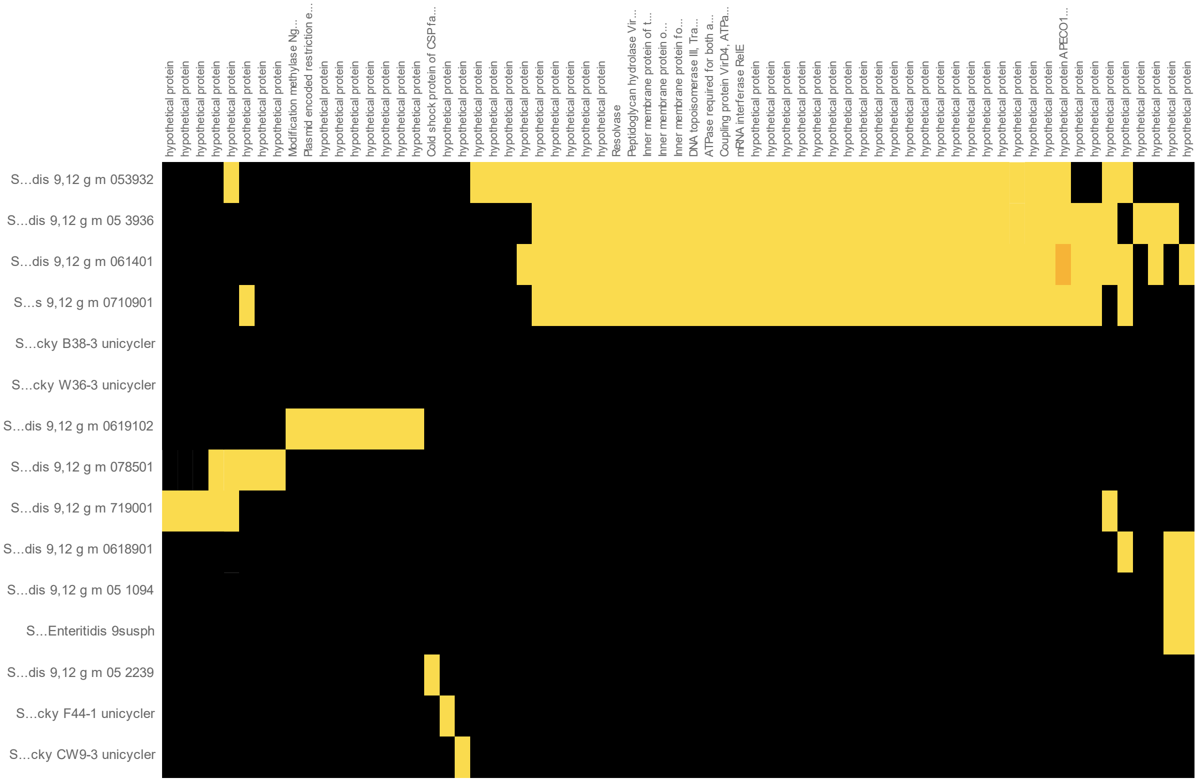

Figure 3-6: Heatmap of the presence of protein-coded genes found in 15 strong SE and SK strains that are absent in all the rest *Salmonella* strains (strains that were intermediate, weak, or non-biofilm forming).

Table 3-3: Screened genes, protein groups associated with biofilm formation, curli and cellulose production, fimbria, flagellum, pilus and type IV secretion system, and their functions (functions were provided by the BV-BRC website).

| | | | **Number of copies** | | |
|---|---|---|---|---|---|
| **Gene category** | **Name** | **Function** | 15 SE | 24 SK | Strain |
| Biofilm regulation protein coding genes | Cyclic-di-GMP-binding biofilm dispersal mediator protein | | 1 | 1 | |
| | BssS/BssR | Biofilm regulator | 1 | 1 | |
| | YjgK | Linked to biofilm formation | 1 | 1 | |
| | BapA | A large cell-surface protein required for biofilm formation by S. Enteritidis | 0 | 0 | |
| Curli production | CsgA | Major curlin subunit precursor | 1 | 1 | |
| | CsgB | Minor curlin subunit, nucleation component of curlin monomers | 1 | 1 | |
| | CsgC | Putative curli production protein | 1 | 1 | |
| | CsgD | Transcriptional regulator for 2nd curli operon | 1 | 0(1/24)<br>1(23/24) | 0 copy: F41-2 non |
| | CsgE/CsgF | Curli production assembly/transport component | 1 | 0(1/24)<br>1(23/24) | 0 copy: F41-2 non |
| | CsgG | Curli production assembly/transport component | 1 | 1 | |
| | OmpR | Two-component system response regulator | 1 | 1 | |
| | RpoS | RNA polymerase sigma factor | 1 | 1 | |
| | Regulator of RpoS | | 1 | 1 | |

| | | | | | |
|---|---|---|---|---|---|
| | Crl | An RpoS-binding factor, binding to RpoS facilitates RNA polymerase holoenzyme formation (EσS) | 0 | 0 | |
| | Curlin genes transcriptional activator | | 1 | 1 | |
| | MlrA | HTH-type transcriptional regulator; Positive regulator of CsgD expression | 1 | 1 | |
| | DdhC | Involved in the synthesis of abequose | 0 | 0 | |
| | WaaG | UDP-glucose:(heptosyl) LPS alpha1,3-glucosyltransferase | 1 | 1 | |
| Cellulose production | BcsA | Cellulose synthase catalytic subunit | 1 | 1 | |
| | BcsB | Cyclic di-GMP-binding protein | 1 | 1 | |
| | BcsC | Cellulose synthase operon protein C | 2(3/15)<br>1(12/15) | 1 | 2 copies: SE 79801, 79901, 715701 (all intermediate) |
| | BcsE/BcsF/BcsG/ BcsQ | Cellulose biosynthesis protein | 1 | 1 | |
| | AdrA (DgcC) | Diguanylate cyclase, positively regulates cellulose synthesis via production of the secondary messenger signaling molecule (3′ -5′ )-cyclic diguanosine monophosphate (c-di-GMP) | 1 | 1 | |
| Fimbriae production | StbC*/StdB*/SthB*/StiC* | Fimbriae usher protein | 1 | 1 | |
| | SthA*/StiB* | Putative fimbrial chaparone | 1 | 1 | |
| | StiA* | Putative fimbrial subunit | 1 | 1 | |

| | | | | |
|---|---|---|---|---|
| SafB* | *Salmonella* atypical fimbria periplasmic chaperone | 1 | 1 | |
| FimI* | Fimbriae-like adhesin | 1 | 1 | |
| FimW* | Fimbriae W protein | 1 | 1 | |
| FimY*/FimZ* | Transcriptional regulator of fimbriae expression | 1 | 1 | |
| BcfA*/BcfD*/BcfE*/BcfF* | Fimbrial subunit | 1 | 1 | |
| BcfB*/BcfG* | Fimbrial chaperone | 1 | 1 | |
| StfA | Major fimbrial subunit | 1 | 1 | |
| StfC | Fimbriae usher protein | 1 | 1 | |
| StfD | Periplasmic fimbrial chaperone | 1 | 1 | |
| StfE/StfF | Minor fimbrial subunit | 1 | 1 | |
| StfG | Minor fimbrial subunit | 1 | 1 | |
| SfmA/SfmH/YehD | Uncharacterized fimbrial-like protein | 1 | 1 | |
| SfmF | Uncharacterized fimbrial-like protein | 1 | 1 | |
| SfmC/YehC | Probable fimbrial chaperone | 1 | 1 | |
| YehA | Uncharacterized fimbrial-like protein | 2(6/15)<br>1(9/15) | 1 | Two copies: Strong (51094, 51095, 52239). Intermediate (79801, 79901, 715701) |
| YadK | Uncharacterized fimbrial-like protein | 0 | 1 | |
| YadU | Uncharacterized protein in stf fimbrial cluster | 1 | 1 | |
| YhcA | Uncharacterized fimbrial chaperone | 0 | 1 | |

| | | | | |
|---|---|---|---|---|
| | EcpD | Fimbria adhesin | 0 | 1 |
| | PilA | Type IV pilin | 1 | 1 |
| | PilB | Type IV pilus assembly, ATPase | 1 | 1 |
| Pilus | PilC | Type IV pilus assembly protein | 1 | 1 |
| | PilM/PilN/PilO/PilP/PilQ | Type IV pilus biogenesis protein | 1 | 1 |
| | FliC | Flagellin | 0 | 2 |
| | FliD | Flagellar cap protein | 1 | 1 |
| | FliE | Flagellar hook-basal body complex protein | 1 | 1 |
| | FliF | Flagellar M-ring protein | 1 | 1 |
| | FliG | Flagellar motor switch protein | 1 | 1 |
| | FliH | Flagellar assembly protein | 1 | 1 |
| | FliI | Flagellum-specific ATP synthase | 1 | 1 |
| | FliJ | Flagellar protein | 1 | 1 |
| Flagella | FliK | Flagellar hook-length control protein | 1 | 1 |
| | FliL | Flagellar basal body-associated protein | 1 | 1 |
| | FliM/FliN | Flagellar motor switch protein | 1 | 1 |
| | FliO/FliP/FliQ/FliR/FliS/FliT | Flagellar biosynthesis protein | 1 | 1 |
| | FlgA | Flagellar basal-body P-ring formation protein | 1 | 1 |
| | FlgB/FlgC/FlgF/FlgG | Flagellar basal-body rod protein | 1 | 1 |
| | FlgD | Flagellar basal-body rod modification protein | 1 | 1 |

| | | | | | |
|---|---|---|---|---|---|
| | FlgE | Flagellar hook protein | 1 | 1 | |
| | FlgH | Flagellar L-ring protein | 1 | 1 | |
| | FlgI | Flagellar P-ring protein | 1 | 1 | |
| | FlgJ | Flagellar protein | 1 | 1 | |
| | FlgK/FlgL | Flagellar hook-associated protein | 1 | 1 | |
| | FlgM | Negative regulator of flagellin synthesis | 1 | 1 | |
| | FlgN | Flagellar biosynthesis protein | 1 | 1 | |
| | FlhA/FlhB | Flagellar biosynthesis protein | 1 | 1 | |
| | FlhC/FlhD | Flagellar transcriptional activator | 1 | 1 | |
| | FlhE | Flagellar protein | 1 | 1 | |
| | MotA/MotB | Flagellar motor rotation protein | 1 | 1 | |
| | YcgR | Flagellar brake protein | 1 | 1 | |
| | flk | Flagellar regulator | 1 | 1 | |
| | RtsA | Type III secretion and flagellar regulator | 1 | 1 | |
| | RtsB | Flagellar regulon repressor | 1 | 1 | |
| | Chemotaxis regulator | Transmits chemoreceptor signals to flagellar motor components CheY | 1 | 1 | |
| | RNA polymerase sigma factor for flagellar operon | | 1 | 1 | |
| Type IV secretion system | NAD(P)H dehydrogenase (quinone) | ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex | 1 | 1 | |
| | VirB1 | Peptidoglycan hydrolase, involved in T-DNA transfer | 1 (4/15)<br>0 (11/15) | 0 | One copy: SE |

| | | | | |
|---|---|---|---|---|
| VirB3 | Inner membrane protein forms channel for type IV secretion of T-DNA complex | 1 (4/15)<br>0 (11/15) | 0 | 53936, 53932, 61401, 710901<br>(all strong) |
| VirB4 | ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex | 1 (4/15)<br>0 (11/15) | 0 | |
| VirB6 | Inner membrane protein of type IV secretion of T-DNA complex | 1 (4/15)<br>0 (11/15) | 0 | |
| VirB10 | Inner membrane protein of type IV secretion of T-DNA complex, TonB-like | 1 (4/15)<br>0 (11/15) | 0 | |
| VirB11 | ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex | 1 (4/15)<br>0 (11/15) | 0 | |
| VirD4 | Coupling protein, ATPase required for T-DNA transfer | 1 (4/15)<br>0 (11/15) | 0 | |

Comparative genomic analysis was conducted to study the genetic differences between *Salmonella* Enteritidis and *Salmonella* Kentucky strains regarding their biofilm formation ability. Our comparative analysis revealed that all 39 *Salmonella* strains harbored almost all of the genes responsible for encoding proteins related to biofilm formation, curli, and cellulose expression, flagella, fimbriae, pilus, and type IV secretion systems regardless of their biofilm category, curli/cellulose production, or isolation sources. Overall, no clear pattern was found between the presence or absence of the genes and their biofilm formation phenotypic results. Several essential genes were absent in all 15 SE and 24 SK strains, including *bapA*, *crl*, and *ddhC*. Some proteins were absent only in the SE strains but present in the SK strains, including YadK, YhcA, and EcpD. Genes *csgDEF* were lacking in a non-biofilm forming SK strain F41-2, and type IV secretion system proteins VirB1, VirB3, VirB4, VirB6, VirB10, VirB11, and VirD4 were absent in all 24 SK strains but present in 4 strong-biofilm forming SE strains.

Interestingly, strain #107, the only bovine strain and the only strain with negative results for both curli and cellulose production, still contained all the screened genes except those type IV secretion system genes, which were only present in 4 strong SE biofilm formers (Table 3-3). Therefore, based on the heatmap analysis of gene presence or absence, no clear conclusions regarding which specific genes contribute to strong-biofilm formation were obtained.

All 39 *Salmonella* strains carried four protein-coding genes linked to biofilm formation regulation: *bssS* and *bssR* (biofilm regulators), YjgK (an uncharacterized protein linked to biofilm formation), and the cyclic-di-GMP-binding biofilm dispersal mediator protein (Table 3-3). The genes *bssS* and *bssR* downregulate the biofilm formation, reducing biofilm mass, surface coverage, and mean thickness of the biofilm in *Salmonella* and *E. coli* (Chin et al., 2017; Domka et al., 2006). Overexpression of *yjgK* represses fimbrial genes, decreasing biofilm formation at 8 hours of incubation but increasing it at 24 hours. Cyclic-di-GMP regulates biofilm dynamics antagonistically: it suppresses motility, single-cell dispersal, and virulence gene expression in the single-cell states while promoting sessility, biofilm formation, and adhesion factor expression in multicellular states (Jenal & Malone, 2006). Notably, BapA, a cell-surface protein, essential for biofilm formation, was absent in all 39 strains. Its deletion eliminates biofilm-forming capacity, while overexpression enhances it. However, Badie et al. reported that the temperature does not directly influence *bapA* expression in *Salmonella* Enteritidis is not directly influenced by temperature (Badie et al., 2021).

The *csg* (<u>c</u>urli <u>s</u>pecific <u>g</u>ene) family, essential for curli and cellulose production (Brombacher et al., 2006; Gerstel & Römling, 2003), was analyzed across SE and SK strains. Genes *csgA*-*csgG* were present in all 15 SE strains and 23 out of 24 SK strains, while genes *csgDEF* were absent in the SK F41-2 strain, a non-biofilm former. Two key operons regulate curli production: *csgBAC* and *csgDEFG* (*agf* in *Salmonella* species is homologous to *csg* in *E. coli* (U. Römling, W. D. Sierralta, et al., 1998)). Among them, *csgA* (major), *csgB* (minor) subunits, and *csgD* (transcriptional regulator) are particularly crucial. Gene *csgA* directly contributes to curli fiber formation, a key factor in biofilm adhesion and stability (Tan et al., 2016), while *csgB* stabilizes *csgA* expression and promotes the initial aggregation of *csgA* to form curli fibers (Hammar et al., 1995). Gene *csgC* and genes *csgEFG* function as accessory genes involved in csgA polymerization (Bhoite et al., 2019) and in the assembly, secretion and structural integrity of curli fibers (Chapman et al., 2002). The absence of *csgD* in SK strain F41-2 explains its non-biofilm-forming phenotype. Several proteins that regulate *csgD* expression were found in our *Salmonella* genomes: RpoS, OmpR, Crl, and MlrA. The transcription of *csgD* was completely dependent on the expression of RpoS and OmpR (U. Römling, Z. Bian, et al., 1998; Römling et al., 2000; U. Römling, W. D. Sierralta, et al., 1998), both of which were present in all 39 *Salmonella* strains. However, Crl, an RopS-binding factor, was absent in all 39 strains. Crl protein interacts with RpoS and it is required for the maximal expression of the *csgD*, *csgB*, *adrA*, and *bcsA* genes, which are essential for the curli and cellulose synthesis (Robbe-Saule et al., 2006). MlrA, present in all stains, is a novel transcriptional regulator of *csgD*, acts as a positive regulator of RpoS-dependent curli production in *Salmonella* Typhi and *E. coli* (Brown et al., 2001). Interestingly, while all non-biofilm formers (except SK F41-2, a non-biofilm former, negative for both curli and cellulose) harbored all seven *csg* genes, only 4 out of 24 SK strains exhibited the red, dry, and rough (RDAR) curli morphotype, which typically emerges during the early stationary growth phase with *csgD* expression (Römling et al., 2000). This discrepancy raises the possibility that some genes were present but non-functional or not expressed. To investigate further, a single nucleotide polymorphism (SNP) variation analysis was conducted to identify potential genomic mutations, which will be discussed in the later sections.

In the present study, genes *bcsABCEFGQ* (responsible for the cellulose production) were present in all 15 SE and 24 SK strains, with three SE strains isolated from poultry (strain 079801, 079901, and 715701), all intermediate biofilm-forming strains) carrying two copies of *bcsC*. BcsC is a periplasmic protein with an N-terminal alpha-helical domain that interacts with peptidoglycan to organize the whole cellulose secretion complex (Römling & Galperin, 2015). Gene duplication can create genetic novelty in organisms and is usually caused by unequal crossing over, retroposition or chromosomal/genome duplication as well as providing new genetic material for mutation (Magadum et al., 2013). Gene duplication can enhance bacterial survival under stress conditions, such as nutrient limitation, extreme temperatures, high salinity, heavy metals, antibiotics, and pesticides (Kondrashov, 2012). In the present study, 3 out of 4 weaker (intermediate) biofilm SE strains carried a duplicated *bcsC* gene, indicating that gene duplication may reduce the biofilm-forming ability of *Salmonella* Enteritidis strains. However, *bcsZ* (encoding an endoglucanase) and *yhjR* (encoding a putative cytoplasmic protein) were absent in all strains. Although *bcsZ* was missing, previous study has shown that *bcsA*, *bcsB*, and *bcsZ* encode for the catalytic subunit of the cellulose synthase: bcsA binds the activator cyclic diguanylic acid, *bcsB* encodes for a cyclic-di-GMP binding protein, and *bcsZ* function as an endoglucanase, respectively (Solano et al., 2002). Both BcsA and BcsB are catalytic subunits required from *bcs* enzyme activity (Römling & Galperin, 2015). Similarly, deletion of *bcsG* significantly decreases bcsA levels and cellulose production (Sun et al., 2018), while deletion of *bcsQ* and *yhjR* resulted in no or reduced cellulose biosynthesis in *E. coli* (Serra et al., 2013). Shi et al. compared *csgD* expression between SE and SK strains and found that SK strains exhibited lower level of gene expression *csgD* expression despite similar *bcsA* expression levels (Shi et al., 2019). This may explain why most SE strains in the present study formed intermediate or strong biofilms, while most SK strains were non-biofilm producers. The *adrA* gene (other name *dgcC* (Hengge et al., 2016) ) encodes a diguanylate cyclase protein was present in all 15 SE and 24 SK stains. The activation of transcription of *adrA* stimulates CsgD and indirectly regulates cellulose production in the biofilms (Robbe-Saule et al., 2006; Römling et al., 2005). Although *bcs* operons are constitutively expressed, cellulose production occurs only when AdrA is produced (Zogaj et al., 2001). SNP variation analysis revealed genetic mutations in several *bcs* genes, which will be discussed in later sections.

Fimbriae and pilus genes were screened in SE and SK strains. Of the 35 fimbriae protein coding genes analyzed, 32 of them were present in all 15 SE and 24 SK strains. However, three proteins, YadK, YhcA, and EcpD, were absent in all SE strains but present in all SK strains (Table 3-3). The adhesion operon Yad has several genes include *yadK* (minor subunit). In *E. coli*, the fimbria Yad contributes to biofilm formation and bladder epithelial cells adherence (Spurbeck et al., 2011). YhcA is an uncharacterized fimbrial chaperone protein, while the *ecp* operon encodes *ecpD*, which is necessity for pilus assembly and functions as a polymerized tip adhesin (Garnett et al., 2012). The absence of these three genes in SE strains remains unexplained. YehA protein was found in both SE and SK, but had different amino acid length and had two copies in 6 SK strains (3 strong biofilm strains and 3 intermediate biofilm strains). Additionally, a total of eight Type IV pilin genes were present in all 15 SE and 24 SK strains, including *pilABCMNOPQ*, which encode bacterial appendages involved in adhesion and motility.

The flagellar filament and its motility in *Salmonella* plays an important role in the early development of biofilms, which is the initial attachment to the surface rather than contributing to the biofilm maturation (Steenackers et al., 2012). In our present study, a total of 45 flagellar-related protein coding genes were found, in which, 18 of them belong to the Fli family (FliC-FliT), 14 of them belong to the Fla family (FlgA-FlgN), 5 Flh proteins (FlhA-FlhE), motor rotation proteins (Mot A and MotB), flagellar regulator proteins (flk, RtsA and RtsB), chemotaxis regulator, and RNA polymerase sigma factor for flagellar operon. All of which were present in all 15 SE and 24 SK strains with one copy of each in each gene (Table 3-3). In the SNP variation analysis, several proteins had mutations in the SE strains, including MotA, MotB, FlgA, FlgB, FlgM, FlhC, FliT, and RtsB, which will be discussed in later sections.

To initiate host infections, *Salmonella* must first attach to and adhere to the surface of host cells. Once adhered, *Salmonella* releases bacterial factors that facilitate its invasion, enable intracellular multiplication, and help the bacteria evade the host's antimicrobial defense (Wiedemann et al., 2015). Virulence factors such as type III secretion system (T3SS) have been studied extensively regarding the biofilm formation in *Salmonella* strains. However, all of the protein coding genes associated with Type III secretion systems were present in all 15 SK and 24 SE strains in our study (Figure 5-1 in the complementary material). Nevertheless, interestingly, type IV secretion system proteins have shown different results. The type IV secretion system (T4SS) plays an important role in mediating the translocation of macromolecules across bacterial cell envelopes (Christie et al., 2005). Out of 8 proteins found in type IV secretion system, 1 of them (NAD(P)H dehydrogenase) was present in all SE and SK strains. Seven of them were only found in 4 strong-biofilm SE strains but absent in the rest of the 11 SE and all 24 SK strains. Those proteins include peptidoglycan hydrolase (VirB1), inner membrane protein (VirB3, VirB6, and VirB10), ATPase (VirB4 and VirB11), and coupling protein VirD4. This result suggests that these groups of type IV secretion system proteins may influence the biofilm formation or its related mechanism. Similar to the *Agrobacterium tumefaciens* VirB/D T4S system, the T4S system in Gram-negative bacteria typically consists of 12 proteins: VirB1-11 and VirD (Christie et al., 2005). Of those 12 core proteins, 7 of them (VirB1, VirB3, VirB4, VirB6, VirB10, VirB11, and VirD4) were detected in 4 of the 15 SE strains, but none was detected in the 24 SK strains (Table 3-3). To date, only a few *Salmonella* serotypes, such as *Salmonella* Enteritidis phage type 34, have been discovered with type IV secretion system (Chen et al., 2009). *Salmonella* Heidelberg strains carrying the VirB/D4 T4SS plasmid can survive in epithelial cells and macrophages better than those without the plasmid, and VirB/D4 T4SS encoding genes can downregulate certain host innate immune response genes which likely contributed to the increased survival within the epithelial cells and macrophages (Gokulan et al., 2013). The functions of type IV secretion system genes were still elusive and needs further investigation.

Based on the results from the comparative genomic analysis, it was hypothesized that some genes might have genetic mutations, preventing them from being expressed or functioning, even though they were present (Figure 3-7). Therefore, a SNP variation analysis was conducted to determine if these genes were mutated.

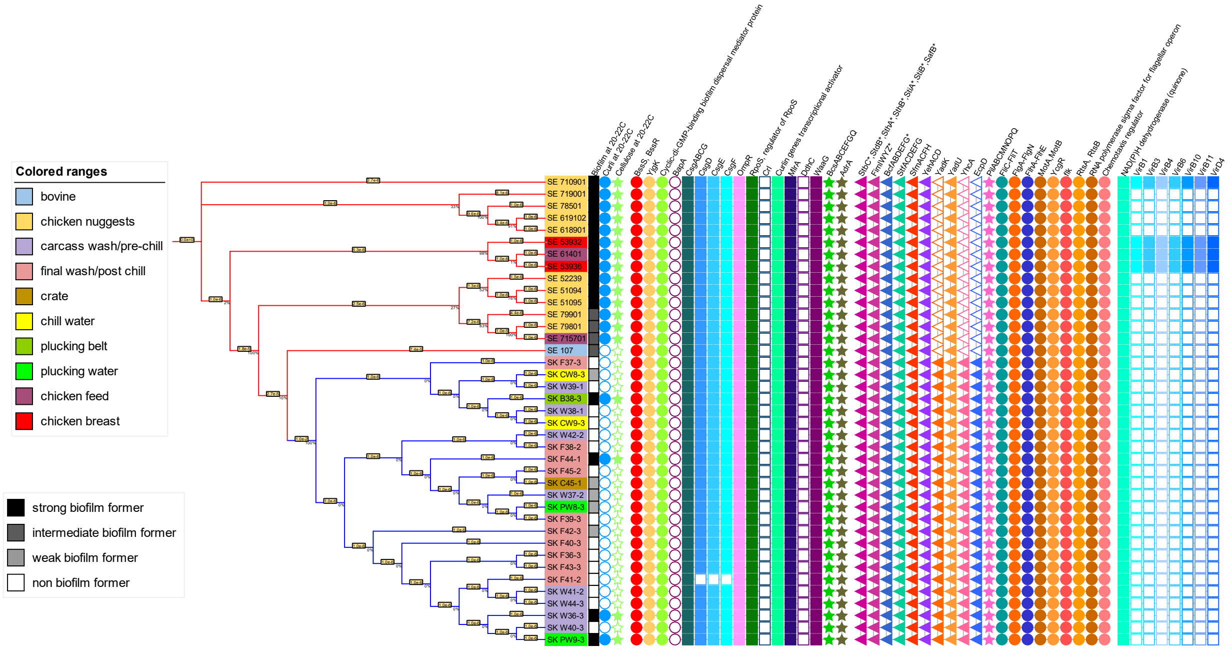

Figure 3-7 Biofilm formation results (the first black and white column) and binary data (starting from the column "Curli 20-22C" and onwards) of the biofilm-related genes in all 15 *Salmonella* Enteritidis strains and 24 *Salmonella* Kentucky strains. A phylogenetic tree from Figure 3-5 was uploaded to the iTol service and the genomes were color-coded to match different isolated sources. For the biofilm development assay (the first column), the darker the squares, the stronger the biofilms were. For the production of curli and cellulose (the second and the third column), a filled shape indicates a positive result and an empty shape indicates a negative result. For the genotypic results, the genes associated with biofilm formation, curli, cellulose, fimbriae, flagella, pilus, and type IV secretion system are shown with the gene names on the top of the image. Solid figures represent gene presence and empty ones, gene absence. The * in the gene name represents the 7 cluster groups in the flagella family.

### 3.4.5. SNP variation analysis

The variation analysis provides a comprehensive report of the potential mutations of the samples using SNPs. Since almost all the essential protein coding genes were present in almost all samples regardless of their biofilm phenotypes, it is vital to check their mutations on their functions, and the products of their upstream and downstream regulatory regions since genes could be present and only partially functioning.

Types of SNP variants may be found in the regulatory regions (RNA-polymerase and ribosomal binding regions), coding regions, and intergenic regions (regions between the genes). Non-coding DNA is DNA that does not encode protein sequences. Some non-coding DNA can be transcribed into RNA, and even though they do not code for proteins, they still are responsible for some regulations in gene expression. Coding region SNPs consist of synonymous and non-synonymous substitutions. Synonymous substitutions change one nucleotide in a codon while still having the same amino acids after translation and the mutations are usually silent. Nonsynonymous substitutions include missense and nonsense. Missense mutation means changing a single base and changing the amino acids possibly causing a malfunctioning protein. Nonsense mutation is a mutation that results in a stop codon causing a premature stop in the translation of a protein which could lead to a nonfunctional protein product.

#### 3.4.5.1. SNP variation analysis of 15 SE strains

The SNP variation analysis report showed the comparison of the 14 multiple-read samples of our *Salmonella* Enteritidis genomes and a closely related reference genome. The SE strain 53932 was selected to be the reference genome due to its strong biofilm-forming ability. The report was categorized into 4 different SNP impacts and 12 specific SNP types.

The results have generated a total of 62 high-impact variants, 417 moderate-impact variants, 243 low-impact, and 179 modifier-impact in all 14 SE strains. High impact variant is described as having a high (disruptive) impact on the protein, probably causing protein truncation, and loss of function. In addition, the high impact could result in one or more of the following consequences: start lost, frameshift variant, missense variant, stop gained, and splice region variant. Within those 62 high-impact variants, 23 were nonsynonymous including 5 stop-gained, 1 start-lost, and 7 stop-lost splice region variants; 16 were insertions with frameshift and missense variant; and 23 were deletions with frameshift variant. Moderate impact included 4 conservative inframe deletions, 4 conservative inframe insertions, 6 disruptive inframe deletions, 1 disruptive inframe insertion, and 401 missense variants. All of the low-impact are synonymous variants, and all modifier impacts are intergenic region variant types.

The functions, upstream and downstream features were found to be associated with biofilm formation, development, or associated with the major component needed for biofilm formation, for example, cellulose. The biofilm categories (strong, intermediate, weak, and non-biofilm producers), the production of curli and cellulose were all statistically significant and strongly associated with the different strains of *Salmonella* Enteritidis used in this study ($p<0.0001$). However, the biofilm categories were not statistically significant or strongly associated with the different SNP types ($p>0.0001$), while the production of curli and cellulose is strongly associated ($p<0.0001$). Neither the biofilm categories nor the production of curli and cellulose were yet strongly associated with the different SNPs impact ($p>0.0001$).

Three of the curli genes were found to have a moderate-impact nonsynonymous missense mutation on curli protein CsgF, its upstream feature CsgG, and its downstream feature CsgE in SE strain 52239 (a strong biofilm-former), changing the codon TCA (codes for Serine) into CCA (codes of Proline). As discussed previously in this thesis, *csgEFG* are all components for curli production assembly and transportation. These are considered accessory genes, which is probably why even with the missense mutation, strain 52239 was still a strong biofilm producer.

This research found genetic mutations in SE strains in essential proteins responsible for cellulose and curli biosynthesis (Table 3-4). As discussed in the cellulose section of this paper, *bcs* (bacterial cellulose synthesis) operon *yhjR-bcsQABZC* encodes gene *yhjR, bcsQ, bcsA, bcsB, bcsZ,* and *bcsC*, and operon *bcsEFG* encodes gene *bcsE, bcsF,* and *bcsG*. Out of 5 mutations associated with cellulose biosynthesis, 1 was stop-gained, 1 was frameshift deletion mutation, and 3 were synonymous variants. BcsG (cellulose biosynthesis protein) and its downstream feature BcsF (cellulose biosynthesis protein) had a low-impact synonymous variation in strain 107 (codon changed from GGG to GGA). BcsQ (cellulose biosynthesis protein) and its upstream feature YhjR (putative cytoplasmic protein) also had a low-impact synonymous variation in strain 107 (codon changed from GGT to GGC). As discussed previously, the deletion of gene *bcsQ* and *yhjR* could result in no or reduced cellulose production in *E. coli* (Serra et al., 2013). However, a synonymous variation should not alter the amino acid sequence (since both amino acids code for glycine). Thus, a variation from codon GGT to GGC does not change the protein being produced and should not change the function or performance of that protein. BcsA (cellulose synthase catalytic subunit), its upstream feature BcsQ (cellulose biosynthesis protein) and its downstream feature BcsB (cyclic di-GMP-binding protein) had a high-impact stop-gained nonsynonymous mutation with a codon changed from TGG to TAG. Stop codons signal the end of the translation process, when it occurs, it can result in a truncated (shortened) protein and disrupt the normal function of this protein. The stop-gained mutation was found in 6 SE strains (715701, 79801, 79901, 52239, 51094, and 51095), in which 3 of them (715701, 79801, and 79901) (Table 3-4) were intermediate biofilm formers. BcsC (cellulose synthase operon protein) had a high-impact frameshift deletion mutation in the 3 strains mentioned above and a low-impact synonymous variation in strain 107. Frameshift deletion mutation is missing nucleotides “cg” in the reading frame of the codons. Interestingly, *bcs* mutations were not seen in SK strains, where non-biofilm formers were predominant. These *bcs* genes were important in synthesizing cellulose in the matrix, as discussed in the cellulose section of this paper.

Yeh, Yad, and Yfc are all core-associated chaperon-usher fimbriae-related proteins that are conserved in *E. coli* and *Salmonella* strains (Wurpel et al., 2013). YehA (Uncharacterized fimbrial-like protein) and its upstream feature YehB (Outer membrane usher protein) had a high-impact frameshift insertion mutation in the same 6 strains mentioned above. Uncharacterized protein YadE, and its upstream feature, putative PTS system IIA component YadI, had a low-impact synonymous variations in strain 107. UPF0126 inner membrane protein YadE, and uncharacterized protein YadU in stf fimbrial cluster had a moderate-impact missense variant in strain 107 and the 3 strains mentioned above. Uncharacterized protein YfcL and YfcC both had low-impact synonymous variations in strain 107.

Flagella is another critical component in the biofilm formation. Several flagellar-associated genes were detected with genetic variations and mutations. Flagellar basal-body P-ring formation protein FlgA, its upstream feature, negative regular of flagellin synthesis FlgM, and its downstream feature, flagellar basal-body rod protein FlgB had a moderate-impact missense mutations in all 6 strains mentioned above, Flagellar motor rotation protein MotA, its upstream feature flagellar motor rotation protein MotB, and its downstream feature flagellar transcription activator FlhC had a low-impact synonymous variations in strain 107. Flagellar biosynthesis protein FliT had a missense mutation and flagellar regulon repressor RtsB had a synonymous variation both in strain 107.

#### 3.4.5.2. SNP variation analysis of 24 SK strains

The variation analysis report showed the comparison of 23 multiple-read samples of our *Salmonella* Kentucky genomes and a closely related reference genome. The SK strain PW9-3 isolated from plucking water was selected to be the reference genome due to its strong biofilm-forming ability. The results have generated a total of 0 high-impact variants, 19 moderate variants (all of which belong to nonsynonymous type missense variant), 53 low-impact variants (all of which belong to synonymous variant), and 39 modifier impact (with 2 insertion, 3 deletions, and the rest unspecified, and all of them belong to the intergenic region type).

From the total of 111 output of genetic variations and mutations with their function, upstream and downstream features generated from BV-BRC from the 24 *Salmonella* Kentucky strains, 43 possible genes, proteins, and enzymes were revealed within not only the functions but also the upstream and downstream that might have caused the mutations. Of those 43 potential candidates, 3 of them were selected upon screening after comprehensive reviews of the literature, and these include *tolA, tolB,* and, *tolR* (Table 3-4).

TolA and TolR are both part of the Tol-Pal system of the *E. coli* envelope which consists of the inner membrane proteins TolQ, TolR, TolA, periplasmic protein TolB, and outer membrane lipoprotein Pal (Cascales et al., 2000) and this system is involved in maintaining the integrity of the outer membrane (Walburger et al., 2002). More importantly, protein TolA can promote survival, biofilm formation, and virulence of avian pathogenic *E. coli* (Su et al., 2024). According to Su et. al., a *tolA* mutation with motility defect results in significantly less biofilm biomass, reduced amount of expression of flagellar biosynthesis gene *fliR*, and weakened resistance to environmental stresses. Additionally, *tolA* mutations in chickens can lead to reduced mortality and lower tissue bacterial burden (Su et al., 2024). The regulation of the fumarate and nitrate reductase (*fnr*) gene (present in all 39 strains, essential in the bacterial growth, motility and virulence of *Salmonella* Typhimurium) controls the expression of TolA (K.C et al., 2021). Therefore, even though *tolA*, *tolB*, and *tolR* genes are present in all 39 *Salmonella* strains with 1 copy in each strain except for the *tolA* gene in SK F43-3 and SK W39-1 (non-biofilm producers) who have 2 copies of this gene), it is possible that the changes in the amino acids in the nonsynonymous mutation (1 out of 5 *tolA* mutations found in SK strains, 3 out of 4 in SE strains) missense variations altered the protein's structure and function, and lead to reduced biofilm formation and the flagellar biosynthesis (Table 3-4). Both Enteritidis and Kentucky strains have found both synonymous and nonsynonymous mutations in them. This could explain the strong biofilm formation in SK B38-3 since it didn't have this mutation and intermediate biofilm formation in SE 715701 and SE 79801 since they have this.

Table 3-4: Gene sequences variation analysis study of the SE and SK strains that could affect the function and performance of some important genes that are associated with biofilm formation, and related components against strain SE 53932 and SK PW9-3 as reference, respectively. Synon=synonymous, Nonsyn=nonsynonymous. In the paratheses: S=strong, I=intermediate, W=weak, N=non-biofilm producers. snpEFF_type=snpEFF variant type, snpEFF_impact=snpEFF variant impact.

| **Serovar** | **strains** | **Ref** | **Var** | **Type** | **Function** | **Upstream_feature** | **Downstream_feature** | **snpEff_type** | **snpEff_impact** |
|---|---|---|---|---|---|---|---|---|---|
| SE | 715701 (I), 79801 (I), 79901 (I), 52239 (S), 51094 (S), 51095 (S) | G | A | Nonsyn | Cellulose synthase catalytic subunit [UDP-forming] (EC 2.4.1.12) | Cellulose biosynthesis protein BcsQ | Cyclic di-GMP-binding protein BcsB | stop gained | High |
| SE | 715701, 79801, 79901 (I) | CCGCGCGCGCT | CCGCGCGCT | Deletion | Cellulose synthase operon protein C | beta-1,4-glucanase (cellulase) (EC 3.2.1.4) | c-di-GMP phosphodiesterase (EC 3.1.4.52) | frameshift | High |
| SE | 715701 (I), 79801 (I), 79901 (I), 52239 (S), 51094 (S), 51095 (S) | AGGGGGGGGTAC | AGGGGGGGGGTAC | Insertion | Uncharacterized fimbrial-like protein YehA | Outer membrane usher protein YehB | Nickel/cobalt homeostasis protein RcnB | frameshift | High |
| SK | F43-3 (N) | AGCGGAG | GGCAGAC | Nonsyn | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller | missense | Moderate |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | repeat protein TolB | | |
| SE | 53936 (S) | C | G | Nonsyn | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller repeat protein TolB | missense | Moderate |
| SE | 52239, 51094, 53936 (S) | C | G | Nonsyn | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller repeat protein TolB | missense | Moderate |
| SE | 719001 (S), 715701 (I), 52239 (S), 51094 (S), 710901 (S), 53936 (S), 618901 (S), 619102 (S) | G | T | Nonsyn | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller repeat protein TolB | missense | Moderate |
| SE | 107 (I) | C | A | Nonsyn | UPF0126 inner membrane protein YadS | Vitamin B12 ABC transporter, substrate-binding protein BtuF | Iron-sulfur cluster insertion protein ErpA | missense | Moderate |
| SE | 107 (I) | G | A | Nonsyn | Glutamate-1-semialdehyde 2,1- | FIG01048481: hypothetical protein | Uncharacterized protein | missense | Moderate |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | aminomutase (EC 5.4.3.8) | | YadU in stf fimbrial cluster | | |
| SE | 715701, 79801, 79901 (I) | G | T | Nonsyn | Glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8) | FIG01048481: hypothetical protein | Uncharacterized protein YadU in stf fimbrial cluster | missense | Moderate |
| SE | 715701 (I), 79801 (I), 79901 (I) 52239 (S), 51094 (S), 51095 (S) | C | T | Nonsyn | Flagellar basal-body P-ring formation protein FlgA | Negative regulator of flagellin synthesis FlgM (anti-sigma28) | Flagellar basal-body rod protein FlgB | missense | Moderate |
| SE | 107 (I) | A | C | Nonsyn | Cytoplasmic alpha-amylase (EC 3.2.1.1) | Flagellar biosynthesis protein FliT | Uncharacterized lipoprotein YedD | missense | Moderate |
| SE | 52239 (S) | A | G | Nonsyn | Curli production assembly/transport component CsgF | Curli production assembly/transport component CsgG | Curli production assembly/transport component CsgE | missense | Moderate |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SK | CW8-3 (W), F38-2 (N), F39-3 (N), F41-2 (N), F42-3 (N), F43-3 (N), F44-1 (S), F45-2 (N), W36-3 (S), W37-2 (N), W38-1 (N), W39-1 (N), W41-2 (N), W42-2 (N), W44-3 (N), F36-3 (N) | G | A | Synon | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller repeat protein TolB | synonymous | Low |
| SK | C45-1 (N), CW8-3 (W), F38-2 (N), F39-3 (N), F41-2 (N), F42-3 (N), F43-3 (N), F44-1 (S), F45-2, W36-3 (S), W37-2 (S), W38-1 (N), W39-1 (N), W41-2 (N), W44-3 (N), F36-3 (N) | A | G | Synon | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller repeat protein TolB | synonymous | Low |
| SK | CW8-3 (W), W38-1 (N), W39-1 (N), W41-2 (N), F36-3 (N) | A | G | Synon | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller repeat protein TolB | synonymous | Low |
| SK | W38-1, W39-1 (N) | AGAG | GGAA | Synon | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller | synonymous | Low |

|  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  |  | repeat protein TolB |  |  |
| SE | 715701 (I), 79801 (I), 52239 (S), 51094 (S), 710901 (S), 53936 (S) | A | G | Synon | TolA protein | Tol biopolymer transport system, TolR protein | Tol-Pal system beta propeller repeat protein TolB | synonymous | Low |
| SE | 107 (I) | C | T | Synon | Cellulose biosynthesis protein BcsG | Small inner membrane protein, YmgF family | Cellulose biosynthesis protein BcsF | synonymous | Low |
| SE | 107 (I) | T | C | Synon | Cellulose biosynthesis protein BcsQ | Putative cytoplasmic protein YhjR | Cellulose synthase catalytic subunit [UDP-forming] (EC 2.4.1.12) | synonymous | Low |
| SE | 107 (I) | C | T | Synon | Cellulose synthase operon protein C | beta-1,4-glucanase (cellulase) (EC 3.2.1.4) | c-di-GMP phosphodiesterase (EC 3.1.4.52) | synonymous | Low |

|  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|
| SE | 107 (I) | A | G | Synon | Uncharacterized protein YadE | Putative PTS system IIA component YadI | Aspartate 1-decarboxylase (EC 4.1.1.11) | synonymous | Low |
| SE | 107 (I) | C | T | Synon | tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase (EC 2.1.1.61) / FAD-dependent cmnm(5)s(2)U34 oxidoreductase | Uncharacterized protein YfcL | 3-oxoacyl-[acyl-carrier-protein] synthase, KASI (EC 2.3.1.41) | synonymous | Low |
| SE | 107 (I) | G | A | Synon | Uncharacterized protein YfcC | Transketolase, C-terminal section (EC 2.2.1.1) | BioD-like N-terminal domain / Phosphate acetyltransferase (EC 2.3.1.8) | synonymous | LOW |
| SE | 107 (I) | G | A | Synon | Flagellar motor rotation protein MotA | Flagellar motor rotation protein MotB | Flagellar transcriptional activator FlhC | synonymous | LOW |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SE | 107 (I) | A | G | Synon | hypothetical protein | Flagellar regulon repressor RtsB | hypothetical protein | synonymous | LOW |

### 3.4.6. SE strains functional genomic comparison (strong vs. intermediate biofilm-forming strains)

To figure out how the intermediate bovine strain was different from the other strong poultry strains, a proteome comparison was conducted. A strong SE biofilm strain 53932 (isolated from poultry) as a reference was blasted against an intermediate SE strain 107 (isolated from bovine). A graph is provided in Figure 3-8, and a detailed table is provided in Table 3-6. Out of 4,765 protein-coding gene sequences that were compared against each other, 4,333 of them were completely the same with 100% identity (shown in the purple area of the ring). A total of 432 proteins showed less than 100% protein sequence identity, within those, 148 genes had 0% identity, indicating that a specific protein was present in the reference genome (the strong biofilm strain) but absent in the comparison genome (the intermediate biofilm strain). Excluding the hypothetical proteins and proteins with unknown functions, 65 proteins remained in the list (shown in Table 3-6). Within those 65 proteins, a group of Type IV secretion system proteins were especially associated with biofilm formation and virulence factors. VirB1, VirB3, VirB4, VirB5, VirB6, VirB9, VirB10, VirB11, VirD2 homolog, and VirD4, were present in the strong biofilm SE strain genome but absent in the intermediate one. According to our results, VirB1, VirB3, VirB4, VirB5, VirB6, VirB10, VirB11, and VirD4 were present in 4 strong SE strains, and absent in the rest of the SE strains and all SK strains. The detailed mechanisms underlying biofilm formation and gene expression involve multiple regulatory pathways that are difficult to fully decipher. As a result, the broader implication of these findings remains elusive. Further investigations are needed in the future studies.

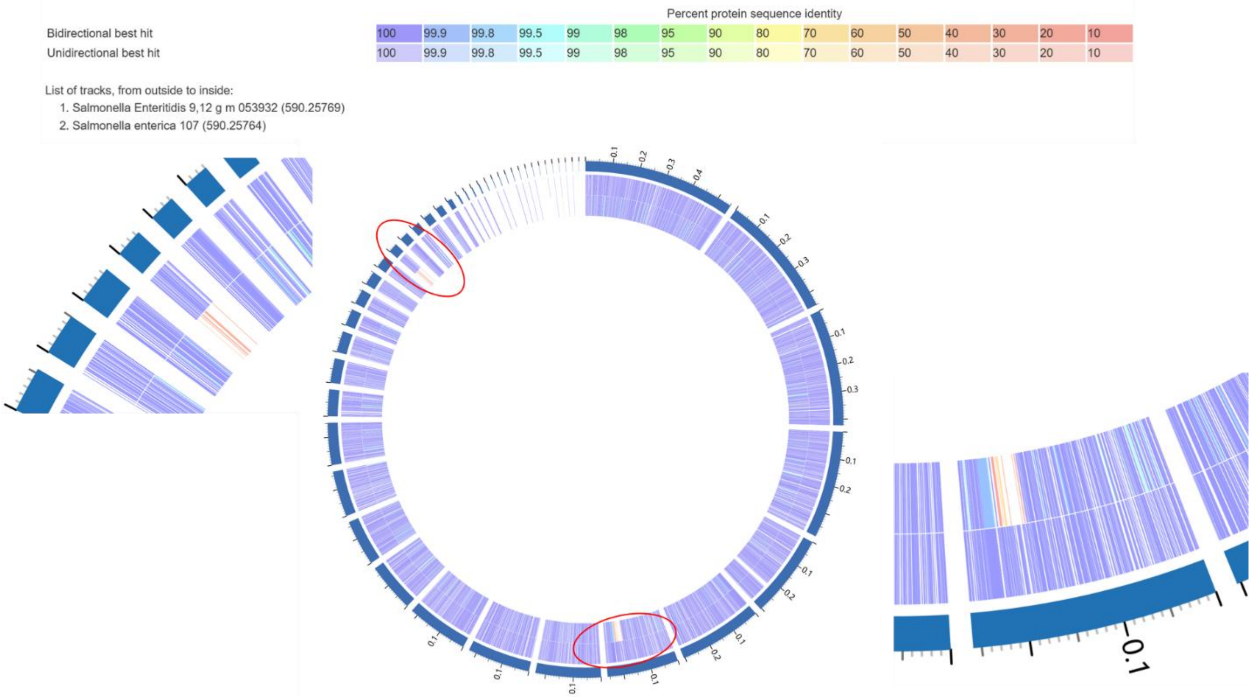

Figure 3-8: Protein coding gene comparison graph showing differences in gene sequence identity between a strong *Salmonella* Enteritidis biofilm strain isolated from poultry product (53932) on the outside of the ring, and an intermediate *Salmonella* Enteritidis strain isolated from bovine (107) on the inside of the ring. The image shows the plot of the bi-directional BLAST hits and a legend that shows the list of tracks and the strength of the BLAST hits, where purple is the strongest and red is the weakest. Two regions of cluster of differences in the red circles were enlarged for better visualization.

Table 3-5: Protein encoding gene comparison conducted in BV-BRC. *=genes associated with the cluster on the bottom of Figure 3-8. **=genes associated with the cluster on the top left of Figure 3-8.

| **Proteins present in SE strong biofilm former 53932, but absent in SE intermediate producer 107, excluding hypothetical proteins and proteins with unknown functions** |
|---|
| Exoenzymes regulatory protein AepA precursor |
| putative e6 protein |
| ail and ompX Homolog |
| Phage repressor protein cI |
| Phage Cox (control of excision) protein |
| Phage activator protein cII |

| |
|---|
| FIL protein |
| Phage replication protein GpB* |
| Phage Orf80 protein* |
| Phage replication protein GpA, endonuclease* |
| Phage protein* |
| DNA-damage-inducible protein I* |
| Phage protein* |
| Phage portal vertex protein GpQ* |
| Phage terminase, ATPase subunit GpP* |
| Phage capsid scaffolding protein GpO* |
| Phage major capsid protein GpN* |
| Phage terminase, endonuclease subunit GpM* |
| Phage head completion-stabilization protein GpL* |
| Phage tail protein GpX* |
| Phage holin* |
| Phage lysis regulatory protein, LysB* |
| Phage tail completion protein GpR* |
| Phage tail completion protein GpS* |
| Phage baseplate assembly protein GpV* |
| Phage baseplate assembly protein GpW* |
| Phage baseplate assembly protein GpJ* |
| Phage tail formation protein GpI* |
| putative inner membrane protein* |
| Phage tail sheath monomer GpFI* |
| Phage major tail tube protein GpFII* |
| Phage tail protein GpE* |
| Phage P2 GpE family protein* |
| Phage tail protein GpU* |
| Phage tail formation protein GpD* |
| Oxaloacetate decarboxylase Na(+) pump, alpha chain (EC 4.1.1.3) |
| Anaerobic sulfite reductase subunit A |
| SdiA-regulated putative outer membrane protein SrgB |

| |
|---|
| Putative cytoplasmic protein |
| Oxaloacetate decarboxylase Na(+) pump, alpha chain (EC 4.1.1.3) |
| RelB/StbD replicon stabilization protein (antitoxin to RelE/StbE)** |
| PI protein** |
| DNA distortion protein 3** |
| Cell division protein FtsH (EC 3.4.24.-)** |
| IncN plasmid KikA protein** |
| Coupling protein VirD4, ATPase required for T-DNA transfer** |
| ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex, VirB11** |
| Inner membrane protein of type IV secretion of T-DNA complex, TonB-like, VirB10** |
| Forms the bulk of type IV secretion complex that spans outer membrane and periplasm (VirB9)** |
| putative conjugal transfer protein |
| Inner membrane protein of type IV secretion of T-DNA complex, VirB6** |
| IncQ plasmid conjugative transfer protein TraG** |
| Minor pilin of type IV secretion complex (VirB5)** |
| Inner membrane protein forms channel for type IV secretion of T-DNA complex, VirB3 / ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex, VirB4** |
| Pilx2 protein** |
| Peptidoglycan hydrolase VirB1, involved in T-DNA transfer** |
| IncQ plasmid conjugative transfer DNA nicking endonuclease TraR (pTi VirD2 homolog)** |
| DNA distortion protein 1** |
| putative membrane protein |
| Phage integrase |
| E3 ubiquitin-protein ligase SspH2 |

# 4. Chapter 4: Conclusions and prospects for future work

In our study, biofilm formation results have shown 11 out of 15 strong biofilm-forming *Salmonella* Enteritidis strains and 4 out of 24 strong biofilm-forming *Salmonella* Kentucky strains, all of which were isolated from chicken-related sources and poultry processing facilities.

Strains isolated from various products and locations resulted in all type of biofilm-forming abilities. However, the strain isolated from bovine (#107) was different than the other SE strains, since it was the only intermediate former with both negative results in the curli and cellulose production. Additionally, 11 genomic variations, including both synonymous and nonsynonymous, were found in this strain.

The results of this research showed no clear pattern of genes or proteins that reliably differentiate between strong and weak biofilm-producing strains. Most strains possess the same core genes related to biofilm formation, including those involved in curli and cellulose production, as well as fimbriae, pilus, and flagella assembly. The majority of the *Salmonella* Kentucky strains were categorized as non-biofilm producers; however, they still possess almost all of the essential genes for biofilm formation, for example, the *csg* genes, while other research papers have shown that biofilm formation is present when those genes were present (Arnqvist et al., 1994; MacKenzie et al., 2015; Tan et al., 2016), as well as the Type IV secretion system proteins.

The next step was to determine if these genes were functional. Using SNP variation analysis, several variations and mutations were found within 39 *Salmonella* strains. However, no significant mutations were found in core genes for biofilm formation (except for *bcs* mutations found in strain #107 and *csg* mutations found in a strong biofilm SE strain 52239). Only three high-impact variants were found in *Salmonella* Enteritidis strains including a nonsynonymous stop-gain mutation in cellulose synthase catalytic subunit in six SE strains (3 intermediate and 3 strong strains); a frame-shift deletion mutation in cellulose synthase operon protein C (BcsC) in 3 intermediate strains; and a frameshift deletion in an uncharacterized fimbrial-like protein YehA in the same six SE strains (3 intermediate and 3 strong strains).

The protein coding genes were also compared between a strong SE strain isolated from poultry and the intermediate SE strain isolated from bovine using the proteome comparison to gain more insights in their genomic differences. Several type IV secretion system proteins, VirB1, VirB3, VirB4, VirB5, VirB6, VirB9, VirB10, VirB11, VirD2 homolog, and VirD4, were present in the strong biofilm SE strain genome but absent in the intermediate one, which were aligned with the genomic comparisons conducted and suggested that type IV secretion systems could play a very important role in the biofilm formation or its related mechanisms. Therefore, type IV secretion system proteins mentioned above should be investigated further to determine their roles in biofilm formation, or its related components.

Examining biofilm formation in *Salmonella* could be complex and challenging due to the intricate genetic regulation, including variations in gene expression and strain, serotype-specific differences, and environmental factors such as temperature fluctuations and nutrient availability that affect the biofilm development. Thus, future research is needed to fully understand the relationship between the genomic and phenotypic results.

This research rejected Hypothesis 1, which proposed that *Salmonella* Enteritidis and *Salmonella* Kentucky strains with different biofilm formation abilities would show different genomic results. However, it partially supported Hypothesis 2, suggesting that genetic variations and mutations may play a role in explaining the contradictory phenotypic and genomic results of Hypothesis 1.

In summary, this thesis has provided more genomic information regarding biofilm formation, and factors that impact the biofilm formation such as curli, cellulose and other important components, and provides a general methodology for genetic analysis of biofilm formation in *Salmonella*. Additionally, the phenotypic results have further emphasized the necessity for proper cleaning and sanitizing in the poultry barns. However, examining biofilm formation in *Salmonella* could be complex and challenging due to the intricate genetic regulation, including variations in gene expression and strain, serotype-specific differences, and environmental factors such as temperature fluctuations and nutrient availability that affect the biofilm development. Thus, future research is needed to fully understand the relationship between the genomic and phenotypic results.

Future studies could include 1) more samples for a more comprehensive comparative genomic analysis and cover more common and important genes found in their genomes. Since so far, only 15 *Salmonella* Enteritidis and 24 *Salmonella* Kentucky strains have been analyzed, those strains may not be representative of all of the ones associated with *Salmonella* outbreaks in poultry. 2) When the resources permit, future studies could also 2) create the mutation genes listed in Table 3-4, such as *bcsG*, *bcsQ*, *yehA*, *flgA*, *motA*, and *csgF*, using the whole genome sequences of those strains, and examine their difference between the genomic results and phenotypic results. 3) Plasmids can also carry accessory genes that influense biofilm formation, therefore, future studies could look into genes encoding proteins associated with conjugative plasmids, especially those related to surface attachment, such as IncF plasmid conjugative transfer pilin proteins.

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# 5. Complimentary material

Figure 5-1: Heatmap of 15 *Salmonella* Enteritidis and 24 *Salmonella* Kentucky strains protein coding genes associated with Type III secretion systems. All of which were present in all strains with one copy. SE 9susph=SE 51095

Table 5-1: Genomes information of the strains used in this study.

CGA annotation information

| *Salmonella* serotypes | Genome ID | Annotation Name | Reference genome | Coarse consistency (%) | Fine consistency (%) | Completeness (%) | Contamination (%) | Contig count | DNA size (bp) | Contigs N50 (bp) | Contigs L50 | Overpresent roles | Underpresent roles | Predicted roles | Completeness roles | Total distinct roles | Protein encoding genes with functional assignment | Protin encoding genes without functional assignment | % protein encoding feature coverage | % features that are hypothetical | % features that are in local protein families |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Enteritidis | 590.25771 | 719001 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 36 | 4681771 | 318415 | 5 | 10 | 2 | 1396 | 82 | 4093 | 3407 | 1306 | 100.67 | 27.71 | 97.35 |
| Enteritidis | 590.25756 | 715701 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 28 | 4667088 | 478633 | 4 | 10 | 2 | 1396 | 82 | 4046 | 3390 | 1278 | 100.02 | 27.38 | 97.58 |
| Enteritidis | 590.25759 | 79801 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 33 | 4665484 | 453071 | 5 | 10 | 2 | 1396 | 82 | 4045 | 3392 | 1284 | 100.23 | 27.46 | 97.52 |
| Enteritidis | 590.25758 | 79901 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 43 | 4719105 | 320547 | 5 | 10 | 2 | 1396 | 82 | 4106 | 3441 | 1319 | 100.87 | 27.71 | 97.33 |
| Enteritidis | 590.2576 | 78501 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 37 | 4691395 | 422764 | 3 | 10 | 2 | 1396 | 82 | 4095 | 3418 | 1305 | 100.67 | 27.63 | 97.42 |
| Enteritidis | 590.25757 | 52239 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 35 | 4716742 | 478423 | 4 | 10 | 2 | 1396 | 82 | 4097 | 3433 | 1312 | 100.6 | 27.65 | 97.39 |
| Enteritidis | 590.25761 | 51094 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 38 | 4720905 | 250378 | 6 | 10 | 2 | 1396 | 82 | 4098 | 3436 | 1213 | 100.62 | 27.66 | 97.56 |
| Enteritidis | 590.25762 | 710901 | 46626.35 | 99.5 | 99 | 100 | 0 | 29 | 4670791 | 422828 | 3 | 10 | 3 | 1396 | 82 | 4066 | 3390 | 1285 | 100.09 | 27.49 | 97.45 |
| Enteritidis | 590.25764 | 107 | 46626.35 | 99.6 | 99 | 100 | 0 | 31 | 4702747 | 489790 | 3 | 11 | 2 | 1396 | 82 | 4098 | 3428 | 1307 | 100.69 | 27.6 | 97.51 |
| Enteritidis | 590.25765 | 53936 | 46626.35 | 99.5 | 99 | 100 | 0 | 31 | 4668114 | 422792 | 4 | 10 | 3 | 1396 | 82 | 4065 | 3391 | 1275 | 99.95 | 27.33 | 97.6 |
| Enteritidis | 590.25766 | 61401 | 46626.35 | 99.5 | 99 | 100 | 0 | 26 | 4669336 | 477399 | 3 | 10 | 3 | 1396 | 82 | 4069 | 3389 | 1292 | 100.25 | 27.6 | 97.52 |
| Enteritidis | 590.25767 | 618901 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 33 | 4682104 | 476619 | 4 | 10 | 2 | 1396 | 82 | 4088 | 3415 | 1299 | 100.47 | 27.56 | 97.48 |
| Enteritidis | 590.2577 | 619102 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 38 | 4690728 | 406118 | 4 | 10 | 2 | 1396 | 82 | 4090 | 3410 | 1306 | 100.54 | 27.69 | 97.26 |
| Enteritidis | 590.25769 | 53932 | 46626.35 | 99.5 | 99 | 100 | 0 | 46 | 4720479 | 304041 | 6 | 10 | 3 | 1396 | 82 | 4116 | 3435 | 1330 | 100.94 | 27.91 | 97.21 |
| Enteritidis | 149539.3315 | 51095 | 46626.35 | 99.6 | 99.1 | 100 | 0 | 31 | 4718888 | 406133 | 4 | 10 | 2 | 1396 | 82 | 4098 | 3434 | 1325 | 100.85 | 27.84 | 97.29 |
| Kentucky | 192955.854 | C45-1 | 46626.35 | 99.9 | 98.9 | 100 | 0 | 65 | 4896569 | 179308 | 8 | 13 | 2 | 1396 | 82 | 4143 | 3499 | 1450 | 101.07 | 29.3 | 97.19 |
| Kentucky | 192955.847 | W44-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 66 | 4899839 | 236975 | 7 | 11 | 2 | 1396 | 82 | 4153 | 3496 | 1441 | 100.76 | 29.19 | 97.27 |
| Kentucky | 192955.845 | W42-2 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 68 | 4901249 | 204427 | 8 | 12 | 4 | 1396 | 82 | 4154 | 3500 | 1444 | 100.87 | 29.21 | 97.25 |
| Kentucky | 192955.846 | W41-2 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 72 | 4896143 | 204426 | 8 | 12 | 4 | 1396 | 82 | 4149 | 3499 | 1447 | 101.02 | 29.26 | 97.35 |
| Kentucky | 192955.843 | W40-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 72 | 4892989 | 2144990 | 8 | 11 | 2 | 1396 | 82 | 4147 | 3498 | 1446 | 101.04 | 29.25 | 97.11 |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kentucky | 192955.842 | W39-1 | 46626.35 | 99.9 | 99 | 100 | 0 | 68 | 4897759 | 204433 | 8 | 12 | 2 | 1396 | 82 | 4147 | 3496 | 1433 | 100.64 | 29.07 | 97.3 |
| Kentucky | 192955.844 | W38-1 | 46626.35 | 99.9 | 99 | 100 | 0 | 63 | 4900309 | 215132 | 8 | 11 | 2 | 1396 | 82 | 4156 | 3499 | 1452 | 101.03 | 29.33 | 97.19 |
| Kentucky | 192955.841 | W37-2 | 46626.35 | 99.9 | 99 | 100 | 0 | 62 | 4899906 | 228134 | 7 | 11 | 2 | 1396 | 82 | 4148 | 3497 | 1449 | 100.94 | 29.3 | 97.21 |
| Kentucky | 192955.839 | W36-3 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 65 | 4897773 | 204340 | 8 | 12 | 4 | 1396 | 82 | 4149 | 3498 | 1443 | 100.88 | 29.2 | 97.25 |
| Kentucky | 192955.84 | PW9-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 63 | 4900394 | 250655 | 7 | 11 | 2 | 1396 | 82 | 4143 | 3495 | 1447 | 100.85 | 29.28 | 97.17 |
| Kentucky | 192955.838 | PW8-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 64 | 4901398 | 215023 | 8 | 11 | 2 | 1396 | 82 | 4145 | 3498 | 1428 | 100.5 | 28.99 | 97.3 |
| Kentucky | 192955.837 | F45-2 | 46626.35 | 99.9 | 98.9 | 100 | 0 | 60 | 4902486 | 255906 | 7 | 13 | 2 | 1396 | 82 | 4141 | 3499 | 1439 | 100.72 | 29.14 | 97.21 |
| Kentucky | 192955.836 | F44-1 | 46626.35 | 99.9 | 99 | 100 | 0 | 62 | 4897486 | 215280 | 7 | 11 | 2 | 1396 | 82 | 4153 | 3497 | 1430 | 100.6 | 29.02 | 97.34 |
| Kentucky | 192955.835 | F43-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 59 | 4901909 | 204333 | 7 | 11 | 2 | 1396 | 82 | 4143 | 3496 | 1446 | 100.82 | 29.26 | 97.31 |
| Kentucky | 192955.834 | F42-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 63 | 4900645 | 236975 | 7 | 11 | 2 | 1396 | 82 | 4143 | 3497 | 1444 | 100.82 | 29.22 | 97.31 |
| Kentucky | 192955.833 | F41-2 | 46626.35 | 99.9 | 99 | 100 | 0 | 62 | 4901436 | 236975 | 7 | 11 | 2 | 1396 | 82 | 4146 | 3495 | 1450 | 100.89 | 29.32 | 97.09 |
| Kentucky | 192955.832 | F40-3 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 68 | 4898435 | 215171 | 7 | 12 | 4 | 1396 | 82 | 4142 | 3496 | 1449 | 100.93 | 29.31 | 97..13 |
| Kentucky | 192955.83 | F39-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 66 | 4898712 | 255853 | 7 | 11 | 2 | 1396 | 82 | 4143 | 3498 | 1439 | 100.78 | 29.15 | 97.27 |
| Kentucky | 192955.831 | F38-2 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 65 | 4902034 | 215242 | 7 | 12 | 4 | 1396 | 82 | 4149 | 3500 | 1449 | 100.96 | 29.28 | 97.21 |
| Kentucky | 192955.829 | F37-3 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 66 | 4897421 | 204426 | 7 | 12 | 4 | 1396 | 82 | 4141 | 3497 | 1436 | 100.73 | 29.11 | 97.3 |
| Kentucky | 192955.827 | F36-3 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 63 | 4901193 | 236975 | 7 | 12 | 4 | 1396 | 82 | 4151 | 3499 | 1444 | 100.85 | 29.21 | 97.23 |
| Kentucky | 192955.828 | CW9-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 68 | 4896666 | 204426 | 10 | 12 | 2 | 1396 | 82 | 4154 | 3502 | 1435 | 100.82 | 29.07 | 97.14 |
| Kentucky | 192955.826 | CW8-3 | 46626.35 | 99.9 | 99 | 100 | 0 | 68 | 4896899 | 236975 | 7 | 11 | 2 | 1396 | 82 | 4151 | 3497 | 1448 | 100.98 | 29.28 | 97.13 |
| Kentucky | 192955.824 | B38-3 | 46626.35 | 99.9 | 98.8 | 100 | 0 | 66 | 4897362 | 204719 | 8 | 12 | 4 | 1396 | 82 | 4142 | 3494 | 1450 | 100.95 | 29.33 | 97.15 |