

Salmonella Enteritidis (SE) Multispecies Biofilm Formation and its Persistence in the
Poultry Environment

By

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A Thesis submitted to the Faculty of Graduate and Postdoctoral Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Food and Human Nutritional Sciences

University of Manitoba

Winnipeg

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Abstract

Introduction: *Salmonella* Enteritidis (SE) is a major food-borne pathogen that forms biofilms which attach to processing surfaces in the laying barns and poultry facilities thereby contaminating eggs and poultry products.

Methods: The aim was to evaluate SE biofilm formation abilities and SE interactions with selected bacteria associated with eggshells (BAE) and Lactic Acid Bacteria (LAB) in multispecies biofilms formed at 10 and 20 °C in 96-well microplates. In the second study, two multispecies combinations M1: *E. coli* + *P. aeruginosa* and M2: *B. cereus* + *P. aeruginosa* were assessed for multispecies biofilm formation with 36SusP a strong biofilm former on galvanized steel (GS) and Plastic (PLA) surfaces under dry (20-30% relative humidity; RH) and wet (50-60% RH) conditions at 20 °C for 6 days. Single-species biofilms were used as control positives, and SE recovery from multispecies biofilms was achieved by enrichment.

Results: It was found that SE biofilm formation increased significantly at 20 °C ($P < 0.001$), forming strong biofilms in comparison to the weak biofilms formed at 10 °C. At 10 °C SE counts were low (between 4.4 to 5.5 log₁₀ CFU/mL) with all multispecies combinations with *E. coli* + *E. faecalis*, *P. aeruginosa* + *E. coli* and *P. aeruginosa* + *B. cereus* showing a slight increase in bacterial counts that was not statistically significant ($P > 0.05$). At 20 °C however, SE counts reduced with the lowest counts recovered from the combination with *P. aeruginosa* + *E. coli* ($P < 0.05$). Regarding the type of surface, SE bacterial growth differed between GS and PLA surfaces under both wet and dry conditions, with PLA having significantly more bacterial retention than GS ($P > 0.0001$). SE recovery was consistent in multispecies biofilms with M1, with enrichment improving recovery in both dry (from 16.7 to 60%) and wet (from 16.7 to

33.3%) conditions, while M2 showed low recovery overall and dropped to 0% even after enrichment in dry and wet conditions.

Conclusion: SE biofilm formation is largely temperature-driven, and species-specific traits determine the extent to which cooperative or competitive interactions develop, controlling which species can occupy and persist within the multispecies biofilm, highlighting that biofilm formation relies on ecological relationships.

Acknowledgment

I want to thank my thesis supervisor, Dr. Claudia Narvaez, for her guidance and support throughout my studies. I am grateful for your patience and for showing me how to think more deeply and ask the right questions.

I would also like to express my appreciation to my committee members, Dr. Nandika Bandara and Dr. Chengbo Yang, for all their feedback. Thank you to Dr. Argenis Rodas-Gonzalez and Dr. Francis Zvomuya for their assistance with the statistical analysis. Thank you to Alison, Yang and Jerry for answering my many questions.

Thank you to my lab mates, Daniel, Rodolfo, Sarah, and Anna, for your companionship and for helping me out with my experiments. Special thanks to my friends Tomer and Alice; meeting both of you was a big highlight in my life. Thank you for bringing me out of my shell, introducing me to new cultures, taking me to see new things, and eating delicious food. I hope we stay friends for a long time.

Thank you to Kavitha, Chow and Aunty Moji for making me a part of your family and treating me like your little sister. Thank you for caring for me and for the extra little siblings I now have through you. Thank you to Philemon and Moyo, my longtime friends, for giving me a safe space to rant all day long.

To my parents, my brother, my aunts, uncles and grandma, you have been my biggest support system and the reason I have carried on as much as I have. Your sacrifices and love can never be forgotten.

Thank you to the Canadian Poultry Research Council and the Manitoba Egg Farmers for sponsoring this project.

Dedication

To my Granduncle, Dr. Ogbemi Omatete, for introducing me to this life of research.

Thesis Organization

This thesis has five chapters, with chapters three and four being manuscripts.

Chapter One is an introduction to the biofilm-forming ability of *Salmonella* Enteritidis (SE) and its persistence in the poultry industry, including the research objectives and hypotheses behind this project.

Chapter Two is a literature review that provides the background of SE, SE biofilm formation and role in the poultry industry, as well as SE multispecies biofilm formation and attachment to surfaces found in poultry facilities.

The first manuscript is Chapter Three titled ”**Multispecies biofilm formation of *Salmonella* Enteritidis strains isolated from poultry**”. This chapter covers the SE single and multispecies biofilm formation in 96-well microplates at 10 and 20 °C. This Manuscript was authored by Oritsetimeyin Ebosa, Argenis Rodas-Gonzalez, Tim McAllister, and Claudia Narvaez-Bravo.

The second manuscript is Chapter Four titled “**Formation of multispecies biofilms by *Salmonella* Enteritidis on contact surfaces found in poultry facilities**”. This chapter discusses SE multispecies biofilms on Galvanized steel (GS) and Plastic (PLA) at 20 °C for 6 days. This Manuscript was authored by Oritsetimeyin Ebosa, Francis Zvomuya, Tim McAllister and Claudia Narvaez-Bravo.

Chapter Five is the final chapter and covers the summary of the thesis as well as future prospects for this research.

Contributions of Authors

For Chapter 3: Claudia Narvaez-Bravo wrote and submitted the project for funding, led the project, supervised graduate students, revised and edited the student manuscript, and wrote the reports for the funding agency. Tim McAllister read the project, provided suggestions and edited the manuscripts. Argenis Rodas-Gonzalez performed the statistical analysis and Oritsetimeyin Ebosa carried out the experiments and drafted the manuscript.

For Chapter 4: Claudia Narvaez obtained funding for the project, led the project, supervised graduate students, revised and edited the student manuscript, and wrote the reports for the funding agency. Tim McAllister read the project, provided suggestions, and edited the manuscripts. Francis Zvomuya performed the statistical analysis and Oritsetimeyin Ebosa carried out the experiments and drafted the manuscript.

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Chapter 1: Introduction

1.1 Introduction

Salmonella Enteritidis (SE) is a major foodborne pathogen typically associated with poultry products, including meat and eggs, and is responsible for significant public health concerns globally (Kumar et al., 2025). As of 2024, SE had the highest number and incidence rate of multistate outbreak-associated isolates in the USA, with food sources accounting for 75.93% of reported *Salmonella* cases (CDC, 2024). According to the National Enteric Surveillance Program (NESP) report of 2022, SE made up 38% of *Salmonella* serotypes reported to cause human salmonellosis in Canada (PHAC, 2022).

The Canadian Food Inspection Agency (CFIA) regulates food and poultry products in Canada to ensure food safety and consumer protection. Strict requirements and guidance are necessary to ensure reduced contamination, targeted public health surveillance, and enhanced coordination within federal health agencies. Up until 2019, Frozen breaded chicken products, like chicken fingers and nuggets, were some of the leading causes of *Salmonella* illnesses in Canada (Carvalho et al., 2020; Currie et al., 2005). To combat this, mandatory industry process controls were implemented, requiring manufacturers of breaded chicken products to ensure that *Salmonella* is completely absent from the products, or the products are fully cooked (CFIA, 2015). A national strategy group was also developed with the aim of reducing human illness due to SE by identifying and addressing the risks found in poultry and egg production (Canada, 2015).

Additionally, the PHAC adopted a more thorough method for identification SE known as whole-genome sequencing (WGS). This method made it easier to distinguish between close

Salmonella isolates and ultimately improved Canadian outbreak investigation and response (Alleweldt et al., 2021; A. Kerr et al., 2024; Nadon et al., 2017). To bolster public safety, federal and provincial agencies provide the general public with public advisories on proper food handling, preparation, and cooking practices to further mitigate the risk of SE (Manitoba, 2015; PHAC, 2019). Even with such extensive policies in place, SE can persist for long periods in poultry facilities even after cleaning and disinfection. Some studies have shown that SE can survive for up to a year in depopulated poultry houses, within dried faeces, litter and feed, and pest vectors (e.g. rodents, mealworms etc.) (Davies & Breslin, 2003; Ethèves et al., 2021; Gast et al., 2015). Incomplete sanitation can lead to the existence of microcolonies known as biofilms on barn equipment and infrastructure, where SE survives and later reinfects a new flock.

Biofilms are microbial aggregates immersed within an extracellular polymeric substance (EPS). The EPS allows microbes to withstand changes in chemical and environmental conditions and facilitates increased resistance to antimicrobial agents (Butt & Khan, 2015; Ciofu & Tolker-Nielsen, 2019). The EPS supports the survival of microorganisms and previous studies have shown that pathogenic bacteria like Shigatoxigenic *Escherichia coli* O103:H2 (STEC) formed on Polyurethane (TPU) under dry conditions (20-50% relative humidity) at 25 °C were viable for up to 60 days, even after the EPS had dried (Yuchen et al., 2022). SE single-species biofilms have also been reported to form denser biofilms at 25 °C, even having increased resistance to antibacterial agents (Yang et al., 2016). Long-term microbe viability in dry conditions and improved biofilm formation at 25 °C pose an issue in poultry facilities, as most barns are maintained at an average temperature and relative humidity of 19-25 °C and 28-70% respectively, conditions which are ideal for SE mesophilic growth (van Staaveren et al., 2018).

In the natural environment, SE biofilms are more likely to exist alongside other microorganisms in multispecies biofilms and SE persistence in poultry facilities can be linked to its ability to form multispecies biofilms, particularly on food-contact surfaces and processing equipment commonly found in barns (Pang et al., 2023). Laying barns contain a blend of organic material like feathers, feces, bedding materials (e.g. wood shavings and sawdust) and even feed material scattered about by poultry which can host both beneficial and pathogenic organisms (Gomes et al., 2022; Gržinić et al., 2023). Cages, perches, feeding and watering equipment, egg and feces conveyors, nesting boxes, and even flooring surfaces can serve as sources of biofilm contamination in poultry barns (Al-Zenki et al., 2007; Biyashev et al., 2025). Poor maintenance and sanitation of farm equipment and structures can lead to material degradation, which may include cracks, abrasions, or other damages that harbour organic material and promote biofilm attachment (Arnold & Silvers, 2000).

The social interactions between bacteria in a multispecies biofilm may manifest as competition for resources (antagonistic), mutually beneficial (synergistic) or neutral (S. Elias & E. Banin, 2012; Luo et al., 2022). There have also been reports that multispecies biofilms have increased resistance to sanitizers and disinfectants often used in the food industry like hydrogen peroxide and quaternary ammonium compounds, requiring concentrations greater than recommended to be effective (Burmølle et al., 2014; Koti et al., 2024).

While there have been studies on SE attachment to the poultry processing surfaces like stainless steel and plastic egg conveyor belts in both single-species and multispecies biofilms (Kusumaningrum et al., 2003; Stocki et al., 2007; Veluz et al., 2012), there is a lack of experimental models that look at environmental barn conditions relative to SE surface attachment. Given these challenges, it is essential to investigate the biofilm-forming potential of

SE under conditions that closely resemble those of the poultry environment. By understanding the multispecies biofilm-forming ability of SE on industrial surfaces and under various environmental conditions, the development of more effective pathogen mitigation strategies in the poultry industry can be achieved.

1.2 Research Hypothesis

SE survival within multispecies biofilms will change depending on environmental conditions like relative humidity (wet (60-70%) and dry (20-40%)), temperature (10 °C and 20 °C), and contact surfaces (Galvanized steel and Plastic).

1.3 Research Objectives

The main objectives of this study were to 1) determine SE biofilm formation under different environmental conditions (wet – 60-70% humidity and dry – 20-40% humidity at 10 °C and 20 °C); 2) evaluate SE survival and possible social interactions within multispecies biofilms formed with BAE and LAB (competitive fitness testing) and 3) Assess SE biofilm formation on galvanized steel (GS) and plastic (PLA).

Chapter 2: Literature Review

2.1 *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE)

2.1.1 SE General Information

Salmonella enterica subsp. *enterica* serovar Enteritidis, commonly known as *Salmonella* Enteritidis (SE), is a Gram-negative, non-spore-forming, facultative anaerobic bacterium that belongs to the *Enterobacteriaceae* family. Excluding *Salmonella enterica* serotypes Typhi, Paratyphi A, Paratyphi B (tartrate negative), and Paratyphi C, SE along with other serotypes of *S. enterica* are referred to as Non-typhoidal salmonellae (NTS) (Napoleoni et al., 2024; Riedel et al., 2019). Classification of *Salmonella* serovars depends on the presence/absence of certain bacterial antigens deduced via serological methods (Franklin et al., 2011). The somatic (O) antigen, which represents the lipopolysaccharide (LPS) outer membrane, the flagellar (H) antigen, representing the presence/ type of flagella and the capsular virulence (vi) antigen for the possession of a capsule on the cell surface of the bacteria (Grimont & Weill, 2007). The combination of these antigens phylogenetically segregates the serovars of *Salmonella* spp. and is particularly useful in epidemiology (Barker & Old, 1989; Grimont & Weill, 2007). SE is known to colonize the gastrointestinal tract of poultry (including chickens, turkeys, ducks, and geese) and is also associated with the contamination of poultry products, such as meat and eggs, during production, processing, and transportation (Hugas & Beloeil, 2014).

2.1.2 Virulence of SE

2.1.2.1 Pathogenicity of SE Infection

Contaminated eggs and poultry products serve as the primary vehicles for SE transmission. The bacteria survive the acidic environment of the stomach and then migrate to the small intestine, where they adhere to intestinal epithelial cells, leading to inflammation and damage of host cells

(Abd El-Ghany et al., 2012). *Salmonella* infections often manifest anywhere between 6 hours to 6 days after infection and can last up to 7 days. Symptoms include, but are not limited to, diarrhea (that can be bloody), stomach cramps, nausea, vomiting, or a headache and most people recover without specific treatment (Andino & Hanning, 2015; Healy & Bruce, 2019).

2.1.2.2 Virulence Factors Associated with SE

The pathogenicity of SE depends on many virulence factors that contribute to cell adhesion, invasion and biofilm formation. The *invA* gene is a part of the *Salmonella* pathogenicity island 1 & 2 (SPI1 and SPI2 respectively), which encodes many invasion-associated genes and type III secretion systems (T3SS), which are essential for epithelial cell invasion into host cells (Sabbagh et al., 2010). SE also carries a virulence plasmid which contains the *spvRABCD* locus. These genes encode an ADP-ribosyltransferase that helps destabilize the cytoskeleton of eukaryotic cells and modulates host signalling (Derakhshandeh et al., 2013; Heithoff et al., 2008; Libby et al., 1997). SE, like other Gram-negative bacteria, produces an endotoxin via lipid A, the toxic portion of the lipopolysaccharide (LPS) outer membrane. The endotoxin triggers an immune response within the host and contributes to SE pathogenesis (Heinrichs et al., 1998; Raetz & Whitfield, 2002). Hair-like structures on the surface of the bacteria, called fimbriae, are also crucial in attachment to both biotic and abiotic surfaces (Kolenda et al., 2019). Composed of repeated protein subunits called fimbrins, fimbriae can be characterized as the type 1 fimbriae (most common and involved in adhesion to epithelial cells and extracellular matrix proteins), curli fimbriae (involved in biofilm formation), SEF14 fimbriae (involved in adhesion to chicken intestinal cells) and the pef fimbriae (involved in adhesion to human intestinal cells) (Barnhart & Chapman, 2006; Edwards et al., 2000; Klemm & Schembri, 2004; Rajashekara et al., 2000). The *fliC* gene gives SE the ability to produce

flagella, which provides motility and aids bacteria in penetrating the outer membrane of host cells as well as in escaping from the host immune cells (van Asten & van Dijk, 2005).

2.1.3 Epidemiology of SE

SE is one of the most common *Salmonella* serotypes isolated from human infections worldwide and outbreaks have been associated with a variety of foods, including eggs, poultry, and produce. Between 2017 and 2019, Canada experienced 16 *Salmonella* outbreaks, 15 of which were caused by SE predominantly isolated from frozen raw breaded chicken products. These outbreaks resulted in 487 confirmed cases with 79 hospitalizations and 2 deaths (Ashley Kerr et al., 2024). Similarly, in the United States, SE remained the leading cause of multistate outbreak-associated isolates in 2024, with food sources responsible for 75.93% of reported *Salmonella* infections. The 2022 National enteric surveillance program (NESP) also reported SE as the predominant serotype in Canada, accounting for 38% of *Salmonella* cases associated with human illness (PHAC, 2022). It is also important to note that some cases of infection are not reported for surveillance due to how self-limiting the infection can be however, this leaves individuals with immunocompromised immune systems, like young children and the elderly, at higher risk for SE infections (Healy & Bruce, 2019). Up until 2019, Frozen breaded chicken products, like chicken fingers and nuggets, were some of the leading causes of *Salmonella* illnesses in Canada (Carvalho et al., 2020; Currie et al., 2005). To combat this, mandatory industry process controls were implemented, requiring manufacturers of breaded chicken products to ensure that *Salmonella* is completely absent from the products, or the products are fully cooked (CFIA, 2015). A national strategy group was also developed with the aim of reducing human illness due to SE by identifying and addressing the risks found in poultry and egg production (Canada, 2015). Additionally, the PHAC adopted a more thorough method for identification SE known as whole-genome sequencing

(WGS). This method made it easier to distinguish between close *Salmonella* isolates and ultimately improved Canadian outbreak investigation and response (Alleweldt et al., 2021; A. Kerr et al., 2024; Nadon et al., 2017). To bolster public safety, federal and provincial agencies provide the general public with public advisories on proper food handling, preparation, and cooking practices to further mitigate the risk of SE (Manitoba, 2015; PHAC, 2019). Even with such extensive policies in place, SE still continues to persist in the poultry industry and this in part is due to the risk of contamination that is present at all levels of production (Ethèves et al., 2021).

2.2 SE in the Poultry Environment

2.2.1 SE Introduction into the Poultry Environment

Vectors and pests like wild birds, insects and rodents can harbor and spread SE to birds in poultry houses (Davies & Breslin, 2003). This can affect the new flock via flock-to-flock transmission, as birds exposed to SE are often asymptomatic and can shed the organism for months, potentially contaminating eggs or transmitting the bacterium to other birds with prolonged contact (Gast & Holt, 1998; Obe et al., 2023). Improper storage of feed and drinking water, contact with wild birds, poor cleaning and sanitation resulting in the use of contaminated farm equipment and contact surfaces, unrestricted access to farm visitors, poor pest control, untrained facility personnel, the use of improper holding temperature during product transport and at the facility, and inadequate vendor vetting, which can lead to the acceptance of SE colonized birds into the facility. (Wang et al., 2023). Contaminated water, feed, and shared equipment between facilities also serve as an avenue for recontamination. This is because persistence is often related to the effectiveness of sanitation and disinfecting practices of the poultry facility and its surroundings (Shaji et al., 2023)

2.2.2 SE Contamination of Laying Hens Environment

Contamination of the poultry environment with SE can occur during the entire production process, from breeding to processing. As mentioned previously, SE colonizes the intestinal tract of hens which remain largely asymptomatic while still shedding bacterial cells in their feces (De Reu et al., 2006). This makes both vertical transmission (passing SE from infected hens to their eggs) and horizontal transmission to contact surfaces possible. It takes about 20 hours for the egg to develop its hard shell and contamination typically occurs during oviposition when the egg passes through the reproductive tract (Gantois et al., 2009). The egg begins as a yolk inside the ovaries, and when matured, it is released into the oviduct which is a long, convoluted tube that runs through the hen's body and is divided into five sections: the infundibulum (where the egg is fertilized by sperm from the rooster if present), magnum (where the egg white (albumen) is added), isthmus (where the two shell membranes are added), uterus (where the hard shell is formed around the outside of the egg), and the vagina which directs the egg towards the cloaca for oviposition (Hrabia et al., 2014). Importantly, the cloaca serves as the intersection between the gastrointestinal and reproductive tracts. It allows the reproductive fluids and fecal material to mix thus creating a microenvironment that is conducive for bacterial transfer allowing SE to contaminate eggs during formation or externally as the eggs are laid (Okamura et al., 2001; Ruiz-De-Castañeda et al., 2011). Inadequate barn sanitation practices, along with improper egg storage and handling, increase the risk of widespread contamination by allowing SE to persist in the poultry environment for extended periods, where it can establish resilient biofilms that are difficult to eliminate.

2.2.3 Biosecurity Measures in Poultry Facilities

Effective SE control on the farm aims to prevent the introduction of SE into the facility and stop its potential spread. Poultry farms and facilities ensure proper upkeep of the poultry barn and

surrounding areas. Sanitary conditions are maintained during feed processing, proper feces and litter disposal, and regular cleaning and refilling of water feeders. All entry points such as windows, doors, and ventilation systems need to be sealed and locked to prevent pests from entering the barn. Access to the barns is also limited and contact with birds by visitors and personnel is minimized where possible. Facilities are also encouraged to source hens from reputable vendors as much as possible (Awad & Ghareeb, 2014; Obe et al., 2023; van de Giessen et al., 1994). Additionally, to promote the well-being and health of the birds, the environmental conditions of laying hen barns must be closely regulated to reduce the susceptibility of the birds to disease. Heat stress in laying hens is characterized by fainting, panting, and reduced feed intake, which inadvertently compromises production parameters like egg quality and meat production. Additionally, reduced immunity of the hens has also been reported (Kocaman et al., 2006; Mashaly et al., 2004). To combat this, ventilation systems comprised of fans and vents are set up to maintain good air quality. The recommended temperature of the barn is between 18-22 °C with a relative humidity ranging from 60-70% (Rosario Díaz et al., 2018). Depending on the local climate, heaters may be introduced in cold weather or overhead misters may be employed in hot weather or low relative humidity to further facilitate temperature control (Renaudeau et al., 2012).

The responsibility of reducing the risk of SE contamination in poultry processing facilities extends beyond the laying barns and continues along the production chain until the eggs and subsequent egg products reach consumers (Agency, 2023; Obe et al., 2023). In Canada, collected eggs are transported to government-approved grading facilities where they are washed multiple times with warm chlorinated water to remove organic matter and dirt, and to reduce the surface contamination by microorganisms (Agency, 2015). Afterwards, the eggs are graded by weight and inspected for cracks, defects and egg quality before refrigeration and transport (Ontario, 2025; Pond, 1961). In

facilities where eggs are processed into liquid egg products, eggs are broken open, and the contents are pooled and filtered into a homogenized batch, which then undergoes pasteurization. Pasteurization is a critical step when processing liquid egg products, as the products are heated to high temperatures for a short time to inactivate pathogenic bacteria like SE without denaturing proteins or altering the product's texture (Cocotine, 2025). The Canadian Food Inspection Agency (CFIA) recommends a minimum pasteurization temperature of 54-63 °C for a duration of 3.5-6.2 minutes depending on the specific product being processed (Agency, 2015).

2.3 Biofilm Forming Ability of SE in the Poultry Environment

2.3.1 General Information on Biofilms

Biofilms are communities of microorganisms that are embedded in a matrix of extracellular polymeric substances (EPS) (Monroe, 2007). The EPS provides many advantages to microorganisms, including but not limited to adhesion to biotic (epithelial tissues, other cells etc.)/ abiotic (plastics, metals, concrete etc.) surfaces, antimicrobial tolerance and resistance, nutrient source, and changes in environment (Butt & Khan, 2015; Ciofu & Tolker-Nielsen, 2019). When immersed in the EPS, bacterial cells are able to exchange genetic information, grow, metabolize nutrients, secrete enzymes and other metabolites, and die all within the EPS. They are in such close proximity that horizontal gene transfer can occur, birthing new generations of resistant cells (Neu & Lawrence, 2010).

2.3.2 The Process of Biofilm Formation

Biofilm formation is a cyclic process that involves various steps and takes place in distinct stages: 1) Initial/reversible attachment, 2) Microcolony formation/irreversible attachment, 3) biofilm development and maturation, and 4) dispersion or detachment (Vogeleer et al., 2014). Poor sanitation practices leave organic residue in food-processing spaces, which promotes the

settlement of planktonic bacteria. Planktonic bacterial cells use physical structures like pili and curli to attach to surfaces, after which the bacterial cells begin to aggregate, forming microcolonies and eventually lose the flagella (irreversible attachment). These cells grow until their cell density reaches the “quorum level,” where cells communicate and begin EPS production (Bassler, 1999). The strength and composition of the EPS is dependent on many factors, such as species composition, environment and nutrient availability (Fagerlund et al., 2016; Rehman et al., 2021). Mature biofilms are made up of many layers of cells and develop channels for the movement of nutrients and genetic material which can lead to competition and starvation amongst cells. The bacteria can then break away from the biofilms to search for more nutrient-rich environments (Sagar et al., 2016).

2.3.3 Role of Curli and Cellulose Production in Biofilm Formation

One of the primary characteristics of biofilms is their sessile nature. Thin extracellular features called curli fimbriae protrude from the surface of many foodborne pathogens, including SE, aiding bacteria in surface attachment and encouraging adhesion to other cells (Barnhart & Chapman, 2006). Curli fimbriae are made up of amyloid fibers and are primarily composed of CsgA protein subunits, encoded by the csgBAC and csgDEFG operons (Römling et al., 2000). The CsgD regulator controls curli biosynthesis and is influenced by environmental cues like surface contact, temperature and nutrient availability. Initial adhesion to abiotic and biotic surfaces is mediated by curli fibers as the amyloid structure confers stability, enabling bacteria to weather drastic changes in environmental conditions (Grantcharova et al., 2010). Curli also regulates cell-to-cell aggregation, facilitating microcolony formation, contributing to the integrity of mature biofilms and enhancing co-adhesion with other bacteria in multispecies biofilms (Austin et al., 1998). Cellulose is another structural polysaccharide that contributes to biofilm formation. A

glucose polymer, cellulose is synthesized by the *bcsABZC* operon, which is also under the regulatory control of CsgD (Arnqvist et al., 1994). Cellulose contributes to the EPS by maintaining hydration, limiting the penetration of chemical agents, and providing stability. Cellulose and curli work in tandem to enhance biofilm adaptability to environmental stressors (Saldaña et al., 2009). Phenotypic manifestations of curli and cellulose can be observed via colony morphology on Congo Red Agar. Curli positive strains can be identified as red, dry and rough (RDAR), pink, dry and rough (PDAR), or brown, dry and rough (BDAR), while curli negative strains are observed to be smooth and white (SAW) (Barnhart & Chapman, 2006). Strains positive for cellulose on the other hand, will fluoresce under UV light (366nm) when cultured with calcofluor dye (Uhlich et al., 2006).

2.3.4 SE Pellicle Formation

In biofilm formation, microbial cells can organize at different interfaces, with the most common being the solid-liquid interface, where cells will colonize submerged solids (e.g. medical devices or water pipes) (Hao et al., 2018; Martí et al., 2011; Mulcahy et al., 2014), the liquid-liquid interfaces between oil and water boundaries (Subbiahdoss & Reimhult, 2020) or the air-liquid interface typically formed at the surface of static cultures (Armitano et al., 2014; Golub & Overton, 2021; Z. S. Xu et al., 2023). Biofilms that develop at the air-liquid interface are referred to as pellicles and provide an oxygen-rich environment that facilitates aerobic metabolism and enhances the production of the EPS (Rainey & Travisano, 1998). Pellicles are usually formed as a way for biofilms to spatially optimize access to both oxygen from the air and nutrients and moisture from the liquid (Z. S. Xu et al., 2023). Pellicle formation promotes survival and persistence in different environments and can be produced by both Gram-positive bacteria (e.g. *B. cereus* and *B. subtilis*) and Gram-negative bacteria (e.g. *P. aeruginosa*, *E. coli*, etc.) (Golub & Overton, 2021; Ude et al.,

2006; Wijman et al., 2007). Pellicle formation by *Salmonella* has been reported widely, with many studies linking a combination of factors such as nutrient and oxygen availability, temperature and pH to the production and strength of biofilms formed (Paytubi et al., 2017; Römling & Rohde, 1999; Sharma et al., 2022; Villa-Rojas et al., 2017). It has also been reported that some *Salmonella* strains isolated from river water and animal feces can form pellicles at the air-liquid interface, which can be affected by environmental factors like pH and salinity (Medrano-Félix et al., 2018). In a study carried out with genetically modified *E. coli* K-12 strains by Serra et al. (2013), it was proven that the elasticity and stability of the pellicle can be attributed to how much curli and cellulose are produced by the bacteria. It was also realized that bacteria that produce more curli than cellulose were able to adhere to surfaces but had brittle pellicles.

2.3.5 SE Attachment to Contact Surfaces

In poultry facilities, common contact surfaces are designed for durability, easy sanitation, and non-porous materials that resist corrosion are typically used. Galvanized steel (GS) is a cost-efficient, lightweight metal that is used in barn infrastructure as it is coated with zinc, which improves its longevity (NMC, 2020; PEB, 2025). In laying barns specifically, GS is the material used for the cages and comes into direct contact with the laying hens, increasing the risk of bacterial deposits on the surface of the steel. When exposed to the environment, GS forms Zinc Oxide (ZnO) and Zinc Hydroxide (Zn(OH)₂), which also provides a layer of protection from corrosion. Research involving Zinc-based nanoparticles has shown that ZnO particles in certain concentrations can kill planktonic cells, disrupt the surface attachment of the biofilms and reduce biofilm mass of foodborne pathogens like *E. coli* O157:H7 and *L. monocytogenes* (Applerot et al., 2012; Fulindi et al., 2023; Jin et al., 2009). Some of the antimicrobial properties of the ZnO particles include, but are not limited to cell dysfunction due to high oxidative processes, prevention

of DNA replication, inhibition of energy metabolism, and cell lysis (Sirelkhatim et al., 2015; TAYEL et al., 2011). Regular maintenance and cleaning of GS materials is important, as some bacteria like *P. aeruginosa* can cause microbially induced corrosion even while living anaerobically in a biofilm eventually promoting biofilm attachment to these surfaces long term (Xu et al., 2023). Other contact surfaces found within the poultry environment include concrete (for flooring and walls), stainless steel (most commonly used in processing stations, slicers, sinks and cutting tables), and plastic (found in nesting boxes, water feeders, egg crates and conveyor belts) (Bach et al., 2025; Giaouris & Nychas, 2006; Joseph et al., 2001). Poor maintenance and sanitation of farm equipment and structures can lead to material degradation, which may include cracks, abrasions, or other damages that harbour organic material and promote biofilm attachment (Arnold & Silvers, 2000).

2.4 SE Multispecies Biofilm Formation

Realistically, SE biofilms formed in the poultry environment are more likely to exist in complex microbial communities known as multispecies biofilms. Unlike single-species biofilms, which are made up of one microbial species, multispecies biofilms consist of two or more microorganisms and are considered the most typical type found in natural environments (Winans et al., 2022). Laying barns contain a blend of organic material like feathers, feces, bedding materials (e.g. wood shavings and sawdust) and even feed material scattered about by poultry which can host both beneficial and pathogenic organisms (Gomes et al., 2022; Gržinić et al., 2023). Cages, perches, feeding and watering equipment, egg and feces conveyors, nesting boxes, and even flooring surfaces can serve as sources of biofilm contamination in poultry barns (Al-Zenki et al., 2007; Biyashev et al., 2025). Microorganisms within the multispecies biofilms exist within the EPS they produce. The social interactions between bacteria in a multispecies biofilm may manifest

as competition for resources (antagonistic), mutually beneficial (synergistic) or neutral (S. Elias & E. Banin, 2012; Luo et al., 2022; Nadell et al., 2016). The protection offered by coexistence was observed in a study by Perlin et al. (2009), where ampicillin-sensitive *Salmonella* Typhimurium strains cocultured with β -lactamase-producing *E. coli* survived exposure to 100 $\mu\text{g ml}^{-4}$ of ampicillin, which had previously been lethal to single-species populations of the same strain. Multispecies biofilms dominated by cooperative interactions pose an increased risk of horizontal gene transfer between pathogenic and non-pathogenic cells. This could eventually lead to a generation of pathogens with increased tolerance to antimicrobial interventions, inadvertently increasing the likelihood of severe foodborne outbreaks (Mitri et al., 2011). There have also been reports of multispecies biofilms having increased resistance to sanitizers and disinfectants often used in the food industry, like Hydrogen peroxide and Quaternary ammonium compounds, requiring concentrations greater than recommended to be effective (Burmølle et al., 2014; Koti et al., 2024; R. Wang et al., 2013). Alternatively, it has been reported that the presence of other bacteria can affect the biofilm-forming abilities of pathogenic foodborne bacteria thereby possibly reducing the impact of their activity (Rendueles & Ghigo, 2012). Many bacteria such as Enterococci, *Bacillus* spp., etc. produce bacteriocins which target and inhibit/kill other bacteria (Ben Braïek & Smaoui, 2019). In the food industry, there has been a shift towards using these antagonistic relationships to develop biological control strategies against foodborne pathogens (Abdelhamid & El-DougDoug, 2020). Woo and Ahn (2013) found that certain bacteria that produced Lactic acid as the by-product of carbohydrate fermentation, known as Lactic Acid Bacteria (LAB), were capable of competitively excluding and, to a degree, inhibiting biofilms formed by pathogens like *Listeria monocytogenes*, through the production of antimicrobial peptides.

Chapter 3: Multispecies Biofilm Formation of *Salmonella* Enteritidis Strains Isolated from Poultry

3.1 Abstract

This study aimed to evaluate the interactions among *Salmonella* Enteritidis (SE), selected bacteria associated with eggshells (BAE) and Lactic Acid Bacteria (LAB) during biofilm formation. Fifteen SE strains were tested for curli expression and cellulose production. All SE, BAE, and LAB strains were assessed for biofilm formation individually using optical density (OD) measured by the Crystal Violet technique. Based on biofilm-forming ability, two SE strains (36SusP and 9SusP) exhibiting strong biofilm formation at 10 and 20 °C were selected to form dual- and multispecies biofilms with 5 BAE: *P. aeruginosa* (ATCC 7700), *B. cereus* (ATCC 14579), *E. coli* (ATCC 11775), *E. faecalis* S36, and 1 LAB: *L. acidophilus* (ATCC 4356) at 10 and 20 °C in 96-well microplates. At 10 °C 6/15 SE strains showed weak biofilm formation while 9 strains formed none. In contrast, at 20°C, 4 SE strains exhibited intermediate biofilm formation, while 11 strains showed strong biofilm formation. It was found that all multispecies combinations including *E. coli* + *E. faecalis* (M1), *P. aeruginosa* + *E. coli* (M2) and *P. aeruginosa* + *B. cereus* (M3), had increased biofilm biomass at 10 °C, although there was no significant increase ($P>0.05$) in SE bacterial counts within those combinations. At 20 °C however, SE counts in multispecies combinations decreased ($P<0.05$) as the higher temperature encouraged competition within multispecies biofilms. SE counts were significantly reduced ($P<0.05$) within M2 (dropped from 7.4 to 5.3 log₁₀ CFU/mL) and in M3 (reduced to 6.5 log₁₀ CFU/mL). Overall, these findings highlight that SE persistence within biofilms is context-dependent and shaped by both environmental conditions and microbial community composition.

3.2 Introduction

Eggs are a highly nutritious food and a staple ingredient in many cultures around the world. However, they can also be a source of *Salmonella enteritidis* (SE) being one of the most common bacterial pathogens associated with eggs (Punchihewage-Don et al., 2022). SE can contaminate poultry products, such as eggs and meat, during production, processing, and transportation (Willey et al., 2011). SE can colonize the intestinal tract of hens, who remain largely asymptomatic while still shedding SE bacterial cells. Colonization allows for both vertical transmission (from infected hens to their eggs) and horizontal transmission (from infected hens to contact surfaces) to occur, (Ruiz-De-Castañeda et al., 2011; Whiley & Ross, 2015).

In the United States, SE remained the leading cause of multistate outbreak-associated isolates in 2024, with food sources responsible for 75.93% of reported *Salmonella* infections. The 2022 National enteric surveillance program (NESP) also reported SE as the predominant serotype in Canada, accounting for 38% of *Salmonella* cases associated with human illness (PHAC, 2022). Furthermore, between 2017 and 2019, Canada experienced sixteen *Salmonella*-related outbreaks, fifteen of which were caused by SE predominantly isolated from frozen raw breaded chicken products. These outbreaks resulted in a total of 487 confirmed cases, with 79 hospitalizations and 2 deaths (Ashley Kerr et al., 2024). Frequent SE foodborne outbreaks reflect the persistence of SE, which could be linked to SE ability to form biofilms (Bai et al., 2021; Chaves et al., 2024).

Biofilms are microbial aggregates embedded into a matrix of extracellular polymeric substances (EPS) (Monroe, 2007). During biofilm formation, microbial cells can organize at different interfaces, with the most common being the solid-liquid interface, where cells will colonize submerged solids (e.g. medical devices or water pipes) (Hao et al., 2018; Martí et al., 2011; Mulcahy et al., 2014), the liquid-liquid interfaces between oil and water boundaries

(Subbiahdoss & Reimhult, 2020) and the air-liquid interface typically formed at the surface of static cultures (Armitano et al., 2014; Golub & Overton, 2021; Z. S. Xu et al., 2023). Biofilms that are developed at the air-liquid interface are referred to as pellicles and provide an oxygen-rich environment that facilitates aerobic metabolism and enhances the production of the EPS (Rainey & Travisano, 1998). It has been reported that some *Salmonella* strains isolated from river water and animal feces can form pellicles at the air-liquid interface, which can be affected by environmental factors like pH and salinity (Medrano-Félix et al., 2018).

Additionally, other major factors that have been found to be involved in *Salmonella* biofilm formation and persistence are the production of curli fimbriae and cellulose expression (Harrell et al., 2020; Simm et al., 2014). Curli fimbriae are amyloid fibres that facilitate bacterial surface adhesion and contribute to biofilm structure. In conjunction with cellulose, it also contributes to the structural integrity of the biofilm, improving durability and resistance to antimicrobial treatments (Austin et al., 1998; Jonas et al., 2007). Phenotypic manifestations of curli and cellulose can be observed via colony morphology on Congo Red Agar. Curli positive strains can be identified as red, dry and rough (RDAR), pink, dry and rough (PDAR), or brown, dry and rough (BDAR), while curli negative strains are observed to be smooth and white (SAW) (Barnhart & Chapman, 2006). Cellulose positive strains on the other hand, will fluoresce under UV light (366nm) when cultured with calcofluor dye (Uhlich et al., 2006). In nature, microorganisms typically exist in complex microbial communities known as multispecies biofilms. These different microorganisms interact and exist within the EPS they produce, thus the microbes might either mutually support and maintain each other's growth (synergistic relationship), suppress and/or eliminate the competition, or cohabit without majorly impacting one another (neutralism) making multispecies biofilms worthwhile to investigate (Nadell et al.,

2016; Winans et al., 2022). When immersed in the EPS, bacterial cells can exchange genetic information as seen with *Pseudomonas aeruginosa* (Gupta et al., 2016). In the EPS, the cells grow, metabolize nutrients, secrete enzymes and other metabolites. As the EPS matures, some cells die within and others split off in search of more nutrient-dense locations. The cells in the EPS are in such proximity to one another that horizontal gene transfer between pathogenic and non-pathogenic bacteria can occur, increasing the likelihood of new generations of cells that are more fit and have increased tolerance to antimicrobials (Neu & Lawrence, 2010).

A study by Yuchen et al. (2022) reported that multispecies biofilms supported the survival of Shiga-Toxigenic *Escherichia coli* (STEC) under dry conditions on thermoplastic polyurethane (TPU) for as long as 30 days at 25 °C. It was also observed that STEC present within those biofilms could be transferred from the contaminated coupons onto meat. Alternatively, antagonistic relationships have also been observed within multispecies biofilms formed with pathogens. Lactic Acid Bacteria (LAB) like *Lactobacillus* spp., *Leuconostoc* spp. and *Bifidobacterium* spp., have been found to aid in controlling the colonization of pathogenic bacteria in the gut of layer hens. This can occur by reducing the immune action of the pathogens (Adhikari et al., 2019), increasing the proliferation of other beneficial bacteria or directly suppressing/limiting the growth of pathogens (Abdelqader et al., 2013; Adhikari et al., 2019; Kobierecka et al., 2017).

Much of the research done on SE biofilms has focused primarily on SE single-species or dual-species biofilm formation and there is not much information on how the environmental conditions encountered at the poultry barn (e.g. temperature), or the influence of multispecies biofilms on SE persistence in the poultry environment. Therefore, this study aimed to 1) investigate the specific environmental conditions under which *Salmonella* Enteritidis (SE)

biofilms can form and persist under conditions representative of the poultry production environment and 2) evaluate the competitive fitness of SE within multispecies biofilms under simulated barn conditions, particularly in the presence of bacteria associated with eggshells (BAE) and lactic acid bacteria (LAB).

3.3 Materials and methods

3.3.1 Bacterial strains

Fifteen *Salmonella enteritidis* (SE) serotypes isolated from poultry feed, chicken breast meat and nuggets were included in this study. Additionally, 5 bacteria genera commonly associated with eggshells (BAE) (*E. coli*, *Enterococcus*, *Staphylococcus aureus*, *Bacillus cereus*, and *P. aeruginosa*) (Alvarez-Fernández et al., 2012; De Reu et al., 2008; Peralta-Sánchez et al., 2012) and 7 lactic acid bacteria (LAB) strains were selected and evaluated to determine their biofilm-forming ability (**Table 3-1**).

SE/BAE strains were grown in Tryptic Soy broth (TSB; Becton, Dickson and Company, MD, USA) for 18-24 hrs at 37 °C (overnight) and cultured on selective media as follows; SE on Xylose Lysine and Dextrose agar (XLD; Hardy diagnostics, Santa Maria, CA, USA), *E. coli* on CHROMagar™ *E.coli* (Kanto Chemical, Saint-Denis, France), Enterococci on Bile Esculin Agar (Hardy diagnostics, Santa Maria, CA, USA), *P. aeruginosa* on *Pseudomonas* isolation agar (Becton, Dickson and Company, Sparks, MD, USA), *B. cereus* on *Bacillus Cereus* Agar (OXOID LTD, Basingstoke, Hampshire, England) supplemented with 50% Egg Yolk Emulsion w/v Egg yolk and 0.85% saline) and 500,000 IU Polymyxin B Sulphate (Fisher Scientific, Ottawa, ON, CA). LAB strains were grown in Lactobacillus De Man Rogosa and Sharpe broth (MRS; OXOID LTD, Basingstoke, Hampshire, England) for 24-48 hrs at 37 °C and streaked onto M.R.S. Agar (MRSA; OXOID LTD, Basingstoke, Hampshire, England). A single colony of each

strain was then inoculated into Luria Broth-No Salt (LB-NS; 10 g/L tryptone and 5 g/L yeast extract) and incubated overnight, and grown to a cell density of 10^8 CFU/ml. All the cultures were obtained from the University of Manitoba culture collection.

Table 3-1: *SE, LAB and BAE bacterial strains assessed for biofilm formation*

Bacterial strain	Strain ID	Source	Category
107	107	Bovine**	SE
710901	07-5386	Chicken nuggets	SE
619102	07-1485	Chicken nuggets	SE
618901	07-1474	Chicken nuggets	SE
79801	07-2789	Chicken nuggets	SE
79901	07-2791	Chicken nuggets	SE
719001	08-0218	Chicken Nuggets	SE
61401	06-1480	Feed	SE
715701	07-5422	Feed	SE
78501	07-2787	Chicken nuggets	SE
9SusPh	05-1095	Chicken nuggets	SE
9SusP	05-1094	Chicken nuggets	SE
33SusP	05-2239	Chicken nuggets	SE
40SusP	05-3936	Chicken breast meat	SE

36SusP	05-3932	Chicken breast meat	SE
<i>Escherichia coli</i>	ATCC 11775	Urine	BAE
<i>E. coli</i>	ATCC 15597	Derived from <i>Escherichia coli</i> K-12	BAE
<i>E. coli</i> *	R508	Bovine/feces	Reference strain
<i>Staphylococcus aureus</i>	ATCC 25923	Clinical Isolate	BAE
<i>Enterococcus faecalis</i>	S36	Unknown	BAE
<i>E. faecium</i>	S34	Unknown	BAE
<i>Bacillus cereus</i>	ATCC 14579	Unknown	BAE
<i>Pseudomonas aeruginosa</i>	ATCC 7700	Well water	BAE
<i>Raoultella</i> *	ENT25-16	Meat-packing plant	Reference strain
<i>Lactobacillus reuteri</i>	ATCC 53608	Feces	LAB
<i>L. rhamnosus</i>	ATCC 7469	Unknown	LAB
<i>L. curvatus</i>	133L	Meat starter culture	LAB
<i>L. sakei</i>	#9	Unknown	LAB
<i>L. acidophilus</i>	ATCC 4356	Human	LAB
<i>Leuconostoc spp.</i>	MPL-A1	Unknown	LAB
<i>L. mesenteriodes</i>	MPL-C	Unknown	LAB

Note: SE: *Salmonella enteritidis*, BAE: *Bacteria associated with eggshells*, and LAB: *Lactic acid bacteria*. “*” denotes positive controls. ** Bovine strain was included as it was the only strain in our collection negative for curli and cellulose, and an intermediate biofilm former at 20 °C.

3.3.2 Single-species biofilm formation

3.3.2.1 Crystal Violet Technique

The Crystal Violet technique (Adator et al., 2018) was used to assess the biofilm-forming ability of all the bacterial strains listed in **Table 3-1**. A single colony was inoculated; 10 g/L tryptone and 5 g/L yeast extract) and incubated overnight, then diluted to a concentration of 10^6 CFU/mL. 20 μ L of the 10^6 CFU/mL culture was transferred to a 96-well flat-bottom non-treated microplate (Falcon®, Corning, NY, USA) containing 180 μ L of LB-NS, achieving a total volume of 200 μ L. Three temperatures were selected to preliminarily test biofilm formation, 10 °C, which is the egg cold storage often used to refrigerate eggs in Canada, 20 °C, which is the temperature of the egg layers barn in Manitoba, and 37 °C, which is the ideal temperature for mesophiles to grow. A group of identical 96-well microplates was prepared for each treatment and incubated at 10, 20, and 37 °C for five days. After 5 days, the 96-wells microplates were washed with 200 μ L of Butterfield’s phosphate buffer (BPB), and air-dried for 15 min in a Biosafety level 2 cabinet, then 200 μ L of methanol was added to each well and left for 15 min. The methanol was aspirated, and 200 μ L of 0.1% crystal violet (CV) was added to each well for 20 min. The microplate was then washed three times with 200 μ L of BPB, and the residual CV in each well was solubilized by adding 200 μ L of 85% ethanol. Biofilm-forming ability was determined indirectly by measuring the optical density of residual CV at 630nm (OD₆₃₀) using a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, VT, USA). Using the optical density of the

negative controls (Odc; sterile media only), each isolate was categorized as non-forming ($OD < ODC$), weak ($ODC < OD < 2ODC$), intermediate ($2ODC < OD < 4ODC$), and strong ($4ODC < OD$) biofilm formers based on the data obtained from the OD readings as previously described (Adator et al., 2018).

3.3.2.2 Curli and Cellulose Expression

All SE strains were also assessed on their capacity to produce curli and cellulose at 10, 20 & 37 °C (Hammar et al., 1996; Uhlich et al., 2006). For curli production, overnight cultures were streaked onto Congo red agar plates (10 g/L casamino acids, 1 g/L yeast extract, and 20 g/L agar) supplemented with 20 µg/mL Coomassie blue dye (Sigma Aldrich, St. Louis, USA) and 40 µg/mL Congo red dye (Sigma Aldrich, St. Louis, USA) (Yuchen et al., 2022). Cellulose production was also evaluated by streaking overnight cultures onto Luria Bertani plates (LB; Hardy diagnostics, Santa Maria, CA, USA) supplemented with 200 mg/L Calcofluor dye (Sigma Aldrich, St. Louis, USA). The plates were then incubated at 10, 20, and 37 °C for 5 days.

3.3.3 Multispecies biofilm formation

3.3.3.1 Dual-species Competitive fitness test

To observe different bacterial interactions, two SE strains, which exhibited strong biofilm formation and curli and cellulose expression at 20 °C, 36SusP (Strong) and 9SusP (Strong), were selected to test for competitive fitness with six BAE (*P. aeruginosa* (ATCC 7700), *B. cereus* (ATCC 14579), *E. coli* (ATCC 11775), *E. coli* (ATCC 15597), *E. faecalis* (S36), and *E. faecium* (S34) selected from 4 bacteria genera commonly associated with eggshells (Alvarez-Fernández et al., 2012; De Reu et al., 2008; Peralta-Sánchez et al., 2012) and 1 LAB (*L. acidophilus* (ATCC 4356)) at 10 and 20 °C (**Table 3-2**). The biofilm-forming abilities of the LAB/BAE strain combinations were assessed in a 96-well flat-bottom non-treated microplate (Falcon[®], Corning,

NY, USA). Mixed-species biofilms were prepared as H.-H. Wang et al. (2013) described with minor modifications. 20µL of LAB and BAE mixed-strain cultures were adjusted to 10^6 CFU/mL and inoculated into a 96-well microplate containing 180µL of LB-NS in each well. Mature biofilms were allowed to form for 5 days at 10 and 20 °C after which the supernatant in each well was aspirated and washed with 200 µL BPB to remove free and loosely attached cells. After washing, 180µL of sterile LB-NS was added to the microplate along with 20µL of an overnight SE culture (adjusted to 10^3 CFU/ml) was also cultured in LB-NS, aliquoted into the designated wells containing biofilms already formed BAE and LAB biofilms; incubated for an additional 5 days and washed three times with 200µL BPB. One plate was used to measure OD (crystal violet) and another for bacterial enumeration which was conducted using the drop-plate method (Herigstad et al., 2001). For enumeration, 200µL of Buffered Peptone Water (BPW; Hardy Diagnostics, Santa Maria, CA, USA) was added to the wells of the microplate after washing then, using sterile pipette tips, the wells were scratched to detach existing biofilms from the walls and bottoms of each well, followed by sonication at 40kHz (Branson 2800, Branson Ultrasonics Co., Danbury, USA) for 1 min. Next, serial dilution in BPW was carried out by taking 20µL of the sample into 180µL of BPW, making a 10-fold dilution. Five 10 µL of the desired dilutions were then plated on Xylose Lysine Deoxycholate agar (XLD), selective for SE, and overlaid with trypticase soy agar (TSA). The plates for OD were prepared as previously described before being observed at 630nm (OD_{630}) using a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, VT, USA).

Mixed biofilm combinations were enumerated on selective media and specific strains were selected based on their antagonistic or synergistic effects to be tested in further experiments. Each trial was replicated three times.

Table 3-2: Dual-species strain combinations with SE

Strain combination	Code
<i>36SusP</i> + <i>E. faecium</i> (S34)	D1
<i>36SusP</i> + <i>E. faecalis</i> (S36)	D2
<i>36SusP</i> + <i>B. cereus</i> (ATCC 14579)	D3
<i>36SusP</i> + <i>P. aeruginosa</i> (ATCC 7700)	D4
<i>36SusP</i> + <i>E. coli</i> (ATCC 11775)	D5
<i>36SusP</i> + <i>E. coli</i> (ATCC 15597)	D6
<i>36SusP</i> + <i>L. acidophilus</i> (ATCC 4356)	D7
<i>9SusP</i> + <i>E. faecium</i> (S34)	B1
<i>9SusP</i> + <i>E. faecalis</i> (S36)	B2
<i>9SusP</i> + <i>B. cereus</i> (ATCC 14579)	B3
<i>9SusP</i> + <i>P. aeruginosa</i> (ATCC 7700)	B4
<i>9SusP</i> + <i>E. coli</i> (ATCC 11775)	B5
<i>9SusP</i> + <i>E. coli</i> (ATCC 15597)	B6
<i>9SusP</i> + <i>L. acidophilus</i> (ATCC 4356)	B7

3.3.3.2 Multispecies Competitive Fitness Test

Bacterial strains for competitive fitness testing were selected based on the results from the crystal violet assay. Preliminary testing at 10, 20 and 37 °C showed that 20 °C was the optimal temperature observed for SE biofilm formation. Strong, intermediate and weak biofilm formers were selected from SE and BAE strains for further multispecies biofilm testing at 10 and 20 °C with four BAE also selected based on their ecological relevance and prevalence in the poultry environment and potential interactions with SE. *P. aeruginosa* (strong) due to its strong biofilm forming ability and persistence under harsh conditions in the environment, *B. cereus* (non-forming) which might allow long-term survival due to the ability to form endospores, *E.*

coli (intermediate) included as an indicator of fecal contamination and intestinal microbiota in poultry, *E. faecalis* S36 (weak) also an intestinal bacteria found in poultry feces and farm environments and one SE: 36SusP (Strong). Ultimately, 3 combinations were formed from the selected BAE strains; **M1**: *E. coli* + *E. faecalis*, **M2**: *P. aeruginosa* + *E. coli* and **M3**: *P. aeruginosa* + *B. cereus* and the multispecies combinations were prepared similarly to the dual-species competitive fitness test.

3.3.4 Statistical Analysis

All the microbial tests were carried out in triplicate. The data obtained was analyzed using Two-way Analysis of variance (ANOVA) on SAS (Cary, NC, United States) with a 5% confidence level.

3.4 Results

3.4.1 SE Single-species Biofilm Formation and Pellicle Formation

To determine the optimal temperature for SE biofilm formation, three temperatures 10, 20 and 37 °C were tested. At 10 °C, 6 out of the 15 SE strains exhibited weak biofilm formation, while 9 strains displayed no biofilm formation ability. In contrast, at 20 °C, 4 strains exhibited intermediate biofilm formation, while 11 strains showed strong biofilm formation. At 37 °C, 5 strains showed intermediate formation, 8 strains displayed weak formation, and 2 strains produced no biofilm (**Figure 3-1**). Interestingly, 14 SE strains were positive at both curli and cellulose at 10 and 20 °C exhibiting strong fluorescence (indicative of positive cellulose expression) and curli production (characterized by red, dry, and rough (rdar) morphology) at 20 °C. 107 (bovine) included as a control for curli and cellulose production, had no fluorescence and a smooth and white (saw) morphology. 107 (bovine) however, showed positive for both

cellulose and curli at 10 °C. All 15 SE strains, including 107(bovine), were negative for cellulose at 37 °C (**Table 3-3**).

During my work with the 96-well microplates, it was noticed that all the SE strains formed biofilms at the air-liquid interface at 20 °C, a phenomenon known as pellicle formation (Armitano et al., 2014). The fragility of the pellicle formed was determined by applying a mild force with a sterile toothpick. Coincidentally, the pellicles formed by the SE strains that were intermediate at 20 °C (107 (bovine), 79801, 79901, 715701) were more brittle, breaking apart after a slight touch of the toothpick. With the other strains that were strong biofilm formers at 20 °C, the pellicle formed did not break apart and even stuck to the toothpick with more force applied. The presence of the pellicle introduced some variability during CV testing, as pellicles formed by strong biofilm-formers were frequently removed entirely during aspiration, resulting in lower biofilm classification (i.e. from strong to intermediate). To address this issue, a sterile pipette tip was used to create small holes in the centre of the pellicle, facilitating easier aspiration of loose cells. None of the SE strains tested produced pellicles at 10 and 37 °C.

Based on the results of the above data, two SE strains that showed stronger biofilm-forming ability ($P > 0.0001$) at 20 °C, 36SusP and 9SusP, were selected for dual-species testing with BAE and LAB.

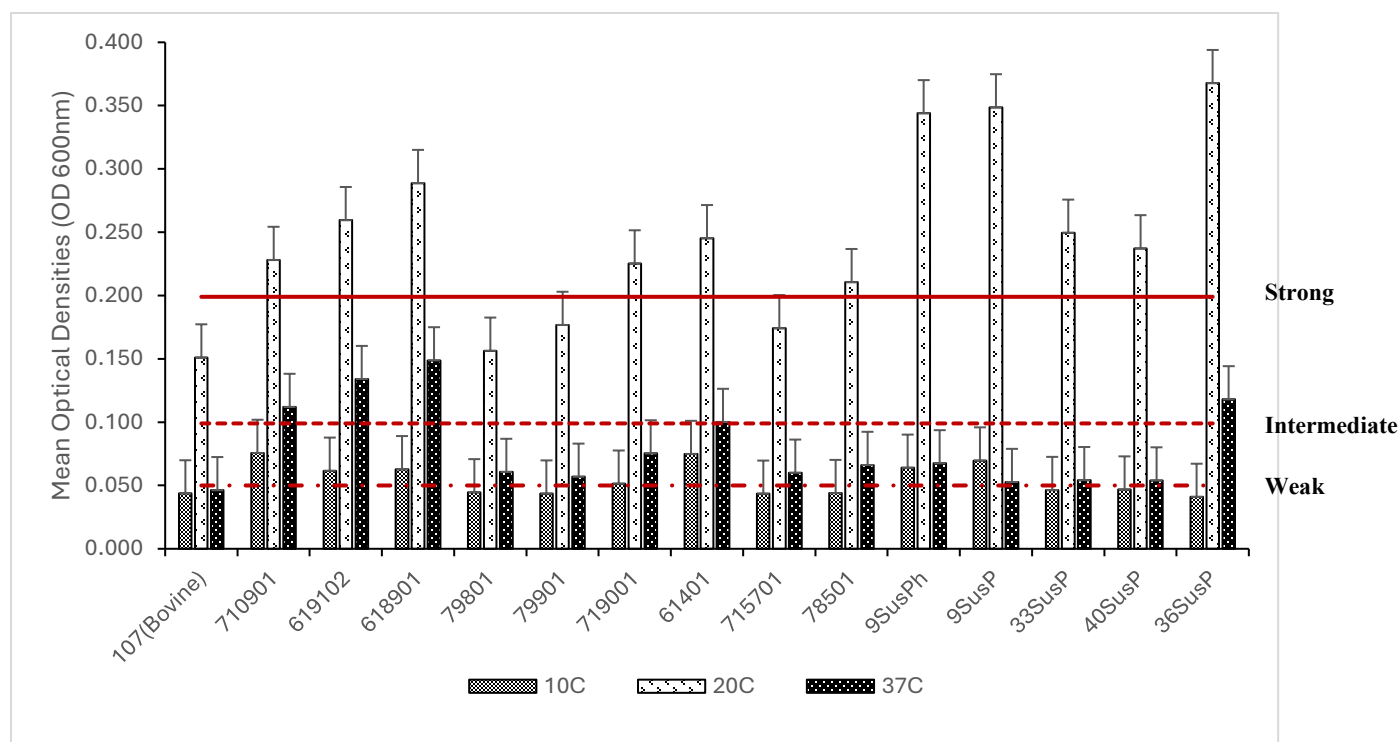


Figure 3-1: Biofilm-forming ability comparison of 15 SE strains formed in 96-well microplates after 5 days at 10, 20 and 37 °C. Mean values were determined using three replicate experiments. Horizontal lines were statistically determined using the negative controls from each temperature and represent division between non-biofilm formers (bottom; $OD < OD_c$), weak (lower middle; $OD_c < OD < 2OD_c$), intermediate (upper middle; $2OD_c < OD < 4OD_c$), and strong (top; $4OD_c < OD$). ($P < 0.0001$). Number of observations per temperature for each strain “N” = 48

Table 3-3: Biofilm-forming ability at 20 °C, Pellicle formation, Curli, and Cellulose production of 15 SE strains at 10, 20 and 37 °C

Strain	Curli production			Cellulose production			Biofilm-forming ability at 20 °C	Pellicle formation at 20 °C
	10*	20	37	10	20	37		
107(Bovine)	+	-	+	+	-	-	Intermediate	+
710901	+	+	+	+	+	-	Strong	++
619102	+	+	+	+	+	-	Strong	++
618901	+	+	+	+	+	-	Strong	++
79801	+	+	+	+	+	-	Intermediate	+

79901	+	+	+	+	+	-	Intermediate	+
719001	+	+	+	+	+	-	Strong	++
61401	+	+	+	+	+	-	Strong	++
715701	+	+	+	+	+	-	Intermediate	+
78501	+	+	+	+	+	-	Strong	++
9SusPh	+	+	+	+	+	-	Strong	++
9SusP	+	+	+	+	+	-	Strong	++
33SusP	+	+	+	+	+	-	Strong	++
40SusP	+	+	+	+	+	-	Strong	++
36SusP	+	+	+	+	+	-	Strong	++

Key: Negative (-), Positive (+), Strong positive (++). *All temperatures (10, 20, and 37) are reported as Celsius (°C).

3.4.2 BAE and LAB Single-species Biofilm Formation

At 10, 20 and 37 °C, *L. sakei*, *L. curvatus* and *L. rhamnosus* did not form biofilms, while *L. acidophilus*, *leuconostoc spp.*, and *L. mensteroides* showed weak biofilm formation at all 3 temperatures. Interestingly, *L. reuteri* demonstrated strong biofilm formation at 37 °C, but weak biofilm formation at 10 and 20 °C. With BAE at 10 °C, *E. faecium* and *P. aeruginosa* strains had weak biofilm formation, while *E. coli* 11775, *E. coli* 15597, *E. faecalis*, *S. aureus*, and *B. cereus* produced no biofilm. At 20 °C, *P. aeruginosa* showed strong biofilm-forming ability, *E. coli* 11775 was intermediate, *B. cereus* and *S. aureus* were non-producers, and the others were weak. At 37 °C, *B. cereus* and 11775 did not produce biofilm, *P. aeruginosa* was intermediate and other strains showed weak biofilm formation (**Figure 3-2**).

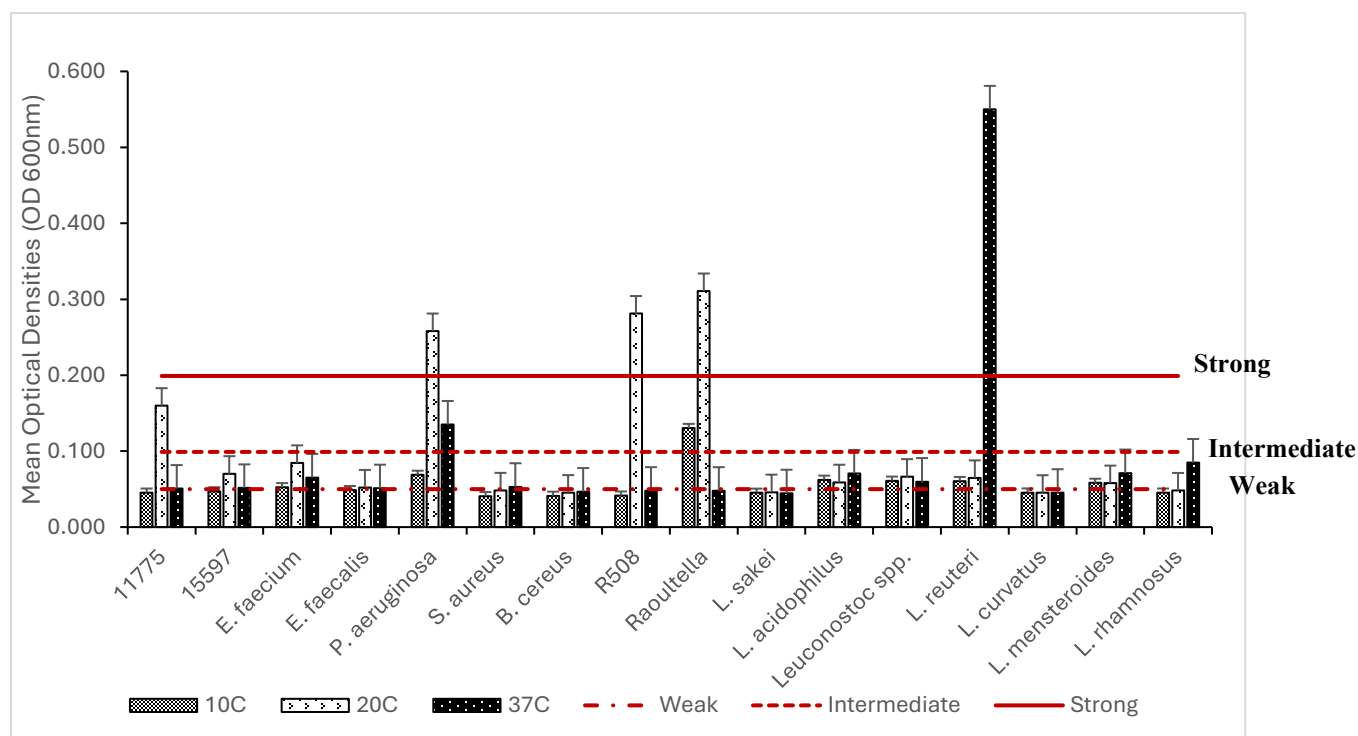


Figure 3-2: Biofilm-forming ability comparison of 7 BAE and 7 LAB strains formed in 96-well microplate after 5 days at 10, 20 and 37 °C. Mean values were determined using three replicate experiments. Horizontal lines were statistically determined using the negative controls from each temperature and represent division between non-biofilm formers (bottom; $OD < OD_c$), weak (lower middle; $OD_c < OD < 2OD_c$), intermediate (upper middle; $2OD_c < OD < 4OD_c$), and strong (top; $4OD_c < OD$). R508 and Raoultella served as positive controls. Number of observations per temperature for each strain “N” = 48

3.4.3 Multispecies biofilm formation

Two SE strains, which exhibited strong biofilm formation and curli and cellulose expression at 20 °C, 36SusP (Strong) and 9SusP (Strong), were selected to test for competitive fitness with six BAE from genera commonly associated with eggshells (Alvarez-Fernández et al., 2012; De Reu et al., 2008; Peralta-Sánchez et al., 2012) ; *P. aeruginosa* (ATCC 7700), *B. cereus* (ATCC 14579), *E. coli* (11775), *E. coli* (ATCC 15597), *E. faecalis* (S36), and *E. faecium* (S34) and 1 LAB; *L. acidophilus* (ATCC 4356). The selections were also based on their strong, intermediate, and/or weak biofilm-forming ability at 10 and 20 °C from preliminary single-species testing.

3.4.3.1 SE dual-species biofilm formation and enumeration with *L. acidophilus* (LA)

Biofilm biomass measured using the Crystal violet assay showed that dual-species biofilms formed by LA + 36SusP (D7) and LA + 9SusP (B7) exhibited significant biofilm formation at 20 °C compared to the same biofilms formed at 10 °C ($P>0.05$). At 10 °C all dual-species combinations were considered non-forming ($OD<OD_c$) with minimal growth. In contrast, at 20 °C, there was a significant increase in biofilm formation as dual-species combinations formed strong biofilms ($4\ OD_c < OD$). LA did not form biofilms at 20 °C (**Figure 3-3**).

Overall, bacterial counts across all dual-species combinations increased with an increase in temperature from 10 to 20 °C. At 10 °C, SE counts across all multispecies combinations had no significant increase ($P>0.05$) in comparison to the controls (3.2 and 3.4 $\log_{10}$ CFU/mL for C1 and C2, respectively). At 20 °C both SE strains experienced a reduction in counts which was not significant ($P>0.05$) in dual-species combinations. SE counts in D7 counts dropped from 8.2 $\log_{10}$ CFU/mL in the controls to 7.6 $\log_{10}$ CFU/mL and in B7, from 8.1 $\log_{10}$ CFU/mL in the controls to 7.8 $\log_{10}$ CFU/mL ($P<0.05$). Similarly, LA counts at 10 °C also showed a non-significant reduction ($P>0.05$) while in dual-species combinations. The increase in LA counts within multispecies biofilms at 20°C was also not significant ($P<0.05$) dropping from 6.9 $\log_{10}$ CFU/mL in the controls to 7.7 $\log_{10}$ CFU/mL in D7 and to 7.9 $\log_{10}$ CFU/mL in B7 (**Figure 3-4**).

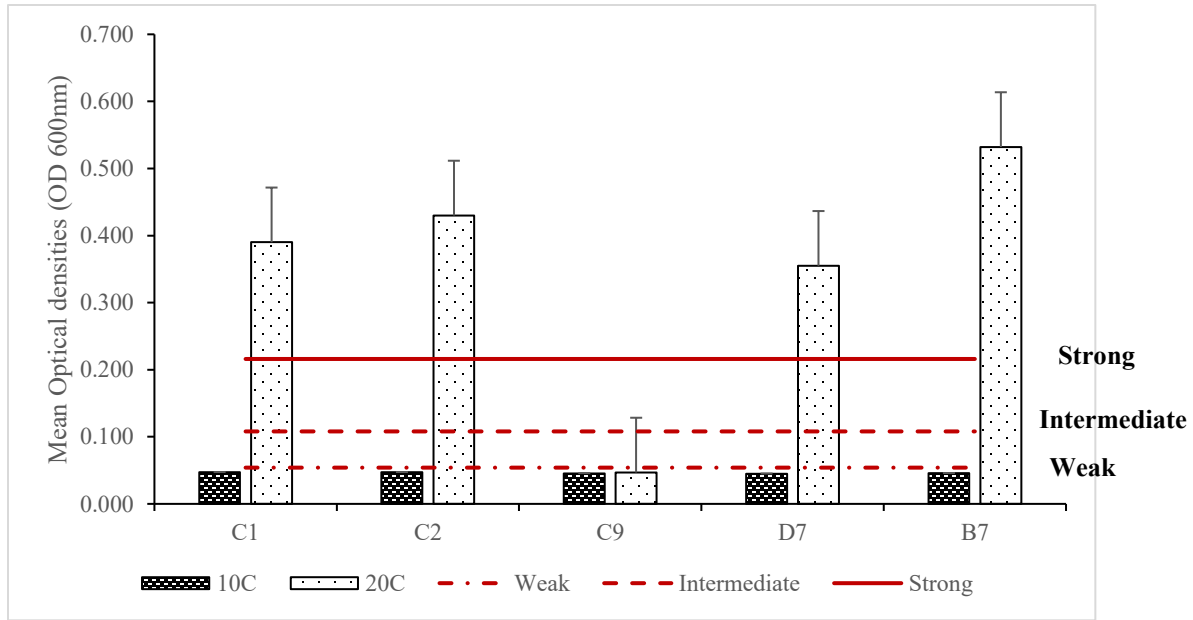


Figure 3-3: Biofilm-forming ability of dual-species biofilms formed with *L. acidophilus*, 36SusP and 9SusP at 10 and 20 °C. Controls are single-species biofilms at both temperatures C1: 36SusP, C2: 9SusP, and C9: *L. acidophilus*. The combinations are as follows D7: *L. acidophilus* + 36SusP, and B7: *L. acidophilus*+ 9SusP. Mean values were determined using three replicate experiments. Horizontal lines were statistically determined using the negative controls from each temperature and represent division between non-biofilm formers (bottom; $OD < OD_c$), weak (lower middle; $OD_c < OD < 2OD_c$), intermediate (upper middle; $2OD_c < OD < 4OD_c$), and strong (top; $4OD_c < OD$). Number of observations per temperature for each strain “N” = 48

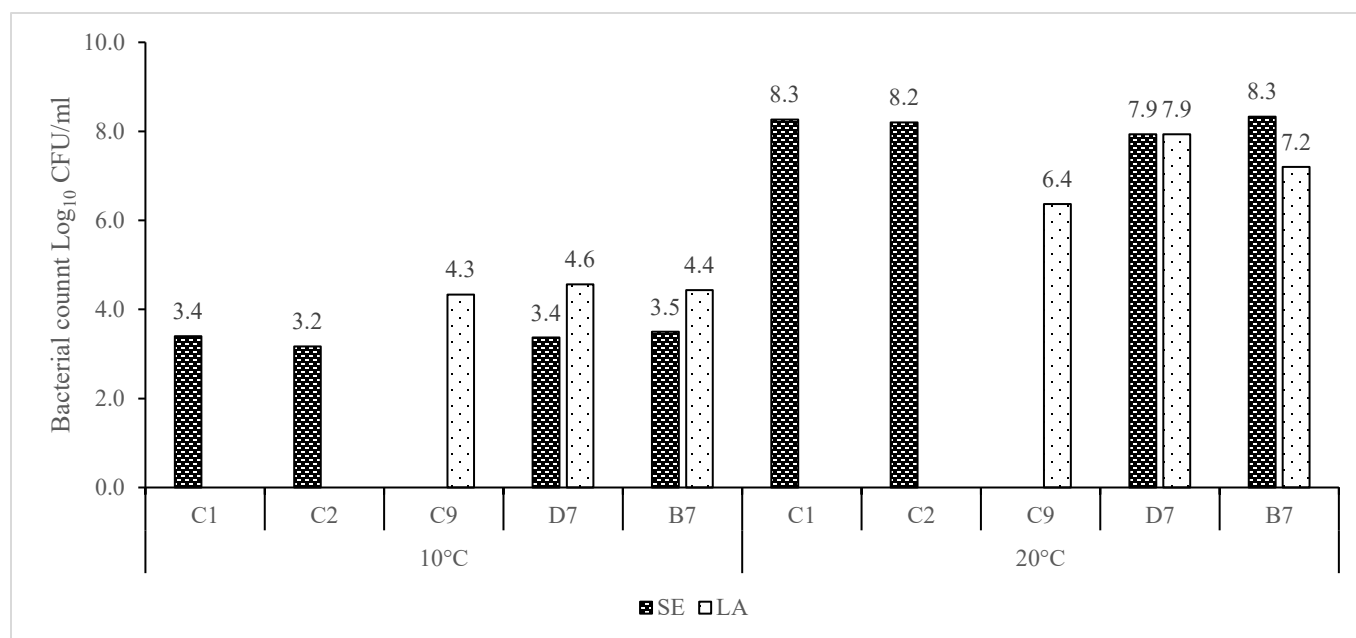


Figure 3-4: Individual bacterial counts of dual-species biofilms formed with *L. acidophilus* (LA), 36SusP and 9SusP at 10 and 20 °C. Controls are counts recovered from single-species biofilms at both temperatures C1: 36SusP, C2: 9SusP, and C9: *L. acidophilus*. The SE counts and *L. acidophilus* counts recovered from both combinations are represented. Mean values were replicated using three replicate experiments. The combinations are as follows D7: *L. acidophilus* + 36SusP, and B7: *L. acidophilus*+ 9SusP. Number of observations per temperature for each strain “N” = 12

3.4.3.2 SE dual-species biofilm formation with BAE

Biofilm biomass measurement showed that at 10 °C, most dual-species biofilm combinations were non-forming (OD<ODc). The exception was the SE + *P. aeruginosa* combination that showed strong biofilm formation (4 ODc < OD) with the 36SusP + *P. aeruginosa* (D4) combination yielding the highest optical density observed at 10 °C (OD=0.605). In contrast, SE + *P. aeruginosa* biofilms formed at 20 °C were all intermediate (2ODc < OD < 4ODc). At 20 °C, there was a general increase in biofilm biomass across all combinations (P<0.05). SE + *E. coli* combinations produced strong biofilms with both 11775 and 15597, with not much variation (P>0.05). The SE + Enterococci combinations all produced intermediate biofilms (2ODc < OD < 4ODc) at 20 °C a contrast to the Enterococci controls, which were all non-forming. The SE + *B. cereus* combinations had the lowest biofilm biomass of all dual-

species combinations tested. Biofilms formed with 36SusP + *B. cereus* (D3) were non-forming (OD=0.052) while those produced by 9SusP + *B. cereus* (B3) were weak (OD=0.062) (Figure 3-5).

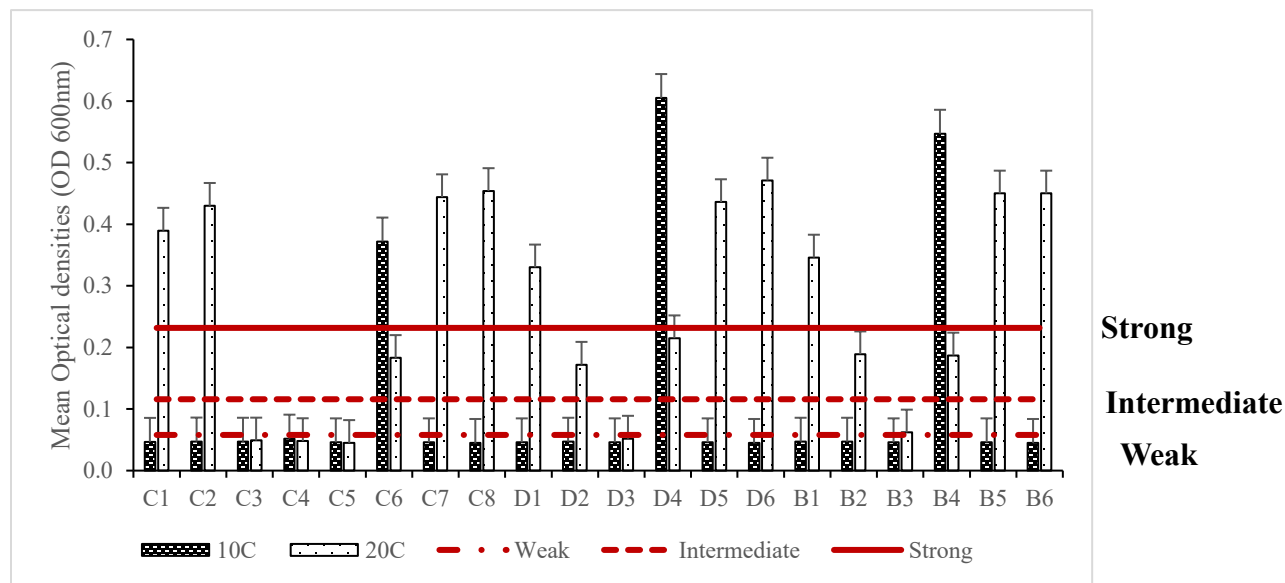


Figure 3-5: Biofilm-forming ability of *E. faecium*, *E. faecalis*, *B. cereus*, *P. aeruginosa*, *E. coli* 11775, *E. coli* 15597, 36SusP and 9SusP dual-species biofilms at 10 and 20 °C. Controls are single-species biofilms at both temperatures C1: 36SusP, C2: 9SusP, C3: *E. faecalis* (S36), C5: *B. cereus*, C6: *P. aeruginosa*, C7: *E. coli* (11775), C8: *E. coli* (15597). The combinations are as follows D1: 36SusP+S34, D2: 36SusP+S36, D3: 36SusP+*B. cereus*, D4: 36SusP+*P. aeruginosa*, D5: 36SusP+11775, D6: 36SusP+15597, B1: 9SusP+S34, B2: 9SusP+S36, B3: 9SusP+*B. cereus*, B4: 9SusP+*P. aeruginosa*, B5: 9SusP+11775, B6: 9SusP+15597. Mean values were determined using three replicate experiments. Horizontal lines were statistically determined using the negative controls from each temperature and represent division between non-biofilm formers (bottom; $OD < OD_c$), weak (lower middle; $OD_c < OD < 2OD_c$), intermediate (upper middle; $2OD_c < OD < 4OD_c$), and strong (top; $4OD_c < OD$). Number of observations per temperature for each strain “N” = 48

3.4.3.3 SE multispecies biofilm formation with BAE

Regarding multispecies biofilms formed by SE and bacteria associated with eggshells, optical density (OD) measurements indicated greater biofilm biomass at 10 °C compared to the single-species SE controls. At this temperature, the combinations M1 (*E. coli* + *E. faecalis*) and M3 (*P. aeruginosa* + *B. cereus*) produced intermediate biofilms (OD=0.134 and OD=0.168

respectively), whereas M2 (*P. aeruginosa* + *E. coli*) formed a strong biofilm (OD = 0.202). At 20 °C, all multispecies combinations were classified as strong biofilm formers. Among them, M1 (OD = 0.407) exhibited the greatest biofilm biomass, exceeding that of the corresponding single-species SE control (OD = 0.351). (**Figure 3-6**).

At 10 °C, SE counts within multispecies biofilms were higher than the control but not significant ($P > 0.05$). SE counts went from 3.9 to 5.5 log₁₀ CFU/mL in M1, 4.6 log₁₀ CFU/mL in M2 and the lowest being 4.4 log₁₀ CFU/mL in M3. SE counts were significantly higher ($P < 0.05$) at 20 °C than at 10 °C however, there was a decrease in counts within all multispecies biofilms. The reduction in M1 was not significant ($P > 0.05$), in contrast to SE counts within M2 (5.3 log₁₀ CFU/mL) and M3 (6.5 log₁₀ CFU/mL), which were significant ($P < 0.05$) in comparison to the controls (**Figure 3-7A**).

To evaluate the influence of the biofilm community on bacterial populations, viable cell counts were quantified within the multispecies biofilms. *P. aeruginosa* exhibited the highest counts in both M2 and M3 at 10 and 20 °C. At 10 °C, although a slight reduction was observed in M2 (from 7.8 to 7.2 log₁₀ CFU/mL), the change was not statistically significant ($P > 0.05$). In contrast, M3 showed a non-significant increase, reaching 9.5 log₁₀ CFU/mL ($P > 0.05$). At 20 °C, *P. aeruginosa* counts remained high; however, no significant differences were detected in M2 (increase from 9.3 to 9.7 log₁₀ CFU/mL) or in M3 (decrease to 8.8 log₁₀ CFU/mL) compared to the respective controls (**Figure 3-7B**).

Similarly, *E. coli* (**Figure 3-7C**), *E. faecalis* (**Figure 3-7D**), and *B. cereus* (**Figure 3-7E**) did not show significant differences in counts within multispecies biofilms compared to single-species controls at either temperature ($P > 0.05$). However, *E. coli* counts were significantly

higher at 20 °C than at 10 °C ($P < 0.05$), indicating a temperature-dependent increase in growth.

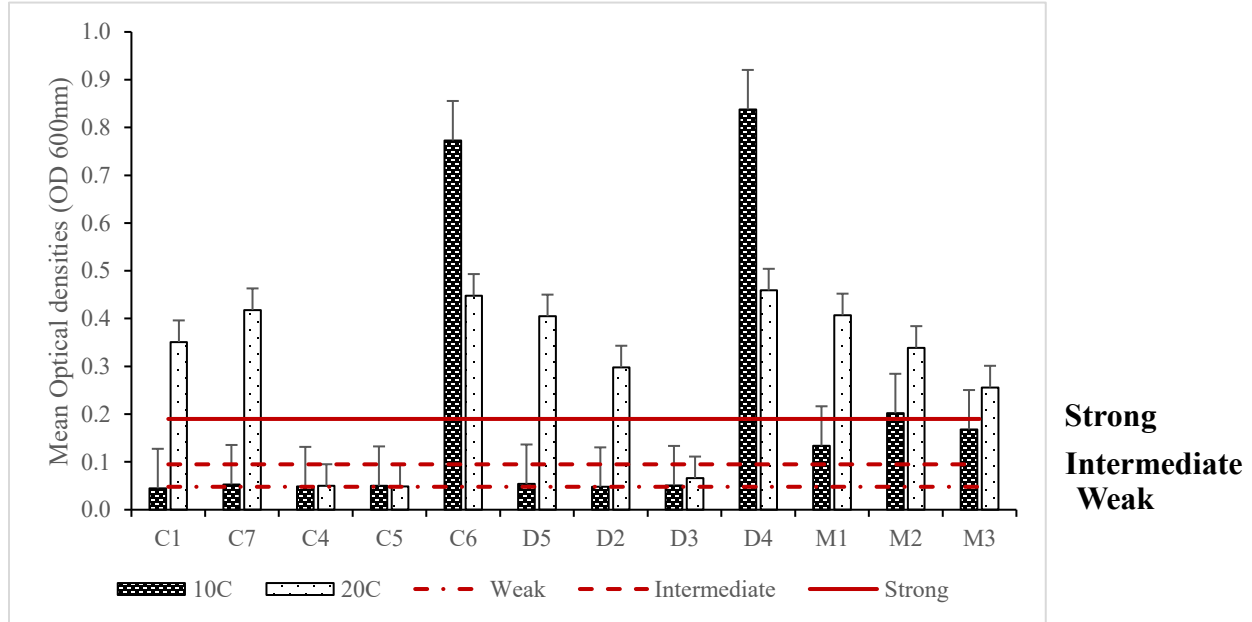


Figure 3-6: Comparison of BAE dual and multispecies biofilms formed with 36SusP in 96-well microplates after 5 days at 10 and 20 °C. Controls are single-species biofilms at both temperatures C1: 36SusP, C4: *E. faecalis* (S36), C5: *B. cereus*, C6: *P. aeruginosa*, C7: *E. coli* (11775). The combinations are as follows: D2: 36SusP+S36, D3: 36SusP+*B. cereus*, D4: 36SusP+*P. aeruginosa*, D5: 36SusP+11775, M1: *E. coli* + *E. faecalis*, B) M2: *P. aeruginosa* + *E. coli* and C) M3: *P. aeruginosa* + *B. cereus*. Mean values were determined using three replicate experiments. Horizontal lines were statistically determined using the negative controls from each temperature and represent division between non-biofilm formers (bottom; $OD < OD_c$), weak (lower middle; $OD_c < OD < 2OD_c$), intermediate (upper middle; $2OD_c < OD < 4OD_c$), and strong (top; $4OD_c < OD$). Number of observations per temperature for each strain “N” = 48

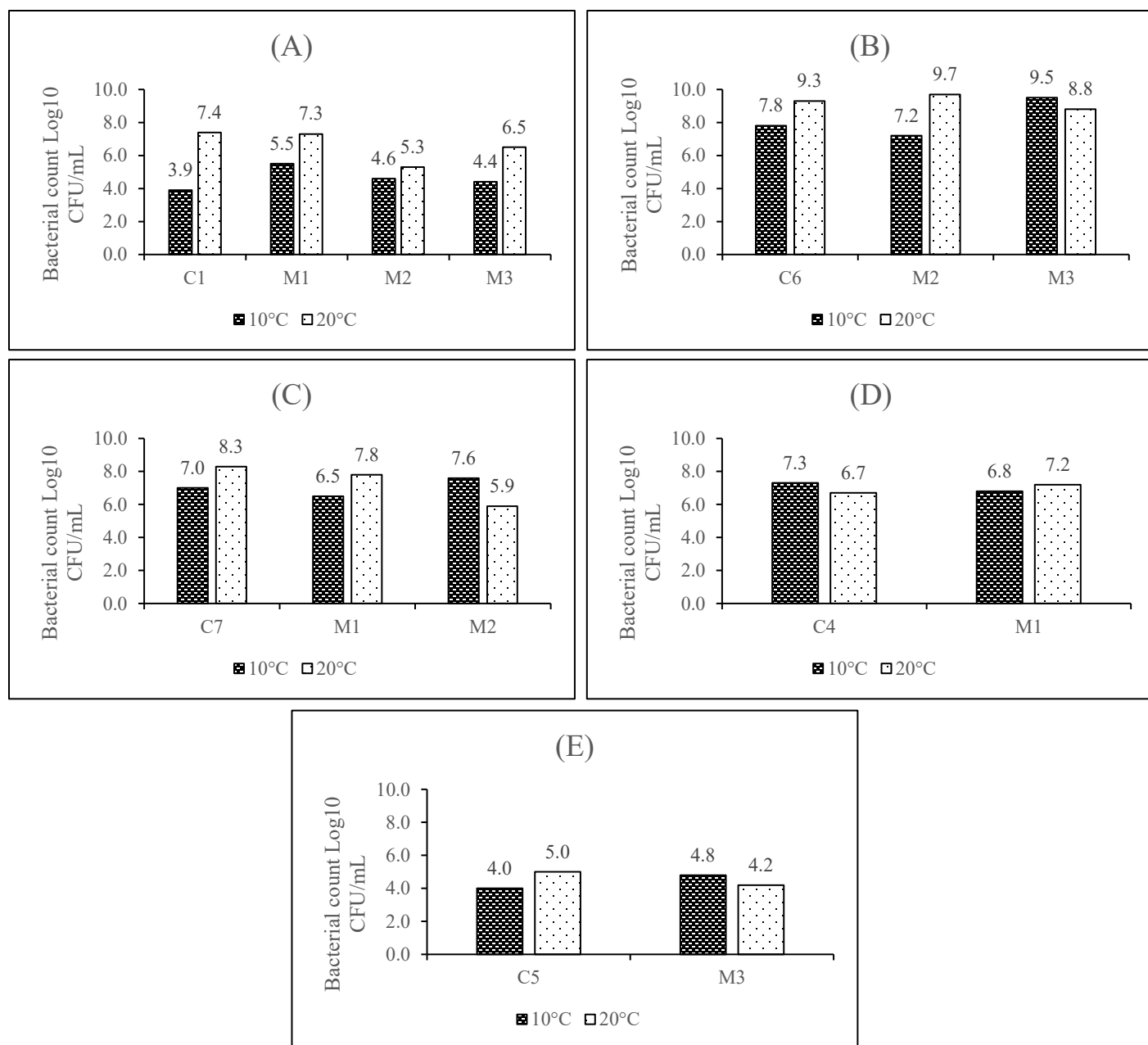


Figure 3-7: Individual bacterial counts of three multispecies biofilm combinations formed in 96-well microplates at 10 and 20 °C. A; *SE*, B; *P. aeruginosa*, C; *E. coli*, D; *E. faecalis*, E; *B. cereus*. The multispecies combinations were; M1: *E. coli* + *E. faecalis*, M2: *P. aeruginosa* + *E. coli* and M3: *P. aeruginosa* + *B. cereus*. Controls are single-species biofilms at both temperatures C1: 36SusP, C4: *E. faecalis* (S36), C5: *B. cereus*, C6: *P. aeruginosa*, C7: *E. coli* (11775). All experiments were completed in triplicate. Number of observations per temperature for each strain “N” = 12

3.5 Discussion

3.5.1 SE Single-Species Biofilm Formation, Curli, And Cellulose Expression Are Temperature Dependent

To reduce the risk of SE proliferation on eggshells, licensed egg facilities in Canada are advised to store eggs at any temperature between 10- 13 °C (Agency, 2015). In the present study, SE biofilm formation at 10 °C was largely limited, with only 6 out of 15 SE strains displaying weak biofilm formation (**Figure 3-1**). These findings are consistent with previous reports indicating that lower temperatures constrain metabolic activity and downregulate genes associated with curli production, cellulose synthesis, and other biofilm-related pathways (White et al., 2006). Reduced biofilm formation at storage temperatures may therefore limit SE persistence on eggshell surfaces and improve the effectiveness of sanitation practices in egg-handling environments (Okamura et al., 2008). In contrast, at 20 °C, SE showed a significant increase in biofilm formation ($P < 0.0001$), with 11 of 15 strains forming strong biofilms. Temperatures within laying barns are commonly maintained between 18 and 22 °C to ensure hen welfare and productivity (Rosario Díaz et al., 2018). The enhanced biofilm formation observed at 20 °C suggests that SE may be well adapted to barn environmental conditions, potentially promoting persistence on equipment and eggshell surfaces. At 37 °C, biofilm formation was reduced relative to 20 °C: five strains formed intermediate biofilms, eight were weak formers, and two strains were categorized as non-biofilm formers. Similar trends have been reported in the literature, where SE strains demonstrate greater surface attachment and biofilm biomass at moderate environmental temperatures (20–28 °C) compared to 37 °C (Dhakal et al., 2019; Shatila et al., 2021). At 37 °C, reduced biofilm formation may reflect a shift toward a planktonic, motile phenotype that facilitates colonization and invasion in the host, rather than environmental

surface persistence (Bisht et al., 2023). Some studies have shown that the biomass of the biofilm decreases with an increase in temperature, as bacteria produce more planktonic cells at higher temperatures (Bisht et al., 2023; Çam & Brinkmeyer, 2020).

3.5.1.1 Curl and Cellulose Expression

Fourteen SE strains expressed both curli and cellulose at 10 and 20°C exhibiting strong fluorescence under UV light (366 nm) (indicative of positive cellulose expression) and curli production (characterized by red, dry, and rough (rdar) morphology). All 15 SE strains, were negative for cellulose at 37°C. Some of the major factors that have been found to be involved in *Salmonella* biofilm formation and persistence are the production of curli fimbriae and cellulose expression (Harrell et al., 2020; Simm et al., 2014). Curli fimbriae are amyloid fibres that facilitate bacterial surface adhesion and contribute to biofilm structure. In conjunction with cellulose, it also contributes to the structural integrity of the biofilm, improving durability and resistance to antimicrobial treatments (Austin et al., 1998; Jonas et al., 2007). These results (**Table 3-3**) indicate that SE curli and cellulose are temperature-dependent and favour the expression of biofilm-associated structures at moderate temperatures or environmental conditions (20 °C) rather than host-associated temperatures (37 °C) (Gualdi et al., 2008). SE 107 (bovine) was included as a control for curli and cellulose production, and it was the only SE strain that had no fluorescence and a smooth and white (saw) morphology, then classified as cellulose and curli negative, respectively. SE 107 at 20°C however, was positive for both cellulose and curli at 10 °C. These findings on curli and cellulose suggest that 107 (bovine) may have temperature-specific regulatory differences that affect biofilm formation at 20 °C however, its ability to express both curli and cellulose at 10 °C indicates a regulatory expression to cold (Counihan et al., 2025). Additionally, 107 (bovine) might be adapted to a temperature, nutrient

composition and availability, and microbial competition that differ from those found in the laying barn, which suppresses traits that might be easily expressed in poultry-associated strains in the same environment (Piras et al., 2015; Yang et al., 2016).

3.5.1.2 SE Pellicle formation

All 15 SE strains formed pellicles at 20 °C; however, notable differences in structural integrity were observed between intermediate and strong biofilm formers (**Table 3-3**). Pellicle strength was qualitatively assessed by applying gentle pressure with a sterile toothpick. Strains classified as intermediate biofilm formers at 20 °C, 107, 79801, 79901, and 715701, produced fragile and less cohesive pellicles that readily disrupted upon contact. In contrast, strains categorized as strong biofilm formers produced robust pellicles that remained intact and adhered to the toothpick even after additional pressure was applied. The presence of pellicles introduced variability during the crystal violet (CV) assay, as pellicles formed by strong biofilm-producing strains were often partially or completely removed during aspiration steps, potentially affecting biomass quantification. Notably, limited literature addressing SE biofilms using the CV technique has discussed the potential impact of pellicle formation on OD measurements. Ivana et al. (2015) tested fourteen SE isolates using the CV technique at 25 and 37 °C and found a correlation between curli morphotype (rdar/bdar/saw) and pellicle strength with OD values. Pellicles formed at 25 °C were thicker, had the rdar morphotype, and higher OD values. Pellicles form at the air-liquid interface to optimize access to oxygen from the atmosphere while maintaining contact with nutrients in the liquid medium (Z. S. Xu et al., 2023). Serra et al. (2013), demonstrated using a genetically modified *E. coli* K-12 strains that pellicle elasticity and stability are largely influenced by the production of curli and cellulose. It is therefore possible that strong SE biofilm formers in this study produced higher levels of curli and cellulose,

resulting in more cohesive and structured pellicles. However, pellicle formation alone should not be considered a definitive indicator of overall biofilm strength, as increased curli expression may primarily facilitate maintenance at the air–liquid interface rather than surface attachment (Paytubi et al., 2014; Sharma et al., 2022). There were no pellicles formed at 10 and 37 °C, indicating temperature suppressed expression of related pellicle formation genes. Pellicle formation by *Salmonella* has been reported widely, with many studies linking a combination of factors such as nutrient and oxygen availability, temperature and pH to the production and strength of biofilms formed (Paytubi et al., 2017; Römling & Rohde, 1999; Sharma et al., 2022; Villa-Rojas et al., 2017). SE strains that produce brittle pellicles might be more susceptible to environmental fluctuations and chemical exposure, disrupting SE adherence to surfaces in poultry facilities and reducing persistence (Scher et al., 2005).

3.5.2 SE Multispecies Biofilm Formation

Previous studies have reported that other bacteria present in the mixed biofilm can increase pathogen survival by nullifying active agents found in commonly used sanitizers and disinfectants or by forming the EPS which is a protective layer that encapsulates the pathogens, offering protection (Bridier et al., 2012; Chitlapilly Dass et al., 2020; Giaouris et al., 2013; Oxaran et al., 2018; van der Veen & Abee, 2011). In the present study, some evidence of such protective interactions was observed at 10 °C. Although SE biofilm formation was generally limited at this temperature in single-species conditions, SE incorporated into multispecies biofilms exhibited increased biofilm biomass across all combinations. Specifically, SE multispecies biofilms formed with M1 (*E. coli* + *E. faecalis*) and M3 (*P. aeruginosa* + *B. cereus*) were classified as intermediate, while M2 (*P. aeruginosa* + *E. coli*) produced strong biofilms (Figure 3-6). Lower temperatures might slow down SE metabolic processes within the

multispecies biofilm, while allowing some bacteria to benefit structurally from the extracellular matrix produced collectively by the community. Surprisingly, although biofilm biomass increased, SE viable counts within multispecies biofilms showed only slight, non-significant increases ($P>0.05$) in bacterial counts in comparison to the controls ($3.9 \log_{10}$ CFU/mL), dropping to $5.5 \log_{10}$ CFU/mL in M1, and $4.6 \log_{10}$ CFU/mL in M2 indicating that SE cells were still affected by the temperature even with the assumed interspecies cooperation (**Figure 3-7A**). This suggests that enhanced matrix production did not directly translate into substantially improved SE proliferation at 10°C . The increased biomass may instead reflect cumulative matrix production by all species rather than a specific survival advantage for SE. Another possibility could be cell-to-cell contact within the multispecies biofilms, facilitated horizontal gene transfer (HGT), a phenomenon well documented in structured microbial communities (Madsen et al., 2012). Combinations M2 and M3 included *P. aeruginosa*, which is opportunistic and metabolically versatile being able to survive under harsh environmental conditions and adapt to exposure to antibiotics (Biswas et al., 2019; Blomquist & Nix, 2021; Motta et al., 2015). Previous studies have reported the phenomenon of horizontal gene transfer (HGT), where bacteria in close proximity within multispecies biofilms exchange genetic material, which might carry genes that encode antimicrobial resistance, increasing persistence in food processing facilities (Abe et al., 2020; Madsen et al., 2012; Michaelis & Grohmann, 2023). It is therefore reasonable to assume that biofilms with similar species composition as M2 and M3 might be able to birth new generations of SE strains well adapted to survival in lower temperatures, prolonging SE persistence within food processing facilities (Alvarez-Ordóñez et al., 2019; Bridier et al., 2015).

In contrast, SE counts were significantly higher ($P < 0.05$) at 20 °C than at 10 °C, with the biomass increasing to produce strong biofilms across all multispecies combinations; however, there was a decrease in SE counts within all multispecies biofilms. SE reduction in M1 was not significant ($P > 0.05$), a difference to SE counts within M2 (5.3 log₁₀ CFU/mL) and M3 (6.5 log₁₀ CFU/mL) which experienced a significant reduction ($P < 0.05$) in comparison to the controls (7.4 log₁₀ CFU/mL) (**Figure 3-7A**). M1 (*E. coli* + *E. faecalis*) interaction with SE suggests that *E. coli* and *E. faecalis* exert comparatively weak antagonistic effects on SE. The higher temperature likely enhanced metabolic activity of all community members, intensifying competition for nutrients, oxygen, and surface attachment sites. Competitive exclusion within biofilms can occur through rapid surface colonization, nutrient depletion, production of inhibitory metabolites (e.g., bacteriocins, enzymes, antibiotics), or interference with quorum sensing (Hol et al., 2015; Rendueles & Ghigo, 2012; Sadiq et al., 2021). Both *P. aeruginosa* and *B. cereus* are known to produce antagonistic compounds that suppress neighbouring species in multispecies environments (Baruzzi et al., 2011; Sumi et al., 2014). The presence of these organisms in M3 likely contributed to the pronounced reduction of SE at 20 °C. Similarly, M2 composed entirely of Gram-negative species (*E. coli*, *P. aeruginosa*, and SE) may have intensified competition due to overlapping metabolic requirements, resulting in the lowest SE counts among combinations at this temperature.

3.6 Conclusion

SE biofilm formation was limited at 10 °C but increased significantly at 20 °C, where most strains produced strong biofilms. Incorporation into multispecies biofilms enhanced overall biomass at 10 °C; however, this did not correspond to a statistically significant increase in SE viable counts. At 20 °C, although biofilm biomass remained high, competitive interactions (with

P. aeruginosa + *B. cereus*, and *P. aeruginosa* + *E. coli*) reduced SE survival in specific multispecies combinations. These findings demonstrate that multispecies biofilm dynamics are both temperature-dependent and species-specific. Increased biofilm biomass does not necessarily equate to enhanced pathogen growth. Temperature acts as a critical abiotic factor regulating metabolic activity, matrix production, and interspecies interactions, while community composition determines whether cooperative or competitive relationships predominate.

Chapter 4: Formation of Multispecies Biofilms by *Salmonella* Enteritidis on Contact Surfaces Found in Poultry Facilities

4.1 Abstract

Salmonella Enteritidis (SE) forms biofilms which attach to processing surfaces in the laying barns and poultry facilities. This study assessed SE interactions with Bacteria Associated with Eggshells (BAE) in multispecies biofilms on galvanized steel (GS) and Plastic (PLA) surfaces. Two combinations (**M1**): *E. coli* + *P. aeruginosa* and (**M2**): *Bacillus cereus* + *P. aeruginosa* were assessed for multispecies biofilm formation with 36SusP, a strong biofilm former under dry (20-30% relative humidity; RH) and wet (50-60% RH) conditions at 20 °C for 6 days. Single-species biofilms were used as control positives. SE growth varied by material with PLA having significantly more bacterial attachment than GS ($P > 0.0001$). SE growth on GS coupons was suppressed across all multispecies biofilms and did not differ significantly from the control ($P > 0.05$), with the lowest counts (ranging from 0 to 0.8 log₁₀ CFU/mL) observed on GS-dry coupons. In contrast, PLA supported higher SE counts regardless of humidity; however, SE counts within multispecies biofilms significantly decreased ($P < 0.05$). With M1, SE counts dropped from 7.1 to 4 log₁₀ CFU/mL and to 5.9 log₁₀ CFU/mL with M2 on PLA-wet coupons. On PLA-dry coupons, counts went from 6.9 to 4.5 log₁₀ CFU/mL in M1 and to 5 log₁₀ CFU/mL in M2. SE survival was consistent in multispecies biofilms with M1, with enrichment improving recovery in both dry (from 16.7 to 60%) and wet (from 16.7 to 33.3%) conditions, while M2 showed low recovery overall and dropped to 0% even after enrichment in dry and wet conditions. SE survival in multispecies biofilms is dependent on the species composition, type of contact surface and humidity. SE reduction was highest when co-cultured with M2 on GS coupons at dry humidities. Controlling these factors can impact SE biofilm formation in poultry facilities.

4.2 Introduction

Salmonella Enteritidis (SE) is a major foodborne pathogen closely linked to poultry products like eggs and chicken meat (Kumar et al., 2025). Frequently present in the gastrointestinal tract of infected poultry, SE can contaminate eggs and meat at various stages of production, processing, and distribution (Willey et al., 2011). In the United States, SE remained the leading cause of multistate outbreak-associated isolates in 2024, with food sources responsible for 75.93% of reported *Salmonella* infections. Similarly, the 2022 National enteric surveillance program (NESP) reported SE as the predominant serotype in Canada, accounting for 38% of *Salmonella* cases associated with human illness (PHAC, 2022).

SE introduction into poultry production systems occurs when biosecurity measures fail or are not properly implemented. Exposure to wild birds, improper storage of feed and drinking water, inadequate cleaning and sanitation, inadequate pest control, and improper temperature control during storage and transport give entry points for SE-colonized birds into the facility (Wang et al., 2023). Infected hens can remain asymptomatic yet continue to shed SE in their feces, facilitating both vertical transmission (transfer of SE from infected hens to their eggs) and horizontal transmission (from infected hens to contact surfaces) (Ruiz-De-Castañeda et al., 2011; Whiley & Ross, 2015). SE persistence in poultry facilities is well documented, with some studies showing that SE can survive for up to a year in depopulated poultry houses, remaining viable within pest vectors (e.g. rodents, mealworms, etc.), dried litter, feed and feces even after cleaning and disinfection (Davies & Breslin, 2003; Ethèves et al., 2021; Gast et al., 2015). Inadequate sanitation at the end of the birds' production cycle can further contribute to the formation of biofilms on barn equipment and infrastructure, allowing long-term survival of SE cells, which can later reinfect a new flock.

Biofilms are microbial communities that are encased within an extracellular polymeric substance (EPS) (Monroe, 2007). The EPS matrix enhances microbial survival by protecting cells from environmental fluctuations and significantly increasing resistance to antimicrobial agents (Butt & Khan, 2015; Ciofu & Tolker-Nielsen, 2019). Within the matrix, bacterial cells exist in close proximity, enabling the exchange of genetic material between pathogenic and non-pathogenic bacteria and promoting the emergence of new, more resistant bacterial populations (Gupta et al., 2016; Neu & Lawrence, 2010). The strength and composition of the EPS are dependent on many factors, such as species composition, environment and nutrient availability (Fagerlund et al., 2016; Rehman et al., 2021). Some studies have shown that SE single-species biofilms develop denser, more antimicrobial-resistant species at 25 °C (Yang et al., 2016). This temperature-dependent improvement in biofilm formation, along with the pathogen's ability to remain viable under dry conditions, presents a challenge in the poultry environment. Most barns are maintained at temperatures between 19-25 °C and relative humidity (rH) levels of 28-70%, which are optimal conditions for SE mesophilic growth (van Staaveren et al., 2018).

Laying barns are often full of organic material that can support diverse microbial communities composed of both beneficial and pathogenic organisms (Gomes et al., 2022; Gržinić et al., 2023). A wide range of structural and operational equipment, like egg and feces conveyors, nesting boxes, cages, feeding and watering systems, and perches, can serve as reservoirs for biofilm development in poultry facilities (Al-Zenki et al., 2007; Biyashev et al., 2025). It is then realistic to assume that SE persistence in poultry facilities is linked to its ability to form multispecies biofilms, particularly on food-contact surfaces and processing equipment commonly found in barns (Pang et al., 2023). In a study conducted by Yuchen et al. (2022), it was discovered that Shigatoxigenic *Escherichia coli* O103:H2 (STEC) multispecies biofilms

formed on Polyurethane (TPU) under dry conditions (20-50% relative humidity) at 25 °C were viable for up to 60 days, even after the EPS had dried. In agriculture, one of the most common materials that can be found in the poultry environment is galvanized steel (GS), a zinc-steel alloy that is cost-effective, durable, and largely resistant to the harsh conditions often found in the farming environment (NMC, 2020). In laying barns specifically, GS is the material used for the cages and comes into direct contact with the laying hens, increasing the likelihood of bacterial deposits on the steel surface (Oyewole et al., 2021). When exposed to the moisture, GS forms Zinc Oxide (ZnO) and Zinc Hydroxide (Zn(OH)₂), which also provides a layer of protection from corrosion. Research involving Zinc-based nanoparticles has shown that ZnO particles in certain concentrations can kill planktonic cells, disrupt the surface attachment of the biofilms and reduce biofilm mass of foodborne pathogens like *E. coli O157:H7* and *L. monocytogenes* (Applerot et al., 2012; Fulindi et al., 2023; Jin et al., 2009). Regular maintenance and cleaning of GS materials is important, as long-term attachment of some bacteria like *P. aeruginosa* can cause microbially induced corrosion even while living anaerobically in a biofilm (L. Xu et al., 2023). It can therefore be inferred that surface composition alone is insufficient to prevent bacterial attachment or biofilm development. Rather, biofilm formation results from the interplay between material properties, environmental conditions, and microbial physiology. These complex connections become particularly relevant when considering SE biofilm formation under real agricultural conditions. SE attachment to poultry processing surfaces such as plastic egg conveyor belts and stainless steel has been well documented in both single-species and multispecies biofilms (Kusumaningrum et al., 2003; Stocki et al., 2007; Veluz et al., 2012), However, SE behavior within multispecies biofilms formed on galvanized steel (GS) or plastic

egg crates under varying environmental conditions, such as changes in relative humidity, remains poorly understood.

4.3 Materials and methods

4.3.1 Bacterial Strains

One *Salmonella* Enteritidis (SE) strain and 3 BAE: *E. coli* (ATCC 11775), *P. aeruginosa* (ATCC 7700), and *B. cereus* (ATCC 14579) strains were assessed in this study (**Table 4-1**). SE/BAE strains were grown in Tryptic Soy broth (TSB; Becton, Dickson and Company, Sparks, MD, USA) for 18-24 hrs at 37 °C (overnight) and streaked on selective media. The bacteria strains were cultured as follows; SE on Xylose Lysine and Dextrose agar (XLD; Hardy diagnostics, Santa Maria, CA, USA), *E. coli* on CHROMagar™ *E. coli* (Kanto Chemical, Saint-Denis, France), *P. aeruginosa* on *Pseudomonas* isolation agar (Becton, Dickson and Company, Sparks, MD, USA), *B. cereus* on *Bacillus Cereus* Agar (OXOID LTD, Basingstoke, Hampshire, England) supplemented with 50% Egg Yolk Emulsion w/v Egg yolk and 0.85% saline) and 500,000 IU Polymyxin B Sulphate (Fisher Scientific, Ottawa, ON, CA). A single colony of each strain was then inoculated into Luria Broth-No Salt (LB-NS; 10 g/L tryptone and 5 g/L yeast extract) and incubated overnight and grown to a cell density of 10⁸ CFU/ml.

All the cultures were obtained from the University of Manitoba culture collection.

Table 4-1: SE and BAE bacterial strains assessed for competitive fitness

Bacterial strain	Strain ID	Source	Category	Biofilm Formation at 20 °C
36SusP	05-3932	Chicken breast meat	SE	Strong

<i>Escherichia coli</i>	ATCC 11775	Urine	BAE	Intermediate
<i>Bacillus cereus</i>	ATCC 14579	Unknown	BAE	Non-Producing
<i>Pseudomonas aeruginosa</i>	ATCC 7700	Well water	BAE	Strong

4.3.2 Multispecies Biofilm Formation on Contact Surfaces

4.3.2.1 Plastic and Galvanized Steel Coupon Preparation

Both 2 cm² disc-shaped galvanized steel (GS) GALV-207L (Pegen Industries Inc., Stittsville, ON, CA) and 1cm x 2cm hand-cut plastic (PLA) (Yosoo, Amazon, CA) egg flat pieces were prepared as previously described with minor adjustments (Adator et al., 2018; Dourou et al., 2011). The coupons were sanitized by soaking in 0.5% Hydrogen peroxide (Accel[®] PREvention^{TM/MC}, DiverseyTM, Fort Mill, USA) for 24 hrs. After sanitation, coupons were then soaked in sterile distilled water for 1hr, sonicated for 20 minutes at 40kHz (Branson 2800, Branson Ultrasonics Co., Danbury, USA) and allowed to dry in a Biosafety Level 2 (BSL2) cabinet.

To test sterility, sample coupons were taken after sterilization and placed into a sterile Petri dish filled with 5mL of sterile LB-NS and incubated at 37 °C for 18-24 hrs, after incubation the samples were tested for microbial growth. All sterile coupons were stored separately in sterile bags (Whirl-pak[®] Standard Sterilized Bags, USA) before the experiment.

4.3.2.2 Multispecies bacteria combinations

Three bacteria genera commonly associated with eggshells (BAE) (*E. coli*, *Bacillus cereus*, and *P. aeruginosa*) (Alvarez-Fernández et al., 2012; De Reu et al., 2008; Peralta-Sánchez et al., 2012) were selected to form multispecies bacteria with SE. These species were evaluated

in combination with SE to assess multispecies biofilm interactions on industrial surfaces. Two strain combinations were tested on the food contact surfaces along with SE (36SusP) **M1**: *E. coli* + *P. aeruginosa* and **M2**: *Bacillus cereus* + *P. aeruginosa*. Fresh cultures of each BAE strain in the combination were prepared according to R. Wang et al. (2013). Briefly, BAE cultures were diluted with sterile LB-NS and adjusted to 10^6 CFU/mL and mixed equally (1:1) to achieve the cocktail subsequently used in the experiments.

4.3.2.3 Dry and Wet Multispecies Biofilm Formation and Enumeration

GS and PLA coupons were placed into separate sterile Petri dishes (60 x 15 mm; VWR International, Radnor, PA, USA) and 5mL of a 1:1 BAE bacterial cocktail (adjusted to 10^6 CFU/mL) was transferred onto each designated coupon and stored at 20 °C for 5 days to develop mature biofilms (Adator et al., 2018). On day 5, the coupons were washed with BPB three times and transferred into new sterile Petri Dishes then, 5mL of the SE culture (adjusted to 10^3 CFU/mL) was added to the pre-formed biofilms and incubated in similar conditions for 5 more days at 20 °C. After washing three times with BPB, the coupons were then transferred into sterile petri dishes and incubated for 6 days. Negative controls consisted of sterile coupons inoculated with 5mL of sterile media, while positive controls consisted of coupons inoculated with single-species bacterial cultures of BAE (adjusted to 10^6 CFU/mL) and SE cultures (adjusted to 10^3 CFU/mL). All coupons were stored at either dry (20-30% relative humidity; RH) or wet (50-60% RH) conditions, and all controls underwent similar treatment as the experimental samples. To maintain humidity All coupons were stored in a box that either had silica gel desiccant packets (Wallaby Goods, USA) (for dry humidity) or wet cheesecloth (for wet humidity) and a digital hygrometer to ensure that humidity levels were kept constant.

Bacteria enumeration was done after the final 6-day incubation. Coupons were washed with 5mL of BPB, placed into sterile petri dishes containing 5mL of BPW, and sonicated for 1 min at 40kHz (Branson 2800, Branson Ultrasonics Co., Danbury, USA) to encourage biofilm detachment. Both sides of the coupon were then scraped with a sterile cell scraper (VWR®, Radnor, USA), sonicated at 40kHz (Branson 2800, Branson Ultrasonics Co., Danbury, USA) for 1 min and 10µL of the sample was then transferred into a 96-well round-bottom non-treated microplate (Falcon®, Corning, NY, USA) containing 180µL to make subsequent 10-fold dilutions as needed. Using the drop-plate technique, five 10 µL drops of the diluted samples were plated onto XLD overlaid with trypticase soy agar (TSA) and incubated for 18-24 hrs at 37 °C to recover stressed SE cells. Each trial was replicated three times.

Bacterial survival of SE cells was assessed after initial enumeration. Samples that showed no growth on XLD plates overlaid with TSA after incubation for 18-24 hrs at 37 °C were enriched in Tryptic Soy Broth (TSB) and incubated at 37 °C for 48 hrs and streaked on XLD medium.

4.3.3 Scanning Electron Microscopy (SEM)

Coupons representative of the experimental and control populations were selected for Scanning Electron Microscopy (SEM) to better visualize biofilms formed. The sample coupons were fixed with 10% Formalin solution (Neutral buffered; Sigma Aldrich, St. Louis, USA) for 2hrs, soaked in BPB for 30 mins and left to dry in a Biosafety Level 2 cabinet. Both GS and PLA coupons were sputter-coated with Gold-Palladium at high vacuum (Denton Vacuum Desk II, Moorestown, USA) and the biofilms were observed using a Quanta 650 FEG environmental SEM (FEI CO., Hillsboro, USA) at 10kV in high-vacuum. SEM was done at the Manitoba Institute of Materials (MIM).

4.3.4 Statistical Analysis

Data obtained from the triplicate experiments were analyzed using SAS (Cary, NC, United States) with a 5% confidence level. To account for undetectable counts, data was log transformed ($\log(x+0.1)$) to carry out the analysis and anti-log transformed for data presentation.

4.4 Results

4.4.1 Multispecies Biofilm Formation on Contact Surfaces

Overall, bacterial growth differed between GS and PLA surfaces under both wet and dry conditions, with PLA having significantly more bacterial retention than GS ($P>0.0001$).

4.4.1.1 *Salmonella* Enteritidis (SE)

SE counts from multispecies biofilms differed across materials and moisture conditions after 6 days at 20 °C, with galvanized steel (GS) showing lower counts and strong moisture dependence, whereas plastic (PLA) consistently supported higher SE counts overall. On GS coupons, SE growth was suppressed, having no significant difference in comparison to the controls (C4), regardless of the relative humidity ($P<0.05$), with counts on GS-dry ranging from 0 to 0.8 log₁₀ CFU/mL and from 1.3 to 1.7 log₁₀ CFU/mL on GS-wet coupons (**Figure 4-1A**). It is also worth noting that dry biofilms formed with *B. cereus* + *P. aeruginosa* (M2) on GS coupons were below the countable range (10^1 CFU/mL) therefore, survival was assessed (**Table 4-2**). PLA supported higher SE counts regardless of humidity; however, there was a significant decrease ($P<0.05$) in SE counts within multispecies biofilms. SE counts went from 6.9 log₁₀ CFU/mL in the controls to 4.5 to 5 log₁₀ CFU/mL within M1 and M2 biofilms respectively, on PLA-dry coupons. Similarly, SE dropped from 7.1 log₁₀ CFU/mL in controls to 4 log₁₀ CFU/mL in M1 and to 5.9 log₁₀ CFU/mL with M2 on PLA-wet coupons (**Figure 4-1B**).

4.4.1.2 *P. aeruginosa*

P. aeruginosa maintained consistently higher values on both contact surfaces. On GS-dry, growth was suppressed, dropping from 4.7 log₁₀ CFU/mL to 4 log₁₀ CFU/mL in M1 and to 4.3 log₁₀ CFU/mL in M2. However, higher relative humidity favoured cell retention as *P. aeruginosa* counts increased from 5.6 log₁₀ CFU/mL to 6.3 log₁₀ CFU/mL in M2 on GS-wet while counts dropped to 4.6 log₁₀ CFU/mL in M1 (**Figure 4-1C**). A similar pattern was observed on PLA, where *P. aeruginosa* counts on PLA-wet reduced from 6.5 to 6 log₁₀ CFU/mL in M1 and increased when in combination with M2 to 7.7 log₁₀ CFU/mL. PLA-dry had no significant change ($P>0.05$) in the combinations M1 and M2 in comparison to the controls (5.3 log₁₀ CFU/mL) (**Figure 4-1D**).

4.4.1.3 *E. coli* and *B. cereus*

When co-cultured on PLA, *E. coli* had a significant reduction ($P<0.0001$) in both M1 dry and wet biofilms. *E. coli* counts reduced from 6.3 log₁₀ CFU/mL in controls to 1 log₁₀ CFU/mL on PLA-dry and from 7.8 to 4.8 log₁₀ CFU/mL on PLA-wet (**Figure 4-1E**). *B. cereus* consistently showed the lowest counts across all combinations, irrespective of humidity. There was no significant change ($P>0.05$) *B. cereus* counts on PLA with both M2 dry and wet biofilms going from 2 and 2.6 log₁₀ CFU/mL, respectively, to less than the countable range (10^1 CFU/mL) in combination with M2 (**Figure 4-1F**). Neither *E. coli* nor *B. cereus* exhibited measurable growth in isolation or in multispecies combinations on GS regardless of the humidity.

Overall, SE reduction was the most severe when co-cultured with M2 on GS coupons at dry humidities. On PLA however, SE reduction was higher in wet biofilms formed with M1.

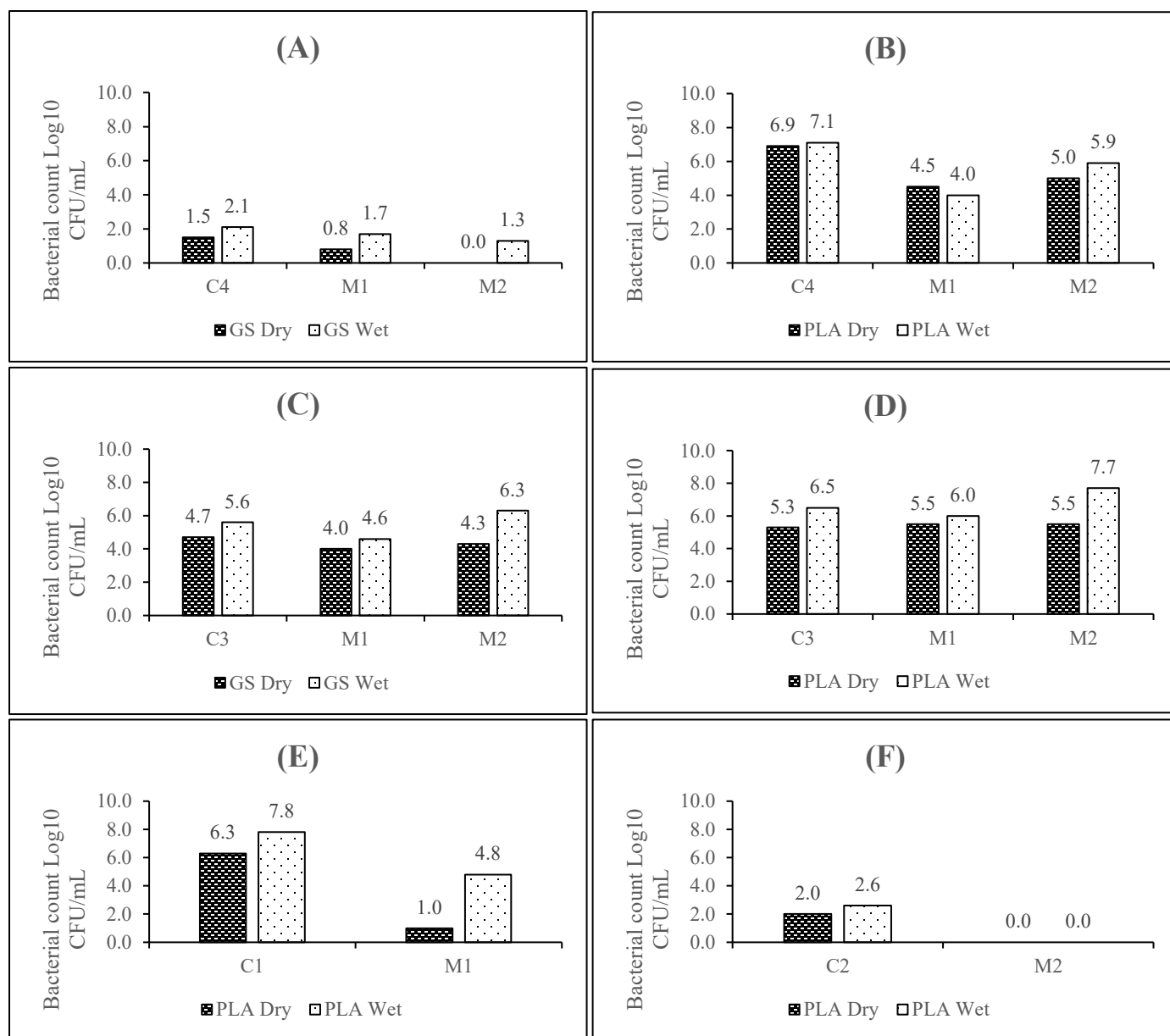


Figure 4-1: A, B; SE counts from multispecies biofilms formed on Galvanized Steel (GS) and Plastic (PLA) in wet and dry conditions at 20 °C for 6 days. C, D; *P. aeruginosa* counts from multispecies biofilms formed on Galvanized Steel (GS) and Plastic (PLA) in wet and dry conditions at 20 °C for 6 days. E, F; *E. coli* and *B. cereus* counts from multispecies biofilms formed on Plastic (PLA) in wet and dry conditions at 20 °C for 6 days respectively. Controls are single-species biofilms formed in both conditions C1: *E. coli*, C2: *B. cereus*, C3: *P. aeruginosa*, and C4: 36SusP. The multispecies combinations were; M1: *E. coli* + *P. aeruginosa* and M2: *Bacillus cereus* + *P. aeruginosa*. Number of observations per surface and humidity for each strain “N” = 12

4.4.2 SE Survival on GS Coupons

SE multispecies biofilms formed on PLA coupons were abundant and quantifiable in wet and dry humidities. However, SE recovery from multispecies biofilms formed on GS coupons was low and, in some cases, only possible with 48 hrs enrichment in Tryptic soy broth at 37 °C (Table 4-2). Under dry conditions on GS coupons, SE recovery was high within multispecies biofilms formed with *E. coli* + *Pseudomonas aeruginosa* (M1), increasing from 16.7% without enrichment to 60.0% with enrichment, with a total recovery of 66.7%. In contrast, multispecies biofilms formed with *Bacillus cereus* + *P. aeruginosa* (M2) had just 33.3% recovery without enrichment but dropped to 0% following enrichment, resulting in a total recovery of 33.3%. A similar pattern was observed under wet conditions, where enrichment enhanced SE recovery in the M1 combination (16.7% to 33.3%, total 50.0%) but had poor recovery in the M2 combination (33.3% without enrichment, 0% with enrichment, total 33.3%).

Table 4-2: SE Survival on GS coupons following incubation at 20 °C under wet and dry conditions with and without 48 hr enrichment

Humidity	Strain Combination	Recovery without enrichment (n/N) %	Recovery with enrichment* (n/N) %	Total Recovery (n/N) %
Dry	<i>E. coli</i> + <i>P. aeruginosa</i> (M1)	(1/6) 16.66%	(3/5) 60.00%	(4/6)66.66%
	<i>B. cereus</i> + <i>P. aeruginosa</i> (M2)	(2/6) 33.33%	(0/4) 0.00%	(2/6)33.33%
Wet	<i>E. coli</i> + <i>P. aeruginosa</i> (M1)	(1/6) 16.66%	(2/5) 33.33%	(3/6)50.00%
	<i>B. cereus</i> + <i>P. aeruginosa</i> (M2)	(2/6) 33.33%	(0/4) 0.00%	(2/6)33.33%

***Note:** Bacteria were incubated in Tryptic Soy Broth (TSB) at 37 °C for 48 hrs.

4.4.3 Scanning Electron Microscopy (SEM)

Under wet conditions, SE single-species biofilms on PLA coupons were strong and well developed across the surface, producing well-distributed, clear rod-shaped cells (Figure 4-3).

However, SE single-species biofilms under dry conditions showed sparsely distributed rod-shaped bacteria across the coupon surface (**Figure 4-2**). Multispecies biofilms formed with *B. cereus* + *P. aeruginosa* (M2) on PLA coupons under dry and wet conditions were robust and showed a good 3-dimensional distribution of rod-shaped bacteria on the coupon surface. Similarly, with *E. coli* + *P. aeruginosa* (M1) on PLA-dry coupons, rod-shaped bacteria were visible and appeared to aggregate into microcolonies across the coupon surface in dry conditions (**Figure 4-2**). On PLA-wet coupons, it was possible to observe cells encapsulated within a matrix with *E. coli* single-species biofilms (**Figure 4-3**).

On GS coupons, cell definition reduced for all bacteria along with an increased roughness of the coupon surface and the presence of crystalline structures confirmed to be deposits composed primarily of Zinc (Zn) (**Figure 4-4**). SE single-species biofilms formed on GS coupons under dry and wet conditions presented individual cells sparsely dotting the surface of the coupons. In contrast, all the multispecies biofilms formed with *B. cereus* + *P. aeruginosa* (M2) and *E. coli* + *P. aeruginosa* (M1) were able to form observable cell aggregates across coupon surface (**Figure 4-5**). Observable *B. cereus* cells on all surfaces were few and far between, with the most prominent structures seeming to be the remnants of damaged or dried-out EPS (**Figure 4-4**).

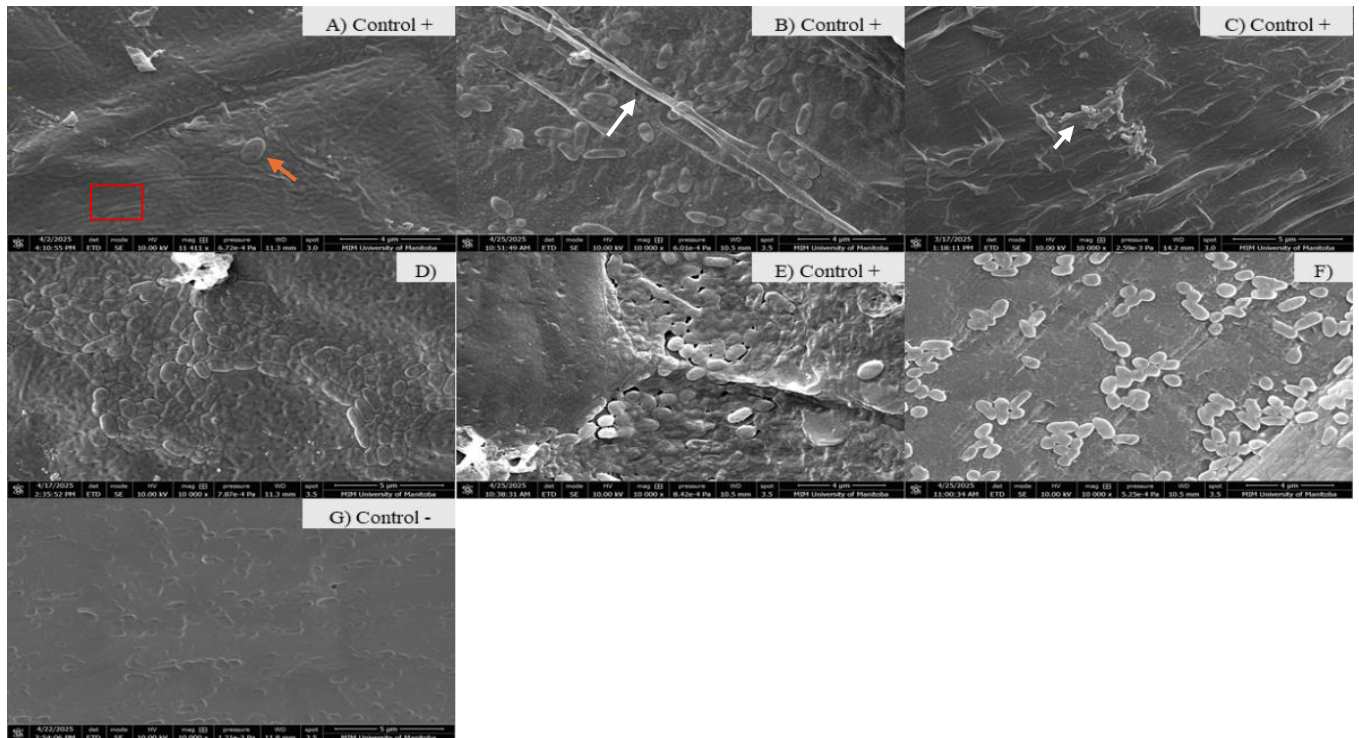


Figure 4-2: PLA coupons: SEM images of 36SusP (A), *P. aeruginosa* (B), *B. cereus* (C), *B. cereus* + *P. aeruginosa* (D), *E. coli*11775 (E), *E. coli* + *P. aeruginosa* (F) Control negative PLA (G) dry biofilms at 20 °C for 6 days . Individual cells are shown (Orange arrow) attached to PLA surface (red box) as well as the visible EPS (white arrow).

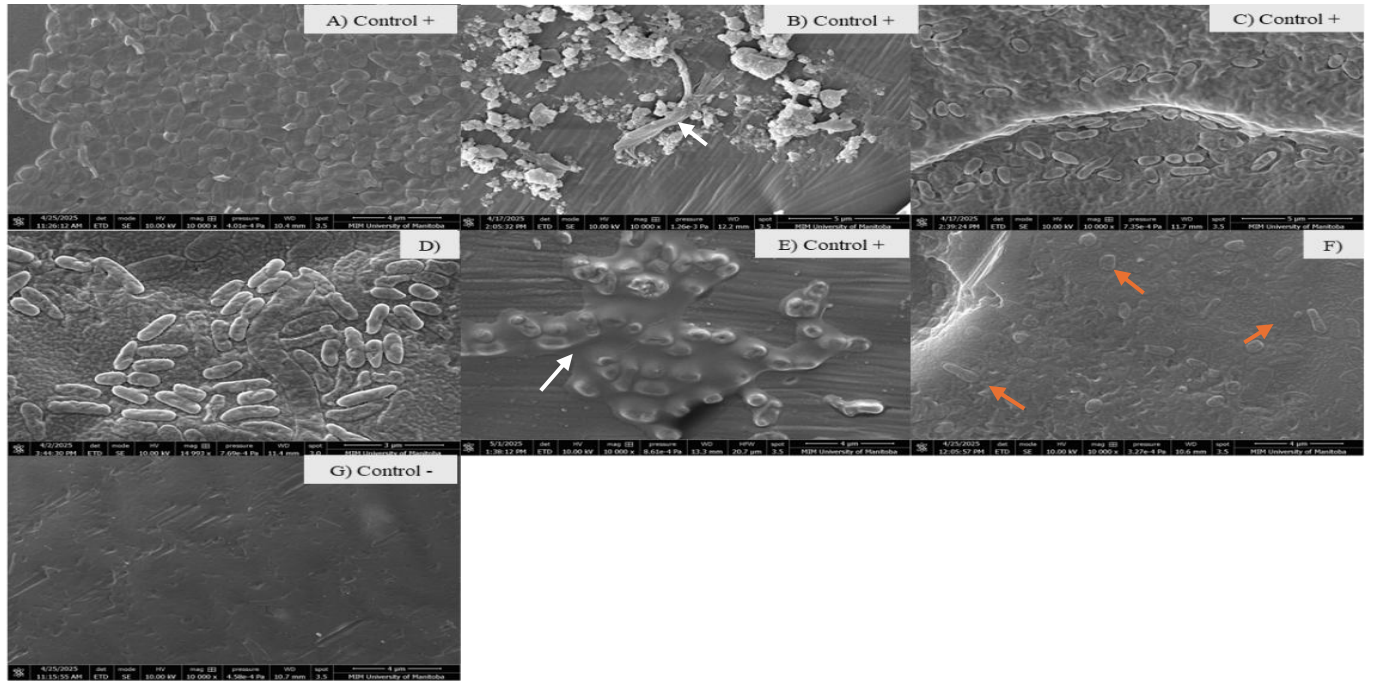


Figure 4-3: PLA coupons: SEM images of 36SusP (A), *B. cereus* (B), *P. aeruginosa* (C), *B. cereus* + *P. aeruginosa* (D), *E. coli*11775 (E), *E. coli* + *P. aeruginosa* (F) Control negative PLA (G) **wet biofilms at 20 °C for 6 days**. Individual cells are shown (Orange arrow) attached to PLA surface (red box) as well as the visible EPS (white arrow).

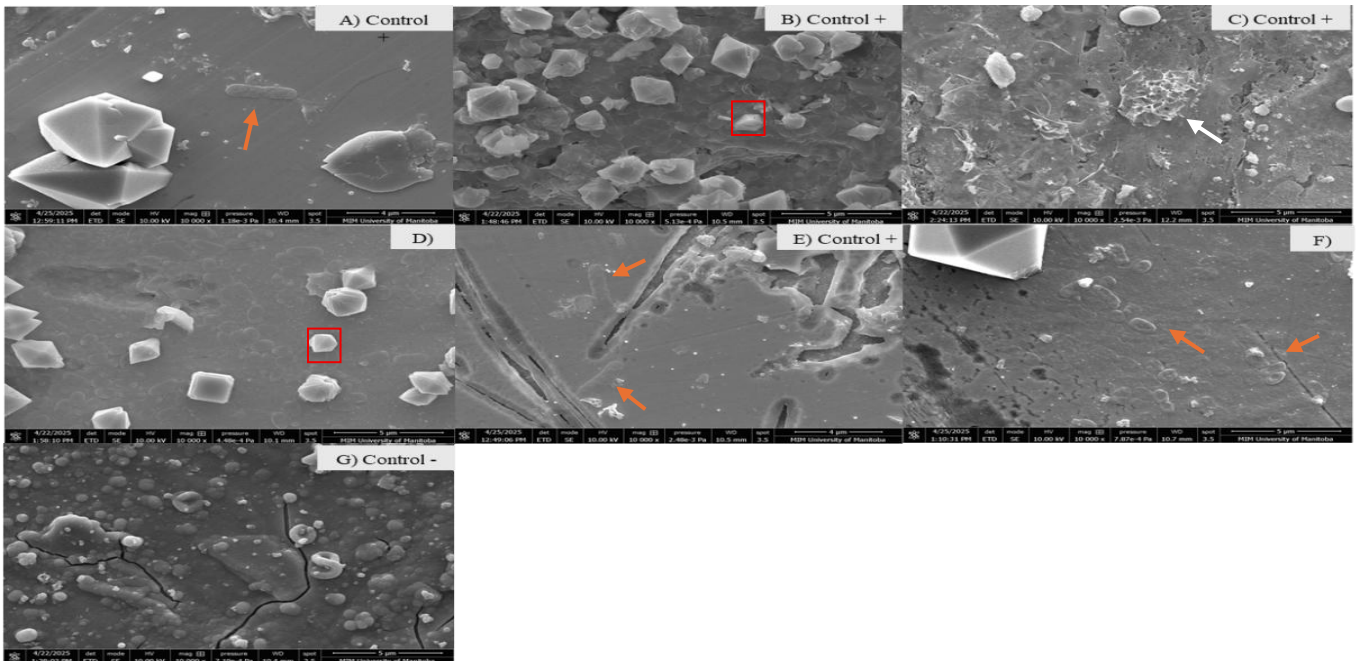


Figure 4-4: GS coupons: SEM images of 36SusP(A), *P. aeruginosa* (B), *B. cereus* (C), *B. cereus* + *P. aeruginosa* (D), *E. coli* 11775 (E), *E. coli* 11775 + *P. aeruginosa* (F) Control negative GS (G) **dry biofilms at 20 °C for 6 days**. Individual cells are shown (Orange arrow) attached to GS surface dotted with Zinc deposits (red box) as well as the visible EPS (white arrow).

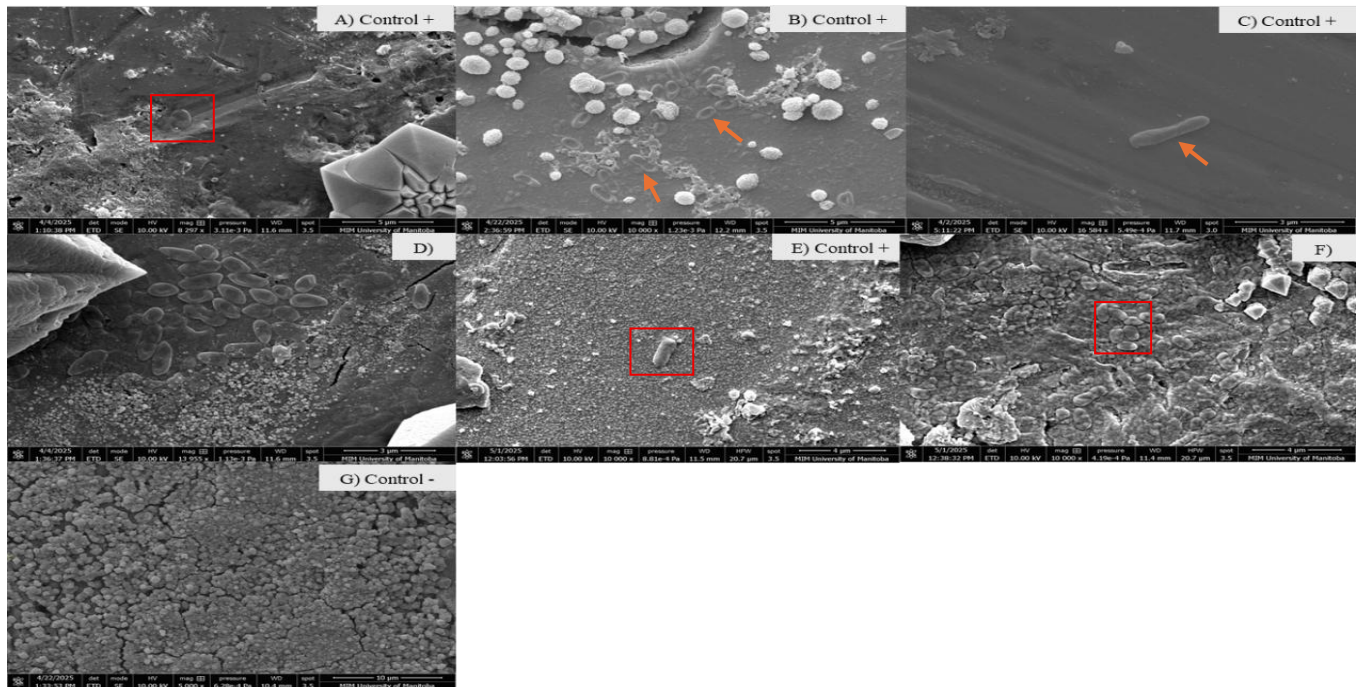


Figure 4-5: GS coupons: SEM images of 36SusP(A), *P. aeruginosa* (B), *B. cereus* (C), *B. cereus* + *P. aeruginosa* (D), *E. coli* 11775 (E), *E. coli* 11775 + *P. aeruginosa* (F) Control negative GS (G) **wet biofilms at 20 °C for 6 days.** Individual cells are shown (red boxes and orange arrows) attached to GS surface.

4.5 Discussion

4.5.1 SE Survival is Influenced by Multispecies Biofilm Composition

SE survival within multispecies biofilms was influenced by the composition of bacteria within the biofilm. In this study, SE recovery from GS coupons had a higher total recovery after enrichment from M1 (*E. coli* + *P. aeruginosa*) than from M2 (*B. cereus* + *P. aeruginosa*). M1 total recovery of SE cells from GS-dry coupons was 66.66%, in contrast to SE recovery from M2 which was 33.33%. Moreover, no SE cells were recovered even after enrichment from M2 under either dry or wet conditions (Table 4-2). The higher recovery of SE cells observed in M1 may be associated with the well-documented biofilm-forming capacity of *E. coli*, which produces

extracellular polymeric substances (EPS) capable of protecting embedded cells from desiccation and environmental stress (Hung et al., 2013; Koti et al., 2024; Reisner et al., 2006; Yuchen et al., 2022). Such structural protection might allow SE cells to remain viable but injured, allowing for recovery after enrichment. Additionally, SE and *E. coli*, both Gram-negative enteric bacteria, share physiological and metabolic similarities that may reduce interspecies competition and promote more stable biofilm architecture (Sivan Elias & Ehud Banin, 2012; Huang et al., 2011; Kolenbrander et al., 2010; Luo et al., 2022). In contrast, the lower recovery of SE from M2 suggests a more antagonistic interaction. *B. cereus*, a Gram-positive bacterium with distinct metabolic requirements, is known to produce antimicrobial compounds such as peptides and degradative enzymes that inhibit competing species (Baruzzi et al., 2011; Sudan & Li, 2022; Sumi et al., 2014). Although antimicrobial activity of the specific *B. cereus* strain used in this study was not evaluated, such mechanisms could contribute to suppression or injury of SE cells beyond recovery during enrichment. Both M1 and M2 multispecies biofilms contained *P. aeruginosa*, known for its persistence, robust biofilm-forming ability and competitive dominance (Blasco et al., 2023; Li et al., 2022; Mulcahy et al., 2014). However, in M1, potential compatibility between SE and *E. coli* may have mitigated competitive pressure from *P. aeruginosa*, whereas in M2, the combined competitive effects of *B. cereus* and *P. aeruginosa* may have enhanced SE exclusion.

4.5.2 Multispecies Biofilm Formation on Contact Surfaces

Notably, in this study, plastic (PLA) surfaces consistently supported significantly higher bacterial loads than galvanized steel (GS), irrespective of humidity conditions ($P < 0.0001$). Although wet conditions generally promoted bacterial growth, the effect of surface material was the predominant factor influencing bacterial recovery. SE growth on GS coupons were

suppressed across all multispecies combinations, SE counts ranged from 0 to $1.7 \log_{10}$ CFU/mL and had no significant difference in comparison to the controls, regardless of the relative humidity ($P < 0.05$) (**Figure 4-1A**). Additionally, GS coupons examined under SEM imaging showed Zn deposits located across the coupon surface (**Figure 4-4**). The performance of SE multispecies biofilms on GS may be directly linked to the production of Zinc byproducts by GS (Thomas et al., 2024). Nanoparticle studies have shown that Zn nanoparticles can inhibit bacterial growth by disrupting cell-surface attachment and killing planktonic cells (Fulindi et al., 2023; Sirelkhatim et al., 2015). In a study by Applerot et al. (2012), glass slides coated with ZnO nanoparticles inhibited biofilm formation of *E. coli* and *S. aureus* cells and increased bacterial susceptibility to antimicrobials. SE performance on GS was worse under dry conditions as dry environments impose stress and can lead to bacterial desiccation (Esbelin et al., 2018). However, a robust EPS can provide a moisture and nutrient-rich buffer against harsh environmental conditions, allowing bacteria to remain viable in dry biofilms over long periods of time (Koti et al., 2024; Yuchen et al., 2022). Scientific research concerning SE biofilm formation on GS is severely lacking. Studies primarily focus on SE attachment to stainless steel, polyethylene, rubber, concrete, nylon or glass (Counihan et al., 2025; Giaouris & Nychas, 2006; Shah et al., 2025; Thames et al., 2023).

PLA surfaces consistently supported higher SE counts (**Figure 4-1B**) however, there was a significant decrease ($P < 0.05$) in SE counts within multispecies biofilms. SE counts dropped from $6.9 \log_{10}$ CFU/mL to 4.5 and $5 \log_{10}$ CFU/mL within M1 and M2 biofilms respectively on PLA-dry coupons. Similarly, SE dropped from 7.1 to $4 \log_{10}$ CFU/mL in M1 and to $5.9 \log_{10}$ CFU/mL with M2 on PLA-wet coupons. The first step of biofilm formation is initial attachment, where bacteria maintain weak intermolecular bonds with the contact surface using structures like

pili and curli to attach (Van Houdt & Michiels, 2010). Unlike GS, PLA surfaces are hydrophobic and have low surface energy, conditions that favour bacterial adhesion as SE possesses hydrophobic components on its outer membrane, which can reduce the energy barrier between SE cells and PLA coupons (Busscher & Mei, 2006; Yuan et al., 2017). These features allow SE to establish stable contact with PLA coupons and might promote early colonization and stimulate EPS production even under dry humidities (Dantas et al., 2018; Ivers et al., 2024). Additionally, PLA coupons gave rise to competition within SE multispecies combinations, causing SE counts to reduce significantly ($P < 0.05$) in both M1 and M2 combinations. However, SE reduction on PLA was the highest on PLA-wet in combination with M1 ($4 \log_{10}$ CFU/mL). A conducive surface for biofilm attachment, along with higher relative humidity, may have increased competition within the M1 combination. M1 consists of *E. coli* + *P. aeruginosa*, both of which are gram-negative bacteria, similar to SE. Shared metabolic pathways and similar nutrient requirements may encourage antagonism within the biofilm, leading to the competitive exclusion of SE (Hol et al., 2015; Rendueles & Ghigo, 2015).

4.6 Conclusion

Biofilm formation varied with surface type, relative humidity and biofilm combination. Overall, these findings demonstrate that SE persistence in poultry environments cannot be predicted solely by environmental factors like relative humidity or surface characteristics. The species composition of SE multispecies biofilms plays a vital role in determining whether SE survives long enough to become a risk. Multispecies biofilms that offer cooperative existence may serve as protective havens for SE proliferation as observed with SE recovery from multispecies biofilms formed with M1 on GS coupons. However, where competition is the dominant interaction, SE can be suppressed naturally by the other bacteria in the biofilms, even

in the presence of favourable conditions for biofilm formation as showcased with M1 combinations on PLA coupons. Additionally, inherent characteristics of the contact surfaces can serve as an extra step in disrupting or preventing SE attachment and subsequent biofilm formation. SE co-cultured with *B. cereus* + *P. aeruginosa* on GS coupons under dry conditions had the highest reduction amongst all multispecies combinations. It is therefore important to understand the role abiotic surfaces play in the interactions between microbes within multispecies biofilms.

Chapter 5: Conclusion and Future Prospects

5.1 Thesis Summary

The main objectives of this study were to 1) determine SE biofilm formation under different environmental conditions (wet – 60-70% humidity and dry – 20-40% humidity at 10 and 20 °C); 2) evaluate SE survival and possible social interactions within multispecies biofilms formed with BAE and LAB (competitive fitness testing) and 3) Assess SE biofilm formation on galvanized steel (GS) and plastic (PLA).

This thesis demonstrated that the persistence of *Salmonella* Enteritidis (SE) in poultry environments is governed by a dynamic interplay between environmental conditions, composition of the microbial community, and the inherent chemical properties of the contact surfaces. SE biofilm formation was highly temperature-dependent, producing strong biofilms at barn-relevant temperature (20 °C) and weaker biofilms at conditions reminiscent of egg storage (10 °C). These findings highlighted the need for strict temperature management within poultry facilities as under conditions typical of the egg barn, SE resilience increases a, becoming more competitive.

Multispecies interactions further revealed that SE survival was not uniform across microbial communities. At 10 °C, SE had the potential to benefit from multispecies biofilms formed with some bacteria associated with eggshells; however, competitive interactions dominated at 20 °C. Both SE combinations with *E. coli* + *P. aeruginosa* and *B. cereus* + *P. aeruginosa* showed pronounced reductions in SE counts across different environmental parameters tested, providing insight into some of the ecological pressures that influence SE social interactions within multispecies biofilms. The species composition of the biofilm

influences SE survival in the poultry environment and identifying bacterial combinations that promote competitive exclusion and competition against SE might generate more opportunities for SE suppression strategies in the poultry industry.

Surface material and humidity also played critical roles in determining SE survival. PLA supported higher SE proliferation, favouring bacterial attachment and biofilm development. In contrast, GS consistently suppressed SE growth, especially under dry conditions. Likely due to the presence of zinc deposits that disrupted biofilm attachment, SE survival was the lowest within multispecies biofilms formed with *B. cereus* + *P. aeruginosa* on GS coupons under dry humidity. It is important that sanitation and disinfection protocols are prioritized in poultry facilities, as proper maintenance of equipment and tools, and moisture control can meaningfully contribute to curbing SE persistence in the poultry environment.

Finally, this thesis demonstrated that SE survival is not an inherent property but an outcome that is shaped by its environment and microbial neighbours. Identifying the environmental conditions and the bacterial communities that reduce SE survival will provide greater insights for designing interventions that limit SE attachment to contact surfaces, disrupt favourable biofilm environments, and ultimately reduce the contamination risk of SE in poultry facilities.

5.2 Future prospects

This study provided a preliminary look into the conditions that affect SE multispecies biofilm formation in poultry environments, and the information provided can be used to explore more facets of SE biofilm formation. Some suggestions are;

1. More multispecies biofilm combinations formed with SE would give greater insight into the possible social interactions with other bacteria present in poultry facilities that have yet to be explored with SE.
2. SE antimicrobial testing could provide insight into how antimicrobial peptides isolated from other bacteria within the poultry facility affect SE biofilm-formation and the influence of zinc on this process.
3. It would be beneficial to explore SE multispecies biofilm formation on more contact surfaces present in poultry facilities like stainless steel (processing tables and sinks) and thermoplastic polyurethane (TPU; conveyor belt) for longer periods of time (30 days, 60 days, etc.).
4. Confocal Laser Scanning Microscopy (CLSM) can be used in conjunction with Scanning Electron Microscopy (SEM) to observe SE spatial distribution and viability within multispecies biofilms as well as surface structure.

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