FATE OF SHIGATOXIGENIC *E. COLI* IN FLOUR-BASED CAKE MIXES AND ASSESSMENT OF POTENTIAL INTERVENTION STRATEGIES

by

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(Under the Direction of Govindaraj Dev Kumar and Abhinav Mishra)

ABSTRACT

Wheat flour and flour-based baking mixes can harbor Shigatoxigenic *E. coli* (STEC) and have been implicated in several outbreaks of foodborne illnesses. Wheat flour and its subsidiary products can become contaminated prior to and during processing, yet no effective post-processing microbial control measures have been identified for these commodities. Rehydration of baking mixes may allow for growth of contaminating bacteria. Ampicillin and streptomycin resistant strains of STEC (O26, O121, and O157:H7) were developed and their behavior in cake batter was monitored under different storage conditions. The population of these STEC strains declined over time in a standard cake batter formulation, but increased rapidly in commercial cake batters. Microencapsulated pelargonic acid was evaluated as a means to prevent the growth of STEC O26, O121, and O157:H7 in commercial cake batter but was found to have no impact on the growth of these strains and is therefore not an effective control measure.

INDEX WORDS: Flour, Shigatoxigenic *E. coli*, antibiotic resistance, low water activity, cake batter, pelargonic acid, novel antimicrobials

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B.S., Michigan State University, 2019

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2021

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August 2021

DEDICATION

This thesis is dedicated to my incredible parents, Dr. D'Anne and Matt Hayman. Thank you for being there on the good days, the bad days, and all the days in between. There is not a chance in the world that I could have done this without you guys. Everything I am is because of who you both are. Thank you for everything.

ACKNOWLEDGEMENTS

First and foremost, I wish to thank my fantastic advisors, Dr. Govindaraj Dev Kumar and Dr. Abhinav Mishra, for the opportunity to be at the University of Georgia and work in not one, but two great labs. Thank you for your guidance and mentorship throughout this project. You have both made me a better scholar, scientist, and person.

I extend my deepest gratitude to my fabulous committee members, Dr. Francisco Diez-Gonzalez, Dr. Harshavardhan Thippareddi, and Dr. Kevin Mis Solval for lending their expertise to this project—it could not have been a success without you all. Thank you for being part of my graduate school journey and for your words of advice and encouragement. Thank you especially to Dr. Mis Solval for sharing your time and lab space for several parts of this project.

Huge thanks to our sensational lab technician, Jye-Yin Liao, for making sure I had everything I needed to complete this project and especially for assisting with the last phase of data collection. I wouldn't have been able to finish this project without your help.

Thank you to Bobby Goss and Boran Yang for helping with my batter cooling study—your willingness to troubleshoot when things weren't going to plan means more than you know. You guys rock.

To my dear lab mate and friend, Ikechukwu Oguadinma—thank you for everything. I couldn't have done this without you.

To my Athens office mates, Xinran Xu, Anna Townsend, Dr. Brittany Magdovitz, Samuel Cimowsky, Dr. Chase Golden, and Elizabeth White—thanks for all the laughs and for talking me off the ledge more than once. I wouldn't have made it through my first year without you all.

Huge extra special thanks to Xinran Xu for dedicating her weekend time to teaching me how to use MATLAB in my final hours. This project would not be complete without you—you're the best!

Finally, a thousand thank-yous to all the friends who saw me through this journey. Thank you for listening to my lamentations and offering words of encouragement (and beer). I wouldn't be here without you guys.

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CHAPTER 1

INTRODUCTION

Starting as early as 1952, there have been more than thirty outbreaks of foodborne illness and recalls around the world linked to wheat flour and products that contain wheat flour, such as baking mixes (Myoda et al. 2019). These incidents result in financial losses due to illnesses, loss of productivity, and recalls. Beyond the financial burden of healthcare for affected individuals, they impact consumer confidence and tarnish the reputation of commercial cake mix brands. The subsequent loss of consumer trust can greatly reduce revenue generated from these products (Hussain and Dawson 2013). Flour is a raw agricultural commodity but is often not considered as such because it is rarely consumed in its uncooked form; usually it is an ingredient in products that are subsequently cooked or baked, or otherwise exposed to heat. It is also a low-moisture food, with $a_w \sim 0.40$, which is too low to support most microbial growth. For this reason, flour has been historically under recognized as a vehicle for bacterial pathogens (Beuchat et al. 2013). It has been recently shown, however, that *Salmonella* is present in 1.23% of flour produced in the U.S., while Shigatoxigenic *E. coli* (STEC) is present at a rate of 0.44% (Myoda et al. 2019). *Salmonella* has been well characterized as a pathogen of concern in low-moisture foods, but STEC has recently emerged as an equal threat to the safety of these products.

One reason pathogens like STEC are such a problem in flour and its subsidiary products like baking mixes is that neither the flour milling process, nor the baking mix manufacturing process are designed to reduce the microbial load of the product (Sabillón and Bianchini 2016). While the water activities of baking mixes are too low to support the microbial growth, STEC possesses specialized molecular mechanisms for surviving in dry environments and is capable of

persisting for long periods of time (Forghani et al. 2018). When these mixes are rehydrated to form a batter, optimum conditions are created for STEC to be revived and regain virulence. At this point, responsibility falls on the consumer to apply a lethality step by baking the batter prior to consumption. However, relying on consumers to do this is not an effective strategy for mitigating the risk posed by uncooked batters. Such batters may encounter time and temperature abuse if a consumer leaves it sitting out too long before baking or tries to refrigerate it for later use, which could allow contaminating bacteria time to proliferate. Further, consumers have been known to taste raw batter and dough prior to baking, or they may undercook these products prior to consumption (Rose et al. 2012; Crowe et al. 2017).

The potential for STEC in raw flour products to cause illness is well documented, and some intervention strategies have been proposed (Sabillón et al. 2017; Dhillion 2007). However, these treatments can only be applied prior to processing of the wheat. An effective antimicrobial measure which could be applied after the flour milling and baking mix manufacturing process has yet to be identified. Pelargonic acid (PA) has demonstrated antimicrobial efficacy against pathogens such as *Salmonella* Newport, Typhimurium, and Javiana and can be microencapsulated to produce a stable, high-quality powder (Dev Kumar 2017; 2020). This antimicrobial powder could be an ideal additive for flour-based mixes which would prevent the proliferation of contaminating bacteria like STEC upon rehydration.

To our knowledge, little work has been done to characterize how STEC survives in products like baking mixes after rehydration, nor have any effective post-processing antimicrobial interventions been developed which could prevent STEC from proliferating in batter. With 15% of all cases of foodborne illness in the U.S. originating in the home, it is critical that these topics be investigated (Centers for Disease Control and Prevention 2011). Thus, the specific objectives

of the present study were to 1) develop antibiotic resistant (ABR) strains of STEC that could be used for detection and enumeration in cake batter without interference from the batter's background microbiota, 2) characterize the survival of these STEC strains in cake batter over time at various storage temperatures, and 3) investigate how microencapsulated pelargonic acid in cake batter would impact the survival of these STEC strains.

CHAPTER 2

LITERATURE REVIEW

2.1 Shigatoxigenic *E. coli*

Escherichia coli are a large and diverse group of bacteria. They are Gram-negative, rod-shaped bacteria which is facultatively anaerobic. They can be found as commensal organisms in the gastrointestinal tract of many humans and animals, but there are also several strains which are highly pathogenic to humans (Behling et al. 2010). Pathogenic *E. coli* is responsible for more than 265,000 cases of illness in the U.S. every year (Centers for Disease Control and Prevention 2019). There are numerous strains of *E. coli* which can cause different illnesses, including intestinal gastroenteritis, urinary tract infections, neonatal sepsis and, occasionally, meningitis (Kaper, Nataro, and Mobley 2004). Strains of *E. coli* which cause gastroenteritis are of the greatest significance in the context of foodborne illness. There are five categories of *E. coli* that cause enteric illness in humans: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and Shigatoxigenic *E. coli* (STEC) (Clements et al. 2012). STEC is the group most frequently implicated in outbreaks of foodborne illness in the U.S. Within this group, serovars O26, O45, O103, O111, O121, O145, and O157:H7 possess the greatest virulence (Gyles 2007). *E. coli* O157:H7 accounts for about 36% of all *E. coli* related illnesses in the U.S. (Centers for Disease Control and Prevention 2019).

STEC causes disease in humans when it is ingested and becomes established in the lower intestine. Here, the bacteria come into contact with the surface of intestinal cells and begin producing components of a Type III secretory system (T3SS), which allows injection of various bacterial effector proteins into the host cell (Gyles 2007). Among these proteins is intimin, which

causes an accumulation of actin that forms a tight bond between the bacterial cell and the host cell. Once the bacterium is securely attached to the surface of the intestinal cell, it will begin producing Shiga toxin (Melton-Cesla et al. 2012). Shiga toxin is a heterohexameric molecule with a single A subunit (32 kDa) comprising a chain of 293 amino acids with an active site at the glutamic acid in position 167. The five identical B subunits (7.7 kDa) contain multiple binding sites which facilitate internalization of the toxin when they bind receptors on the host cell (He et al. 2012). Upon internalization, the toxin moves to the Golgi complex and endoplasmic reticulum, where it interrupts the action of mRNA and inhibits protein synthesis. This results in cell death through apoptosis (Castro et al. 2017). There is limited information regarding the lethal dose of Shiga toxin in humans, but Russo et al. (2014) have estimated that a dosage of 0.1 mg/kg of body weight may be sufficient to cause illness. These authors further suggest the possible presence of pre-formed Shiga toxin in contaminated food, which could additionally contribute to disease.

Symptoms of STEC infection manifest as watery diarrhea and abdominal cramping after a short incubation period of 3-4 days. This often turns to bloody diarrhea within another few days as a result of the degradation of intestinal cells and subsequent damage to surrounding endothelial cells (Karch 2001). In severe cases, this damage to nearby blood vessels will cause extreme cell swelling and reduced blood supply to the kidneys, which severely impairs their function (Castro et al. 2017). This is known as hemolytic uremic syndrome (HUS); this condition causes death in 5% of cases, and serious long-term complications such as chronic renal failure, renal hypertension, and neurological disorders in most other cases (Jubelin et al. 2018).

STEC infections cannot be treated with antibiotic medications as most other bacterial infections can. This is because antibiotics have been shown to increase patients' risk of developing HUS by increasing the production and release of Shiga toxin (Mor and Ashkenazi 2014). This is

mediated by a few factors. Antibiotics create an advantageous environment for STEC in the patient's gastrointestinal system by eliminating the native gut microbiota that would normally compete with the infecting bacteria for resources. Additionally, antibiotics that function by lysing bacterial cell walls can cause a release of pre-formed Shiga toxin from STEC cells, drastically increasing the amount of toxin that can interact with intestinal cells (Kakoullis et al. 2019). Thus, the primary means of treating STEC infections currently is simply supportive therapy, such as rehydration and dialysis when necessary (Mühlen and Dersch 2020). STEC requires only a low infectious dose to cause disease (50-100 cells) and is estimated to be responsible for an annual total of 2.8 million cases of illness and 230 deaths worldwide, making it a very serious threat to human health (Castro et al. 2017; Majowicz et al. 2014).

2.2 Wheat flour and its subsidiary products

2.2.1 Wheat

Wheat (*Triticum aestivum*) is the third largest crop produced in the U.S., with more than 800 billion bushels being grown on 50 million acres of cropland. While wheat planting has been on the decline due to changes in government incentives for farmers and increased global competition, the U.S. maintains a solid share of the global wheat market, accounting for 15% of global wheat exports (U.S. Department of Agriculture 2020).

A wheat grain is a small ovoid kernel, measuring 5-9 mm in length and weighing 35-50 mg. The kernel is the seed from which the wheat plant grows and is composed of three primary parts: the bran, the endosperm, and the germ (Figure 2.1) (NZ Flour Milling Association 2020). The bran is the tough, shell-like outermost layer of the kernel which protects the soft inner portions and accounts for about 15% of the total kernel. It is primarily composed of insoluble fibers such

as cellulose and lignin (Onipe, Jideani, and Beswa 2010). The bran is largely a by-product of the milling process, which is used for animal feed as its tough, fibrous nature is not ideal for most flours, however it is occasionally milled with the rest of the kernel to produce whole wheat flour (Serna-Saldivar 2010). The endosperm is the soft, starchy inner part of the kernel which makes up 80-85% of the total kernel; this is the portion which primarily comprises flour. The endosperm is mostly made of proteins and carbohydrates, as well as vitamins such as riboflavin, niacin, and thiamine (Khan and Shewry 2009). The germ is the embryo of the seed, located at the bottom innermost part of the kernel. It is usually excluded from the flour milling process due to its high fat content (10%), which makes it susceptible to rancidity and would thus limit the shelf-life of flour (Brandolini and Hidalgo 2011).

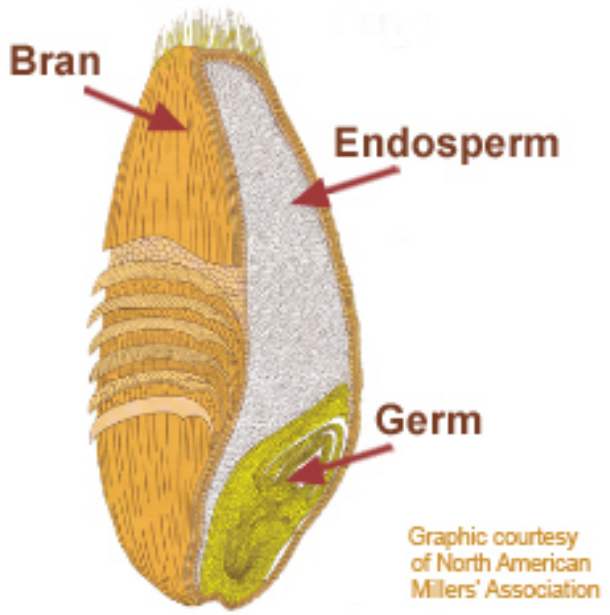

Figure 2.1: A diagram of the structure of a typical wheat kernel. Reprinted from the North American Miller's Association, 2021.

There are six classes of wheat grown in the U.S., which are differentiated based on their color, hardness, and growing season: hard red winter, hard red spring, soft red winter, soft white, hard white, and durum wheat. These varieties and their subsequent flours have many different

qualities which make them suited for different types of end products—for example, the low protein content of soft white wheat makes it best for use in delicate baked goods like cakes and cookies, while the hardness and higher protein content of durum wheat is ideal for pasta products and tougher breads (U.S. Wheat Associates 2020). The standard all-purpose flour used by most consumers is made from a blend of hard and soft wheat, as the combined qualities make it universally suitable for any end product.

In the Code of Federal Regulations (21:137), the U.S. Food and Drug Administration defines flour as "the food prepared by the grinding and bolting of cleaned wheat" (U.S. Food and Drug Administration n.d.). Flour production begins with the harvest and delivery of wheat kernels to the flour mill. After various tests for quality are performed, the wheat kernels proceed through a dry-cleaning process which uses sieves, controlled air flow, disc separators, and plate magnets to remove impurities such as sticks, stones, dirt, metal fragments, and any other debris that may have accumulated during harvest and transport. Once clean, the kernels are conditioned with water to adjust their moisture to a desired level in a process called "tempering." This makes subsequent separation of the components of the kernels easier and maximizes flour extraction (Catterall and Cauvain 2007; Posner and Hibbs 2005). When the right moisture content is achieved, the kernels move on to the break system, which is a series of corrugated rollers that begin the process of grinding open the kernels and separating the bran from the endosperm. The broken fractions proceed through a series of sieves to separate out the finest portions of the endosperm, which then go through a reduction system. This is a series of smooth rollers which grind down the size of the endosperm particles into fine, soft flour (Doblano-Maldonado et al. 2012). An overview of the complete milling process is shown in Figure 2.2. Notably, the milling process is not designed to

reduce the microbial load of the wheat kernels or flour, nor is an antimicrobial step applied before distribution to consumers, making it a raw product (Sabillón and Bianchini 2016).

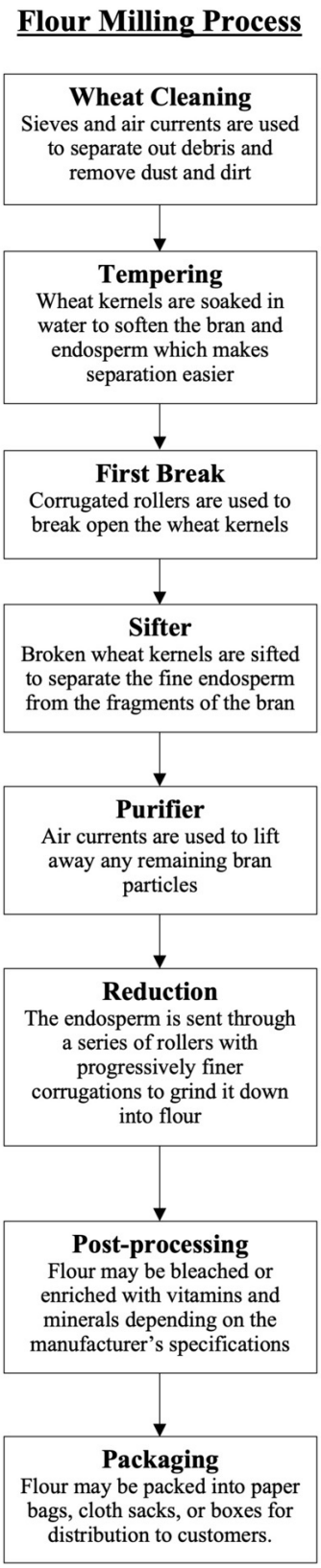

Figure 2.2: A simplified overview of the flour milling process (Doblano-Maldonado et al. 2012).

2.2.2 Commercial baking mixes

Flour is used in the homes of millions of people for a variety of different purposes. One of its most popular functions is as a foundational ingredient in doughs and batters for breads, cookies, cakes, and other baked items. These goods may be homemade, or they may come from commercial pre-made mixes. Such mixes have been around for more than 100 years—one of the first debuted in 1889, when the owners of a milling company created a just-add-water pancake mix to deal with a surplus of flour during a lull in demand. The mix included wheat flour, corn flour, phosphates, baking soda, and salt (Firkser 2016). Today, commercial baking mixes are formulated similarly, albeit with the inclusion of more modern ingredients such as shortening, emulsifiers, flavors, and antioxidants like butylated hydroxytoluene or citric acid. Their convenient appeal remains the same, however, with most requiring only the addition of water, oil, and eggs (Park 2013). Like flour, these products do not undergo a lethality treatment during their manufacture and the responsibility falls on the consumer to apply a kill step by baking the product before consumption (Sabillón and Bianchini 2016). When these products are rehydrated to form a batter prior to baking, especially in the presence of other ingredients such as sugars, proteins, and fats, optimal conditions for bacterial proliferation are created (Wu et al. 2017). Further, these macromolecules can protect the bacteria from stresses encountered later on when the batter is baked or washed off kitchen utensils (Li, Kuda, and Yano 2014).

2.3 Nutritional factors that may impact STEC survival in cake batter

The nutritional profile of cake batter may exacerbate the issue of bacterial persistence. Fat, for example, is known to exert a protective effect on bacteria and can increase their tolerance to a number of stresses. Brar et al. (2018) found a negative correlation between the fat content of ground beef and D-values for STEC at 55°C. This is in agreement with earlier works such as that

of Juneja and Eblen (2000), who found that higher levels of fat in ground beef resulted in increased heat tolerance of *Salmonella.* The protective effect of fat on bacteria is attributed to the decreased water activity of high fat environments, which is known to inhibit the efficacy of antimicrobial treatments like heat (Jay 1993).

2.4 Outbreaks associated with STEC in flour and related products

Flour is a low-water activity food, with a_w generally ~0.40, and was previously considered microbiologically safe since its low moisture content was thought to be an adequate barrier to the growth of pathogenic bacteria (Beuchat et al. 2013). On the contrary, flour has been increasingly implicated in outbreaks of foodborne illness in recent years (Myoda et al. 2019). While many of these outbreaks were caused by *Salmonella*, which has long been known for its ability to survive in dry environments, STEC has emerged as an equally serious threat to flour safety (Forghani et al. 2018).

One of the first documented and most notable outbreaks of STEC in a flour-containing food product was *E. coli* O157:H7 linked to Nestle Toll House refrigerated raw cookie dough sold in the U.S. Seventy-seven people were sickened and 35 of them were hospitalized; 10 of these patients developed HUS. The outbreak strain was isolated from the raw cookie dough and flour was identified as the most likely source of contamination, although this was not definitively determined (Centers for Disease Control and Prevention 2009). In 2016, the first confirmed outbreak of STEC in flour occurred in the U.S. Sixty-three people across 24 states reported illness after using flour to make dough or batter in their home; the flour was found to be contaminated with *E. coli* O121 or O26. Nineteen out of 38 ill persons who were interviewed reported sampling the raw homemade batter or dough. Traceback revealed that the implicated brands of flour had all been produced at the same facility in Kansas City, Missouri. The outbreak also triggered a recall

of several popular boxed cake mixes that were made with flour from the supplier (Centers for Disease Control and Prevention 2016). In 2017, 27 cases of illness due to *E. coli* O121 in flour were reported in Canada. The flour was sold by several different brands but was traced back to a single supplier (Entis 2017). In this outbreak, 11 out of 22 ill persons interviewed reported sampling raw dough during their period of exposure to the contaminated flour (Morton et al. 2017).

Most recently, in 2019, an outbreak of *E. coli* O26 in flour in the U.S. caused 21 illnesses and 3 hospitalizations. The flour, sourced from a single supplier, was sold by itself and in brownie and cookie mixes, which were part of extensive recalls (U.S. Food and Drug Administration 2019). These outbreaks clearly demonstrate the ability of STEC to persist in flour and flour-based baking mixes and, further, illustrate the risk these products pose when consumers use them to prepare doughs and batters.

2.5 Sources of STEC contamination in flour and related products

Wheat flour is made from wheat kernels, which are grown in a traditional agricultural setting and thus tend to carry a high native microbial load. These organisms include bacteria, which primarily belong to families such as *Pseudomonadaceae, Micrococcaceae, Enterrobacteriaceae, Lactobacillaceae,* and *Bacillaceae,* as well as fungal species including *Alternaria, Fusarium, Helminthosporium, Aspergillus, Penicillium,* and *Cladosporium* (Laca et al. 2006). Bacteria are generally concentrated on the surface of the wheat kernel, although some may become internalized within the kernel due to physical damage during cultivation (Los, Ziuzina, and Bourke 2018).

In addition to many typical environmental organisms, it has been shown that flour may carry pathogens such as *Salmonella* and STEC (Myoda et al. 2019). These organisms can be introduced to wheat kernels via dust (Dev Kumar et al. 2016), contaminated irrigation water (Dev

Kumar, Patel, and Ravishankar 2020), insects, and animal feces (Sabillón and Bianchini 2016). Several of these factors are known vehicles for pathogenic bacteria and, considering the number of recent flour-related outbreaks of illness, it is highly likely that these introductory routes do indeed play a role in compromising the safety of wheat flour. Further, additional microbial contamination may be introduced to wheat kernels during harvest and subsequent handling and storage (Doyle and Buchanan 2013).

As an agricultural product, wheat is exposed to many naturally occurring microbiological threats such as soil, water, insects, and animal feces (Sabillón and Bianchini 2016). STEC has been isolated from all of these sources. Cattle are well-known reservoirs of *E. coli* O157:H7, which is shed in their feces. This material is often used to fertilize crops like wheat, and thus can transfer bacteria to them (Ferens and Hovde 2011). Crop fields tend to attract wild animals such as deer, hogs, mice, rats, and rabbits, which are also known to carry pathogenic *E. coli* in their gastrointestinal tract. Their defecation in crop areas allows *E. coli* to persist in the soil and offers another route of transmission of these bacteria to wheat plants (Brar and Danyluk 2018). STEC may also be present in irrigation water that includes runoff from nearby animal pastures (Jacobsen and Bech 2012). Thus, most bacterial contamination in wheat is introduced before it is even harvested or handled. STEC can adhere to and remain on the outer shell of wheat kernels until they reach the milling facility.

Although the kernels undergo a cleaning step prior to milling, this has little effect on their overall microbial load (Sabillón and Bianchini 2016). Therefore, STEC can be easily spread to flour extracted from the kernels when they enter the break system (Myoda et al. 2019). While the water activity of flour is too low to support microbial growth, STEC has several mechanisms which

allow it to adapt to low-moisture environments and persist for as long as nine months at room temperature (Forghani et al. 2018; Chen and Goulian 2018).

2.6 Mechanisms of STEC survival in low-water activity foods

2.6.1 Stabilization of bacterial cell membrane with compatible solutes

When STEC is exposed to a low-moisture environment, water leaves the bacterial cell due to osmotic pressure. This leads to a loss of turgor pressure and molecular crowding in the cell (Minton 2001; Burgess et al. 2016). Without intervention, this would cause cell lysis. STEC can combat this by accumulating compatible solutes, such as sucrose and trehalose. These sugars "replace" water by forming hydrogen bonds with the cell's membrane phospholipids, effectively stabilizing the membrane, and preventing damage due to desiccation (Crowe et al. 1987). Trehalose is known to be a superior stabilizer of proteins compared to other solutes in the cell environment. A study of recombinant *E. coli* engineered to overexpress genes for trehalose synthesis showed that increasing trehalose production resulted in significantly improved desiccation survival (Miller and Ingram 2008). Accumulation of trehalose in *E. coli* is a result of upregulation of its biosynthesis via the OtsAB pathway. Here, UDP-glucose and glucose-6-phosphate are condensed to form trehalose-6-phosphate, which is dephosphorylated to yield trehalose (Ruhal et al. 2013). Trehalose is then used to preserve the lipid bilayer of the cell's membrane by forming hydrogen bonds between its own OH groups and the lipid heads of the bilayer (Tang et al. 2007).

2.6.2 Mitigation of oxidative stress

Membrane disruption is not the only damage that must be avoided when STEC enters a desiccated state. During drying, bacterial cells will greatly reduce their metabolic activity,

including respiratory pathways, in an effort to conserve energy and resources (Lebre, De Maayer, and Cowan 2017). This leads to an accumulation of reactive oxygen species (ROS), which impose oxidative stress on the cell. One of the most potent ROS, hydrogen peroxide, is able to penetrate bacterial membranes and be converted to a hydroxyl radical in the presence of iron, which is concentrated inside the bacterial cell during drying (Amano 2011). Hydroxyl radicals can damage the cell's critical components; for example, they can hydroxylate purine and pyrimidine bases in DNA and create lesions or oxidize free thiol residues on protein structures to form disulphide bonds and destroy the proteins' functionality (Kranner and Birtic 2005). STEC can prevent this damage from occurring through the production of a DNA-binding protein called Dps. Dps is able to bind and chelate Fe ions in the cell to stop their participation in the production of hydroxyl radicals. Additionally, Dps binds to the cell's DNA and protein bodies to prevent their modification by hydroxyl radicals (Karas, Westerlaken, and Meyer 2015).

2.6.3 General stress response of E. coli

The OtsAB pathway and the production of Dps are under the transcriptional control of the general stress response regulator in *E. coli*, *rpoS* (Lebre, De Maayer, and Cowan 2017; Schellhorn et al. 2016). *RpoS* regulates approximately 10% of the *E. coli* genome, and its induction during times of cellular stress results in the upregulation of more than 140 genes related to various stress responses (Battesti, Majdalani, and Gottesman 2011). Induction of *rpoS* begins when the cell senses that its environmental conditions are suboptimal. This triggers a regulatory cascade that results in the activation of *rpoS* and the production of RpoS. RpoS then mediates the binding of RNA polymerases to the promoters of *rpoS*-dependent genes and thus facilitates their transcription (Sheldon et al. 2012; Battesti, Majdalani, and Gottesman 2011). These genes encode proteins that all share a similar purpose: to stabilize the cell membrane, DNA, and proteins. This offers

protection from most unfavorable environmental conditions. Thus, the benefit of the general stress response is that when it is activated by one stress, in confers upon the cell resistance to a number of other stressors (Sheldon et al. 2012). For example, *E. coli* AW1.7 which was exposed to heat and osmotic stress prior to treatment at 60 ℃ for 30 min was only reduced by 1 log CFU/ml, compared to the un-stressed control strain which was reduced by 4 log CFU/ml (Pleitner et al. 2012). As such, desiccated STEC surviving in raw flour products may possess increased tolerance to antimicrobial treatments encountered later on when the product is prepared as a batter—for example, exposure to heat during baking or sanitizing agents used to clean utensils involved in batter preparation.

2.7 Risks posed by STEC-contaminated batter in the home

Consumer mishandling of batter in the home can exacerbate the issue of bacterial contamination in flour-based baking mixes. Once the mix is reconstituted, and other ingredients like sugar, eggs, and fats are added, optimal conditions are created for surviving bacteria to proliferate (Wu et al. 2017). If the consumer leaves the batter to sit at ambient temperatures prior to baking, or stores it in a refrigerator for later use, sufficient time may be given for the bacteria to grow. With some strains of *E. coli* having a doubling time as short as 20 minutes in ideal conditions, even a low level of original contamination in a dry flour mix can quickly become a big problem when it is made into batter (Fossum, Crooke, and Skarstand 2007). Little work has been done to characterize the survival of STEC in raw batter over time. A previous study has shown that *E. coli* O157:H7 inoculated into commercial, ready-to-bake cookie dough was only reduced by 3.0 log CFU/g after 8 weeks of storage at 4°C; however, this study used a liquid inoculum, which does not reflect the dry-stressed state of STEC when inhabiting a low-moisture environment such as a baking mix (Wu 2016). As bacteria exposed to prior stress are known to gain cross-

resistance to other stresses, desiccated STEC may have an increased ability to persist in a reconstituted baking mix during storage.

2.7.1 Prevalence of raw flour product consumption

An additional risk related to contaminated wheat products in the home is that consumers frequently sample raw or undercooked batter. In 2005, the U.S. Food and Drug Administration declared that dry cake mix cannot be considered a ready-to-eat food in response to an outbreak of *Salmonella* in ice cream sold by Cold Stone Creamery, which added cake mix to the ice cream to create a "cake batter" flavor (U.S. Food and Drug Administration 2005). Since then, the U.S. Food and Drug Administration and Centers for Disease Control and Prevention have both made many efforts to encourage consumers to refrain from eating raw batter. Consumers do not always adhere to these warnings, however, as evidenced by a 2011 survey of 1,032 individuals in the U.S. which found that 58% of consumers admitted to tasting cookie dough before baking, while 80% said they had licked beaters after mixing batter for cakes, brownies, or muffins (Rose et al. 2012). The potential for these consumer habits to lead to illness is clearly illustrated by the number of individuals involved in recent outbreaks related to flour products who reported consuming uncooked batter. Relying on consumers to apply a lethality step by adequately baking these products and not ingesting them before that point is not an effective risk mitigation strategy (Sabillón et al. 2016). Thus, a control measure which could be applied to baking mixes after processing to prevent bacterial proliferation despite mishandling of batter during preparation would be a valuable tool in reducing incidences of foodborne illness.

2.7.2 Raw flour products as a vehicle for cross-contamination

STEC in raw batter can also contribute to illness in the home when residual batter is not adequately removed from kitchen utensils after they are used for preparation. Organic soil is known to aid in the accumulation and attachment of bacteria to food contact surfaces. Upon adhesion, such bacteria tend to exhibit increased resistance to cleaning and disinfecting agents, allowing for their long-term persistence on these surfaces (Mafu et al. 2011). Thus, utensils which are used to prepare batter and not sufficiently cleaned afterward may harbor bacteria, and these bacteria could be subsequently transferred to other kitchen surfaces or foods (Lee et al. 2006). Once again, STEC which has adapted to survive in a low-moisture food like a baking mix could pose a unique risk in this situation, as its exposure to dry stress may confer cross-resistance to other stresses which would be encountered during the cleaning of dishes such as hot water or detergents. Ultimately, this exacerbates the risk posed by raw flour batters in the home—even if consumers do not eat the raw batter, they may not realize that soiled utensils could lead to cross-contamination of other ready-to-eat foods made in the same environment.

2.8 Intervention strategies for STEC persistence in raw batter

Considering the myriad opportunities for microbial contamination during wheat kernel handling and flour milling, it is critical that control measures are implemented in order to reduce the potential for pathogen presence; however, few such measures exist right now in the wheat industry. The primary means of intervention currently available to wheat processors is simply cleaning wheat kernels prior to flour milling, however this process is largely targeted at removing extraneous material such as sticks, stones, insects, attached soil, and other kinds of field debris that may have been picked up during harvest (Posner and Hibbs 2005). While the removal of foreign matter may reduce sources of contamination, this cleaning does little to reduce the microbial load

of the kernels themselves (Sabillón and Bianchini 2016). Manthey et al. (2004) showed that typical kernel cleaning processes only reduced aerobic bacteria as well as yeast and mold counts by 1.0 log CFU/g, while Seiler (1986) reported similarly low reductions in bacteria and mold when evaluating the efficacy of different cleaning methods.

Some potential intervention methods have been suggested for use during the tempering stage of wheat processing, where kernels are soaked in water in order to reach a desired moisture content (Posner and Hibbs 2005). Similar to other produce washing practices, oxidizers such as peroxyacetic acid and chlorine have been added to wheat tempering water in an effort to remove contamination from the surface of the kernels (Dhillion 2007). Both are commonly used in the food industry but have many drawbacks. Chlorine, for example, is easily impeded by the presence of organic matter and requires frequent monitoring of concentration and pH (White et al. 2021). Further, chlorine is known to leave behind surface residues which may be harmful to human health. For this reason, chlorine use is largely unfavorable among consumers (Papachristodoulou et al. 2017). Peroxyacetic acid (PAA) is more efficacious than chlorine in produce washes but is far more expensive and is also highly corrosive, which constitutes a significant health hazard for workers (Herdt and Feng 2009).

Other tempering water treatments have been investigated, including the use of ozonation and organic acids. Ozone is a powerful oxidizer which is known to be effective against both pathogenic and spoilage microorganisms and, unlike chlorine, leaves no chemical residue (Rose et al. 2012). Despite its superiority in this aspect, ozone used in tempering water has limited efficacy against bacteria. A study which used ozone at a concentration of 11.5mg/L in tempering water for hard red and soft white wheat found only a 1-2 log CFU/g decrease in the total bacteria, yeast, and mold counts for both varieties of wheat (Ibanoglu 2002). Similarly, Dhillion (2007)

used ozone in tempering water at a concentration of 16 mg/L for durum and hard red spring wheat and achieved a <1 log CFU/g reduction in the bacterial load of both varieties.

Sabillon et al. (2016) have investigated the use of organic acids in combination with salt in tempering water. This study added acetic, citric, lactic, or propionic acid (1.0, 2.5, or 5.0% v/v) to tempering water alone and in tandem with NaCl (26 or 52% w/v) and found that the 5.0% concentration of acetic, propionic, and lactic acids resulted in a 2.3 log CFU/g reduction in *Enterobacteriaceae* (Eb), while the synergistic interaction of 5.0% lactic acid and 52% NaCl increased Eb reduction to 4.7 log CFU/g. A later study by the same group looked at the impact of this treatment on the functional properties of the flour produced by the treated wheat kernels and found that the acid content of the flour was significantly increased as compared to that of flour from untreated kernels. Further, it was found that this acid-saline treatment resulted in reduced gluten strength in doughs made from the treated flour. Ultimately, these findings demonstrate that, despite the promising antimicrobial effect of using organic acids and salts in the tempering of wheat kernels, these treatments may detrimentally affect the functional characteristics of the resulting flour. This is the same reason why other, more common antimicrobial treatments such as heat cannot be applied to flour—reduced flour functionality will lead to reduced consumer acceptance and lower profitability of these flour products.

Consequently, it is of increasing interest to the wheat industry to find new, effective antimicrobial interventions for wheat that will not impact flour quality. A survey of industry attitudes toward such an antimicrobial was conducted in collaboration with the University of Georgia's Innovation Gateway organization to gather insight from 32 representatives of the food industry regarding the ideal qualities of a bacterial control measure for wheat. Responses outlined that, since a true kill step does not currently exist for flour production, a treatment that could be

applied to wheat flour after milling in order to mitigate any bacterial hazards introduced during processing would be extremely valuable. Representatives also noted that, in line with recent growing consumer demands for transparent and minimal processing of foods, an antimicrobial that could be classified as natural, organic, and freely usable without regard to toxicity limits or chemical residues would be ideal for wheat.

2.8.1 Pelargonic acid and its antimicrobial potential

Pelargonic acid (PA) is a nine-carbon fatty acid which is commonly used as an antifungal agent (National Center for Biotechnology Information 2020). PA is approved for use as a food or feed additive by the U.S. Food and Drug Administration. Fatty acids like PA have shown significant antimicrobial activity against foodborne pathogens such as *E. coli*, *Salmonella,* and *C. perfringens* (Gómez-García et al. 2019). Fatty acid mechanisms of action against bacterial cells include the disruption of cell membrane lipids, alteration of membrane fluidity, and promotion of hydroperoxide formation, which imposes oxidative damage on the cell's critical components (Kim and Rhee 2016). Dev Kumar and Micallef (2017) showed that pelargonic acid at a concentration of 125 mM will inhibit the growth of *Salmonella* serotypes Newport, Typhimurium, and Javiana, and visualization of these cells after treatment using confocal microscopy revealed that PA had disrupted the cells' membranes and caused cell leakage.

Despite the notable antimicrobial activity of fatty acids such as PA, their use as sanitizers and food additives is limited by their poor solubility in water and subsequent phase separation in aqueous media (Dev Kumar and Micallef 2017). The same study has shown, however, that emulsification of PA with a surfactant such as Quillaja saponin, which is a triterpene glycoside from the bark of the *Quillaja saponaria* Molina tree (Yang et al. 2013), will improve its dispersion in water and antimicrobial activity. Further, the emulsion can be microencapsulated in

maltodextrin and spray dried to form a powder, which has potential for use as an additive in products like baking mixes to prevent bacterial proliferation upon rehydration (Dev Kumar et al. 2020).

2.8.2 Improving stability and applicability of pelargonic acid through microencapsulation

The microencapsulation of fatty acids is an effective means of improving their stability in food products. Encapsulation entails the entrapment of an active ingredient, referred to as the core material, within another coating substance, called the wall material. The wall material protects the core material from environmental effects such as light, oxygen, and humidity, thus conferring improved stability and handling ability to the core material, as well as expanding its application range (Kaushik et al. 2015). Microencapsulation is commonly accomplished through spray drying, which is cost-effective and easy and prized for its ability to produce powders which are of high quality and low water activity (Jiang et al. 2020; Carneiro et al. 2013). Wall material selection is an important component of maximizing spray drying efficiency, and maltodextrin is known to be an ideal encapsulant due to its low cost and low viscosity at high solids concentrations (Carneiro et al. 2013).

Dev Kumar et al. (2020) have demonstrated that PA can be effectively encapsulated in maltodextrin and spray dried to form a powder. They have also shown that this powder retains the antimicrobial activity of PA, as evidenced by its ability to inhibit growth of *S.* Newport, *S.* Typhimurium, and *S.* Oranienburg, and thus could be effective against other pathogens such as STEC. Due to its powdered format, this encapsulated PA would be an ideal post-processing additive in products like flour-based baking mixes and could inhibit bacterial growth in cases of consumer mishandling when the mix is reconstituted to form a batter.

CHAPTER 3

VALIDATION OF ANTIBIOTIC MARKER IN SHIGATOXIGENIC *E. COLI* STRAINS FOR USE IN FOOD SAFETY[1]

[1]Hayman, K.M., Dev Kumar, G., and Mishra, A. To be submitted to *Journal of Food Safety*

ABSTRACT

Wheat flour and its subsidiary products are known to carry a high native microbial load, which could interfere with successful detection and enumeration of target organisms in such matrices. Shigatoxigenic *Escherichia coli* (STEC) serogroups O26, O121, and O157:H7 were transformed with the pGFPuv plasmid encoding ampicillin resistance (+Amp) and green fluorescent protein (GFP) and were additionally conferred resistance to streptomycin (+Amp+Strep) through exposure to incremental concentrations of the antibiotic. Growth characteristics of these antibiotic resistant strains were compared to those of the non-resistant native strains (NR). The supplementation of ampicillin and ampicillin + streptomycin in growth media was evaluated for its ability to suppress growth of the native microbial load of three different commercial cake mixes. Antibiotic supplementation in growth media was successful in suppressing the native microbiota of the cake mixes, while the growth characteristics of the +Amp+Strep variants of the three STEC strains did not differ significantly from the NR strains ($p>0.05$). These results indicate that STEC strains with ampicillin and streptomycin resistance markers can be used for traceability studies in wheat flour and wheat flour derived products such as cake batters as their detection and enumeration can occur without interference from background organisms.

3.1 Introduction

Microbiological analysis of samples taken from environmental sources or raw agricultural products can be complicated due to the robust indigenous microbiota of these materials. Wheat flour, for example, is capable of carrying a bacterial load of up to 8.20 log CFU/g (Manthey et al. 2004). Such significant microbial background can interfere with detection and enumeration of organisms inoculated into such materials for challenge studies.

One solution is the use of antibiotics in microbiological media to suppress background microbiota. Used in tandem with an antibiotic-resistant (ABR) inoculum, recovery of target bacteria without interference from background organisms can be achieved (Ha et al. 1995). Erickson, Zabala-Diaz, and Ricke (2001) used basal growth media containing ampicillin and novobiocin to suppress the background bacterial and fungal load of animal feed, which can be present at rates of up to 5-7 log CFU/g. Growth of their target organism, *E. coli* with conferred resistance to ampicillin, was not inhibited. Similarly, Franz et al. (2007) used ampicillin-resistant *E. coli* O157:H7 to inoculate the surface of fresh lettuce and sorbitol MacConkey agar containing ampicillin as the recovery medium. Effective suppression of the lettuce's native microbiota was achieved.

In addition to ABR, fluorescence can be used to improve bacterial detection. Fluorescence in bacteria can be achieved through the conferral of a green fluorescent protein (GFP) isolated from jellyfish (Shimomura, Johnson, and Saiga 1962). GFP has been used as a marker to track bacterial cells in numerous survival studies in both ecological settings and in food matrices due to the fact that it can be easily detected upon excitation with UV light (Binet et al. 2018). For example, Aspira et al. (2000) used GFP-tagged *Streptococcus gordonii* to visualize the spatial organization of this organism during biofilm formation, and Leff and Leff (1996) transformed *E. coli* with a

GFP plasmid to monitor its survival in aquatic environments. In both studies, the authors reported the advantage of being able to visualize and quantify cells through fluorescent microscopy as opposed to relying on culture-based techniques. GFP-transformed bacteria are also very useful for food safety studies. Cabello, Espejo, and Romero (2005) used GFP in *Vibrio parahaemolyticus* to trace this pathogen's internalization and persistence in fresh oyster tissues, while Gandhi et al. (2001) used GFP in *Salmonella* to monitor the presence of the bacteria in alfalfa sprouts during germination and sprouting. Similarly, Dev Kumar et al. (2017) used bioluminescent *Salmonella* to visualize surface presence and internalization of the pathogen in tomatoes and their blossoms.

Some issues could arise with the use of GFP-tagged strains; for example, expression of the gene which encodes the protein may result in an additional metabolic burden on the bacteria which could alter its growth characteristics (Rang et al. 2003). Therefore, the transformed bacteria may not accurately represent the behavior of the wild-type strain. In contrast, Ma, Zhang, and Doyle (2011) used several strains of pathogenic bacteria which are significant in food safety contexts (*Listeria monocytogenes, Salmonella,* and *E. coli* O157:H7) to show that transformation with a GFP plasmid had no significant effect on the growth rates of these bacteria. Further, they found that the plasmid remained extremely stable in *E. coli* O157:H7 through several generations. This is in agreement with several previous works which also showed that incorporation of the GFP plasmid into various strains of *E. coli* did not alter their growth rates (Allison and Sattenstall 2007; Skillman et al. 1998).

Similar potential issues may arise when using antibiotics to suppress the growth of non-target organisms—there are research works which suggest that conferring ABR to some bacterial strains may alter their growth characteristics compared to the non-resistant parent strain. For example, Heß and Gallert (2016) found that environmental *E. coli* isolates grown in the presence

of antibiotics such as anhydroerythromycin, clindamycin, cotrimoxazole, and ciprofloxacin resulted in longer lag phases and lower growth rates as compared to the same isolates grown without exposure to the antibiotics.

One of the primary goals of this research project was to characterize the risk of STEC survival in raw cake batter; as such, it was critical that our experimental settings accurately represented the growth conditions of this pathogen *in situ*. Therefore, the goal of this study was to determine whether conferring ABR to our selected strains of STEC would significantly impact their growth behavior. Additionally, the amount of background microbiota in three commercial cake mixes was assessed, as well as the efficacy of antibiotic media in suppressing the growth of these organisms.

3.2 Materials and methods

3.2.1 Bacterial strains

STEC serogroups O26 (strain 3012-03) and O121 (strain TW08980) from a 2016 Missouri flour outbreak and serogroup O157:H7 (ATCC 43895) from a 1982 ground beef outbreak were obtained from the University of Georgia Center for Food Safety culture collection. Frozen cultures were thawed and a loopful of each was revived in 10 ml of tryptic soy broth (TSB; Neogen, Lansing, MI) at 37 °C for 24 h. A loopful of this culture was transferred to fresh TSB under the same conditions and, finally, a loopful of this subculture was streaked onto tryptic soy agar (TSA; Neogen, Lansing, MI) for isolation. A single well-isolated colony was used to perform the API 20E identification test (Biomeriuex, Marcy-l'Étoile, France) for confirmation, and was additionally confirmed by streaking on eosin methylene blue agar (EMB; Neogen, Lansing, MI) and CHROMAgar (CHROMAgar Microbiology, Inc., Paris, France). Stock cultures of the confirmed

strains were prepared by streaking each to TSA and incubating at 37 ℃ for 24h. Colonies were scraped from the plates with a sterile loop, suspended in phosphate-buffered saline (PBS; VWR International, Radnor, PA) and adjusted to a concentration of ca. 6 log CFU/ml.

3.2.2 Development of antibiotic resistance

The selected bacterial strains were transformed with a green fluorescence and ampicillin resistance (100 µg/ml; Fisher Scientific, Fair Lawn, NJ) plasmid (pGFPuv, Clontech, Mountain View, CA) through electroporation according to the method described by Dev Kumar et al. (2017) with modifications. Competent cells were prepared by inoculating 1 ml of an overnight culture of each of the strains into 45 ml of TSB followed by incubation at 37 ℃ for 4 h or until an optical density (OD_{600}) of 0.8 was reached. Following this, cultures were placed on ice for 15 min, then centrifuged at 1400 g for 10 min using the Corning LSE high-speed microcentrifuge (Corning, Inc., Corning, NY). Cell pellets were washed three times and resuspended with ice-cold 15% glycerol, then stored at -80 ℃ until use. Transformation was accomplished using the Gene Pulser II system (Bio-Rad, Hercules, CA) with electroporation conditions of 2.5 kV, 25 µF, and 400 Ω in a 0.2 cm cuvette.

Further resistance to streptomycin (100 µg/ml; Fisher Scientific, Fair Lawn, NJ) was conferred chromosomally by sequential transfer (10 µg increments) of the newly transformed strains into TSB with increasing concentrations of streptomycin, until growth at a concentration of 100 µg/ml was achieved.

Stock cultures of the ABR strains were prepared by streaking each to TSA with the appropriate antibiotic supplements and incubating at 37 ℃ for 24h. Colonies were scraped from

the plates with a sterile loop, suspended in PBS, and adjusted to a concentration of ca. 6 log CFU/ml.

3.2.3 Cake mix procurement

Three different commercial cake mixes (A, B, and C; n=5 for each) which have previously been implicated in outbreaks were purchased from a local grocery store. Mixes were stored in their original packaging placed inside a Whirl-Pak bag and kept in their boxes at room temperature until use. A list of the ingredients in the cake mixes is displayed in Table 3.1.

3.2.4 Stability of pGFPuv transformed STEC serogroups in cake batter

To prepare each cake mix as a batter, the ingredients were mixed according to the manufacturers' instructions using a sterile KitchenAid countertop standing mixer (Whirlpool Corporation, Benton, MI). The batter was mixed for two min, with the mixing bowl scraped with a sterile rubber spatula as necessary, until homogeneity was achieved. Individual samples of the batters were prepared by aliquoting 1 g portions into sterile Whirl-Pak bags; these were refrigerated until use. Inoculation of samples with the transformed STEC strains was accomplished by delivering 100 µl of a 4 log CFU/ml stock solutions of each bacterial strain into individual 1-g batter samples. The samples were hand-massaged for 30 s to create a homogenous 3 log CFU/g initial bacterial load. Samples were stored at 35 °C and sampled at regular intervals over a 24 h period. At the time of sampling, 10 ml PBS were added to each sample bag and hand-massaged for 30 s to mix, then spread plated on TSA, TSA with 100 µg/ml ampicilli(TSA-A), and TSA with 100 µg/ml ampicillin and 100 µg/ml streptomycin (TSA-AS). Plates were incubated at 37 °C for 24 h. Recovered colonies were observed for fluorescence upon excitation with UV light at 365nm

and those which fluoresced were considered to carry the plasmid. Colony counts were transformed to log CFU/g accordingly.

3.2.5 Assessment of cake mix background microbiota

The cake mixes were measured in 4 g portions into individual sterile Whirl-Pak bags, and 40 ml of PBS were added. The contents of the bags were stomached for 30 s, then spread plated on plate count agar (PCA; Neogen, Lansing, MI) for a total aerobic plate count, violet red bile agar (VRB; MilliporeSigma, Burlington, MA) for coliforms, and dichloran rose-bengal chloramphenicol agar (DRBC; Difco, Sparks, MD) for yeasts and molds. PCA and VRB plates were stored at 37 ℃ for 24 h, while DRBC plates were stored at room temperature for five days. Colony-forming units on each media were enumerated and transformed to log CFU/g accordingly. The limit of detection (LOD) of the assay was 1.00 log CFU/g in accordance with the lowest number of colony-forming units (one) observed at the lowest dilution (10^{-1}).

3.2.6 Suppression of cake mix background microbiota with antibiotic media

A 4 g portion of Mix C was measured into a sterile Whirl-Pak bag and 40 ml PBS were added. The sample was hand-massaged for 30 s to mix, then spread plated on TSA, TSA-A, and TSA-AS. Plates were incubated at 37 ℃ for 24 h. The LOD of the assay was 1.00 log CFU/g.

3.2.7 Assessment of growth of non-resistant and antibiotic resistant STEC strains

The growth rates of the transformed ABR STEC strains as compared to their non-resistant parent strains were determined by measuring the increase in turbidity in the growth medium over a duration of 24 h. This was accomplished by inoculating 20 µl of each strain—non-resistant (NR), with ampicillin resistance (+Amp), and with streptomycin and ampicillin resistance (+Amp+Strep)—at a concentration of 5 log CFU/ml into 180 µl of TSB in a 96-well plate. The

Bio-Tek Cytation 3 image reader (BioTek Instruments, Inc., Winookski, VT) was used to measure optical density of these strains at 600nm over a 24 h period at 37 °C.

3.2.8 Mathematical modeling of growth behavior

The modified Gompertz model (Gibson, Bratchell, and Roberts 1988) modified by Begot et al. (1996) was fitted to the growth curve of these bacterial strains using MATLAB software (version R2021a, The MathWorks, Inc. Natick, MA). The model can be described by the following equation, where N is the bacterial population at a given time, N_0 is the initial bacterial population, $O.D._{min}$ is the lowest O.D. value above the detection threshold, A is the logarithmic increase of bacterial population, L is the lag time, μ is the maximum growth rate, and t is time:

$$log_{10}\left(\frac{N}{N_0}\right) = log_{10}\left(\frac{(\Delta O.D.)_t}{\Delta O.D._{.min}}\right)$$

$$= A \cdot exp\left(-exp\left(\frac{\mu \cdot e}{A} \cdot (L - t) + 1\right)\right)$$

The growth parameters assessed were change in bacterial population in log CFU/ml (A), lag phase duration in hours (L), maximum growth rate in log CFU/h (μ_{max}), and generation time in hours (T), which was calculated according to the following equation:

$$T = log_{10}\left(\frac{log_{10}(2)}{\mu}\right)$$

3.2.9 Statistical analysis

The experiments were performed in triplicate. JMP Pro statistical software (v15.0.0, SAS Institute, Cary, NC) was used to compare the growth parameters of these strains and their ABR

variants through a one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test (HSD) (α=0.05).

3.3 Results

3.3.1 Background microbiota of commercial cake mixes

Three commercial cake mixes (A, B, and C; n=5 for each) were enumerated on PCA for a total aerobic plate count, VRB for coliforms, and DRBC for yeasts and molds (Table 3.1). Mixes A and B contained 1.16±0.22 and 1.14±0.76 log CFU/g, respectively, of aerobic bacteria, and no detectable bacteria on VRB. Mix C contained 3.00±0.09 log CFU/g aerobic bacteria and 1.42±0.28 log CFU/g bacteria on VRB. None of the mixes contained any detectable yeasts or molds. When Mix C was plated on TSA, 2.32±0.37 log CFU/g bacterial growth was observed. When plated on TSA-A and TSA-AS, no growth was observed, indicating a significant reduction in the recovered bacterial load ($p \leq 0.05$) (Table 3.2).

3.3.2 Stability of pGFPuv transformed STEC serogroups in cake batter

The growth of the transformed STEC serogroups was monitored over 24 h in cake batter at 35 ℃ to observe the stability of the pGFPuv plasmid in the bacteria. Over 24 h, the population of all transformed STEC serogroups increased by 1-2 log CFU/g, and all recovered bacteria expressed fluorescence, indicating the stability of the pGFPuv plasmid regardless of the presence of antibiotic pressure.

3.3.3 Growth rate of E. coli O26, O121, and O157:H7 non-resistant and antibiotic resistant variants

Growth characteristics of *E. coli* strains O26, O121, and O157:H7 with or without resistance (NR) to ampicillin (+Amp) or ampicillin and streptomycin (+Amp+Strep) were assessed over a 24 h period at 37 ℃ using a turbidimetric technique. The change in bacterial population (*A*), lag phase duration (*L*), maximum growth rate (μ_{max}), and generation time (*T*) were the growth parameters of interest, which are displayed in Table 3.3. Goodness-of-fit statistics for the predicted growth models are shown in Table 3.4. The *L* for all strains was approximately 7-8 h with the longest being *E. coli* O26+Amp (7.92±1.04 h) and the shortest being *E. coli* O121 NR (6.75±0.01 h). Only these two strains differed significantly ($p \leq 0.05$); overall, no differences existed within strains among the non-resistant and resistant mutants ($p > 0.05$) (Table 3.3). Similarly, there were no significant differences between the μ_{max} of all strains—all values were close to 0.40 log CFU/h ($p > 0.05$). The only significant difference within a strain type was between the *A* values of *E. coli* O121 NR (1.31±0.01 log CFU/h) and *E. coli* O121+Amp (1.15±0.07 log CFU/g), with the increase in the population of the non-resistant strain being significantly greater ($p \leq 0.05$). *T* values did not differ between any strain type or variant, with all values falling in a range of approximately 0.70-0.85 h ($p > 0.05$). Notably, no significant differences were observed in any growth parameters of the non-resistant strains and their +Amp+Strep resistant variants ($p > 0.05$).

3.4 Discussion

The study evaluated the suitability of STEC strains with ABR for their traceability in cake batters. Cake mixes can have fungi and pathogenic bacteria (Myoda et al. 2019). The populations of these native organisms can increase in number when cake batters are stored for extended durations. The presence of a high population of background microbiota can hinder enumeration on

plating media. While the use of selective media is a commonly used strategy to reduce background microbiota, several disadvantages could be encountered. These could include the incomplete exclusion of native microorganisms as well as increased stress on the target organism due to selective chemical agents such as bile salts (Stephens and Joynson 1998). ABR markers have been used in foodborne pathogens to improve traceability (Dev Kumar, Patel, and Ravishankar 2020; Tomás-Callejas et al. 2011) and were hence evaluated for their applicability to detect STEC in cake batters.

All three cake mixes used in this study contained a detectable aerobic bacterial load, which is in accordance with what is known about the native microbiota of ingredients in cake mix, such as wheat flour. Mix C contained a far greater microbial load compared to Mix A and B and thus was chosen as the test matrix for determining the efficacy of antibiotic media in suppressing growth of native microorganisms. On TSA, more than 2 log CFU/g bacteria were recovered from Mix C, while no growth was observed on TSA-A or TSA-AS, demonstrating that these antibiotics were able to effectively suppress the background microbiota of the cake mix (Figure 3.1).

No significant differences were observed between the maximum growth rate or generation time of all STEC strains studied, regardless of ABR. This is in contrast to what has been found in other works, where *E. coli* with conferred resistance to or grown in the presence of certain antibiotics were shown to have different growth characteristics as compared to their non-resistant counterparts. Previous studies in our lab have shown that *E. coli* O157:H7 with resistance to ampicillin and streptomycin grown in TSB over 24 h had a significantly longer lag phase and higher maximum cell concentration as compared to the same strain with no antibiotic resistance. Similarly, Heß and Gallert (2016) found that *E. coli* isolates grown in the presence of anhydroerythromycin, clindamycin, cotrimoxazole, and ciprofloxacin had longer lag phases and

lower growth rates compared to the same isolates grown without exposure to the antibiotics, indicating slower overall growth in the presence of antibiotics. This can be explained by the fact that the strains used in those studies are different than those used here—the isolate used in our previous studies was a human fecal O157:H7 isolate from a lettuce outbreak, while those used by Heß and Gallert were typical environmental isolates from raw sewage and river water. The strains used in this study were O26, O121, and O157:H7 isolated from two wheat flour and ground beef outbreaks, respectively. As different bacterial strains tend to have different phenotypic and morphological attributes, they often have different growth patterns as well. Sekse et al. (2012) investigated differences in the growth characteristics of several different types of *E. coli* grown in standard nutrient broth and found that enteroinvasive strains of *E. coli* grew significantly slower probiotic and commensal strains, while enteroaggregative strains grew significantly faster ($p \leq 0.05$).

While the overall population increase in *E. coli* O121 NR was greater than that of *E. coli* O121+Amp ($p \leq 0.05$), there was no difference in this parameter or any other for all non-resistant strains and their +Amp+Strep resistant variants ($p > 0.05$). Further, there were no differences observed in the morphology of these strains. This indicates that the +Amp+Strep resistant variants were highly similar to the native bacterial strains, and thus could be used in this study to accurately model the conditions under which STEC contamination would persist in cake batter outside of a laboratory setting.

Table 3.1: List of three commercial cake mix ingredients.

Common ingredients	Mix A	Mix B	Mix C
Enriched bleached flour, sugar, baking soda, sodium aluminum phosphate, salt, propylene glycol esters of fatty acids, monoglycerides, cellulose gum, xanthan gum, yellow 5	Wheat starch, canola oil, dextrose, corn starch, sodium stearoyl lactylate, red 40, **citric acid**, **BHT**	Monocalcium phosphate, corn starch, palm oil, dicalcium phosphate, yellow 6	Dicalcium phosphate, monocalcium phosphate monohydrate, dextrose, palm oil, sodium stearoyl lactylate, wheat starch, red 40

Note: Common ingredients are shared by all three cake mixes; ingredients listed under each mix are unique. Ingredients which are commonly used as antioxidant agents are bolded.

Table 3.2: Background microflora for three commercial cake mixes.

	Bacterial counts (log_{10} CFU/g)		
Cake mix	**PCA**	**VRB**	**DRBC**
A	1.16±0.22	ND	ND
B	1.14±0.76	ND	ND
C	3.00±0.09	1.42±0.28	ND

PCA=Plate count agar, VRB=Violet red bile agar, DRBC= Dichloran rose-bengal chloramphenicol agar. ND=No growth detected. Results reported as CFU/g. LOD=1.00 log CFU/g.

Table 3.3: Results for Mix C plated on TSA, TSA-A, and TSA-AS.

	Bacterial counts (log_{10} CFU/g)		
Cake mix	**TSA**	**TSA-A**	**TSA-AS**
C	2.32±0.37[a]	ND[b]	ND[b]

ND=No growth detected. Results reported as CFU/g. LOD=1.00 log CFU/g. Values within a row sharing the same superscript are not significantly different.

Table 3.4: Comparison of population change (*A*), lag time (*L*), maximum growth rate (μ_{max}), and generation time (*T*) for *E. coli* O26, O121, and O157:H7 non-resistant strains and ABR variants.

Strain	Growth parameters			
	A **(log_{10} CFU/h)**	***L*** **(h)**	μ_{max} **(log_{10} CFU/h)**	***T*** **(h)**
E. coli O26 NR	1.13±0.05[b]	7.28±0.12[ab]	0.41±0.08[a]	0.74±0.16[a]
E. coli O26+Amp	1.09±0.10[b]	7.92±1.04[a]	0.45±0.05[a]	0.67±0.08[a]
E. coli O26+Amp+Strep	1.18±0.03[ab]	7.33±0.13[ab]	0.39±0.02[a]	0.76±0.06[a]
E. coli O121 NR	1.31±0.01[a]	6.75±0.01[b]	0.40±0.04[a]	0.74±0.08[a]
E. coli O121+Amp	1.15±0.07[b]	7.51±0.13[ab]	0.39±0.01[a]	0.75±0.02[a]
E. coli O121+Amp+Strep	1.19±0.02[ab]	7.17±0.33[ab]	0.38±0.04[a]	0.78±0.09[a]
E. coli O157:H7 NR	1.23±0.01[ab]	7.41±0.05[ab]	0.36±0.02[a]	0.82±0.04[a]
E. coli O157:H7+Amp	1.12±0.05[b]	7.66±0.08[ab]	0.41±0.02[a]	0.73±0.05[a]
E. coli O157:H7+Amp+Strep	1.18±0.03[ab]	7.46±0.01[ab]	0.35±0.03[a]	0.85±0.09[a]

NR=non-resistant, +Amp=ampicillin resistant, and +Amp+Strep=ampicillin and streptomycin resistant. Values within a column sharing the same superscript are not significantly different. Values are not compared across rows.

Table 3.5: Goodness-of-fit statistics for predicted growth models of *E. coli* O26, O121, and O157:H7 non-resistant strains and ABR variants.

Strain	Statistic		
	SSE	**R^2**	**RMSE**
E. coli O26 NR	0.08±0.04	0.98±0.01	0.06±0.02
E. coli O26+Amp	0.32±0.23	0.95±0.02	0.12±0.04
E. coli O26+Amp+Strep	0.07±0.01	0.99±0.01	0.05±0.01
E. coli O121 NR	0.03±0.01	0.99±0.01	0.03±0.01
E. coli O121+Amp	0.08±0.05	0.98±0.01	0.06±0.01
E. coli O121+Amp+Strep	0.06±0.04	0.99±0.01	0.05±0.02
E. coli O157:H7 NR	0.05±0.01	0.99±0.01	0.04±0.01
E. coli O157:H7+Amp	0.11±0.12	0.98±0.01	0.06±0.03
E. coli O157:H7+Amp+Strep	0.11±0.05	0.98±0.01	0.06±0.02

NR=non-resistant, +Amp=ampicillin resistant, and +Amp+Strep=ampicillin and streptomycin resistant.

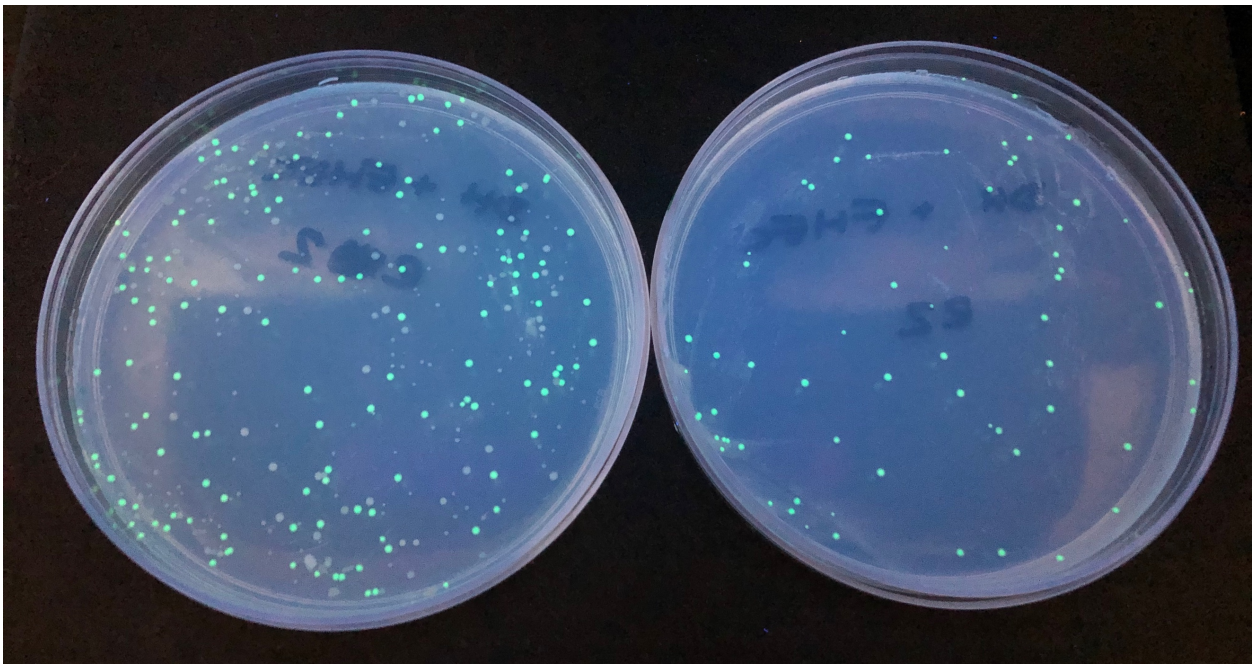

Figure 3.1: Cake Mix C inoculated with a cocktail of the +Amp+Strep resistant STEC strains plated on TSA (L) and TSA-AS (R), demonstrating inhibition of the cake mix's background microbiota.

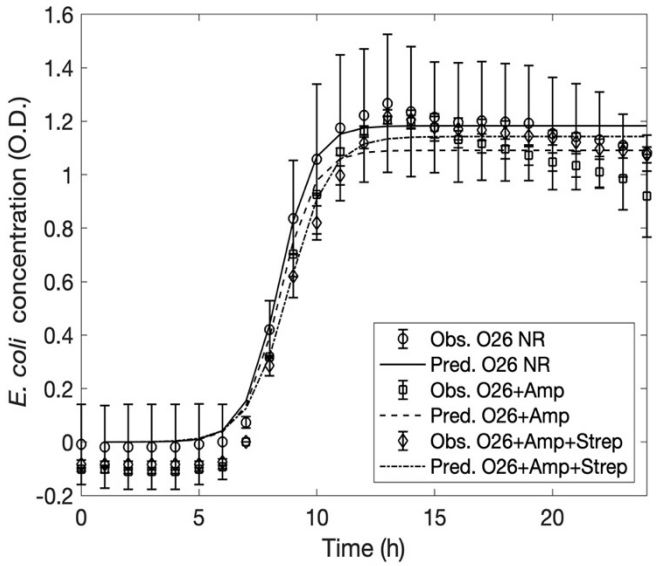

Figure 3.2

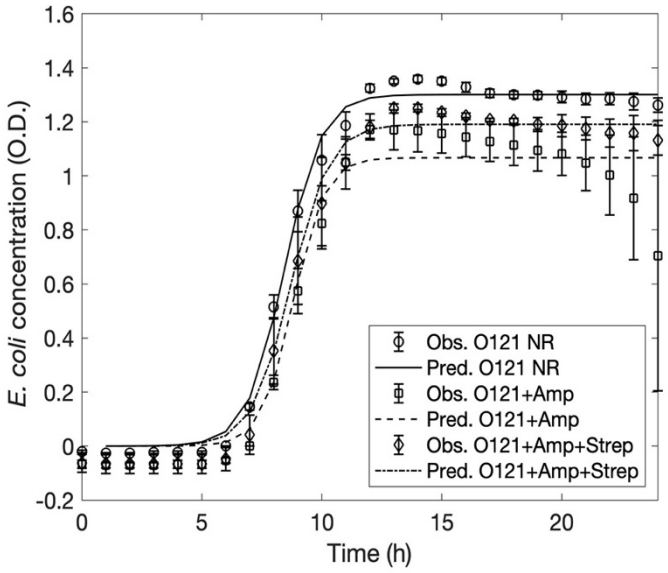

Figure 3.3

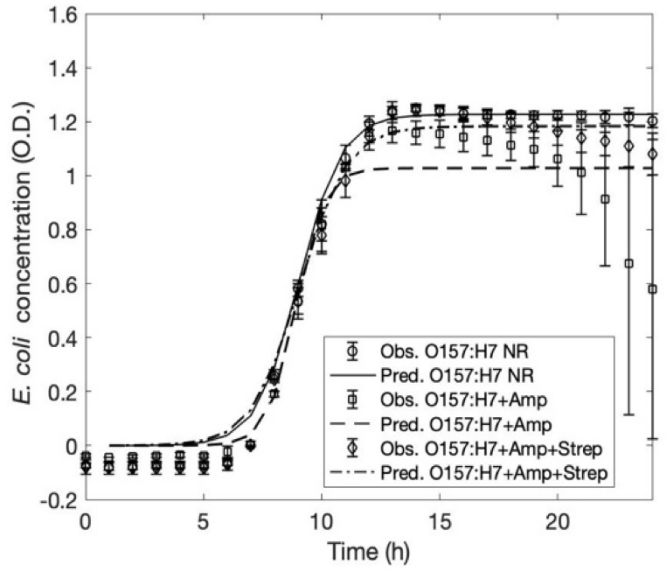

Figure 3.4

Figures 3.2-3.4: Observed growth of antibiotic resistant and non-resistant strains of *E. coli* O26, O121, and O157:H7 and predicted growth models for each.

CHAPTER 4

GROWTH AND SURVIVAL MODELING OF SHIGATOXIGENIC *E. COLI* SURVIVAL IN CAKE BATTER[1]

[1]Hayman, K.M., Dev Kumar, G., and Mishra, A. To be submitted to *Food Microbiology*

ABSTRACT

Shigatoxigenic *Escherichia coli* (STEC) may be introduced to wheat kernels at several points prior to harvest and may persist through harvest, storage, milling, and subsequent processing. While the low water activity of flour products will prevent the growth of STEC, these bacteria are capable of surviving for long periods of time in a desiccated state. When flour-based products like cake mixes are rehydrated to make batter and other ingredients like fat, sugar, and eggs are added, ideal conditions for bacterial proliferation are created, drastically increasing the chances of consumer illness if the raw batter is consumed. Little information is currently available about the behavior of STEC in a matrix like cake batter. This study examined the fate of STEC serogroups O26, O121, and O157:H7 in standard and commercial cake batter formulations under storage conditions which simulated refrigerated, ambient, and abusively high temperatures. All three strains declined gradually over time in the standard batter formulation but remained detectable for up to three weeks at refrigerated temperatures. The strains grew rapidly during the first 48 h of storage and then declined sharply in commercial cake batters at ambient and abuse temperatures but remained detectable for up to a week. Overall, STEC was shown to survive and proliferate in cake batter, which demonstrates the risk posed by this commodity in the home of a consumer.

4.1 Introduction

Low-moisture food powders such as cake mix have been increasingly implicated in outbreaks of foodborne illness, notably as novel vehicles for Shiga-toxin producing *Escherichia coli* (STEC). Wheat flour is a foundational element of cake mixes, and its status as a raw agricultural product is often overlooked because it is infrequently consumed in uncooked foods. A gap in information needs to be bridged to elucidate the behavior of STEC in a matrix like cake mix, especially when it is rehydrated to prepare batter. Additionally, time and temperature abuse may exacerbate the risk of STEC in cake batter.

STEC causes illness in humans through the production of Type 1 (Stx1) and Type 2 (Stx2) Shiga toxins. These bacterial exotoxins are taken up by epithelial intestinal cells, where they inhibit protein synthesis and ultimately cause cell death. This degradation of intestinal tissue often results in the bloody diarrhea which is characteristic of STEC infection; in severe cases, this can lead to partial or complete kidney failure (known as hemolytic uremic syndrome) and even death (Castro et al. 2017). STEC requires only a low infectious dose to cause disease (50-100 cells) and sickens more than 2 million people every year worldwide (Majowicz et al. 2014).

Foods with low water activity pose a unique concern as vehicles for foodborne pathogens and such foods have been implicated in several recent outbreaks of foodborne illness. STEC is an emerging pathogen for these foods. There are several opportunities for this pathogen to be introduced to low water activity foods during their production. Once established, STEC can employ several mechanisms to endure desiccation and osmotic stress for long periods of time and subsequently resist eradication by many common industrial antimicrobial treatments. Intervention strategies that can be implemented to control the risk posed by this pathogen in low-moisture foods are currently limited.

Wheat flour is a raw agricultural commodity with a robust native microbiome. In addition to many typical environmental organisms, it has been shown that flour may carry pathogens such as *Salmonella* and STEC (Myoda et al. 2019). Flour is the foundational ingredient in popular products like dry baking mixes that can be used to prepare cakes, cookies, brownies, and many other baked goods. Because the flour milling process is not designed to reduce the microbial load of the wheat kernels or flour, any bacterial contamination in flour is readily introduced to these mixes. Once the mix is reconstituted by the consumer to make a batter, and other ingredients like sugar, eggs, and fats are added, optimal conditions are created for bacteria to proliferate (Wu et al. 2017). Fat, in particular, is known to confer a protective effect on bacteria which can aid them in surviving environmental stresses (van Asselt and Zwietering 2006). If the consumer leaves the batter to sit at ambient temperatures prior to baking, or stores it in a refrigerator for later use, sufficient time may be given for the bacteria to grow. With some strains of *E. coli* having a doubling time as short as 20 minutes in ideal conditions, even trace amounts of contamination in a dry flour mix can become a very serious health threat upon rehydration (Fossum, Crooke, and Skarstand 2007).

Four different cake batters were used in this study to evaluate STEC survival. Three were made with popular commercial mixes, and one was prepared according to the Association of Analytical Cereal Chemists' (AACC) Method 10-90, which is used to evaluate the baking quality of cake flour (Cereals and Grains Association 2021).

Understanding the behavior of STEC in a nutritionally complex matrix like cake batter under different storage conditions will provide critical insight on the risk incurred by consumers when handling this commodity. Thus, the goal of this study was to observe and quantify the growth

of STEC in different kinds of cake batter over time at storage temperatures similar to refrigeration, ambient conditions, and abusively high temperatures.

4.2 Materials and methods

4.2.1 Bacterial strains

STEC serogroups O26, O121, and O157:H7 were revived from storage and confirmed as described in Section 3.2.1.

4.2.2 Development of antibiotic resistance

The selected STEC strains were transformed with fluorescence as well as resistance to ampicillin and streptomycin as described in Section 3.2.2. Stock cultures of the transformed strains were prepared by streaking each to TSA with the appropriate antibiotic supplements and incubating at 37 ℃ for 24h. Colonies were scraped from the plates with a sterile loop, suspended in PBS, and adjusted to a concentration of ca. 4 log CFU/ml.

4.2.3 Cake mix procurement and batter preparation

Three different commercial cake mixes (A, B, and C) were procured, stored, and prepared as batter as described in Sections 3.2.3 and 3.2.4. The standard cake batter as described by the AACC Method 10-90 was prepared in the same way, with all ingredients combined in the amounts listed in Table 4.2. The water activity of each batter was measured (AquaLab 3TE, Decagon Devices, Pullman, WA) as well as the pH (Oakton pH/CON 510 Benchtop Meter, Oakton Instruments, Vernon Hills, IL). Individual samples of the batters were prepared by aliquoting 1 g portions into sterile Whirl-Pak bags; these were refrigerated until use. The nutritional components

of each of the prepared batters on a per-gram basis was calculated based on the nutritional facts provided by the manufacturers (Table 4.3).

4.2.4 Sample inoculation and treatment conditions

Inoculation was accomplished by delivering 100 µl of the 4 log CFU/ml stock solutions of each bacterial strain into individual 1 g batter samples. The samples were hand-massaged for 30 s to create a homogenous 3 log CFU/g initial bacterial load. The AACC batter samples were then subjected to storage at 5 ℃, 10 ℃, 15 ℃, 25 ℃, and 35 ℃ with appropriate sampling times selected based on the predicted growth rate of the STEC inoculum. The commercial cake batter samples, meanwhile, were stored at 25 ℃ and 35 ℃ and sampled at 24 h intervals over a 120 h period (five days).

4.2.5 Microbiological analysis

At the designated time, samples were retrieved from storage and 10 ml PBS were added to the sample bag. The contents of the bag were hand-massaged for 30 s to homogenize. A 1 ml portion of this suspension was extracted and used to prepare appropriate ten-fold dilutions in PBS, which were plated on TSA-AS using the droplet plating method described by Miles and Misra (1938). Plates were incubated at 37 ℃ for 24 h. TSA was selected as the base growth medium as opposed to other media which are typically used for the recovery of *E. coli* in order to avoid the additional stress of selective agents, which could hinder recovery.

4.2.6 Mathematical modeling of growth and survival behavior

Using the USDA's IPMP software, the three-phase growth and survival models described by Buchanan and Golden (1995) were fitted to the bacterial populations over time in cake batter. For models showing growth, the maximum specific growth rate (μ_{max}; log CFU/h) and maximum

bacterial population (Y_{max}; log CFU/g) were assessed. This model can be described by the following equation, where Y_0 is the initial bacterial population, t is time, lag is lag phase duration, μ_{max} is the maximum specific growth rate of the bacteria, t_{max} is the time at which the maximum bacterial population is reached, and Y_{max} is the maximum bacterial population:

$$Y = Y_o, if\ t < lag$$

$$Y = Y_o + \mu_{max}(t - lag), if\ lag \leq t \leq t_{max}$$

$$Y = Y_{max}, if\ t \geq t_{max}$$

For models showing decline, the k value (time needed to achieve 1 log CFU reduction; h) and shoulder phase duration (h) were the parameters of interest. This model can be described by the following equation, where Y_0 is the initial bacterial population, t is time, $t_{shoulder}$ is the duration of the shoulder phase, k is the time required to achieve 1 log CFU reduction in the bacterial population, t_{tail} is the time at which the tail phase begins, and Y_m is the final bacterial population:

$$Y = Y_o, if\ t \leq t_{shoulder}$$

$$Y = Y_o - \frac{t - t_{shoulder}}{k}, if\ t_{shoulder} \leq t \leq t_{tail}$$

$$Y = Y_m, if\ t \geq t_{tail}$$

4.2.7 Statistical analysis

Three replicates were prepared for each experimental sample (n=3). JMP Pro v15.0.0 statistical software was used to compare the growth parameters of these STEC strains across different batter types and storage temperatures for significant differences via ANOVA and Tukey's HSD test (α=0.05).

4.3 Results

4.3.1 Survival of STEC in a standard cake batter

The survival curves of *E. coli* strains O26, O121, and O157:H7 in the AACC standard cake batter at 5 °C, 25 °C, and 35 °C are shown in Figures 4.1-4.3 and goodness-of-fit statistics for the predicted survival models are shown in Table 4.6. The k values and shoulder phase durations of each strain at each temperature were compared. Survival curves for the 10 °C and 15 °C storage treatments are not shown as the bacterial population of all three strains did not significantly change over the storage period at these temperatures. All samples had an initial bacterial load of 3-4 log CFU/g which declined to below the limit of detection (2.69 log CFU/g) by the end of the sampling period. The limit of detection was calculated according to the method described by Miles and Misra (1938) where the lowest number of colony-forming units (one) observed at the lowest dilution (10^{-1}) is multiplied by the volume of sample plated (20 µl).

There was no significant difference in the k value of the STEC strains compared against each other within each storage temperature treatment ($p>0.05$) (Table 4.4). At 5 °C, the average k value for *E. coli* O26 was the highest (1254.19±648.96 h), while that of *E. coli* O121 and O157:H7 were slightly lower at 902.35±275.96 h and 908.69±233.51 h, respectively. In contrast, at 25 °C, the k value of *E. coli* O157:H7 was the highest at 123.00±17.88 h, followed by *E. coli* O121 (121.41±21.61 h), and *E. coli* O26 (93.63±30.84 h). The k value of *E. coli* O157:H7 was highest again at 35 °C (90.56±70.93 h), while that of *E. coli* O121 and O26 were lower, though not significantly so, at 66.34±27.45 h and 50.89±22.40, respectively ($p>0.05$).

In contrast, significant differences were observed in the k value of these STEC strains when compared across the storage temperature treatments. The k value of *E. coli* O26 at 5 °C

(1254.19±648.96 h) was significantly higher than it was at 25 ℃ (93.63±30.84 h) and 35 ℃ (66.34±27.45 h) ($p \leq 0.05$). Similar results were seen for *E. coli* O121, where the k value was again significantly higher at 5 ℃ (902.35±275.96 h) than it was at 25 ℃ (121.41±21.61 h) and 35 ℃ (66.34±27.45 h) ($p \leq 0.05$). The same was observed for *E. coli* O157:H7, with the highest k value at 5 ℃ (908.69±233.51 h) which was significantly greater than that at 25 ℃ and 35 ℃ (123.00±17.88 h and 90.56±70.93 h, respectively) ($p \leq 0.05$). No significant differences existed between the k values for any strains at 25 ℃ and 35 °C ($p > 0.05$).

There was slightly more notable variation in the shoulder phase duration of these strains. At 5 °C, neither *E. coli* O26 or O121 had a discernable shoulder phase, while that of *E. coli* O157:H7 was 474.213±160.89 h ($p \leq 0.05$). There was no difference in shoulder phase duration among the strains at 25 °C (103.99±13.85, 83.44±12.68, and 93.45±22.23 h for O26, O121, and O157:H7, respectively) or at 35 °C (93.62±9.55, 51.77±17.68, and 54.40±40.76 h, respectively) ($p > 0.05$). Within strain types, only *E. coli* O157:H7 had a significantly longer shoulder phase at 5 °C compared to 25 °C and 35 °C ($p \leq 0.05$).

While tail values for all strains were given by the predicted survival models, these are not compared as they were all the same—the bacterial populations for all strains at all storage temperatures declined to below the limit of detection (2.69 log CFU/g) near the end of the storage period.

4.3.2 Growth of STEC in commercial cake batters

The growth curves of *E. coli* O26, O121, and O157:H7 in three commercial cake batters (A, B, and C) stored at 25 ℃ are shown in Figures 4.4-4.6 and goodness-of-fit statistics for the

predicted growth models are shown in Table 4.7. The μ_{max} and Y_{max} of each strain were compared within and across batter types. All samples had an initial bacterial load of 3-4 log CFU/g.

No significant difference was observed in the average μ_{max} of any of the STEC strains when examined across batter types ($p>0.05$). In contrast, when comparing the μ_{max} of all three strains within a single batter type, the μ_{max} of *E. coli* O26 was significantly higher (0.24±0.17 log CFU/h) than *E. coli* O157:H7 (0.04±0.01 log CFU/h) in Mix C ($p\leq0.05$).

No differences were observed in the Y_{max} values for *E. coli* O26 or O157:H7 when compared across batter types ($p>0.05$). In contrast, the Y_{max} of *E. coli* O121 was significantly higher in Mix A (8.34±0.11 log CFU/g) than in Mix B or C (7.31±0.22 log CFU/g and 6.59±0.14 log CFU/g, respectively) ($p\leq0.05$). Additionally, when examined within batter types, the Y_{max} of *E. coli* O121 was significantly higher (8.34±0.81 log CFU/g) than *E. coli* O157:H7 (6.74±0.25 log CFU/g) in Mix A ($p\leq0.05$).

Growth curves of the STEC strains in the commercial cake batters stored at 35 °C are shown in Figures 4.7-4.9. Due to the erratic growth pattern of these bacteria at this temperature, no model could be fitted to this data. As such, no growth parameters could be compared for these samples.

4.3.3 Water activity and pH of prepared batters

The water activity of the standard cake batter (0.92±0.01) was significantly lower than that of the batters prepared with Mix A, B, and C (0.96±0.00 for all) ($p\leq0.05$). The pH of the standard cake batter (5.78±0.07) was also significantly lower than all three commercial batters (6.44±0.03, 6.60±0.08, and 6.64±0.09 for Mix A, B, and C, respectively) ($p\leq0.05$). These results are displayed in Table 4.1

4.4 Discussion

The survival dynamics of STEC serogroups O26, O121, and O157:H7 were evaluated in cake batters made according to a standard formulation and three commercial mixes. Previous studies have examined the survival rate of *E. coli* and *Salmonella* in raw wheat, but a gap in information exists when it comes to subsidiary products like flour-based cake batters. The risk of STEC contamination may be exacerbated by time and temperature abuse of cake batter, which can occur in the home of a consumer. Surveys have shown that consumers frequently engage in the practice of sampling raw batter and dough prior to baking. Thus, the goal of this study was to understand the influence of time and temperature on the survival of STEC in cake batter.

The bacterial populations of *E. coli* strains O26, O121, and O157:H7 declined gradually over time in the standard cake batter. The k values of these STEC strains did not significantly differ when compared within storage temperatures of 5 °C, 25 °C, or 35 °C, indicating that STEC strain type had no effect on survival at these temperatures ($p>0.05$). This is in accordance with what was observed in our earlier work which examined differences in the morphology and growth rate of these antibiotic resistant strains—no notable discrepancies were seen. Alternatively, storage temperature had a significant effect on the survival of these strains. At 5 °C, a duration of 900-1,200 h was needed to achieve a 1 log CFU reduction in all three strains, while an average of only approximately 100 h or less was needed to achieve this reduction in these strains at 25 °C and 35 °C ($p\leq0.05$). This is consistent with what has been previously established in literature—bacteria which are stressed by unfavorable conditions, such as low temperature, will enact survival mechanisms such as slowed metabolic rates and membrane fortification in order to preserve themselves (Shivaji and Prakash 2010). Thus, stressed bacterial cells are often equipped to survive

longer than cells in more favorable environments, which explains why the STEC strains studied here declined more slowly at 5 ℃ than at higher temperatures.

Models were not fitted to the data generated by the 10 ℃ and 15 ℃ storage temperatures because none of the bacterial populations declined significantly from their initial load of 3-4 log CFU/g under these conditions and, as such, no useful survival parameters could have been calculated. Further, the total sampling period for these temperatures (more than one week) was well beyond a reasonable estimate for the shelf life of cake batter—it is unlikely that a consumer would keep batter for this long. Had sampling continued beyond the established timeframe, the concentration of these STEC strains would have eventually declined to the limit of detection as they did at 5 ℃ storage. Secondary models were not constructed for the survival data because of the lack of notable population decline at 10 ℃ and 15 ℃. The gap between the available data at 5 ℃ and 25 ℃ introduces a significant degree of variability and thus would create difficulty in fitting secondary models. A future study which could create these models would be a useful tool for more accurate risk assessment of STEC persistence in cake batter.

For commercial cake batters stored at 25 °C, batter type had no effect on the growth rate of any of the STEC strains ($p>0.05$); however, *E. coli* O26 had a higher growth rate than O121 and O157:H7 in Mix C ($p\leq0.05$). These results suggest that strain type does affect the growth of these bacteria in some kinds of commercial cake batter. Similarly, *E. coli* O121 achieved higher maximum bacterial population compared to *E. coli* O157:H7 in Mix A ($p\leq0.05$), providing further evidence for the impact of strain type on growth characteristics. Additionally, the maximum population of *E. coli* O121 in Mix A was higher in Mix than in Mix B and C ($p\leq0.05$), suggesting that batter type also impacts the growth of these bacteria. Mix A contained the greatest amount of

fat between the three commercial mixes, which may explain the higher bacterial population as fat is known to shield bacteria from stress.

While the erratic growth patterns of the STEC strains at 35 ℃ in the commercial cake batters could not be assessed through mathematical modeling, they still provided insight on how these bacteria behave in a complex matrix like cake batter at an elevated temperature. In some of the strains studied, a decline in cell concentration to near the limit of detection was observed after approximately 60 h of storage, but around 100 h, the concentration had increased to nearly 4 log CFU/g. It is likely that the stress induced by the high storage temperature in combination with the high sugar content of the batter led to the implementation of survival mechanisms by these bacteria, which are known to affect growth behavior. For example, a notable spike in growth is observed in the first 48 h for all three STEC strains in all three batters, followed by a relatively quick decline. Elevated temperatures have been associated with increased rates of bacterial metabolism (Russell 2003; Hall, Neuhauser, and Cotner 2008). Specifically, Soini et al. (2005) showed that *E. coli* exposed to a temperature upshift from 30 ℃ to 42 ℃ experienced a rapid and significant increase in cellular ATP concentration ($p \leq 0.05$), which was connected to a longer-lasting period of increased rate of cellular respiration and glucose uptake. In the present study, the rapid initial growth of the STEC strains likely resulted from a similar increase in cellular metabolism, which led to a quick depletion of available nutrients in the batter matrix. Once these were used up, the concentration of the bacterial strains declined rapidly.

The high concentration of sugar in the cake batter may have also played a role in the rapid initial growth of the STEC populations at 35 °C. Cheng, Huang, and Liu (2011) studied the effects of sucrose concentrations on the growth rate of *E. coli.* Activity of the β-galactosidase enzyme, which is used by *E. coli* to hydrolyze lactose, was observed as an indicator of cellular activity and

growth. Some, but not all, strains of *E. coli* studied showed increased β-galactosidase activity when exposed to increasing concentrations of sucrose ($p \leq 0.05$). Alternatively, the high sugar content of the batter may have had the exact opposite effect on the STEC strains upon nutrient depletion. High concentrations of external solutes like sucrose and salt are known to induce osmotic stress on *E. coli* cells, wherein the concentration gradient of the environment surrounding the bacterial cell leads to an efflux of water from the cell and, ultimately, cell death (Minton 2001). The osmotic pressure experienced by the STEC strains in the cake batter, in tandem with the stress of limited nutrient availability after a period of rapid growth, may have caused the sudden decline in cell concentration.

One particularly notable difference in the behavior of the STEC strains between the cake batters is that all three strains declined gradually over time in the standard cake batter at all storage temperatures, but in the commercial cake batters, the bacterial populations increased. This could be explained by the formulation differences between the batter types. For example, the standard cake batter contained sodium acid pyrophosphate, which is known to have an antibacterial effect against *E. coli* (Rathgeber and Waldroup 1995). Additionally, both the water activity and pH of the standard cake batter were significantly lower than that of the three commercial batters. This could have further contributed to the decline of the STEC populations.

These results further suggest that survival studies for other food matrices performed under abuse temperatures could yield similarly inconsistent growth patterns. The use of culture-based analysis techniques like those used in this study may exacerbate this issue. A potential solution would be the use of non-culture based analytic techniques, such as microscopy or other kinds of cell imaging. This could allow more direct and accurate visualization of cell concentration.

Table 4.1: Water activity (a_w) and pH measurements of four prepared cake batters.

Batter	a_w	pH
Mix A	0.96±0.00[a]	6.44±0.03[b]
Mix B	0.96±0.00[a]	6.60±0.08[ab]
Mix C	0.96±0.00[a]	6.64±0.09[a]
AACC	0.92±0.00[b]	5.78±0.07[c]

Values within a column sharing the same superscript are not significantly different. Values are not compared across rows.

Table 4.2: Formulation of cake batter according to AACC Method 10-90 (Cereals and Grains Association 2021).

AACC Method 10-90 Formula	
Ingredient	**Amount (g)**
Flour	100.00
Sugar	140.00
Vegetable shortening	50.00
Nonfat dry milk	12.00
Dried egg whites	9.00
Salt	3.00
Sodium bicarbonate	2.10
Monocalcium phosphate	0.35
Sodium acid pyrophosphate	2.52
Water	145.00

Table 4.3: Comparison of nutritional components across all four cake batter formulations.

	Amount of nutrient in prepared batter (g/g)			
	AACC	**Mix A**	**Mix B**	**Mix C**
Calories	3.03	3.16	2.95	2.95
Fat	0.11	0.17	0.15	0.15
Sodium	0.00	0.00	0.00	0.00
Carbohydrate	0.49	0.36	0.38	0.37
Fiber	0.01	0.00	0.00	0.00
Total sugar	0.32	0.19	0.19	0.20
Protein	0.04	0.03	0.04	0.03

Note: Nutrients are presented as amount of nutrient in grams per gram of prepared batter with the exception of calories, which are presented as calories per gram of prepared batter.

Table 4.4: Inactivation parameters of *E. coli* O26, O121, and O157:H7 in a standard cake batter formulation at storage temperatures of 5 °C, 25 °C, and 35 °C.

		Inactivation parameter	
Strain	**Temperature**	**k value (log CFU reduction/h)**	**Shoulder (h)**
E. coli O26	5 °C	1254.19±648.96^{a}	0.00±0.00^{c}
	25 °C	93.63±30.84^{c}	103.99±13.85^{b}
	35 °C	50.89±22.40^{bc}	93.62±9.55^{b}
E. coli O121	5 °C	902.35±275.96^{ab}	0.00±0.00^{c}
	25 °C	121.41±21.61^{c}	83.44±12.68^{b}
	35 °C	66.34±27.45^{c}	51.77±17.68^{b}
E. coli O157:H7	5 °C	908.69±233.51^{ab}	474.213±160.89^{a}
	25 °C	123.00±17.88^{c}	93.45±22.23^{b}
	35 °C	90.56±70.93^{c}	54.40±40.76^{b}

Values within a column sharing the same superscript are not significantly different. Values are not compared across rows.

Table 4.5: Maximum specific growth rate (μ_{max}) and maximum cell concentration (Y_{max}) of *E. coli* O26, O121, and O157:H7 in three commercial cake batters during storage at 25 °C.

		Growth parameter	
Strain	**Mix**	**μ_{max} (log CFU/h)**	**Y_{max} (log CFU/g)**
E. coli O26	A	0.15±0.07^{ab}	8.34±0.11^{ab}
	B	0.17±0.05^{ab}	7.88±0.26^{abc}
	C	0.24±0.17^{a}	7.35±0.84^{bc}
E. coli O121	A	0.06±0.01^{ab}	8.34±0.81^{a}
	B	0.11±0.03^{ab}	7.31±0.22^{bc}
	C	0.07±0.01^{ab}	6.59±0.14^{c}
E. coli O157:H7	A	0.09±0.05^{ab}	6.74±0.25^{bc}
	B	0.05±0.01^{ab}	6.38±0.69^{c}
	C	0.04±0.01^{b}	6.42±1.02^{c}

Values within a column sharing the same superscript are not significantly different. Values are not compared across rows.

Table 4.6: Goodness-of-fit statistics for the predicted inactivation models of *E. coli* O26, O121, and O157:H7 in a standard cake batter formulation during storage at 5 °C, 25 °C, and 35 °C.

		Statistic		
Strain	**Temperature**	**SSE**	**MSE**	**RMSE**
E. coli O26	5 °C	0.23±0.08	0.03±0.01	0.18±0.03
	25 °C	0.55±0.32	0.06±0.04	0.25±0.07
	35 °C	0.87±0.21	0.11±0.02	0.32±0.03
E. coli O121	5 °C	0.38±0.01	0.05±0.01	0.23±0.01
	25 °C	0.30±0.10	0.03±0.01	0.19±0.03
	35 °C	0.44±0.43	0.07±0.07	0.24±0.13
E. coli O157:H7	5 °C	0.40±0.45	0.05±0.05	0.21±0.11
	25 °C	0.54±0.16	0.06±0.02	0.26±0.04
	35 °C	0.35±0.28	0.05±0.04	0.21±0.12

Table 4.7: Goodness-of-fit statistics for the predicted growth models of *E. coli* O26, O121, and O157:H7 in three commercial cake batters during storage at 25 °C.

		Statistic		
Strain	**Mix**	**SSE**	**MSE**	**RMSE**
E. coli O26	A	1.02±0.97	0.51±0.48	0.65±0.35
	B	3.46±2.67	1.73±1.33	1.203±0.66
	C	7.56±8.53	3.78±4.27	1.69±1.17
E. coli O121	A	1.11±1.06	0.42±0.35	0.60±0.35
	B	0.37±0.48	0.18±0.24	0.37±0.27
	C	0.85±0.38	0.42±0.19	0.64±0.14
E. coli O157:H7	A	2.81±1.52	1.25±0.81	1.08±0.34
	B	1.06±1.01	0.53±0.50	0.67±0.34
	C	3.64±1.81	1.73±1.05	1.26±0.46

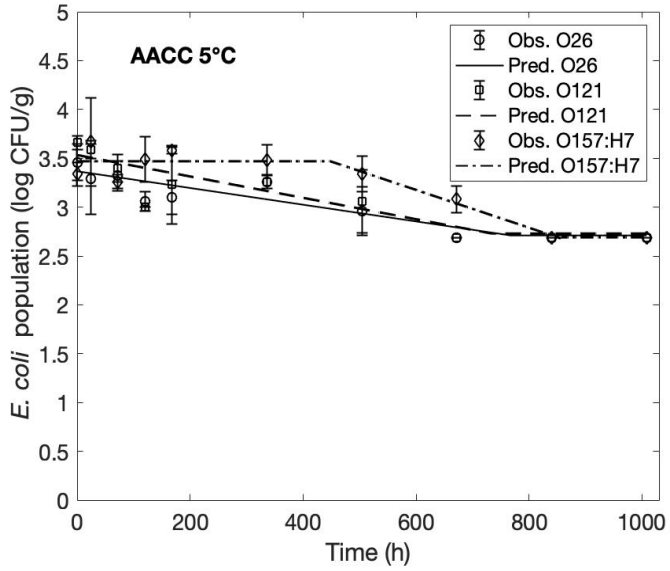

Figure 4.1

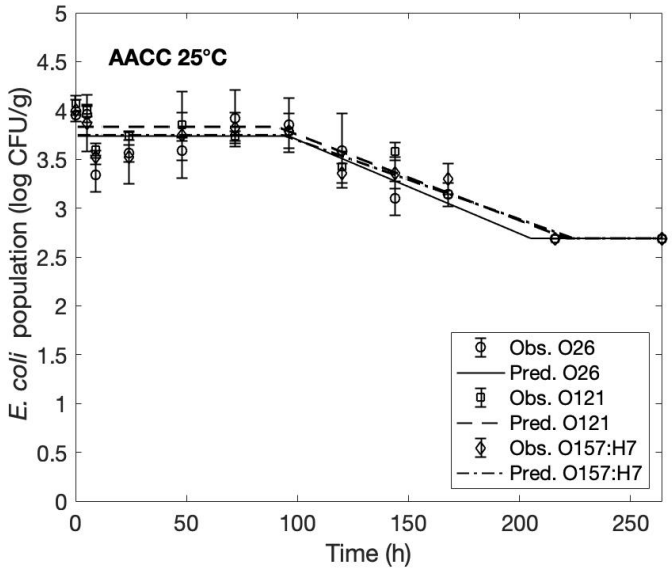

Figure 4.2

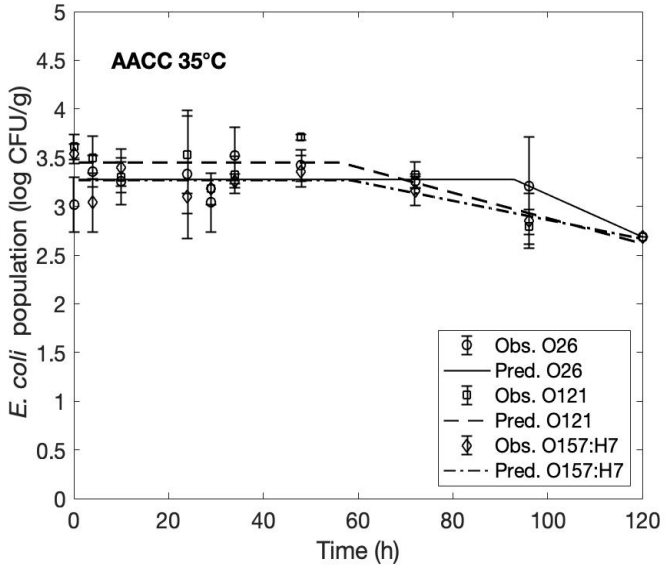

Figure 4.3

Figures 4.1-4.3: Inactivation of *E. coli* O26, O121, and O157:H7 in a standard cake batter formulation at storage temperatures of 5 °C, 25 °C, and 35 °C.

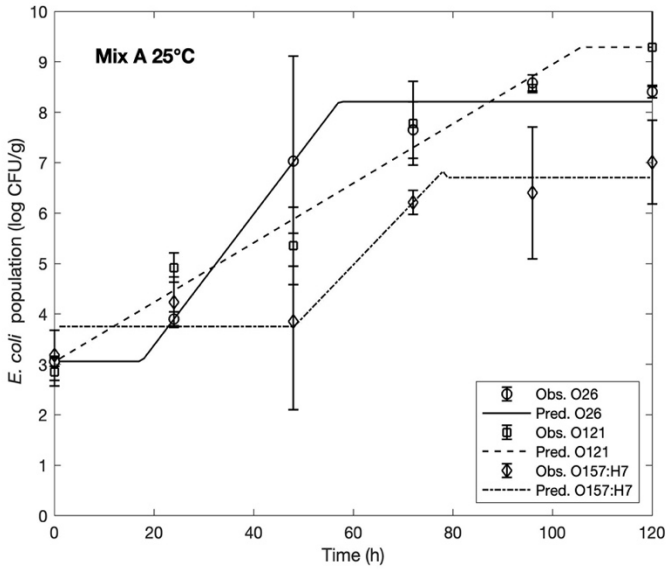

Figure 4.4

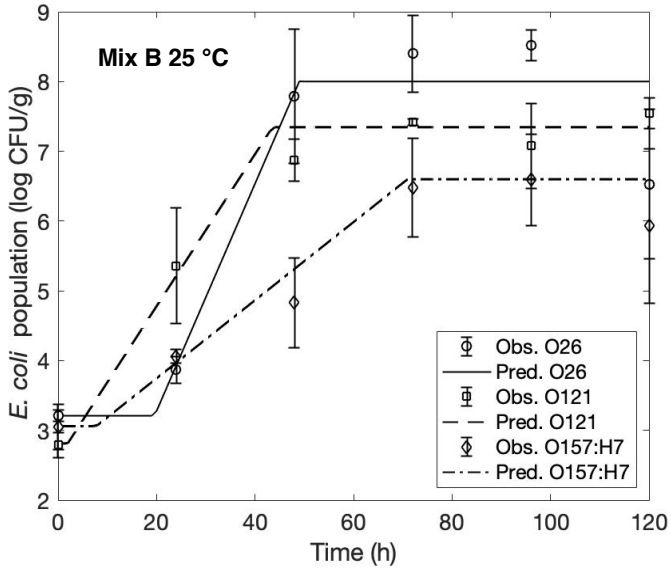

Figure 4.5

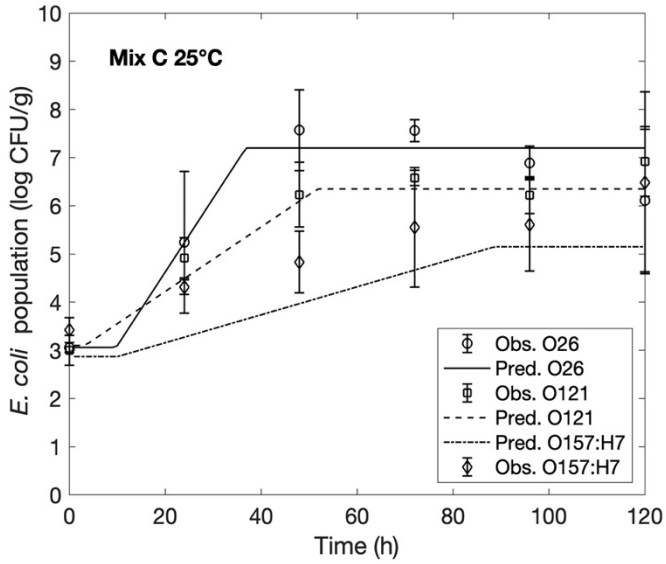

Figure 4.6

Figures 4.4-4.6: Growth of *E. coli* O26, O121, and O157:H7 in commercial cake batter formulations during storage at 25 °C.

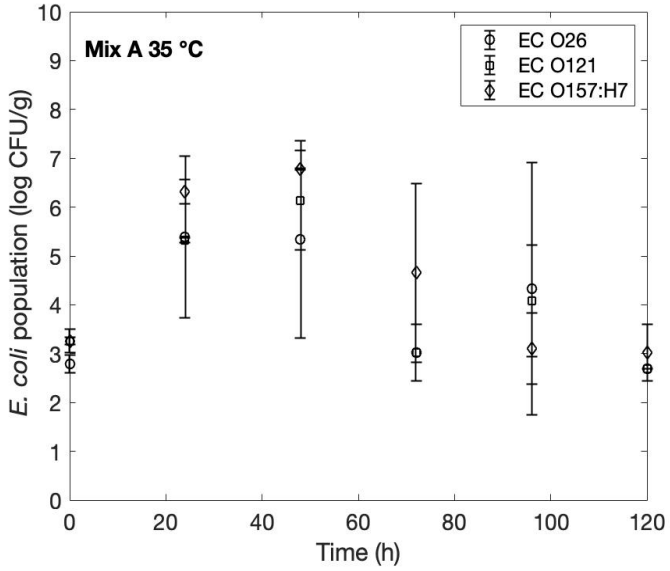

Figure 4.7

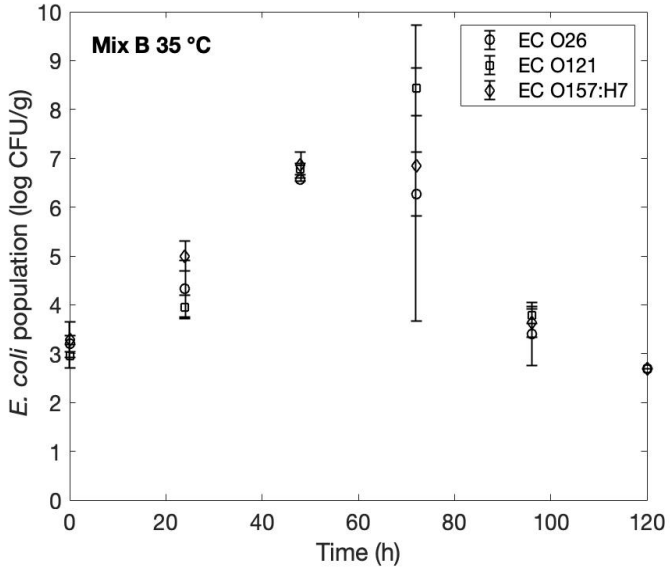

Figure 4.8

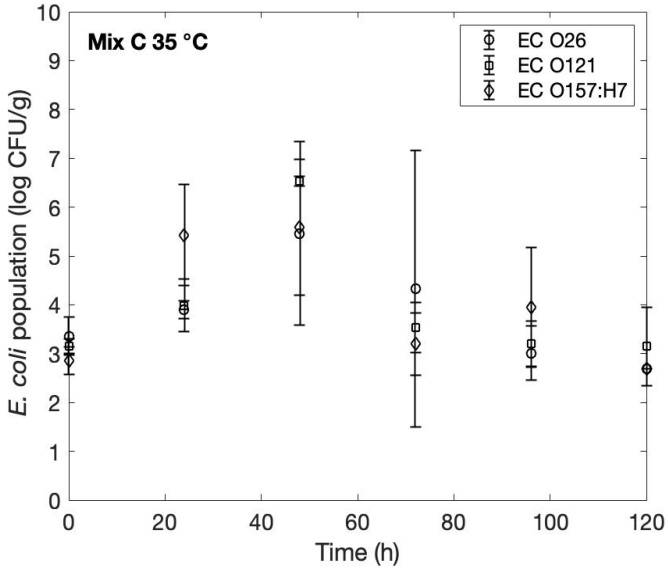

Figure 4.9

Figures 4.7-4.9: Growth of *E. coli* O26, O121, and O157:H7 in commercial cake batter formulations during storage at 35 °C.

CHAPTER 5

EVALUATION OF PELARGONIC ACID FOR THE MITIGATION OF SHIGATOXIGENIC *E. COLI* IN CAKE BATTERS

[1]Hayman, K.M., Dev Kumar, G., and Mishra, A. To be submitted to *LWT*

ABSTRACT

Few control measures currently exist for mitigating the risk of Shigatoxigenic *Escherichia coli* (STEC) persistence in raw flour commodities like cake mix. Some antimicrobial treatments have been investigated for use during the tempering stage of flour milling but have shown limited efficacy. A treatment which could be administered to raw flour and its subsidiary products would be valuable for the wheat industry. Microencapsulated pelargonic acid (PA) is a stable, high-quality powder that can be easily incorporated in cake mixes and has demonstrated potential against several foodborne pathogens. Three commercial cake batters were formulated with encapsulated PA or a liquid PA emulsion and the populations of STEC serogroups O26, O121, and O157:H7 were monitored in these batters over 24 h of storage at 25 °C. No significant differences ($p>0.05$) were observed in the population increase of these three strains in cake batter regardless of the presence of PA, indicating that neither encapsulated nor emulsified liquid PA had an antimicrobial effect on STEC in cake batter.

5.1 Introduction

Shiga toxin producing *Escherichia coli* (STEC) have been known to persist in raw flour products. Recent outbreaks have indicated that STEC contaminated wheat flour resulted in illnesses in individuals who had consumed products made with contaminated flour. These products included cookie and brownie mixes. Some consumers who fell ill reported to having handled and eating raw dough (U.S. Food and Drug Administration 2019). The consumption of raw cookie dough and cake batter remains a common trend in the U.S. despite the efforts of the U.S. Food and Drug Administration and the Centers for Disease Control and Prevention to warn against these practices due to the ability of STEC to persist in wheat flour for extended durations of time.

Forghani et al. (2018; 2019) found that several strains of STEC were detectable in wheat flour itself after more than nine months of storage at ambient temperature, while Wu (2016) showed that *E. coli* O157:H7 survived in raw cookie dough for more than eight weeks in refrigerated storage. Further, consumers are known to eat raw flour products prior to cooking or baking. In a survey of 1,032 U.S. individuals, 58% admitted to eating raw cookie dough, while an alarming 80% reported licking spatulas and beater paddles used to prepare cake and brownie batters (Rose et al. 2012). Thus, flour products contaminated with STEC can lead to illnesses in consumers.

There are some novel antimicrobial interventions which have been studied and have shown potential for use in the production of flour products. Such treatments involve the addition of organic acids and salt solutions to water that is used for tempering wheat kernels prior to milling, to prevent contamination on the kernels from being introduced to the extracted flour (Sabillón et al. 2016). Ozone has also been shown as a mildly effective microbial control measure for wheat when added to tempering water (Ibanoglu 2002; Dhillion 2007). However, these treatments can

only be applied prior to processing of the wheat. An effective antimicrobial measure which could be applied after the flour milling and baking mix manufacturing process has yet to be identified.

Pelargonic acid (PA) presents a viable solution. The fatty acid has demonstrated antimicrobial efficacy against pathogens such as *Salmonella* Newport, Typhimurium, and Javiana even at very low concentrations (125 mM) (Dev Kumar and Micallef 2017). These authors have shown that PA can be emulsified with Quillaja saponin as a surfactant in order to improve its dispersion in water as well as its antimicrobial activity—the minimum inhibitory concentration of the emulsion against the same *Salmonella* serotypes was 31.25 mM. Further, Dev Kumar et al. (2020) found that emulsified PA can be microencapsulated in maltodextrin through spray drying, which creates a stable, high-quality powdered form of PA that retains its antimicrobial efficacy against pathogenic *Salmonella*. PA can be naturally present in the cuticle of tomatoes (Dev Kumar and Micallef 2017) and is certified by the U.S. Food and Drug Administration as an additive in foods, which is in line with the growing consumer demands for transparent, minimal, and natural methods of food processing. Thus, powdered PA would be ideal additive in flour-based mixes that could prevent the proliferation of contaminating bacteria like STEC upon rehydration.

The antimicrobial properties of PA can be influenced by its micelle size and concentration when emulsified (Dev Kumar et al. 2020). Further, it is well known that exposure to sublethal concentrations of antimicrobials can cause bacteria to develop resistance. Therefore, it was critical to determine the minimum inhibitory concentration (MIC) of PA against the STEC strains used in this study. Once this was accomplished, the ability of microencapsulated PA to inhibit growth of STEC in cake batter was assessed. The goal of this study was to determine if the presence of microencapsulated PA or a liquid PA emulsion would significantly affect the growth of STEC in commercial cake batter during storage at 25 °C over a 24 h period.

5.2 Methods and materials

5.2.1 Bacterial strains

The STEC strains were revived from storage and confirmed as described in Section 3.2.1.

5.2.2 Development of antibiotic resistance

The selected bacterial strains were transformed with fluorescence as well as resistance to ampicillin and streptomycin as described in Section 3.2.2. Stock cultures of the transformed strains were prepared by streaking each to TSA with the appropriate antibiotic supplements and incubating at 37 ℃ for 24h. Colonies were scraped from the plates with a sterile loop and suspended in PBS to create a ca. 8 log CFU/ml stock solution, which was then adjusted to a concentration of ca. 4 log CFU/ml. A cocktail of all three ABR strains was also prepared by adding 1 ml of each 8 log CFU/ml stock solution to 7 ml of PBS and vortexing to mix. The cocktail was then adjusted to a final concentration of ca. 4 log CFU/ml.

5.2.3 Cake mix procurement and batter preparation

Three different commercial cake mixes (A, B, and C) were procured, stored, and prepared as batter as described in Sections 3.2.3 and 3.2.4.

5.2.4 Preparation of microencapsulated PA

A PA emulsion was prepared for encapsulation as described by Dev Kumar et al. (2020). Briefly, 10 g of Quillaja saponin and 158 ml of PA were added to 1 L of sterile deionized water, along with 100 g of maltodextrin (dextrose equivalent of 9-13). The mixture was processed by an ultra-shearing device (OMNI, OMNI PHD, Omni International, Kennesaw, GA) fitted with a 32-55 mm generator probe for three min at 10,000 rpm to produce a stable emulsion. The emulsion

was then spray dried at 160 ℃ inlet air temperature under concurrent drying conditions using a pilot plant-scale spray dryer equipped with a rotary atomizer (Anhydro, PSD 52, Denmark). The resultant PA powders were stored in a desiccator at room temperature until needed.

5.2.5 MIC determination

The MIC of the encapsulated PA was determined using the 96-well plate Resazurin assay described by Sarker, Nahar, and Kumarasamy (2007) with modifications described by Dev Kumar et al. (2020). Briefly, 1 g of the encapsulated PA was added to 10 ml sterile DI water, then serially diluted in Iso-sensitest broth (Oxoid Ltd., Hants, UK) containing Resazurin (Biotium, Hayward, CA) at 0.001 g/ml in a 96-well plate. The concentrations of encapsulated PA tested were 50, 25, 12.50, 6.25, 3.12, 1.61, 0.86, and 0.43 mg/ml. Each of the three STEC strains were inoculated into individual wells at ca. 5 log CFU/ml. The 96-well plates were incubated at 37 ℃ for 24 h, then observed for color change. Wells which had changed from blue to pink indicated bacterial growth. Thus, the lowest dilution at which no growth was observed was considered the MIC of the encapsulated PA. This experiment was performed in triplicate and the highest average concentration of PA required to inhibit growth between the three STEC strains was used to determine the amount of PA that would be added to the cake batters.

5.2.6 Sample preparation, inoculation, and treatment conditions

A 12.5 g portion of the encapsulated PA was stirred into the dry cake mixes, which were then prepared as batter in 500 g portions to achieve a concentration of 25 mg/g. Individual samples of the batters were prepared by aliquoting 1 g portions into sterile Whirl-Pak bags. Inoculation was accomplished by delivering 100 µl of the 4 log CFU/ml stock solutions of each bacterial strain into individual batter samples. The samples were hand-massaged for 30 s to create a homogenous 3 log

CFU/g initial bacterial load. Samples were then stored at 25 ℃ and sampled at regular intervals over a 24 h period.

One commercial cake batter (Mix C) was additionally prepared with a liquid PA emulsion. Emulsions at concentrations of 25, 75, and 100 mM were added to individual 100 g portions of the batter and mixed for two min with a KitchenAid standing mixer until homogeneity was achieved. Individual samples of the batters were prepared by aliquoting 1 g portions into sterile Whirl-Pak bags. Inoculation was accomplished by delivering 100 µl of the 4 log CFU/ml STEC cocktail into individual batter samples. The samples were hand-massaged for 30 s to create a homogenous 3 log CFU/g initial bacterial load. Samples were then stored at 25 ℃ and sampled at regular intervals over a 24 h period.

5.2.7 Microbiological analysis

At the designated time, samples were retrieved from storage and 10 ml PBS were added to the sample bag. The contents of the bag were hand-massaged for 30 s to homogenize. A 1 ml portion of this suspension was extracted and used to prepare appropriate ten-fold dilutions in PBS, which were plated on TSA-AS using the droplet plating method described by Miles and Misra (1938). Plates were incubated at 37 ℃ for 24 h.

5.2.8 Statistical analysis

All experiments were performed in triplicate. JMP Pro v15.0.0 statistical software was used to compare the MIC values and average increase in the population of the STEC strains in cake batters with and without encapsulated PA or liquid PA after 24 h of storage at 25 °C via ANOVA and Tukey's HSD test (α=0.05).

5.3 Results

5.3.1 MIC of encapsulated PA against STEC

The average MIC values of encapsulated PA against the selected STEC strains are shown in Table 5.1. A concentration of 10.42±3.61 mg/ml was required to inhibit the growth of *E. coli* O26, which was the lowest among the three STEC serogroups. *E. coli* O121 required a concentration of 12.50±0.00 mg/ml for growth inhibition. The STEC serogroup that was most tolerant to the encapsulated PA was *E. coli* O157:H7, which was inhibited by a significantly higher concentration of 25.00±0.00 mg/ml encapsulated PA ($p \leq 0.05$).

5.3.2 STEC survival in cake batter amended with encapsulated pelargonic acid

There was an increase of approximately 1-2 log CFU/g in all bacterial populations in all three batters both with and without PA. Table 5.2 shows the population increase in *E. coli* O26, O121, and O157:H7 in commercial cake batters with and without the inclusion of encapsulated PA after 24 h of storage at 25 °C. *E. coli* O26 increased by 0.85±0.14, 0.66±0.12, and 2.18±1.38 log CFU/g in Mix A, B, and C without PA, respectively. These values were not significantly different ($p > 0.05$). In the same batters with PA, the average population increase was about the same—1.31±0.38, 1.41±0.52, and 1.23±0.36 log CFU/g, respectively. These values were not significantly different from each other, nor were they significantly different from the population increases of *E. coli* O26 observed in the batters without PA ($p > 0.05$). Similar results were observed for *E. coli* O121, which had statistically equivalent increases of 2.07±0.56, 2.56±0.95, and 1.93±0.42 in Mix A, B, and C, respectively ($p > 0.05$). With the inclusion of PA, the average increase of *E. coli* O121 in each batter was slightly less, but not significantly so ($p > 0.05$). The average increase in *E. coli* O157:H7 changed very little across the three batters or between

treatments, with average growth of approximately 1 log CFU/g in all batters regardless of the presence of PA ($p>0.05$).

5.2.3 STEC survival in cake batter amended with liquid pelargonic acid emulsion

There was an increase of approximately 1.5-2 log CFU/g in the population of the STEC cocktail in the commercial cake batter formulated with liquid PA concentrations ranging from 25-100 mM as well as the control batter (no PA) (Table 5.3). In the control batter, the STEC population increased by 1.55±0.09 log CFU/g. In the batter containing PA at 25 mM, the STEC population increased by 1.78±0.026 log CFU/g. An equivalent increase was observed in the batters containing 75 and 100 mM PA (1.90±0.15 and 1.73±0.26 log CFU/g, respectively) ($p>0.05$). There were no significant differences in the population increase of STEC in any of the batters regardless of the presence or concentration of the liquid PA emulsion ($p>0.05$).

5.4 Discussion

Pelargonic acid has demonstrated potential as an antimicrobial agent against several pathogenic bacteria in several *in vitro* studies. For example, Dev Kumar and Micallef (2017) used a Resazurin-based MIC assay to show that PA at a concentration of 125 mM inhibited the growth of *Salmonella* serotypes Newport, Typhimurium, and Javiana via disruption of the bacterial cells' membranes which caused cell leakage. Sahin, Kula, and Erdogen (2006) used a similar broth microdilution susceptibility assay to show that PA at a concentration of 32 μg/ml inhibited visible growth of *Bacillus cereus, Salmonella typhimirium,* typical *E. coli,* and *Candida utilis* and prevented germination of *Streptomyces* spores. Despite these promising results, only one previous study has evaluated the efficacy of PA in a food matrix. White et al. (2021) used 30 mM and 50

mM PA emulsions as a produce wash to reduce *Salmonella* contamination on the surface of tomatoes by up to 6 log CFU/g after 7 days of storage.

Determining the MIC of an antimicrobial compound is important because bacteria can develop resistance upon exposure to sublethal concentrations of such compounds, which exacerbates the issue of their persistence. For example, Dev Kumar, Macarisin, and Micallef (2019) have shown that exposing *Salmonella* to PA at concentrations below 31.25 mM resulted in filament formation, which is a mechanism used by many bacteria to evade stress. A bacterial filament is an aggregation of cells which have failed to divide and thus create one large, elongated multinucleated cell that has reduced susceptibility to environmental damage and, further, can evade diagnostic testing (Rizzo, De Plano, and Franco 2020). Upon return to more favorable conditions, filaments can fragment into individual cells and regain their virulence (Dev Kumar, Macarisin, and Micallef 2019). Therefore, it was critical to determine the concentration of PA that would be optimally effective against STEC serogroups O26, O121, and O157:H7.

The present study endeavored to demonstrate the antimicrobial potential of PA against STEC in cake batter—a commodity which lacks microbial control measures almost entirely and thus presented a unique matrix for evaluating the efficacy of encapsulated PA as a novel antimicrobial food additive. In contrast with what has been found in previous *in vitro* studies, PA did not inhibit the growth of *E. coli* O26, O121, or O157:H7 in any of the three commercial cake batters tested. There were no significant differences in the average population increase of any of these three pathogens after 24 h in cake batters formulated with encapsulated PA when compared to those without, indicating that encapsulated PA has no notable antimicrobial effect on these bacteria in cake batter ($p>0.05$).

A secondary study was conducted to determine if the delivery method of PA into cake batter influenced its antimicrobial activity. Liquid PA emulsions at concentrations of 25, 75, and 100 mM were added to a commercial cake batter inoculated with a cocktail of the three selected STEC strains and their growth in the batter was monitored over 24 h. Again, none of the concentrations evaluated produced any significant effect on the population increase of the STEC cocktail in the cake batter—growth of approximately 1-2 log CFU/g was observed across all treatments, including the control batter ($p>0.05$).

The insignificant antimicrobial activity of the PA in cake batter was most likely due to the high fat environment of the batter. Fat can confer a protective effect on bacteria and increase their tolerance to many environmental stresses. For example, Brar et al. (2018) found that increasing the fat content of ground beef resulted in longer treatment at 55°C needed to achieve reduction in several strains of *E. coli*. Similarly, Juneja and Eblen (2000), found that the heat tolerance of *Salmonella* in ground beef increased as the fat content of the meat increased. The protective effect of fat on bacteria is attributed to the decreased water activity of high fat environments, which is known to inhibit the efficacy of antimicrobial treatments like heat (Jay 1993).

Notably, Gaysinsky et al. (2007) conducted an experiment very similar to ours and produced similar findings. They investigated the antimicrobial activity of a microemulsion of eugenol in surfactant micelles against *Listeria monocytogenes* and *E. coli* O157:H7 in cow's milk with varying levels of fat. It was reported that, in the milk with the highest fat content (4%), the eugenol microemulsion was unable to inhibit the growth of both pathogens. The authors suggest that this phenomenon can be explained by the complexity of a multiphase food system which contains a lipid phase. The antimicrobial contained within the surfactant micelle may have a higher affinity for the lipid phase of the food matrix than the aqueous phase, and thus may leach out of

the micelle. In doing so, the surfactant micelle loses its ability to promote interaction of the antimicrobial compound with bacterial cell membranes and efficacy is drastically reduced. It is possible that the fat contained in the cake batters used in our study similarly inhibited the antimicrobial action of emulsified PA. This could be evaluated by testing the efficacy of PA alone against STEC in cake batter, without the inclusion of surfactants and other emulsion components.

Additionally, the global stress response of the STEC strains studied here may have contributed to their tolerance of PA. The presence of solutes such as sugar and salt in the cake batters may have induced osmotic stress on the bacterial cells; the mechanisms employed by *E. coli* to survive this kind of osmotic pressure are regulated by genes which are expressed in response to general stress (Battesti, Majdalani, and Gottesman 2011). The RpoS regulon controls roughly 10% of the genes in *E. coli* which are related to stress response; among these are genes involved in the OtsAB pathway, which synthesizes trehalose (Klauck, Typas, and Hengge 2007). Besides its ability to stabilize bacterial cell membranes to prevent damage due to osmotic stress, trehalose can also preserve cell proteins by hydrogen bonding with their hydroxyl groups and polar residues. This interaction stabilizes the proteins and prevents them from being denatured during exposure to heat and other stresses (Elbein et al. 2003; Pleitner et al. 2012). Other cytoplasmic solutes that are accumulated or synthesized in response to osmotic stress include glucose and sucrose. These osmolytes further contribute to the cross-resistance of *E. coli* by protecting its cell proteins from the effects of pressure (Ruan et al. 2003). Thus, the mechanisms of tolerance invoked by *E. coli* in response to osmotic stress can offer protection from several other environmental stresses, including the antimicrobial activity of compounds like PA.

Table 5.1: Average MIC values of encapsulated PA against *E. coli* O26, O121, and O157:H7 as determined by the modified 96-well plate Resazurin assay (Dev Kumar et al. 2020).

Strain	MIC (mg/ml)
E. coli O26	10.42[a]
E. coli O121	12.50[a]
E. coli O157:H7	25.00[b]

Values within a column sharing the same superscript are not significantly different.

Table 5.2: Increase in *E. coli* O26, O121, and O157:H7 populations in three commercial cake batters with and without the inclusion of encapsulated PA after 24 h of storage at 25 ℃.

		Population increase (log CFU/g)		
Strain	**Treatment**	**Mix A**	**Mix B**	**Mix C**
E. coli O26	Control	0.85±0.14[a]	0.66±0.12[a]	2.18±1.38[a]
	PA	1.31±0.38[a]	1.41±0.52[a]	1.23±0.36[a]
E. coli O121	Control	2.07±0.56[a]	2.56±0.95[a]	1.93±0.42[a]
	PA	1.88±0.25[a]	1.30±0.87[a]	1.20±0.40[a]
E. coli O157:H7	Control	1.04±0.83[a]	1.00±0.42[a]	0.90±0.11[a]
	PA	1.00±0.30[a]	1.64±0.25[a]	0.93±0.13[a]

Values within a column sharing the same superscript are not significantly different. Values are not compared across rows.

Table 5.3: Increase in STEC cocktail population in one commercial cake batter with and without the inclusion of a liquid PA emulsion at increasing concentrations after 24 h of storage at 25 ℃.

PA concentration (mM)	Population increase (log CFU/g)
0	1.55±0.09[a]
25	1.78±0.26[a]
75	1.90±0.15[a]
100	1.73±0.26[a]

Values within a column sharing the same superscript are not significantly different.

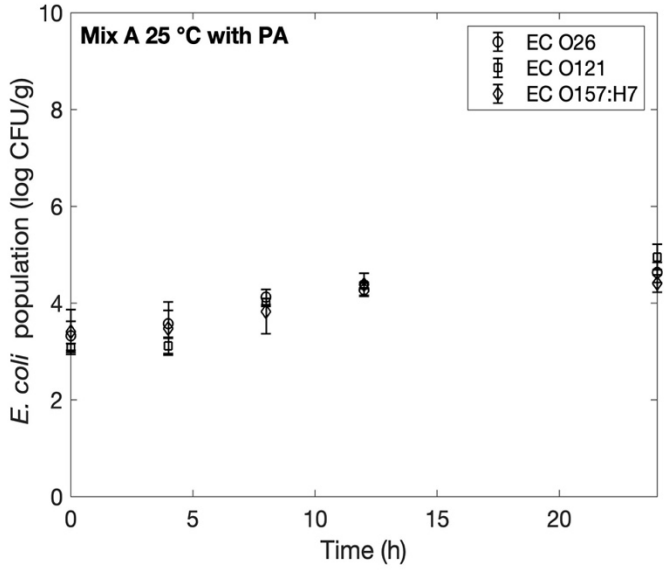

Figure 5.1

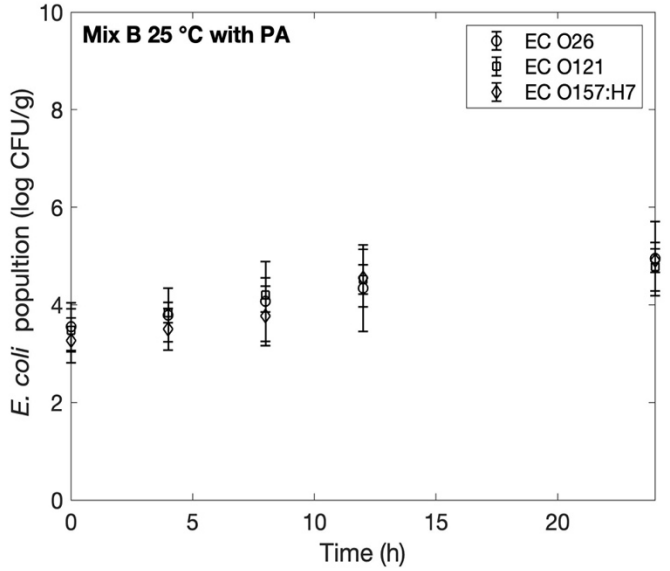

Figure 5.2

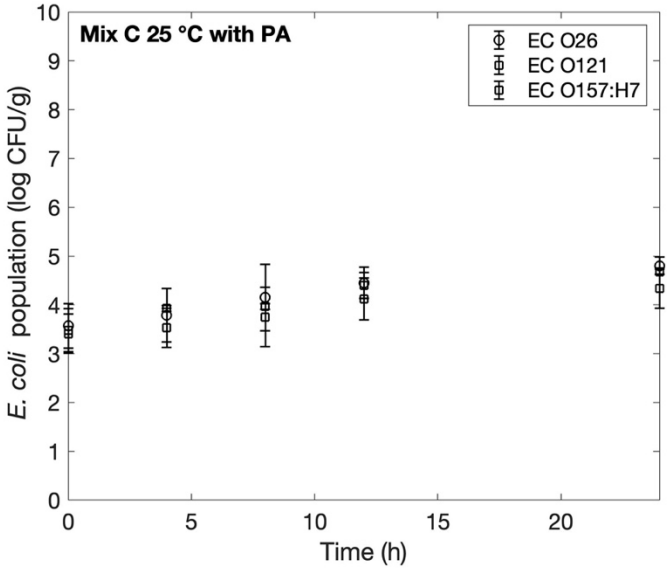

Figure 5.3

Figures 5.1-5.3: Growth of *E. coli* O26, O121, and O157:H7 in three commercial cake batters over 24 h of storage at 25 °C with encapsulated PA at a concentration of 25 mg/g.

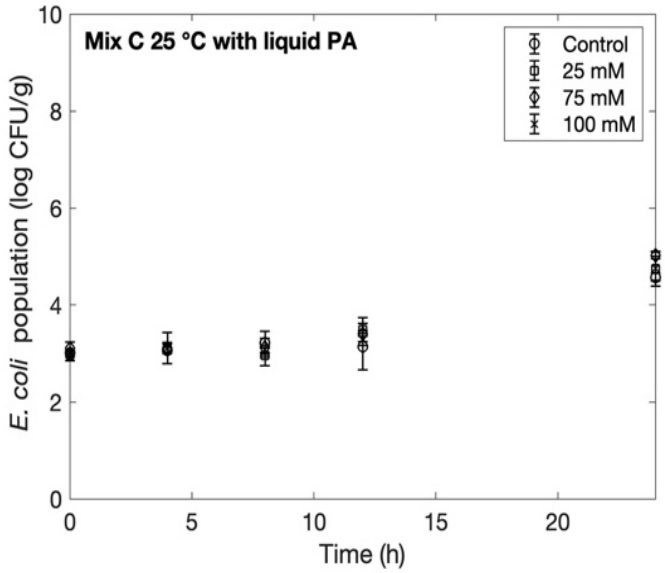

Figure 5.4

Figure 5.4: Growth of a cocktail of *E. coli* O26, O121, and O157:H7 in a cake batter over 24 h of storage at 25 °C with a liquid PA emulsion at concentrations of 25, 75, and 100 mM.

CHAPTER 6

CONCLUSIONS

As a raw agricultural commodity, wheat flour and its subsidiary products like cake mix have great potential to carry pathogenic bacteria like STEC and have been implicated in numerous recent outbreaks of foodborne illness. These outbreaks not only affect the health and wellbeing of the sickened individuals; they also have a detrimental impact on the reputation and revenue of manufacturers of these products. Intervention strategies for controlling STEC in these products are currently limited and, consequently, it is of great interest to producers of flour-based products to elucidate new and effective antimicrobial treatments.

This study showed that STEC can survive in a standard cake batter formulation for more than 3 weeks in refrigerated storage. Higher storage temperatures resulted in more rapid inactivation of STEC, but these bacteria remained detectable up to a week under those conditions. In commercial cake batters, STEC grew rapidly during the first 48 h of storage at ambient and elevated temperatures. This confirmed the expectation that these bacteria would proliferate in a nutritionally rich matrix like cake batter after time and temperature abuse, which may occur during consumer handling. This is highly likely to lead to illness if a consumer were to sample the abused batter prior to baking, which is a common practice. It is possible that differing intrinsic factors between the cake batters resulted in the bacterial decline observed in the standard cake batter formulation, as opposed to the growth observed in the commercial cake batters.

Microencapsulated pelargonic acid was evaluated as a potential control measure for the persistence of STEC in cake batter; despite its promising ability to inhibit growth of many foodborne pathogens in previous *in vitro* studies, no notable differences were observed in the

population increase of STEC in cake batters formulated with or without the antimicrobial. This is likely a result of the high fat content of cake batter.

These findings shed light on the risk posed by flour-based cake batters in the home of consumers and can be used to inform stakeholders of the challenges that exist in controlling microbial contamination of this commodity. Encapsulated pelargonic acid has little potential for success as a novel antimicrobial additive in cake batters and, while a successful intervention strategy was not identified in this study, our results illustrate the complexity of this matrix and may assist in the development of more appropriate control measures in the future.

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