A NOVEL SELECTIVE ENRICHMENT MEDIUM FOR CULTURING THE HUMAN PATHOGEN *ESCHERICHIA ALBERTII*

A Thesis

by

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ABSTRACT

Escherichia albertii, first observed in 2003 as the causative agent in a diarrheal infection of a child in Bangladesh, is commonly misidentified as other Gram-negative pathogens such as *E. coli*, *Shigella* spp., and *Salmonella enterica* spp. *E. albertii* misidentification can lead to a mis-estimation of its impact in the food industry and on food safety. The goal of this research was to develop a novel selective enrichment broth to aid in its isolation and recovery from broiler tissue. Isolates underwent a NARMS Panel screening, identifying sulfamethoxazole, piperacillin, and trimethoprim as selective agents to be utilized in a selective enrichment formulation. Growth kinetics of *E. albertii* isolates 0065, ATCC 10457, 3542, 4085, and 3033 were assessed using varying concentrations of each listed antibiotics, then combinations of each antibiotic and bile salts. Data from growth kinetic experiments were used to plot a growth curve from which isolate doubling times were derived. Doubling times and visual observations of log/exponential phases produced were used to determine two selective enrichment formulations. *E. albertii* isolates were paired with other pathogens and inoculated into both formulation A (30.0 g dehydrated TSB, 2.5 g of bile salts, 34.0 µg/mL sulfamethoxazole, and 4.0 µg/mL trimethoprim) and formulation B (30.0 g TSB, 2.5 g bile salts, 4.0 µg/mL of piperacillin, and 4.0 µg/mL trimethoprim) to determine which medium produced better recovery. Samples positive for

E. albertii were determined by a multiplex-PCR and biochemical assays. Results from multiplex PCR revealed no significant difference between the formulations (p=0.4775), although formulation A produced a higher rate of detection (6.02%) of *E. albertii* over formulation B. Recovery of *E. albertii* through biochemical assay resulted in 6.63% more positive *E. albertii* identifications using formulation A over B. The selective agent combination of sulfamethoxazole and trimethoprim is commonly utilized in clinical treatment for infections caused by enteric pathogens, which could be why formulation A produced better selectivity for *E. albertii*. The greater utility of selective enrichment formulation A will help in producing better recovery of *E. albertii* in food vehicles and the environment.

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1. INTRODUCTION

Ensuring that consumers have access to high quality foods is extremely important for the food industry. Quality can be attributed to the control of the flavor, texture, color, and other desirable properties of a product. Food safety is complementary to total food quality as the final product should not cause risk of consumer foodborne illness or death. Foodborne illness is a widespread issue that impacts the lives of consumers. It has been reported by the U.S. Centers for Disease Control and Prevention (CDC) that about one in six American consumers develop a foodborne illness each year (40). That is about 48 million people annually, and of those, roughly 130,000 are hospitalized and 3,000 illnesses result in death (40).

Microbiological foodborne illness can be simply defined as illness caused by food contaminated with bacteria, viruses, parasites, or microbially produced toxins (5). Microorganisms that can cause disease and are transmitted through food consumption are known as foodborne pathogens, and there are many points along the food chain where they can contaminate food. Their contact with food and food products during production, however, does not guarantee the final product purchased by consumers will remain contaminated with them, or that disease in the consumer will necessarily occur. This is do to various steps/procedures within a food processing environment that should decrease the chances of the finished good/final product being contaminated with adulterants. These steps can include sanitation methods, cook/heating times and temperatures, packaging, non-heat lethality treatments, and other steps/procedures.

The impact of foodborne illness is also very costly, ranging from medical costs to production losses. It has been estimated that foodborne illness can accrue upwards to

more than $15.6 billion annually in costs related to resolving foodborne illness outbreaks (38). The U.S. Department of Agriculture (USDA) Economic Research Service (ERS) has been able to estimate the cost of foodborne illness by pathogen. There are about 31 pathogens of concern as it relates to public health in the food industry. These pathogens have been heavily studied and are routinely connected to the food industry. In 2013, E. coli O157:H7 was estimated to have cost $271,418,690 in foodborne illness-related expenses (38). The projected costs ranged from testing for the illness to treating acute and chronic disease, such as hemolytic uremic syndrome (HUS). In the same year, non-typhoidal Salmonella-related foodborne illness resulted in $3,666,600,031 in medical expenses (38).

Reports have shown that Campylobacter spp., Salmonella spp. (non-typhoidal), Shigella spp., Shiga toxin-producing E. coli (STEC), both the O157 and non-O157 STEC, Clostridium perfringens, Yersinia enterocolitica, and Listeria monocytogenes are of the highest threat to public health among the 31 listed pathogens regarding frequencies of illness and illness resulting in hospitalizations and death (40). Pathogens transmitted to consumers through fresh produce, particularly leafy green vegetables, generate the most foodborne illnesses and second most hospitalizations due to illness (37). Meat and poultry have the highest rates of mortality due to foodborne illness, with poultry leading at 19%. The major causative agents associated with death due to consuming contaminated poultry were L. monocytogenes and Salmonella (37).

The consumption of poultry has rapidly grown over the past five decades with broilers (chicken) carrying most of the growth in demand. The National Chicken Council approximated 34 lbs. per capita of poultry was consumed in 1960; for 2018, it was

reported that consumption had risen to about 110 lbs. per capita with 2019 and 2020 predictions being around 112 and 114 lbs. per capita, respectively (9). With an increase in consumption, this may provide the opportunity for a higher incidence of foodborne illness. This means that there is a need for more research on how to lessen the potential for foodborne illness to occur (transmission vehicles, hurdle technologies, etc.) and that research begins with gaining information on the pathogenic organisms that present a risk.

2. LITERATURE REVIEW

1.1 *Escherichia albertii*

Escherichia albertii is an emerging and under-studied pathogen that was first linked to diarrheal infections in young children *(1, 2, 21)*. The pathogen has been recovered from avian species (carcasses and living animals), potable water, and soil in both developing and industrialized countries *(20, 30, 33)*. Studies have shown that *E. albertii* has been misidentified as *E. coli, Salmonella enterica, Shigella* spp. and other Gram-negative bacteria, which could mean that *E. albertii* has been responsible for more foodborne illnesses than previously accounted for *(22, 41)*. Epidemiological research conducted on *E. albertii* relies heavily on its identification by using whole genome sequencing (WGS) and polymerase chain reaction (PCR), as there are no standard media in use for its identification, isolation, and enumeration. Its prevalence in U.S. food production and processing systems remains unknown.

1.2 Genotypic and Phenotypic Properties

When it was first discovered in Bangladesh by John Albert and his collaborators, *E. albertii* was thought to be an atypical *Hafnia alvei*, capable of operating in the manner of enteropathogenic *E. coli* (EPEC) isolates *(2)*. It was not until 1999, where studies revealed that the strains in question belonged to the genus *Escherichia (26)*. Researchers reported *E. albertii* as a Gram-negative non-motile rod with an oxidative and fermentative metabolism producing both acid and gas from glucose *(21)*. It was further described as cytochrome oxidase-negative and catalase-positive with growth after 24-hour incubation between 35–37°C on tryptic soy agar (TSA) *(21)*.

Hafnia alvei was suspected as a possible cause of the diarrheal infection in the child in the first case of disease caused by *E. albertii*, due to it being the only organism cultured with high colony count *(3)*. It was not until further analysis of the isolate that distinct differences between *H. alvei*, *E. coli*, and the case isolate of *E. albertii* were noted. For instance, *E. albertii* displays weak to moderate L-prolineaminopeptidase activity, versus *H. alvei*, which expresses extraordinarily strong activity in only thirty minutes *(3)*. *Escherichia albertii* can be differentiated from *E. coli* and other *Escherichia* species due to its inability to utilize a variety of carbohydrates: L-rhamnose, α-D-melibiose, D-xylose, D-cellobiose, D-sorbitol, D-arabitol, D-sucrose, D-glucoside, D-raffinose, lactose, dulcitol, salicin, amygdalin, erythritol, inositol, methyl, and adonitol *(26)*. *Escherichia albertii* tests positive for lysine and ornithine decarboxylases, methyl red reaction and nitrate reduction, but is negative for arginine dihydrolase, tryptophan production, DNase, urease, gelatinase and the Voges–Proskauer test *(1, 21)*. It shows no growth in potassium cyanide broth (KCN) and will utilize acetate as a carbon source, though it cannot use citrate or malonate *(1, 21)*. It produces acid from L-arabinose and D-mannitol *(1, 21)*. Its fermentation of D-maltose and D-trehalose is variable *(21)*.

Escherichia albertii belongs to a group of human pathogens capable of producing attaching and effacing (A/E) lesions in the colonized gastrointestinal tract; this group of pathogens includes the EPEC and enterohemorrhagic *E. coli* (EHEC) *(28, 41)*. The production of these lesions aid virulence of the pathogens for humans, furthering illness potential. This group of pathogens forms lesions on intestinal epithelial cell surfaces by the combined action of an outer membrane protein, an intimin-encoding *eae* gene, and type III secretion system (T3SS) effectors *(12)*. These groups of pathogens utilize T3SS to inject

effector proteins, such as translocated intimin receptor (Tir), directly into the host cell *(12)*. Translocated intimin receptor acts as a receptor for intimin *(45)*. The T3SS inclusive of Tir and intimin-encoding genes are in the locus of enterocyte effacement (LEE), a pathogenicity island *(45)*. While there are similarities between the two sets of genes, studies by Ooka et al. revealed several key differences between *E. albertii* and *E. coli (34)*. There was shown to be a size difference between *E. albertii* genomes ranging from 4.5-5.1 Mbp versus those of *E. coli*, ranging from 4.6-5.6 Mbp *(34)*. There are fewer copies of non-LEE effectors secreted by LEE-encoded T3SS produced by *E. albertii* as compared to EPEC and EHEC *(34)*. Studies also revealed a possible second T3SS in *E. albertii* that is inactivated in *E. coli (34)*.

The virulence capabilities of *stx2a* and STEC also harboring *eae* lesions are displayed through *E. albertii*'s ability to possess *stx2a* gene, indicating the pathogen's ability to causes severe diseases such as hemolytic uremic syndrome *(15)*. These pathogens can produce Shiga toxin due to a temperate lambdoid *stx*-phage that carries the toxin operon that includes the genes for toxin production from *Shigella* prophages to some *E. coli (4)*. A 48-year-old Norwegian patient infected with *stx2a*-positive *E. albertii* experienced bloody diarrhea, showing that *E. albertii* may produce clinical disease symptoms associated with severe illness *(15)*.

1.3 Recovery of *E. albertii* from Differing Geographies

A study completed by Oaks et al. examined the prevalence of *E. albertii* in dead birds from Alaska, Canada, Australia, and Scotland: redpoll finches, *Carduelis flammea*; chicken, *Gallus gallus*; gyrfalcon, *Falco rusticulos*; pine siskin, *Carduelis pinus*; magpie, *Gymnorhina tibicen*; honeyeater, *Melithreptus brevirostris*; wren, *cyaneus*; and fantail, *Rhipidura fulginosa (32)*. Data from virulence genes analyses revealed that all avian isolates,

mirroring clinical isolates reported by Hyma et al., were positive for *eae* and *cdtB* but negative for *stx1, stx2,* and *sta (23, 32)*. Multi-locus sequence typing (MLST) data provided three main clades of *E. albertii* that were based on nucleotide variation at 6 conserved housekeeping loci in the genome, as isolates were not grouped by origin, host type, and disease association *(32)*. Results from pulsed-field gel electrophoresis (PFGE) suggest the recovered isolates were due to single clone expansion *(32)*. Host related differences between isolates revealed that isolates from birds produced indole from tryptophan whereas clinical isolates were indole negative *(32)*. It was concluded *E. albertii* may be able to sub-clinically colonize various species of wild birds globally and can be associated with sporadic disease in bird populations *(32)*. Oh et al. *(33)* examined *eae*A-positive *E. coli* and *E. albertii* in wild birds from Korea to further determine their epidemiological characteristics. Between 2009 and 2010, 1,204 cloacal swab samples were collected from birds across different sites in Korea. Thirty-one *eaeA*-positive strains were identified as *E. coli* by biochemical testing using the VITEK 2 system (bioMérieux sa. 69280, France)*(33)*. Of those, 9 (29.0%) were then identified as *E. albertii* strains through the detection of genes *clpX, lysP*, and *mdh* by diagnostic multiplex PCR *(33)*.

Asoshima et al. investigated the incidence of *E. albertii* in chicken sashimi and raw chicken meat sold at retail stores in Fukuoka City, Japan *(13)*. A total of 220 samples over the course of five months in 2013 and two *Escherichia coli* (EC) broth-recovered cultures of raw chicken meat samples were PCR-positive for *eae*. After further analysis, *lysP*, *mdh,* and *clpX* genes were detected suggesting that *E. albertii* strains existed in the two raw chicken meat samples *(13)*.

Maheux et al. *(30)* aimed to characterize *E. fergusonii* and *E. albertii* isolated from potable water from Quebec, Canada, utilizing three housekeeping genes (*adk, gyrB,* and *recA*) for their ability to discriminate among *Escherichia* species. *E. albertii* isolates were identified as *E. coli, E. hermanii, E. fergusonii* or as low discriminatory organism with VITEK 2 analysis *(30)*. Using the Colilert® method (IDEXX Laboratories Canada Corp., Toronto, ON, Canada), approximately 86.5% of 527 isolates resulted in presumptive identification of *E. coli* due to their expression of either β-glucuronidase and/or *uidA* PCR *(21, 30)*. The remaining isolates were identified through 16S rRNA analysis. Further analysis for the identified *E. albertii, E. fergusonii*, and atypical *E. coli* strains showed the presence of intimin, cytolethal distending toxin (CDT) and Shiga toxin *(30)*. This research was able to determine the potential risk of *E. albertii* presence in environments shared by animals and humans as the presence of intimin, CDT, and Shiga toxin are important virulence factors regarding foodborne disease in humans.

A study conducted in Zigong City, China, investigated the prevalence of *eae*-positive, lactose non-fermenting *E. albertii* in raw red and poultry meats. Over the course of 15 months between 2013 and 2014, samples from beef, pork, mutton, chicken, and duck meat along with chicken and duck intestines were examined *(43)*. Results from the study showed about 6.73% of the samples were identified as contaminated with *eae*-positive, lactose non-fermenting *E. albertii*, with 56.7% isolated from chicken intestines and 20.0% from duck intestines *(43)*. These strains were positive for *E. albertii*-specific alleles of *lysP, mdh,* and the *clpX* genes. Thirty *E. albertii* strains were identified to fit within 10 distinct sequence types with a range of 99.7% to 100% similarity, with 99.6% identity to the *E. albertii* type strain LMG 2097 *(43)*.

1.4 Disease Epidemiology

As previously discussed, the incident that led to the discovery of *E. albertii* was due to a 9-month-old Bangladeshi girl. Her illness was report as experiencing vomiting, mild dehydration, fever, abdomen distention, and watery diarrhea that lasted 3 days in 2003 *(3)*. After rice-based rehydration fluids and a 20-hour hospital stay, the young girl was released.

In May of 2011, 48 people became ill after attending one of two parties hosted in a restaurant in Kumamoto, Japan. Infected individuals complained of diarrhea (83%), abdominal pain (69%), and nausea (29%) *(36)*. The average onset of infection was 19 h post-exposure. Analysis of fecal samples prematurely determined that *E. coli* O183:H18 and lactose negative, O and H antigens bearing, non-motile atypical strains of *E. coli* were the causative agents. The atypical strains that were *eae*-positive and lactose-utilization negative were further analyzed through biochemical and phenotypic methods. It was revealed that these strains exhibited similar properties to those listed for *E. albertii*: non-motility, inability to ferment xylose and lactose, and inability to produce β-D-glucuronidase *(36)*. A sample group of presumptive *E. albertii* strains underwent multilocus sequence typing (MLST) analysis and sequencing the *eae* gene for its intimin subtype. Results revealed strains had genetic features like *E. albertii*: intimin σ which is rarely identified in ETEC and STEC pathogroups; the LEE was integrated into the *pheU* tRNA gene; and the *cdtB* gene of the II/III/V subtypes were identified *(36)*. This group and others have previously reported intimin σ the *cdtB* gene of the II/III/V subtypes as differences in genetic features between *E. albertii* and *E. coli (32, 35)*. These subtypes are rarely seen in *eae*-positive *E. coli (14, 35).*

In 2015, there was a case of bacteremia in a 76-year-old woman living in residential care. She had a febrile illness: an oral temperature peaking at 38.7°C, tachycardia at 139

beats per minute, a respiratory rate of 26 breaths per minute, but no features of gastrointestinal infection *(24)*. It was also noted that she suffered convalescing from a pelvic fracture, she had an abnormal growth polyp of the gastric fundus, and she had epilepsy and hypertension *(24)*. Blood culture collected during her hospital stay were first assayed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (MALDI-TOF; Biotyper, Bruker Daltonik, Germany), and results presumptively identified *E. coli (24)*. Her recovery was assisted by treatments of piperacillin/tazobactam intravenously for 72 hours and then oral ciprofloxacin 500 mg twice daily *(24)*. Further analysis of blood cultures revealed discrepancies, as a molecular film array method (BioFire, bioMérieux, France) and 16 rRNA gene sequence was utilized to determine samples were consistent with reports of *E. albertii (15, 24)*. Due to the inability for routine identification of *E. albertii*, its epidemiological impact on public health and threat assessment to the food industry is widely unknown. This suggests a need for continued research on its structure, function, and mechanism of virulence as it is an emerging pathogen of concern to the industry.

1.5 Microbiological Tools for the Selective Enrichment and Detection of *E. albertii*

As previously stated, research must be done on potential food safety hazard risks to heavily consumed foods and food products. *E. albertii* has shown itself to be a potential risk to the poultry industry and consumer, and the development of an assay to aid in its identification is vital. One of the first steps in creating such an assay for detection and identification is developing media to ensure its recovery during sampling. A selective enrichment medium is purposed to provide a microorganism with the nutrients it needs for

growth while reducing competition from other microorganisms that may share a similar environment (i.e., sample tissue/fluid) or otherwise be present in the sample along with targeted organisms. The goal is to have a fluid sample of viable cells from a specific type or group of microorganisms be the dominant organism after an incubation period. Salts, detergents, and antimicrobial agents are often added to an enrichment medium to increase its selectivity. These agents can alter pH, enzymatic activity, and other important factors that promote the growth of the desired type of group of microorganisms while halting the growth of others.

Tryptic soy broth (TSB) is a commonly used enrichment fluid medium for bacterial growth support *(7)*. Both Gram-positive and Gram-negative bacteria can utilize the nutrients available *(7)*. Generally, TSB is composed of pancreatic digest of casein, papaic digest of soybean meal, sodium chloride, dipotassium hydrogen phosphate, and glucose. Both the casein and soybean meal serve as nitrogen sources. Sodium chloride is utilized as an osmo-stabilizing agent, while dipotassium hydrogen phosphate is used as acid-buffering agent. Glucose serves as a carbohydrate that is commonly used by many bacteria. These are mixed with water (typically distilled/deionized), and steam-sterilized for use. When needed, the addition of bile salts to a formulation of TSB will inhibit the growth of many Gram-positive bacteria and germinated spores, while allowing the growth of *Enterobacteriaceae*. This modified TSB (mTSB) is primarily used to select for *E. coli* and *Salmonella* species from environments where the bacteria are not isolated from other microorganisms.

The USDA Food Safety and Inspection Service (FSIS) and the Food and Drug Administration (FDA) utilize formulations of mTSB to recover foodborne pathogens from food and environmental samples. FSIS uses mTSB to selectively enrich meat, poultry, and

environmental samples in order to recover *Salmonella* (MLG 4.10) and STEC (MLG 5C) *(42)*. The formulation as provided by the FSIS Microbiology Laboratory Guidebook (MLG) also includes the addition of casamino-acids to mTSB as an additional source of nitrogen and novobiocin as an antibiotic *(42)*. The FDA Bacteriological Analytical Manual (BAM) incorporates four formulations of mTSB that utilize bile salts as the selective agent. Cell recovery is provided by agents such as ferrous sulfate, sodium chloride, sodium pyruvate, or yeast extract *(8)*.

1.6 Rationale and Significance

Despite the relatively few outbreaks, *E. albertii* presents a potential to function as a high-risk foodborne hazard, and there is need for continued research to determine effective ways to identify it from other enteric pathogens. Using conventional biochemical identification systems such as API 20E and Vitek, *E. albertii* has been falsely identified as *E. coli, H. alvei, Shigella, Salmonella,* and even *Yersinia* spp. *(1)*. *Escherichia albertii* may be responsible for a select fraction of the 38.4 million foodborne illnesses caused by unspecified agents *(39)*. Current studies are based mainly on reference strains, the presence of the *eae*-producing genes, lack of motility, and an inability to utilize a host of carbohydrates. However, phenotypic, and genetic descriptions are dependent on where the isolates are sourced *(43)*. Being that *E. albertii* closely resembles *E. coli*, a selective enrichment medium/process based around their biochemical differences may assist in its recovery from a food sample. Any microorganism serving as a potential food safety hazard needs to be distinguishable from others, as its recovery can aid in properly surveying microbiological foodborne hazards. For this reason, the development of a selective

enrichment for the recovery of *E. albertii* is vital to efficiently progress in research of this pathogen.

3. RESEARCH OBJECTIVES AND HYPOTHESIS

The purpose of this research was to develop a selective enrichment fluid medium to assist in the recovery of *E. albertii* from food tissue. The objectives of this research were: 1) to survey the biochemical and molecular properties of *Escherichia albertii*; and 2) to develop a selective enrichment for the isolation and identification of *Escherichia albertii*. It is believed that the development of a selective enrichment broth utilizing a combination of antibiotics will assist in the selective recovery and identification of *E. albertii* from inoculated food sample tissue.

4. METHODOLOGY

1.7 *E. albertii* Confirmation and Culture Preparation

E. albertii isolates were provided by Texas A&M University, the CDC, and Cornell University (Table 1). For isolates originating from the CDC and Cornell University, isolates were transferred to Texas A&M University under a material transfer agreement between the supplier and Texas A&M AgriLife. Organisms were revived from cryo-storage (-80°C) by aseptically inoculating a sterile 5.0 mL volume of TSB (Becton, Dickinson and Co., Sparks, MD) with a bacterial isolate, and then incubating at 37°C for 18-24 h. After the primary incubation, isolates were then individually aseptically transferred into new test tubes containing 5.0 mL sterile TSB and incubated for 18-24 h at 37°C. After the completion of the secondary incubation procedure, each isolate was aseptically streaked onto two individual tryptic soy agar plates (TSA; Becton, Dickinson and Co.) and incubated for 48 h at 37°C to isolate colonies. Post-incubation, isolated colonies underwent Gram staining to confirm if the isolate was a Gram-negative rod. Individual colonies from the second TSA plate were selected for analysis by Accugenix Microbial ID and Strain Typing (Charles River Laboratories, Wilmington, MA). Isolates confirmed as *Escherichia* spp. or *E. albertii* underwent the two-step culture revival process as previously mentioned. Isolated colonies were then inoculated into Sulfur Indole Motility medium (SIM; Hardy Diagnostics, Santa Maria, CA). Isolates identified as Gram-negative rods, negative for hydrogen sulfide (H_2S) production, and/or non-motile growth by the appearance of flagella were listed as *E. albertii*. After the second incubation period, isolates were aseptically streaked onto TSA slants, incubated for 48 h at 37°C, and then stored at 5°C until needed for use in subsequent experiments.

1.8 Non-*E. albertii* bacteria selection and preparation

Table 1 displays the organisms used in further experiments to assist in screening selective agents for a selective enrichment formulation and were obtained from several different sources: American Type Culture Collection (ATCC; Manassas, VA), TAMU Departments of Animal Science and Poultry Science, and USDA Agriculture Research Service (ARS). All the Gram-negative organisms were revived and stored in a similar fashion to the *E. albertii* isolates, whereas *Enterococcus faecium* NRRL B-2354, a Gram-positive organism, was revived from -80°C cryo-storage by aseptically inoculating a sterile 5 mL volume of TSB with a bacterial isolate and then incubating at 25°C for 18-24 h. After the primary incubation, the isolate was aseptically transferred to a fresh 5 mL of sterile TSB and then incubated at 25°C for 18-24 h. The following day, the isolate was aseptically streaked onto TSA slants, and incubated for 48 h at 37°C. All isolates were stored at 5°C until needed for use in subsequent experiments.

1.9 NARMS Panel Screening

To determine the antibiotic resistance of *E. albertii*, isolates underwent the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) panel screening (Thermo Fisher Scientific, Waltham, MA). The screening was used to determine which antibiotics could be utilized as selective agents in an enrichment broth, as antibiotics are commonly utilized as selective agents due to how they are able to stop vital functions necessary for cell growth and/or replication. The NARMS program functions as a partnership between the CDC, FDA, and USDA for monitoring antibiotic/antimicrobial resistance in pathogenic bacteria. Using a loop, 10.0 µL of bacteria was aseptically transferred to 5.0 mL of sterile TSB from a TSA slant. Cultures were incubated for 18-24 h

at 37°C and streaked for isolation onto TSA plates. After 24 h incubation at 37°C, three and five isolated colonies were picked and emulsified in 5.0 mL sterile deionized water and were adjusted to a 0.5 McFarland Standard. From the emulsification, 10.0 µL of the McFarland suspension was transferred into 11.0 ml volume of Muller Hinton broth (Becton, Dickinson and Co.). Next, 50.0 µL of the broth suspension was aseptically pipetted into a GN2F Sensititre Gram™ Negative Plate (Thermo Fisher Scientific, Waltham, MA).

The NARMS plate contained the following antibiotics at varying concentrations: amikacin, ampicillin, ampicillin/aulbactam 2:1 ratio, aztreonam, cefazolin, cefepime, cefotetan, ceftriaxone, ceftazidime, Cefuroxime, ciprofloxacin, gatifloxacin, meropenem, gentamicin, imipenem, nitrofurantoin, cefoxitin, piperacillin, piperacillin/tazobactam constant, ticarcillin/clavulanic acid, trimethoprim/sulfamethoxazole, tobramycin, and cefpodoxime. Once all the samples were loaded, plates were covered with the provided adhesive seal and then incubated at 36°C for 24 h. Wells were visually observed for turbidity compared to the positive and negative control wells. Positive control wells were produced by inoculating 50.0 µL of broth suspension into the wells labeled for the positive control. Sterile distilled water was used as negative controls. The NARMS Panel Screenings were completed in triplicate and antibiotic susceptibility for each isolate assayed was recorded.

1.10 Growth Kinetics

To determine how well various selective enrichment formulations would work, growth curves of the isolates were developed to evaluate growth over an 18-24 h period. Isolates 3033, 0065, 10457, 4085, and 3542 were chosen to serve as a representative group of *E. albertii* in the collection, based on weak or strong resistance to antibiotics observed during NARMS Panel trials (3033, 4085, 3542), the source of the isolate (0065 and 10457

were provided from different areas compared to all the CDC isolates), and to ensure that all varying isolate types were included in experimental trials.

From each of their respective TSA slants, 10.0 µL of each isolate was aseptically transferred into 10.0 mL of Luria-Bertani broth (LB; Becton, Dickinson and Co.) and incubated at 37°C for 18-24 hr. Next, 10.0 µL of the culture was added to 10.0 mL of sterile LB broth and vortexed. Then, 50.0 µL of the inoculated broth suspension was dispensed into a 96-well plate (Falcon®, Corning, Inc., Corning, NY). Plates were covered with the provided lid and placed into a BioTek® Cytation™ 5 (BioTek Instruments, Inc., Winooski, VT) plate reader. Using the BioTek® Gen 5 microplate reader software, the assay parameters were as follows: incubate at 37°C for 24 h with absorbance readings taken every 10 min at 600 nm, low orbital shaking for 10 sec prior to reading.

1.11 Antibiotic Stock Preparation

Results from the NARMS panel trials identified potential antibiotics that could be used in the development of a selective enrichment. Piperacillin, trimethoprim, and sulfamethoxazole were selected for further screening as *E. albertii* was observed to have the greatest resistance to varying concentrations of these antibiotics. To prepare stocks, 0.1 g of a selected antibiotic was mixed with 10.0 mL of an appropriate solvent until fully dissolved. Piperacillin was dissolved in 0.3mM of sodium hydroxide, trimethoprim was dissolved in 10% dimethyl sulfoxide (DMSO), and sulfamethoxazole was dissolved in 70% ethanol *(35)*. The solution was then filter sterilized into a 10.0 mL conical vial using a 0.45 mm syringe filter, resulting in a 10 mg/mL concentration of the antibiotic. These stocks were prepared weekly during trials and kept at 5°C to be used in subsequent experiments.

1.12 Antibiotic Resistance Testing in LB broth

To further assay the selected *E. albertii* isolates' resistance to the selected antibiotics (Section 4.5), 10.0 µL of culture from a TSA slant was aseptically transferred into 5 mL of LB broth and incubated for 18-24 h at 42°C. Antibiotic concentrations in LB broth were developed by taking the desired volume of the antibiotic stock and adding to the corresponding volume of sterile LB broth. Next, 50.0 µL volumes of the LB broth were added to a 96-well plate. One µL of inoculum from each overnight culture was added to each of the corresponding wells, respectively. The plate was covered with a lid and incubated in a similar fashion as the growth kinetics experiments previously described in 4.4.

1.13 Bile Salts Testing in LB broth

The *E. albertii* isolates 0065, 3033, 10457, 3542, and 4085 were exposed to two concentrations of bile salts: 1.5 g/L and 2.5 g/L. Bile salts are commonly used in selective enrichments for Gram-negative bacteria as it can thwart the growth of many Gram-positive organisms, yeasts, and molds. Both the FSIS and the FDA utilize 1.5 g/L of bile salts in their mTSB formulations to selectively enrich for *Salmonella enterica* spp. and STEC. Isolates were cultured overnight by aseptically transferring 10.0 µL of each isolate from a TSA slant into an individual tube of sterile 10.0 mL of LB broth and incubated at 37°C for 18-24 h. Two separate formulations of LB broth were prepared: one with 1.5 g/L bile salts and the second with 2.5 g/L bile salts. This would allow for the comparison of the utility of each concentration as a selective agent in a broth for *E. albertii.* For both formulations, the bile salts (Oxoid™, Thermo Fisher Scientific) were added along with the dehydrated LB broth mix to one liter of deionized water. The solution was then sterilized at 121°C for 15 min and cooled to room temperature. Next, 50.0 µL of each broth was added to a 96-well plate,

respectively. Using disposable sterile loops, 1.0 µL of the overnight culture was added to each of the corresponding wells. The plate was covered with a lid and incubated in a similar fashion as the growth kinetics experiments previously described in 4.4.

1.14 Selective Enrichment Medium Formulation

Two formulations were developed as potential selective enrichment media for *E. albertii*. Formulation A utilized 30 g of dehydrated TSB (Oxoid™, Thermo Fisher Scientific), 2.5 g of bile salts, 34.0 µg/mL sulfamethoxazole (Alfa Aesar, Haverhill, MA), and 4.0 µg/mL trimethoprim (Alfa Aesar). Formulation B utilized 30.0 g TSB, 2.5 g bile salts, 4.0 µg/mL of piperacillin (Alfa Aesar), and 4.0 µg/mL trimethoprim.

Sulfamethoxazole, 4-amino-N-(5-methyl-1,2-oxazol-3-yl) benzenesulfonamide, is a water-insoluble synthetic bacteriostatic antibiotic*(4)* . It inhibits the conversion of *p*-aminobenzoic acid by dihydropteroate synthase preventing the formation of dihydropteroic acid *(4)*. Trimethoprim, 5-[(3,4,5-trimethoxyphenyl)methyl]pyrimidine-2,4-diamine, belongs to the anisole class of antibiotics *(18)*. It is a synthetic derivative of trimethoxybenzyl-pyrimidine that works as an inhibitor bacterial dihydrofolate reductase by blocking the production of tetrahydrofolic acid from dihydrofolic acid *(18)*. Piperacillin, (2S,5R,6R)-6-[[(2R)-2-[(4-ethyl-2,3-dioxopiperazine-1-carbonyl)amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, is a broad-spectrum semisynthetic that inhibits bacterial cell growth by binding to enzymes that catalyze the synthesis of peptidoglycan *(18)*.

Both selective enrichments were prepared by mixing the dehydrated broth and bile salts with 1.0 L deionized water in a flask. After allowing the medium to boil, both media were sterilized at 121°C for 15 min. After sterilization, each selective enrichment was cooled

to room temperature and placed onto a magnetic hot plate with only the magnetic stirrer on low. Next, filter-sterilized solutions of the antibiotic combinations were dispensed into their respective selective enrichment broth. Following the inclusion of antibiotics, the pH of each selective enrichment formulation was recorded. Lastly, each broth was poured into its own sterile glass bottle and stored at 5°C until it needed to be used. Selective enrichment broths were only kept for 5-7 days before being disposed and remade.

1.15 Selective Enrichment Testing

To determine the efficiency of the two formulations developed, each *E. albertii* isolate (3033, 4085, 10457, 0065, and 3542) was enriched alongside other organisms (Table 1). All isolates were revived in 5.0 mL of TSB as described in 4.1. The two selective enrichment formulations were prepared as previously described and 10.0 mL of each broth was aseptically transferred into sterile test tubes. Next, 10.0 µL of each *E. albertii* isolate was individually inoculated into both broths, respectively. Then, 10.0 µL of one of each of the non-*E. albertii* organisms was inoculated into the same tubes the *E. albertii* isolates were inoculated in. The tubes were incubated at 42°C for 18-24 h, streaked onto TSA plates, and then the plates were incubated at 37°C for 24-48 h. Plates were observed for colony growth, individually wrapped with parafilm, and stored at 5°C for PCR confirmation and biochemical testing.

1.16 PCR Confirmation of Recovered Cells Using the Selective Enrichment Formulations

To determine which of the formulations would potentially work better as a selective enrichment, a multi-plex PCR assay was used to confirm isolated colony identity. The PCR assay was adapted from the multi-plex protocol developed by Lindsey et al. *(29)*. Individual

colonies from TSA plates were selected and individually inoculated into 5.0 mL LB broth. Samples were incubated at 25-37°C for *E. faecium* NRRL B-2354 for 18-24 h. The following day, 1.5 mL of overnight growth was used for PCR. Primers were purchased from Integrated DNA Technologies 2020 (Coralville, IA). Table 5 shows the gene targets, primer sequence, and amplicon size for each of *Escherichia* spp. used in the multiplex. Bacterium DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), which was performed according to the manufacturer protocol. Isolated DNA samples were amplified using Veriti® Thermal Cycler (Applied Biosystems, Thermo Fisher) and PCR kits supplied by HotStarTaq® Master Mix Kit (QIAGEN). The final reaction mixture contained 10.0 µL of lysate, 10.0 µL of 10x PCR buffer, 1.0 µL of 25.0 nM dNTP, 1.0 µL of each primer, and 0.5 µL HotStarTaq®. The PCR amplification was performed as described by Lindsey et al. *(29)*: one cycle at 95°C for 10 min; 30 cycles of 92°C for 1 min, 57°C for 1 min, and 72°C for 30 s; and one final cycle at 72°C for 5 min. 10.0 µL of PCR products were diluted in 3.0 µL of 6x purple gel loading dye and electrophoresed on a 1% agarose gel prepared with 1x Tris Acetate-EDTA buffer (TDA; VWR International, Avantor®, Radnor Township, PA). Electrophoresis was performed at a constant voltage of 100 V for 90 min, and the agarose gel was stained with ethidium bromide. Agarose gels were imaged under UV light using the Gel Doc™ XR + system (Bio-Rad, Hercules, CA) and analyzed for band sizes as compared to the 100 bp DNA ladder (New England BioLabs, Ipswich, MA).

1.17 Biochemical Confirmation

Due to unforeseen issues experienced during multiplex PCR trials; the decision was made to use a biochemical assay to select for *E. albertii* against the other organisms (Table 1) used throughout the project. Isolates were pulled from the TSA plates used to preserve

samples during selective enrichment testing (Section 4.9) and compared to controls that were assayed in identical fashion (Table 3).

MXgMAC is a novel medium currently in development for the selection and differential identification of *E. albertii*. It utilizes peptone, proteose peptone, bile salts, sodium chloride (NaCl), neutral red, and agar. The bile salt is inhibitory to Gram-positive microorganisms while the NaCl serves as osmotic stabilizer. The neutral red is a pH indicator. Melibiose (6-O-α-D-Galactopyranosyl-D-glucose) serves as the carbohydrate source and is only metabolized by some enteric and lactic acid bacteria and other microbes. The chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is used for the enzyme ß-galactosidase and forms an intense blue precipitate, serving as the differential agent for bacteria showing ß-galactosidase activity.

1.18 Data Analysis

NARMS Panel screening. Resistance was visually determined by growth in test wells as compared to positive and negative controls for each isolate. Wells positive for growth were recorded indicating the minimum concentrations where resistance was identified from over three trials. As many of the *E. albertii* isolates were shown to be susceptible to the panel of antibiotics, resistance levels were grouped to provide a general outlook of potential antibiotics to further screen rather than specific concentrations of each antibiotic. Low resistance was displayed by isolates showing between 1-33% growth, moderate resistance between 34-66% growth, and strong resistance between 67-100% growth.

Growth curve experiments. Trials were completed in triplicate for each isolate. Absorbance readings were subtracted from the baseline reading of the negative control and the means were plotted in Microsoft Excel (Microsoft Corp., Redmond, WA) against time

to develop a growth curve. Growth curves were visually observed for differences in the rate of growth over the 24 h period. For each sample, the exponential slope and R^2 value was recorded for growth during the log/exponential phase. Using the exponential curve fitting equation $y = Ae^{Bx}$, the value of B was used to determine doubling time in the equation $\mu = LN(2)/t_d$ where $B = \mu, the\ growth\ rate\ constant$ *(44)*. The duration of the stationary phase was also recorded for selective agent determination trials as an indicator of how long each isolate could withstand a lack of nutrients after growth *(17)*.

Selective Enrichment Testing I. Samples from the selective enrichment formulation trials were confirmed using PCR. Results from the multiplex PCR were analyzed using a Fisher's Exact Test to compare the selective enrichments. A *P*-value greater than 0.05 indicated no statistical difference between the two selective enrichment broths' effectiveness in selecting for *E. albertii*. Statistical analysis was completed using JMP (JMP® Pro 15, SAS Institute, Inc., Cary, NC).

Selective Enrichment Testing II. Biochemical confirmations testing was completed in triplicate and visually observed for resulting indicating positive results. *E. albertii* detection confirmations were based off results from positive controls from pure isolate cultures assayed in identical fashions as selective enrichment formulation trials. Table 5 provides the descriptions for positive *E. albertii*. Positive results were given the value of 1.0 and negative results were given the value of 0.0. These numerical values were then used to predict efficiency outcomes of antibiotics that could be used in a selective enrichment formulation.

5. RESULTS AND DISCUSSION

1.19 *E. albertii* Isolate Confirmation

The *E. albertii* isolates were cultured and underwent assaying to confirm their identities for subsequent study. First, isolates were Gram stained (Table 4); two *E. albertii* isolates, 9194 and 19982, initially stained Gram-positive, leading researchers to believe that those isolates were not *Escherichia* spp. Isolates were then sent off to Accugenix Microbial ID and Strain Typing (Table 5). Figures 1-6 provide the full identification reports for each isolate. Microbial ID confirmed that isolates 9194 and 19982 were not *Escherichia* spp. They were identified as *Listeria monocytogenes* and *Bacillus cereus*, respectively. Isolate 1823B was identified as *Enterobacteriaceae*. After their identification was determined to be primarily *Escherichia* spp., they were removed from the stock of *E. albertii*, and were not included in further studies. Isolate 0065 was identified as being either *E. coli, E. fergusonii, Shigella dysenteriae, Shigella flexneri,* or *Shigella sonnei*. Due the ability of *E. albertii* to be routinely misidentified as *E. coli*, 0065 was kept in the isolate stock. This would allow for data to be gathered on the likelihood of *E. albertii* to exhibit behaviors identical to *E. coli*.

Further assaying of the confirmed isolates revealed differences between clinical (human) and non-clinical isolates (plants, animals, water, etc.). All isolates were negative for H_2S production when inoculated on SIM medium. Of these isolates, 46.2% of isolates were positive for motility. Comparatively, 53.8% of the isolates were positive for indole production. *E. albertii* has been commonly reported to be negative for indole *(1)*. However, it has also been reported that *E. albertii* isolated from humans and wild birds can produce indole from tryptophan *(30-32)*.

1.20 Isolates NARMS Panel

To determine which antibiotics could be usefully incorporated into a selective enrichment broth formulation, *E. albertii* and the non-*albertii* isolates underwent a NARMS antibiotic resistance screening. Figure 7 provides the resistance of all isolates to 23 antibiotics on the panel. Weak to strong resistance was shown in *E. albertii* isolates to ampicillin, ampicillin/sulbactam 2:1 ratio, aztreonam, cefazolin, ceftriaxone, ceftazidime, and meropenem. However, trials also revealed *K. pneumoniae*, *E. faecium* NRRL B-2354, and *S.* Typhimurium 2582 displayed resistance to several antibiotics including those previously mentioned above. All isolates proved susceptible to the piperacillin/tazobactam antibiotic concentration combinations. Due to the poor overall resistance exhibited by *E. albertii* (Figure 7), antibiotics were selected based on at least three isolates showing resistance compared to no more than 2 of the non-*E. albertii* isolates showing resistance to those same antibiotics. Piperacillin and the trimethoprim/sulfamethoxazole combinations were the two groups of antibiotics that met that requirement. This provided three antibiotics that were then further assessed for their utility in a selective enrichment formulation.

1.21 *Escherichia albertii* Growth Curves

Bacteria have four major growth phases: lag, logarithmic/exponential, stationary, and death. Table 6 displays the growth kinetic parameters for *E. albertii* 3033, 3542, 4085, 0065 and 10457. Fresh cultures were grown in LB broth over a 24 h period. Growth rates and doubling times were calculated by dividing the value of the y-intercept (μ) with the natural log of two. Determining the y-intercept was done by plotting the optical densities (x-axis) against the time (y-axis) where the optical densities were seen to begin doubling. R^2 values between 0.94-1.00 were recorded to ensure at least three points were used in determining

values. The *E. albertii* growth curves from inoculated cultures in non-selective broth (Figures 20-24) were compared to the curves developed from selective agent exposure experiments. Isolates 0065 and 10457 had similar growth rate constants (0.1587 and 0.1184) compared to isolates 3033, 3542, 4085 (0.3547, 0.4142, and 0.4437). This led to 0065 and 10457 exhibiting doubling times between 24-43 min longer than the other isolates.

1.22 Selective Agent Determination

Potential selective enrichment formulations were determined by exposing isolates 3033, 3542, 4085, 0065, and 10457 to varying concentrations of selective agents. The antibiotics were chosen from the NARMS Panel screening. Bile salts are utilized in mTSB, so two concentrations of bile salts were screened. All selective agents were first screened separately in LB broth. *E. albertii* and non-*E. albertii* isolates were grown in two enrichments of LB broth, one containing bile salts at 1.5 g/L and another at 2.5 g/L, over a 24-hour growth period. The duration of logarithmic/exponential and stationary phases of growth were observed (Figures 25-38), and doubling times were calculated in similar fashion to the growth curve data.

The stationary phase of bacterial growth can be defined as the phase of growth following log/exponential phase where the OD is constant due the exhaustion of nutrients *(17)*. During the stationary phase, the number of viable cells is consistent with the relationship between the rate of bacterial cell growth and the rate of cell death *(25)*. Tables 7-11 display those results. It was observed that stationary phases were shorter for higher concentrations of antibiotics, especially 76.0 µg/mL sulfamethoxazole, where isolates almost immediately entered death phase after exponential phase. The longer the stationary phase was, the better equipped the organism maintains stability after nutrient availability

is diminished *(17)*. Stationary phase is understood to be a state that bacteria have been able to adapt to being due to how uncommon perfect growth conditions are in nature. Adaptions to maintain stability prior to death phase include but are not limited to cell size reduction and changes to gene expression *(25)*.

Exposure to trimethoprim resulted in stationary phases that lasted upwards of 6.0 h for *E. albertii* isolates, which led to the decision to use a concentration 4.0 µg/mL in formulations. This decision was due in part to needing to develop a combination of antibiotics that would not cause an overload of stress on *E. albertii*. The utilization of 34 µg/mL sulfamethoxazole produced logarithmic and stationary phases lasting upwards of 5.0 h in *E. albertii* isolates. Exposure to all concentrations of piperacillin produced uniformity in growth rates among the *E. albertii* isolates at 4.0 µg/mL. As other formulations of mTSB already utilize 1.5 g/L of bile salts, the decision was made to increase the bile concentration from 0.15% to 0.25% to further ensure Gram-positive organisms from being able to replicate during enrichment.

1.23 Selective Enrichment Formulation

Two selective enrichment broths were developed as potential options to aid in the selection of *E. albertii*. Selective enrichment formulation A was made with 30.0 g TSB, 2.5 g/L bile salts, 34.0 µg/mL sulfamethoxazole, and 4.0 µg/mL trimethoprim. Formulation B contained 30 g TSB, 2.5 g/L bile salts, 4.0 µg/mL of piperacillin, and 4.0 µg/mL trimethoprim. The mean pH of the media was 7.07±0.01and 7.09±0.01, respectively. The pH values of the selective enrichment broths were shown to be significantly different at 95% confidence (p=0.001) by two-tailed t-test for unequal variances. However, it is suggested that a 0.02 difference in pH was not biologically significant.

Figures 9 and 10 depict the results of the 24 h growth trials conducted using each selective enrichment broth. Formulation A produced longer doubling times for *E. albertii*, *S. enterica*, and *K. pneumoniae* isolates than Formulation B. Assumptions made by Gibson et al. suggest that stressors in the environment cause bacteria to behave differently, increasing their doubling time *(18)*. Selective agents apply stress to bacteria during growth, so the assumption could be applied here with the two formulations when compared to *E. albertii* grown in a non-selective enrichment (Table 11). Both formulations relied on equal amounts of trimethoprim (4 µg/L) and bile salts (2.5 g/L) as stressors for the isolates. Sulfamethoxazole (34 µg/L; Formulation A) and piperacillin (4 µg/L; Formulation B) were the differences in stressors for each selective enrichment. Sulfamethoxazole's ability to prevent dihydropteroic acid formation aids in the hindrance of folic acid which is vital to bacterial growth *(6)*. In Formulation B, the stressor is due to piperacillin halting peptidoglycan synthesis which then halts cell wall synthesis *(11)*.

1.24 Multiplex PCR Confirmation

A subset of samples from the selective enrichment test trials were confirmed using a multiplex PCR. Results from the PCR proved there was no significant difference between the two selective enrichment formulations (p =0.4775) at a 95% confidence interval. Of the samples, 15.7% and 9.68% were confirmed as *E. albertii* from being inoculated in Selective enrichments A and B, respectively. Selective enrichment B produced one positive confirmation for *E. coli*. However, that sample was isolate 0065 paired with isolate TDCC47 (*S. enterica*). This led to concerns about the accuracy of the multiplex protocol. It was then discovered that procedural issues such as primer dimerization, DNA concentration, and DNA purity may have introduced opportunities for errors for the PCR amplification and

electrophoresis. The issues with PCR led to the decision to incorporate biochemical testing to gather more data on the efficiency of the selective enrichment formulations.

1.25 Biochemical Confirmation

To determine the efficiency of the selective enrichment formulations, *E. albertii* isolates were paired with non-*E. albertii* isolates (Table 1) and inoculated into both Selective enrichment broths A and B. After a 24-hour incubation period at 42°CC, samples were streaked onto TSA and incubated. The following day, individual colonies were inoculated in sterile tubes of LB for further assaying with various biochemical assays. Samples inoculated into selective enrichment A resulted in 51.3$\pm$0.27% positive *E. albertii* confirmations while selective enrichment B resulted in 44.87$\pm$0.34% positive confirmations. Table 12 displays the results of the positive *E. albertii* by the medium used for confirmation. These results show that selective enrichment A was between 17-26% better at selecting *E. albertii* against *E. coli, E. fergusonii, E. faecium,* and *K. pneumoniae* than selective enrichment B. However, enrichment B was more effective at selecting *E. albertii* against *Salmonella enterica.* As *E. albertii* is commonly misidentified as *E. coli*, a selective enrichment that is more effective in selecting for *E. albertii* over *E. coli* would provide utility in an assay designed to select and identify *E. albertii* from food tissue.

Sulfamethoxazole and trimethoprim (SXT) are commonly used together in clinical environments *(6, 10, 19)*. These antibiotics work as a bacteriostatic when alone, but together they are bactericidal by blocking two steps in the biosynthesis of essential nucleic acids and protein *(27)*. The combination of sulfamethoxazole and trimethoprim is commonly used to treat a wide variety of bacterial illness such as traveler's diarrhea which is caused by ETEC *(16, 27)*. As SXT is used to aid in fecal illnesses, this means that it works against fecal

pathogens such as STEC, making combinations of the antibiotics useful in a selective enrichment. The efficiency together could explain why formulation A resulted in more positive *E. albertii* confirmations.

1.26 Further Observations of *E. albertii* Isolates

The five isolates selected for use in selective enrichment formulation trials further revealed differences between isolates of *E. albertii*. These isolates were acquired from different places (Table 1). Isolate 0065 was identified as *E. coli* (Table 5, Figure 1) through microbial ID but was kept in the entire sample of isolates due to the understanding that *E. albertii* can commonly be misidentified. Isolates 3033, 3542, and 4085 (Table 5, Figure 3) were a part of the group of clinical isolates provided by the CDC and were identified as *E. albertii* along with other *Escherichia* spp. and *Shigella* spp. Isolate 10457 (Table 5, Figure 4) was the only isolate to be confirmed as *E. albertii*. Throughout the remaining experiments it was routinely observed that 0065 and 10457 provided similar results, leading investigators to believe that 0065 was indeed *E. albertii*.

Isolate 0065 was then routinely observed for distinct characteristics of *E. coli*. SIM testing revealed that isolates 0065, 10457, 3542, and 4085 all possess the enzyme tryptophanase. Isolates 0065, 10457, 3033, and 3542 all were positive for motility which does not support the various reports of *E. albertii* being a non-motile organism. These are all phenotypic properties of *E. coli*, however biochemical testing of the isolates (Table 3) revealed that these isolates produce different morphologies when assayed on the plating medium designed to differentiate *E. coli* (specifically STEC) from other organisms. When tested, isolates 0065 and 10457 produced similar morphologies on *E. coli* count plates and

MXgMAC agar. These differed from isolates 3033, 3542, and 4085 who produced different morphologies than the STEC organisms tested on those same media.

6. CONCLUSION

Making selective enrichment media available to assist in the identification of foodborne pathogens is vital to properly survey their potential impact on public health, since they can be better detected within the food supply. Collecting accurate data on outbreaks of foodborne illness can lead to better prevention of them. This research had the goal of producing a novel selective enrichment to select for *E. albertii*, as it is commonly misidentified as other pathogens such as *E. coli*. Microbial ID and biochemical testing of isolates displayed that *E. albertii* shares a variety of similar characteristics with many other pathogens, like *E. coli*. This furthered proved that there is need to develop a selective enrichment for its identification.

The novel formulations presented in this research utilized combinations of antibiotics and bile salts aimed to slow and/or stop the growth of the non-*E. albertii* enteric bacteria. Screening of antibiotic resistance identified sulfamethoxazole, trimethoprim, and piperacillin as potential selective agents. Modified TSB is frequently utilized to selectively enrich *Salmonella* spp. and STEC, so bile salts were also incorporated into the formulation. Concentrations of these selective agents were chosen for combinations when 24 h growth periods were assessed. Specific attention was paid to logarithmic/exponential and stationary phases of growth.

A multiplex PCR was used as a primary method for confirming the identification of *E. albertii* from non-*E. albertii* organisms. Due to troubleshooting issues with the multiplex, several biochemical assays were enlisted to provide secondary identity confirmation when multiplex PCR identification was confounded. Neither the PCR nor the biochemical assays resulted in statistically different results in selective enrichment formulations A and B, but

Formulation A produced higher percentages of positive confirmations of *E. albertii*. Selective enrichment A was also shown to provide better selectivity of *E. albertii* against *E. coli*. These two pathogens share many characteristics, so there is more utility in using and refining the formulation for selective enrichment A over enrichment B.

7. REFERENCES

1. Abbott, S. L., J. O'Connor, T. Robin, B. L. Zimmer, and J. M. Janda. 2003. Biochemical properties of a newly described Escherichia species, Escherichia albertii. *J Clin Microbiol*. 41:4852-4.

2. Albert, M. J., K. Alam, M. Ansaruzzaman, J. Montanaro, M. Islam, S. M. Faruque, K. Haider, K. Bettelheim, and S. Tzipori. 1991. Localized adherence and attaching-effacing properties of nonenteropathogenic serotypes of Escherichia coli. *Infection and Immunity*. 59:1864-1868.

3. Albert, M. J., K. Alam, M. Islam, J. Montanaro, A. S. Rahaman, K. Haider, M. A. Hossain, A. K. Kibriya, and S. Tzipori. 1991. Hafnia alvei, a probable cause of diarrhea in humans. *Infect Immun*. 59:1507-13.

4. Allison, H. E. 2007. Stx-phages: drivers and mediators of the evolution of STEC and STEC-like pathogens. *Future Microbiol*. 2:165-74.

5. Anonymous. Date, Foodborne Illness: What Consumers Need to Know. Available at: https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/foodborne-illness-and-disease/foodborne-illness-what-consumers-need-to-know/ct_index. Accessed.

6. Anonymous. Date, PubChem Compound Summary for CID 5329, Sulfamethoxazole Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Sulfamethoxazole. Accessed September 18, 2020.

7. Anonymous. Date, Tryptic Soy Broth. Available at: https://assets.fishersci.com/TFS-Assets/LSG/manuals/IFU112730.pdf. Accessed, 2020.

8. Anonymous. 1998. Bacteriological Analytical Manual *In* F.a.D. Administration (ed.).

9. Anonymous. Date, 2019, Per Capita Consumption of Poultry and Livestock, 1960 to Forecast 2021, in Pounds. Available at: https://www.nationalchickencouncil.org/about-the-industry/statistics/per-capita-consumption-of-poultry-and-livestock-1965-to-estimated-2012-in-pounds/. Accessed.

10. Anonymous. Date, 2020, PubChem Compound Summary for CID 5578, Trimethoprim. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Trimethoprim#section=Drug-and-Medication-Information. Accessed 2020, September 17.

11. Anonymous. Date, 2020, PubChem Compound Summary for CID 43672, Piperacillin. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Piperacillin. Accessed September 17, 2020.

12. Asoshima, N., M. Matsuda, K. Shigemura, M. Honda, H. Yoshida, H. Hiwaki, K. Ogata, and T. Oda. 2014. Identification of Escherichia albertii as a Causative Agent of a Food-Borne Outbreak Occurred in 2003. *Japanese Journal of Infectious Diseases*. 67:139-140.

13. Asoshima, N., M. Matsuda, K. Shigemura, M. Honda, H. Yoshida, T. Oda, and H. Hiwaki. 2015. Isolation of Escherichia albertii from Raw Chicken Liver in Fukuoka City, Japan. *Japanese Journal of Infectious Diseases*. 68:248-250.

14. Blanco, M., S. Schumacher, T. Tasara, C. Zweifel, J. E. Blanco, G. Dahbi, J. Blanco, and R. Stephan. 2005. Serotypes, intimin variants and other virulence factors of

eae positive Escherichia coli strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (eae-eta2). *BMC microbiology*. 5:23-23.

15. Brandal, L. T., H. S. Tunsjø, T. E. Ranheim, I. Løbersli, H. Lange, and A. L. Wester. 2015. Shiga Toxin 2a in Escherichia albertii. *Journal of Clinical Microbiology*. 53:1454-1455.

16. Ericsson, C. D., H. L. DuPont, J. J. Mathewson, M. S. West, P. C. Johnson, and J. A. M. Bitsura. 1990. Treatment of Traveler's Diarrhea With Sulfamethoxazole and Trimethoprim and Loperamide. *JAMA*. 263:257-261.

17. Gefen, O., O. Fridman, I. Ronin, and N. Q. Balaban. 2014. Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. *Proceedings of the National Academy of Sciences*. 111:556-561.

18. Gibson, B., D. J. Wilson, E. Feil, and A. Eyre-Walker. 2018. The distribution of bacterial doubling times in the wild. *Proceedings. Biological sciences*. 285:20180789.

19. Gleckman, R., S. Alvarez, and D. W. Joubert. 1979. Drug therapy reviews: Trimethoprim-sulfamethoxazole. *American Journal of Hospital Pharmacy*. 36:893-906.

20. GrillovÁ, L., I. SedlÁČEk, G. PÁChnÍKovÁ, E. StaŇKovÁ, P. ŠVec, P. HolochovÁ, L. MicenkovÁ, J. BosÁK, I. SlaninovÁ, and D. ŠMajs. 2018. Characterization of four Escherichia albertii isolates collected from animals living in Antarctica and Patagonia. *The Journal of Veterinary Medical Science*. 80:138-146.

21. Huys, G., M. Cnockaert, J. M. Janda, and J. Swings. 2003. Escherichia albertii sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. *International Journal of Systematic and Evolutionary Microbiology*. 53:807-810.

22. Hyma, K., D. Lacher, A. Nelson, A. Bumbaugh, J. M. Janda, N. Strockbine, V. Young, and T. Whittam. 2005. Evolutionary genetics of a new pathogenic Escherichia species: Escherichia albertii and related Shigella boydii strains. *Journal of bacteriology*. 187:619-628.

23. Hyma, K. E., D. W. Lacher, A. M. Nelson, A. C. Bumbaugh, J. M. Janda, N. A. Strockbine, V. B. Young, and T. S. Whittam. 2005. Evolutionary Genetics of a New Pathogenic Escherichia Species: Escherichia albertii and Related Shigella boydii Strains. *Journal of Bacteriology*. 187:619-628.

24. Inglis, T. J. J., A. J. Merritt, N. Bzdyl, S. Lansley, and M. N. Urosevic. 2015. First bacteraemic human infection with Escherichia albertii. *New Microbes and New Infections*. 8:171-173.

25. Jaishankar, J., and P. Srivastava. 2017. Molecular Basis of Stationary Phase Survival and Applications. *Frontiers in Microbiology*. 8.

26. Janda, J. M., S. L. Abbott, and M. J. Albert. 1999. Prototypal diarrheagenic strains of Hafnia alvei are actually members of the genus Escherichia. *J Clin Microbiol*. 37:2399-401.

27. Kemnic, T., and M. Coleman. Date, 2020, Trimethoprim Sulfamethoxazole. Available at: https://www.ncbi.nlm.nih.gov/books/NBK513232/. Accessed, 2020.

28. Konno, T., J. Yatsuyanagi, S. Takahashi, Y. Kumagai, E. Wada, M. Chiba, and S. Saito. 2012. Isolation and Identification of Escherichia albertii from a Patient in an Outbreak of Gastroenteritis. *Japanese Journal of Infectious Diseases*. 65:203-207.

29. Lindsey, R. L., L. Garcia-Toledo, D. Fasulo, L. M. Gladney, and N. Strockbine. 2017. Multiplex polymerase chain reaction for identification of Escherichia coli,

Escherichia albertii and Escherichia fergusonii. *Journal of microbiological methods*. 140:1-4.

30. Maheux, A. F., D. K. Boudreau, M. G. Bergeron, and M. J. Rodriguez. 2014. Characterization of Escherichia fergusonii and Escherichia albertii isolated from water. *Journal of Applied Microbiology*. 117:597-609.

31. Nimri, L. 2013. Escherichia albertii, a newly emerging enteric pathogen with poorly defined properties. *Diagnostic microbiology and infectious disease*. 77:91-95.

32. Oaks, J. L., E. B. Thomas, T. W. Seth, M. G. David, B. B. Kimberlee, A. B. Kathy, J. H. Gary, S. B. Dan, O. Lindsey, R. R. Fred, A. D. Margaret, D. Greg, and S. W. Thomas. 2010. Escherichia albertii in Wild and Domestic Birds. *Emerging Infectious Disease journal*. 16:638.

33. Oh, J.-Y., M.-S. Kang, H.-T. Hwang, B.-K. An, J.-H. Kwon, and Y.-K. Kwon. 2011. Epidemiological investigation of eaeA-positive Escherichia coli and Escherichia albertii strains isolated from healthy wild birds. *The Journal of Microbiology*. 49:747-752.

34. Ooka, T., Y. Ogura, K. Katsura, K. Seto, H. Kobayashi, K. Kawano, E. Tokuoka, M. Furukawa, S. Harada, S. Yoshino, J. Seto, T. Ikeda, K. Yamaguchi, K. Murase, Y. Gotoh, N. Imuta, J. Nishi, T. A. Gomes, L. Beutin, and T. Hayashi. 2015. Defining the Genome Features of Escherichia albertii, an Emerging Enteropathogen Closely Related to Escherichia coli. *Genome Biology and Evolution*. 7:3170-3179.

35. Ooka, T., K. Seto, K. Kawano, H. Kobayashi, Y. Etoh, S. Ichihara, A. Kaneko, J. Isobe, K. Yamaguchi, K. Horikawa, T. A. T. Gomes, A. Linden, M. Bardiau, J. G. Mainil, L. Beutin, Y. Ogura, and T. Hayashi. 2012. Clinical Significance of Escherichia albertii. *Emerging Infectious Diseases*. 18:488-492.

36. Ooka, T., E. Tokuoka, M. Furukawa, T. Nagamura, Y. Ogura, K. Arisawa, S. Harada, and T. Hayashi. 2013. Human Gastroenteritis Outbreak Associated with Escherichia albertii, Japan. *Emerging Infectious Diseases*. 19:144-146.

37. Painter, J., R. Hoekstra, T. Ayers, R. Tauxe, C. Braden, F. Angulo, and P. Griffin. 2013. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbreak Data, United States, 1998–2008. *Emerging Infectious Disease journal*. 19:407.

38. Sandra Hoffmann, B. M., and Michael Batz 2015. Economic Burden of Major Foodborne Illnesses Acquired in the United States. *In* United States Department of Agriculture, Economic Research Service.

39. Scallan, E., P. Griffin, M., F. J. Angulo, R. V. Tauxe, and R. M. Hoekstra. 2011. Foodborne Illness Acquired in the United States—Unspecified Agents. *Emerging Infectious Disease journal*. 17:16.

40. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Disease journal*. 17:7.

41. Sharma, M., K. E. Kniel, A. Derevianko, J. Ling, and A. A. Bhagwat. 2007. Sensitivity of Escherichia albertii, a Potential Food-Borne Pathogen, to Food Preservation Treatments. *Applied and Environmental Microbiology*. 73:4351-4353.

42. Staff, L. Q. A. 2013. Media and Reagents. *In* U.D.o. Agriculture (ed.) Food Safety and Inspection Service, Athens, Georgia.

43. Wang, H., Q. Li, X. Bai, Y. Xu, A. Zhao, H. Sun, J. Deng, B. Xiao, X. Liu, S. Sun, Y. Zhou, B. Wang, Z. Fan, X. Chen, Z. Zhang, J. Xu, and Y. Xiong. 2016. Prevalence of

eae-positive, lactose non-fermenting Escherichia albertii from retail raw meat in China. *Epidemiology and Infection*. 144:45-52.

44. Widdel, F. 2007. Theory and Measurement of Bacterial Growth 1-11.

45. Yamamoto, D., R. T. Hernandes, A. M. A. Liberatore, C. M. Abe, R. B. de Souza, F. T. Romão, V. Sperandio, I. H. Koh, and T. A. T. Gomes. 2017. Escherichia albertii, a novel human enteropathogen, colonizes rat enterocytes and translocates to extra-intestinal sites. *PLoS ONE*. 12:e0171385.

8. APPENDIX A: TABLES

Table A.1 Pathogen Isolate List		
Organism	Isolate Identification	Source
Escherichia albertii	ATCC 10457, ATCC 9194, ATCC 19982	Texas A&M Department (Dept) of Animal Science (ANSC)
	0065	Cornell University
	3866, 4015, 4750, 4180, 4143, 3542, 1823B, 4312, 4085, 5188, 3449, 3033	CDC
Escherichia coli O103:H11	BAA2215	ATCC
Escherichia coli O157:H7	P41	TAMU Dept of ANSC
	2488	USDA ARS
Escherichia fergusonii	85469	ATCC
Salmonella enterica	Typhimurium 2582	ATCC
	Typhimurium TDCC47	TAMU Dept of Poultry Science
	Enteritidis	
Enterococcus faecium	NRRL B-2354	ATCC
Klebsiella pneumoniae		TAMU Dept of ANSC

Table A.2 *Escherichia* spp. Primer Information			
Organism	Target Genes	Primer sequence (5′ to 3′)	Amplicon Size
E. albertii	*cdgR*, AW869_22935 from MG1655, CP014225	GTAAATAATGCTGGTCAGACGTTA AGTGTAGAGTATATTGGCAACTTC	393 bp
E. coli	EAKF1_ch4033 from genome KF1, CP007025	CCAGGCAAAGAGTTTATGTTGA GCTATTTCCTGCCGATAAGAGA	212 bp
E. fergusonii	EFER_0790 from genome ATCC 35469, CU928158	AGATTCACGTAAGCTGTTACCTT CGTCTGATGAAAGATTTGGGAAG	757 bp

Table A.3 Biochemical Identification for a *E. albertii* Confirmation					
Medium Used	Typical Use	Positive	Isolate Tested	Negative	Isolate Tested
ECC Plate	Enumeration of *E. coli* coliforms	Red colonies/film Gas or no gas production	*Ea* 3033, *Ea* 3542, *Ea* 4085	Reddish purple colonies/film with gas production	STEC
		bluish-purple colonies/film with no gas production	*Ea* 0065, *Ea* 10457		
Sorbitol MacConkey Agar	Detection of *E. coli*	Yellow agar with pale colonies	*Ea* 3542	Pink mucoid colonies	*K. pneumonia*
		pink non-mucoid colonies	*Ea* 10457		
Purple Broth + 5% Xylose	Carbohydrate Fermentation Test	Purple broth color	*Ea* 3033, *Ea* 3542, *Ea* 4085	Yellow broth color	*E. fergusonii*
		yellow broth color	*Ea* 0065, *Ea* 10457		
XLT4	Identification of *Salmonella* spp.	Pinkish-white colonies	*Ea* 3033, *Ea* 3542, *Ea* 4085	Black colonies	*S*. Tphymirurim
		yellow agar with small white colonies	*Ea* 0065, *Ea* 10457	White colonies with black centers	*S*. Enteritidis
MXgMAC	Identification of *E. albertii*	Colorless colonies	*Ea* 3033, *Ea* 4085	Dark blue with a halo	P41
		light blue colonies	*Ea* 3542	pinkish-purple with a dark center	2488, O103
		dark blue colonies with a halo	*Ea* 0065, *Ea* 10457	pink colonies	*Salmonellae*
Kenner Fecal Streptococcus agar without antibiotic supplement	Enumeration of *Enterococcus* spp.	No growth	*Ea* 3033, *Ea* 4085, *Ea* 0065	Yellow agar with white colonies	*E. faecium*

Table A.4 *E. albertii* Gram Staining Result			
Isolate	Result	Isolate	Result
0065	Gram-	4085	Gram-
10457	Gram-	4143	Gram-
1823B	Gram-	4180	Gram-
3033	Gram-	4312	Gram-
3499	Gram-	4750	Gram-
3542	Gram-	5188	Gram-
3866	Gram-	**9194**	**Gram+**
4015	Gram-	**19982**	**Gram+**

Table A.5 Growth Kinetic Parameters for *E. albertii* Isolates Cultured in LB broth at 37℃ for 24 hours

Isolate	R^2	μ	t_d	min
0065	0.998	0.159	4.368	**43.68**
10457	0.983	0.118	5.854	**58.54**
4085	0.943	0.355	1.954	**19.54**
3542	0.964	0.414	1.673	**16.73**
3033	0.962	0.444	1.562	**15.62**

R^2 values were recovered from linear regression equations determining the maximum specific growth rate from the log-linear component of the growth curve plotted from mean optical densities from three identically completed replicates for each isolate. The growth rate constant (μ) was determined by the exponential curve fitting equation $y = Ae^{Bx}$, where B is the growth factor. This value was then used to determine doubling time where t_d=LN(2)/μ

TABLE A.6 GROWTH KINETIC PARAMETERS FOR *E. ALBERTII* 0065 CULTURED IN SELECTIVITY ENRICHED LB BROTH AT 42℃ FOR 24 HOURS

SELECTIVE AGENT	R^2	μ	t_d	min
SULFAMETHOXAZOLE 76	0.973	0.240	2.888	28.88
SULFAMETHOXAZOLE 34	0.987	0.261	2.652	26.52
SULFAMETHOXAZOLE 19	0.999	0.276	2.516	25.16
SULFAMETHOXAZOLE 10	0.987	0.238	2.912	29.12
TRIMETHOPRIM 4	0.998	0.205	3.386	33.86
TRIMETHOPRIM 2	0.994	0.313	2.212	22.12
TRIMETHOPRIM 1	0.996	0.357	1.943	19.43
TRIMETHOPRIM 0.5	0.997	0.334	2.075	20.75
PIPPERACILIN 16	0.996	0.215	3.221	32.21
PIPPERACILIN 8	0.997	0.306	2.267	22.67
PIPPERACILIN 6	1.000	0.272	2.549	25.49
PIPPERACILIN 4	0.999	0.3162	2.192	21.92
BILE SALTS 2.5	0.948	0.022	31.08	310.83
BILE SALTS 1.5	0.933	0.026	26.26	262.56

R^2 values were recovered from linear regression equations determining the maximum specific growth rate from the log-linear component of the growth curve plotted from mean optical densities from three identically completed replicates for each isolate. The growth rate constant (μ) was determined by the exponential curve fitting equation $\boldsymbol{y = Ae^{Bx}}$, where B is the growth factor. This value was then used to determine doubling time where t_d=LN(2)/μ

TABLE A.7 GROWTH KINETIC PARAMETERS FOR *E. ALBERTII* 10457 CULTURED IN SELECTIVITY ENRICHED LB BROTH AT 42℃ FOR 24 HOURS

SELECTIVE AGENT	R^2	μ	t_d	min
SULFAMETHOXAZOLE 76	1.000	0.265	2.613	26.13
SULFAMETHOXAZOLE 34	0.999	0.272	2.547	25.47
SULFAMETHOXAZOLE 19	0.997	0.252	2.751	27.51
SULFAMETHOXAZOLE 10	1.000	0.259	2.674	26.74
TRIMETHOPRIM 4	0.999	0.234	2.962	29.62
TRIMETHOPRIM 2	1.000	0.310	2.240	22.40
TRIMETHOPRIM 1	0.996	0.316	2.194	21.94
TRIMETHOPRIM 0.5	0.999	0.260	2.667	26.67
PIPPERACILIN 16	0.991	0.310	2.237	22.37
PIPPERACILIN 8	0.999	0.378	1.833	18.33
PIPPERACILIN 6	1.000	0.207	3.355	33.55
PIPPERACILIN 4	0.999	0.323	2.147	21.47
BILE SALTS 2.5	0.991	0.127	5.466	54.66
BILE SALTS 1.5	0.991	0.347	2.000	20.00

R^2 values were recovered from linear regression equations determining the maximum specific growth rate from the log-linear component of the growth curve plotted from mean optical densities from three identically completed replicates for each isolate. The growth rate constant (μ) was determined by the exponential curve fitting equation $y = Ae^{Bx}$, where B is the growth factor. This value was then used to determine doubling time where t_d=LN(2)/μ

TABLE A.8 GROWTH KINETIC PARAMETERS FOR *E. ALBERTII* 10457 CULTURED IN SELECTIVITY ENRICHED LB BROTH AT 42℃ FOR 24 HOURS

SELECTIVE AGENT	R^2	μ	t_d	min
SULFAMETHOXAZOLE 76	1.000	0.265	2.613	26.13
SULFAMETHOXAZOLE 34	0.999	0.272	2.547	25.47
SULFAMETHOXAZOLE 19	0.997	0.252	2.751	27.51
SULFAMETHOXAZOLE 10	1.000	0.259	2.674	26.74
TRIMETHOPRIM 4	0.999	0.234	2.962	29.62
TRIMETHOPRIM 2	1.000	0.310	2.240	22.40
TRIMETHOPRIM 1	0.996	0.316	2.194	21.94
TRIMETHOPRIM 0.5	0.999	0.260	2.667	26.67
PIPPERACILIN 16	0.991	0.310	2.237	22.37
PIPPERACILIN 8	0.999	0.378	1.833	18.33
PIPPERACILIN 6	1.000	0.207	3.355	33.55
PIPPERACILIN 4	0.999	0.323	2.147	21.47
BILE SALTS 2.5	0.991	0.127	5.466	54.66
BILE SALTS 1.5	0.991	0.347	2.000	20.00

R^2 values were recovered from linear regression equations determining the maximum specific growth rate from the log-linear component of the growth curve plotted from mean optical densities from three identically completed replicates for each isolate. The growth rate constant (μ) was determined by the exponential curve fitting equation $\boldsymbol{y = Ae^{Bx}}$, where B is the growth factor. This value was then used to determine doubling time where t_d=LN(2)/μ

TABLE A.9 GROWTH KINETIC PARAMETERS FOR *E. ALBERTII* 3542 CULTURED IN SELECTIVITY ENRICHED LB BROTH AT 42℃ FOR 24 HOURS

SELECTIVE AGENT	R^2	μ	t_d	min
SULFAMETHOXAZOLE 76	1.000	0.243	2.855	28.55
SULFAMETHOXAZOLE 34	1.000	0.258	2.690	26.90
SULFAMETHOXAZOLE 19	1.000	0.270	2.568	25.68
SULFAMETHOXAZOLE 10	1.000	0.330	2.099	20.99
TRIMETHOPRIM 4	0.999	0.113	6.161	61.61
TRIMETHOPRIM 2	0.997	0.097	7.175	71.75
TRIMETHOPRIM 1	0.997	0.099	6.987	69.87
TRIMETHOPRIM 0.5	0.994	0.099	7.009	70.09
PIPPERACILIN 16	0.999	0.284	2.444	24.44
PIPPERACILIN 8	0.998	0.293	2.363	23.63
PIPPERACILIN 6	0.998	0.290	2.393	23.93
PIPPERACILIN 4	0.994	0.249	2.789	27.89
BILE SALTS 2.5	0.997	0.308	2.250	22.50
BILE SALTS 1.5	0.997	0.239	2.927	29.27

R^2 values were recovered from linear regression equations determining the maximum specific growth rate from the log-linear component of the growth curve plotted from mean optical densities from three identically completed replicates for each isolate. The growth rate constant (μ) was determined by the exponential curve fitting equation $\boldsymbol{y = Ae^{Bx}}$, where B is the growth factor. This value was then used to determine doubling time where t_d=LN(2)/μ

TABLE A.10 GROWTH KINETIC PARAMETERS FOR *E. ALBERTII* 4085 CULTURED IN SELECTIVITY ENRICHED LB BROTH AT 42℃ FOR 24 HOURS

SELECTIVE AGENT	R^2	μ	t_d	min
SULFAMETHOXAZOLE **76**	0.988	0.298	2.324	23.24
SULFAMETHOXAZOLE **34**	0.999	0.323	2.143	21.43
SULFAMETHOXAZOLE **19**	0.997	0.326	2.129	21.29
SULFAMETHOXAZOLE **10**	0.995	0.300	2.309	23.09
TRIMETHOPRIM **4**	1.000	0.329	2.1091	21.09
TRIMETHOPRIM **2**	0.999	0.287	2.417	24.17
TRIMETHOPRIM **1**	0.999	0.263	2.636	26.36
TRIMETHOPRIM **0.5**	0.998	0.312	2.225	22.25
PIPPERACILIN **16**	0.998	0.292	2.322	23.22
PIPPERACILIN **8**	0.999	0.335	2.067	20.67
PIPPERACILIN **6**	0.991	0.347	2.000	20.00
PIPPERACILIN **4**	0.999	0.328	2.115	21.15
BILE SALTS **2.5**	0.998	0.226	3.071	30.71
BILE SALTS **1.5**	0.999	0.235	2.945	29.45

R^2 values were recovered from linear regression equations determining the maximum specific growth rate from the log-linear component of the growth curve plotted from mean optical densities from three identically completed replicates for each isolate. The growth rate constant (μ) was determined by the exponential curve fitting equation $\boldsymbol{y = Ae^{Bx}}$, where B is the growth factor. This value was then used to determine doubling time where t_d=LN(2)/μ

TABLE A.11 POSITIVE IDENTIFICATION OF *E. ALBERTII* BY PLATING MEDIUM

MEDIUM	Selective Enrichment A		Selective Enrichment B	
	# of total samples	% positive[a]	# of total samples	% positive[a]
ECC PLATE	15	60.0±0.2	15	46.7±0.4
SORB MC	6	83.3±0.2	6	66.7±0.5
PBX	15	53.3±0.2	15	33.3±0.2
XLT4	15	33.3±0.2	15	46.7±0.4
MXGMAC	18	38.9±0.3	18	44.4±0.4
KFS	9	66.7±0.3	9	44.4±0.4

aValues depict mean percentages and standard deviations of positive *E. albertii* samples from each selective enrichment broth when plated on various selective and selective/differential media from three identically completed replicates.

9. APPENDIX B: FIGURES

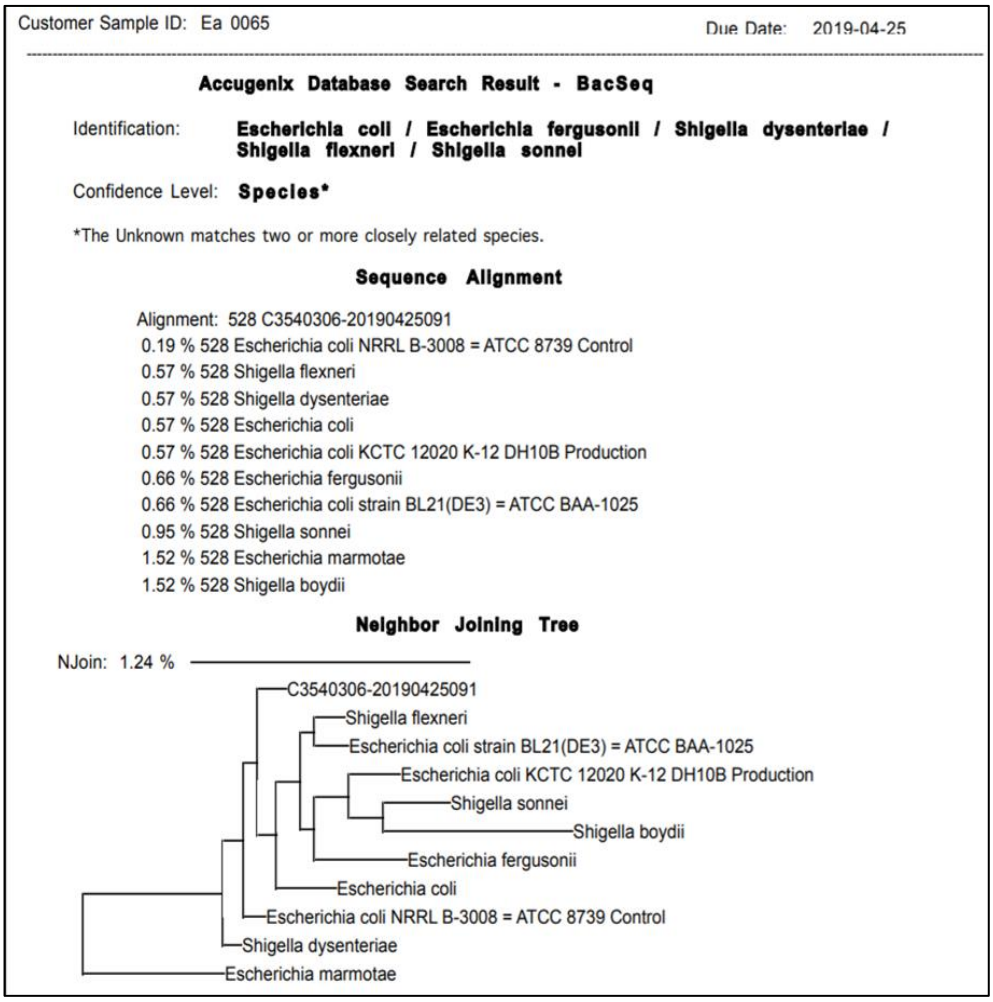


Figure B.1. Microbial ID for *E. albertii* 0065

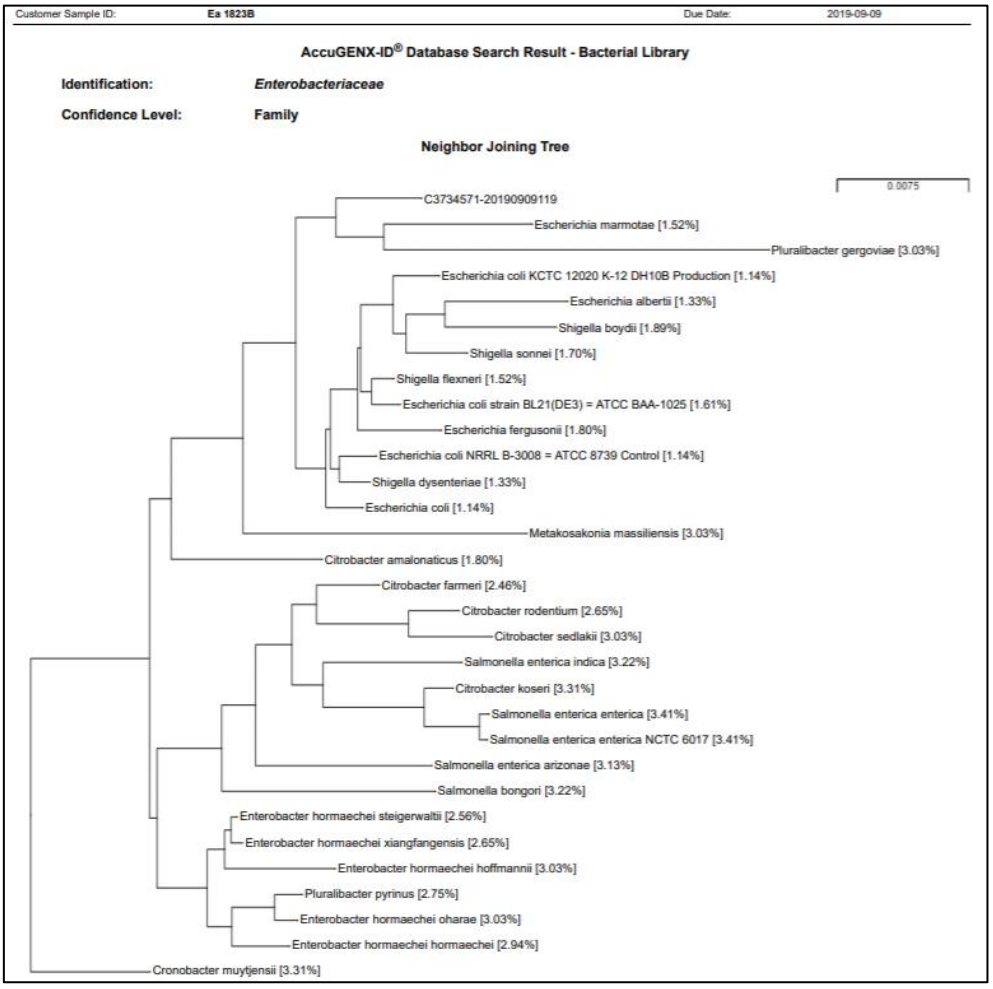


Figure B.2. Microbial ID for *E. albertii* 1823B

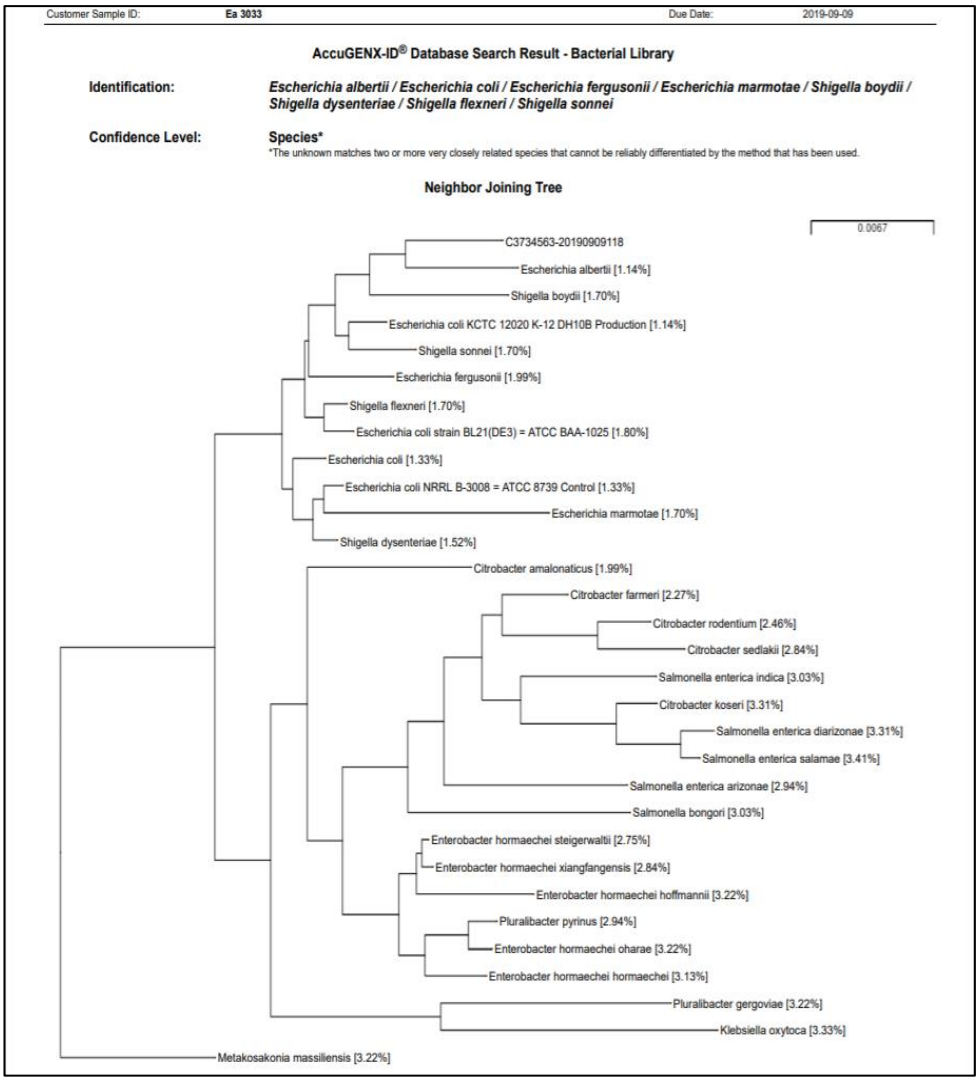


Figure B.3. Microbial ID for *E. albertii* 3866, 4015, 4750, 4180, 4143, 3542, 4312, 4085, 5188, 3449, 3033

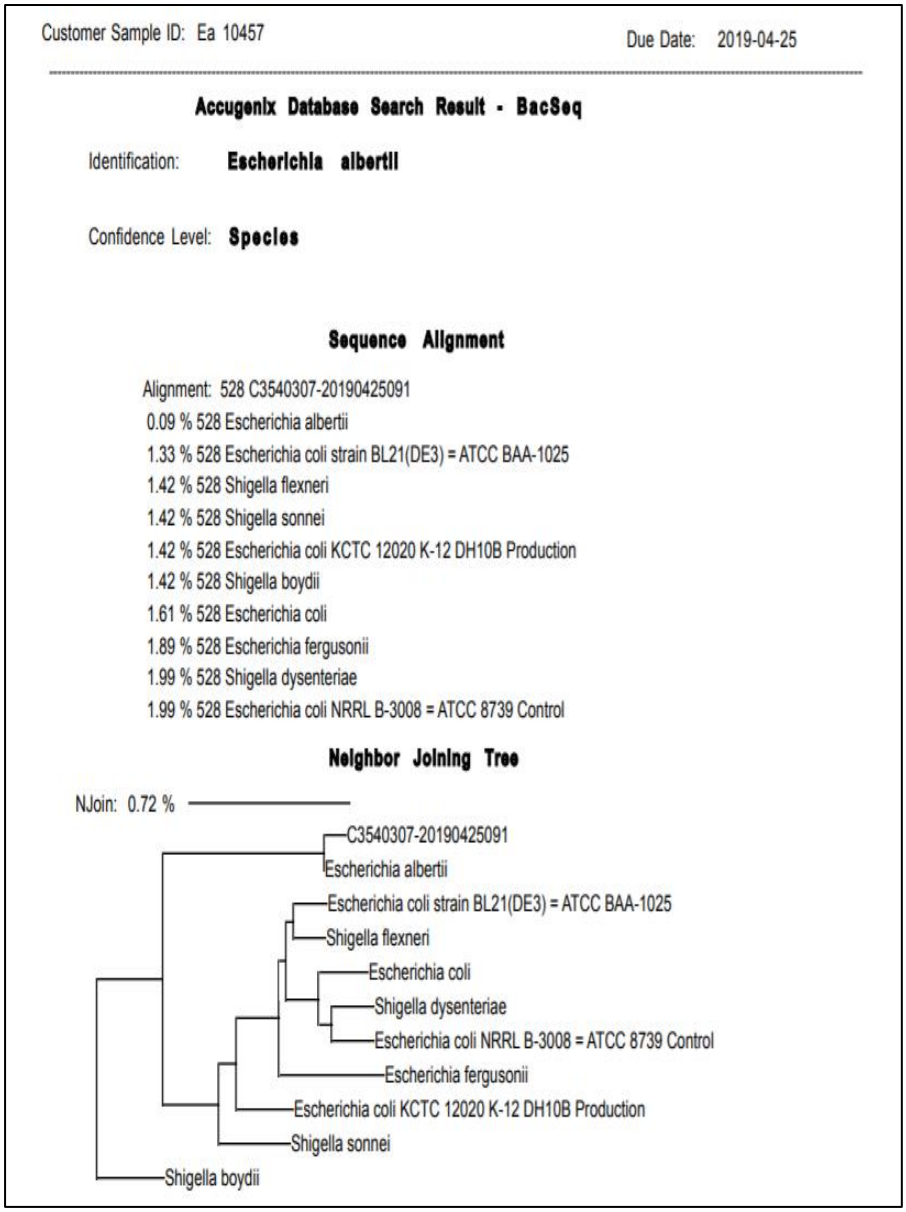


Figure B.4. Microbial ID for *E. albertii* 10457

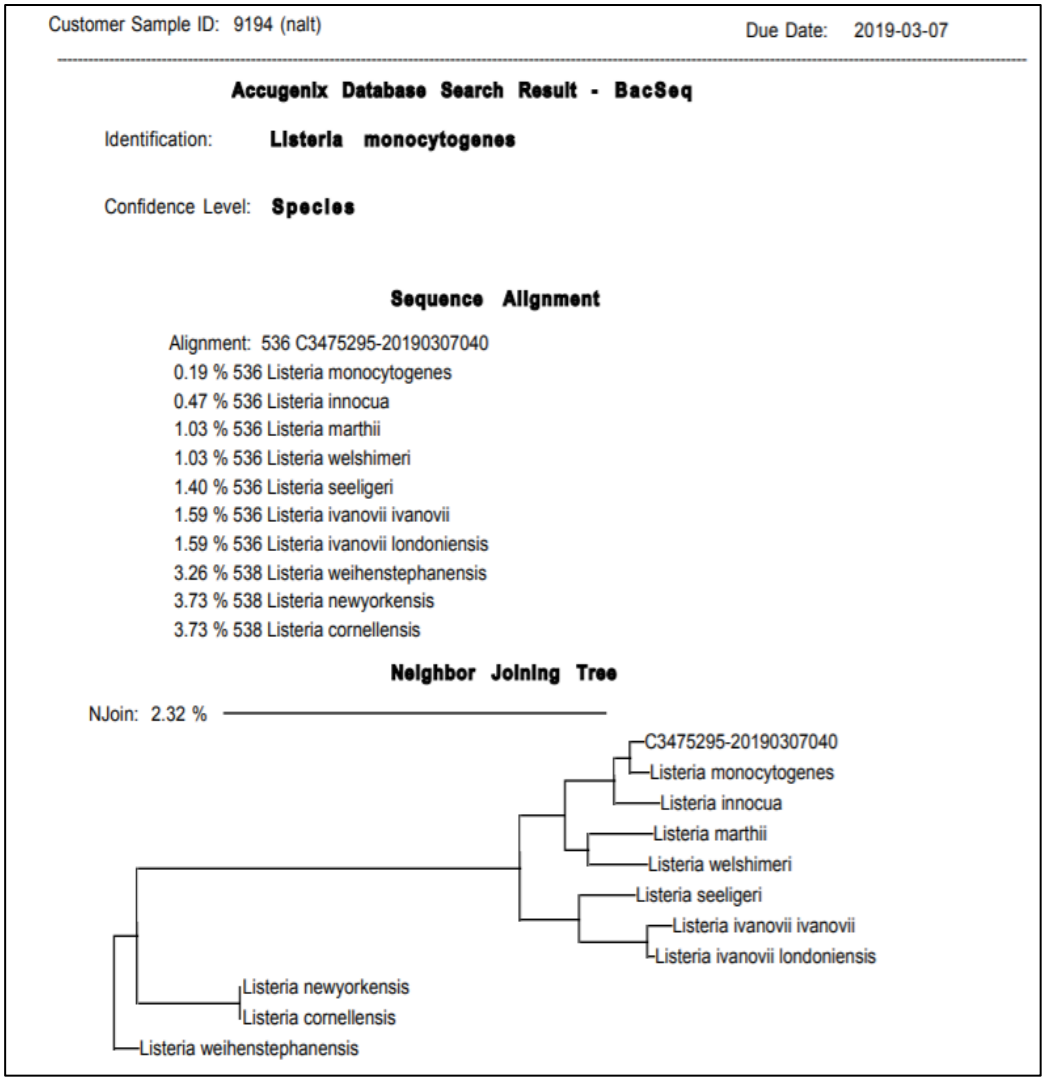


Figure B.5. Microbial ID for *E. albertii* 9194

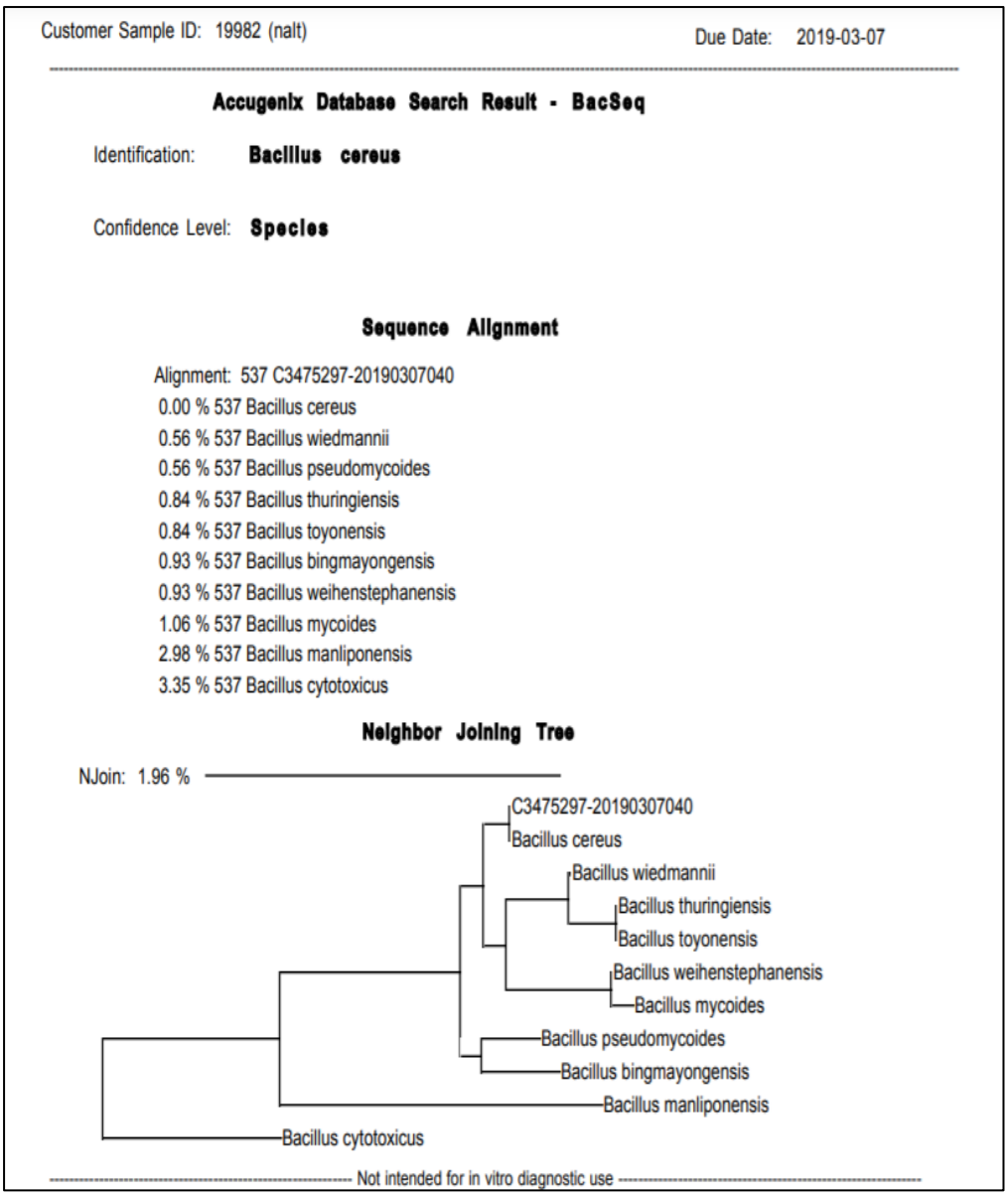


Figure B.6. Microbial ID for *E. albertii*

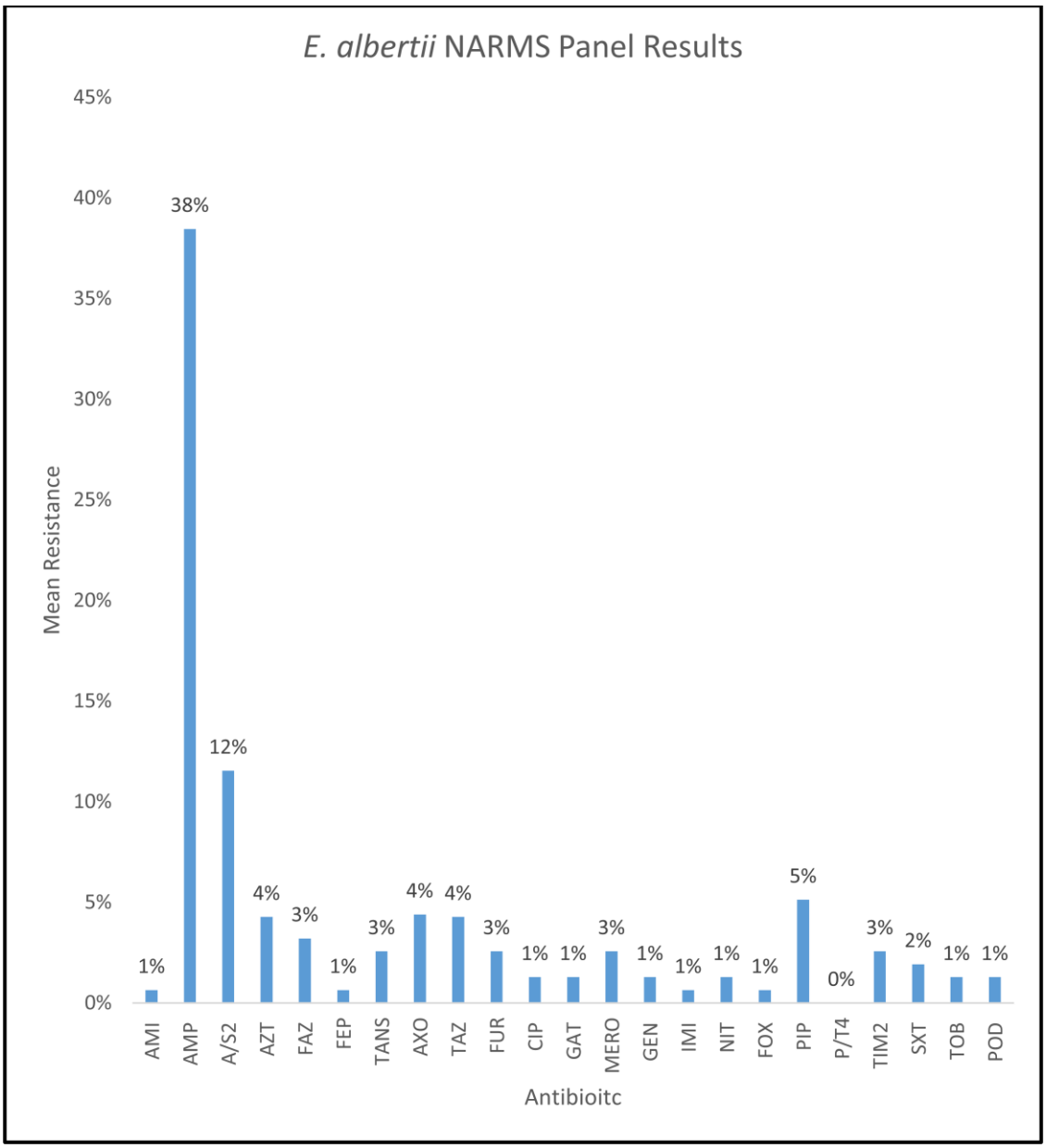


Figure B.7. *E. albertii* NARMS Panel Results
Graph depicts overall resistance to the antibiotics (see below) provided in the NARMS panel. This data was used in determining potential selective agents for a selective enrichment formulation.
AMI Amikacin; AMP Ampicillin; A/S2 Ampicillin / sulbactam 2:1 ratio; AZT Aztreonam; FAZ Cefazolin; FEP Cefepime; TANS Cefotetan; AXO Ceftriaxone; TAZ Ceftazidime; FUR Cefuroxime; CIP Ciprofloxacin; GAT Gatifloxacin; MERO Meropenem; GEN Gentamicin; IMI Imipenem; NIT Nitrofurantoin; FOX Cefoxitin; PIP Piperacillin; P/T4 Piperacillin / tazobactam constant; TIM2 Ticarcillin / clavulanic acid constant; SXT Trimethoprim/Sulfamethoxazole; TOB Tobramycin; POD Cefpodoxime

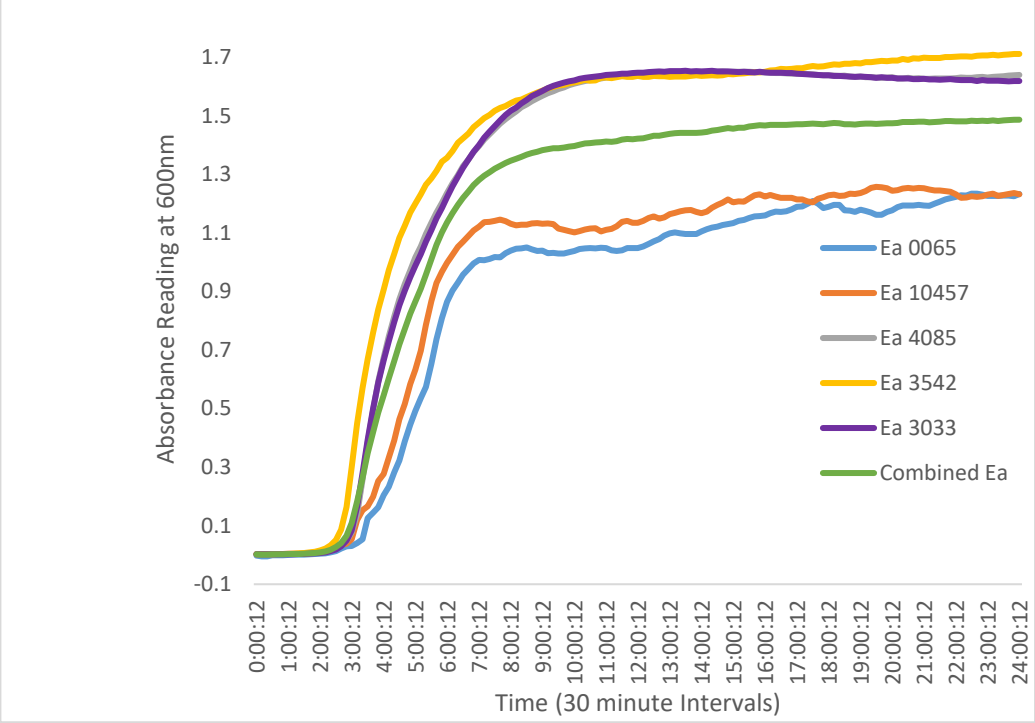


Figure B.8. *E. albertii* growth over 24h in LB broth. Graph displays the mean 24hr growth of *E. albertii* plotted from three replicates.

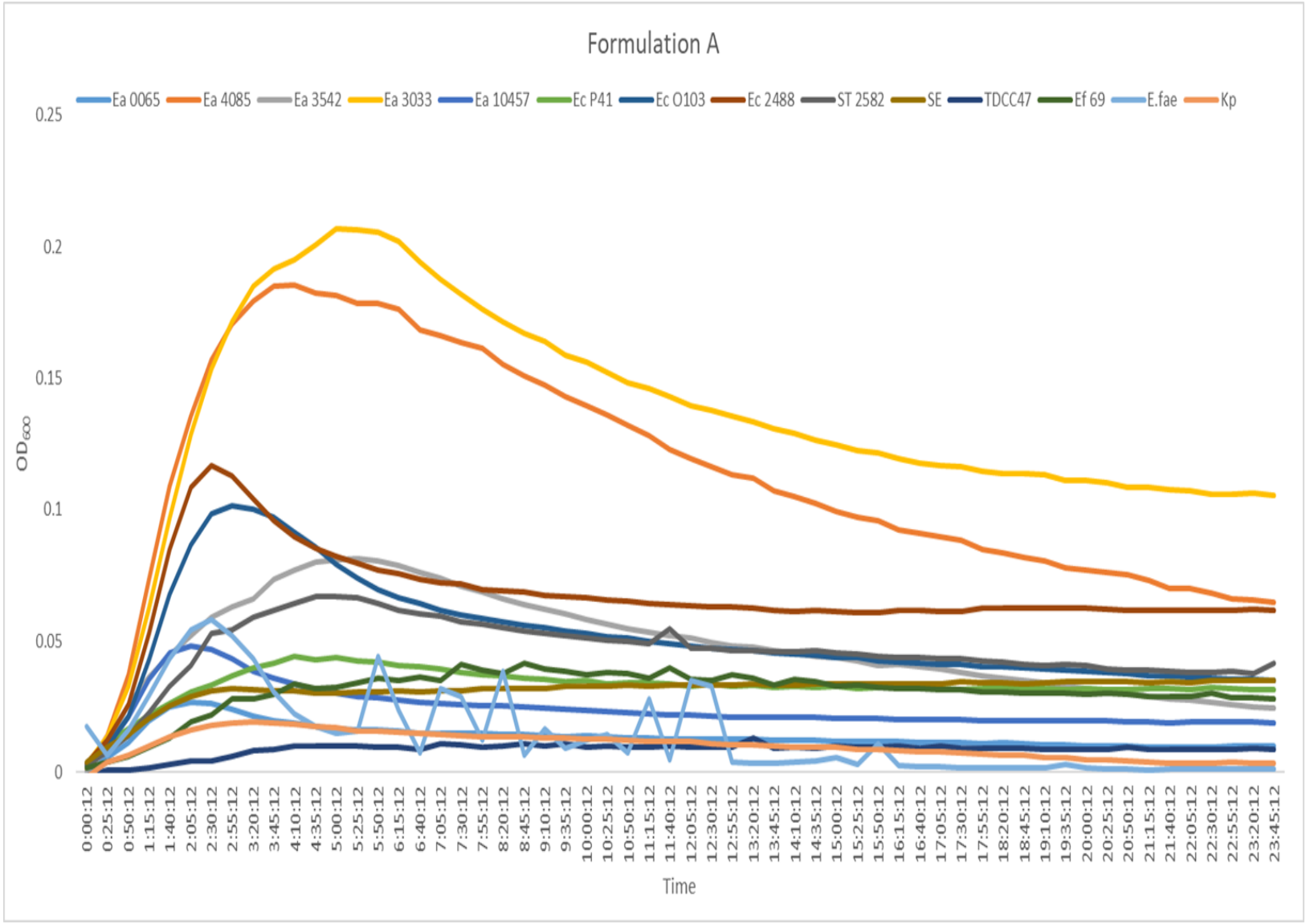


Figure B.9. Isolate 24 Hour Growth in Selective Enrichment A

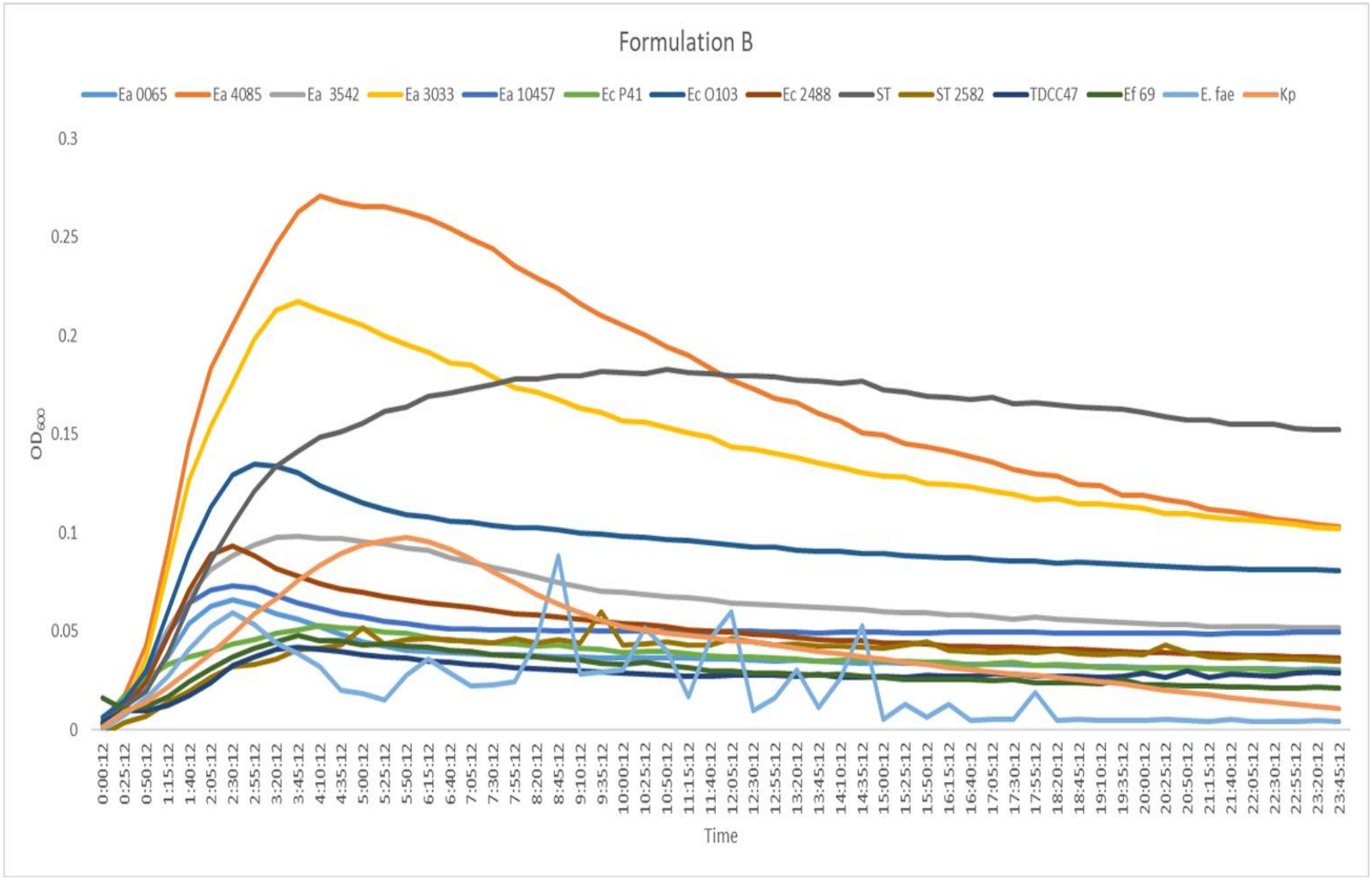


Figure B.10. Isolate 24 Hour Growth in Selective Enrichment

10. APPENDIX C: ADDITIONAL FIGURES

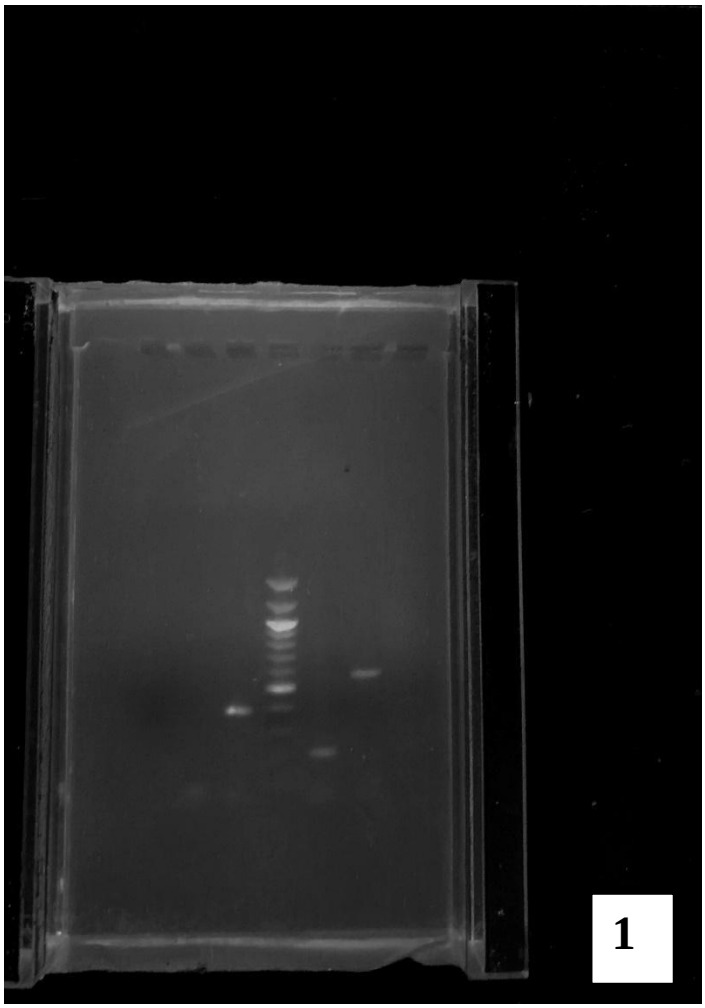
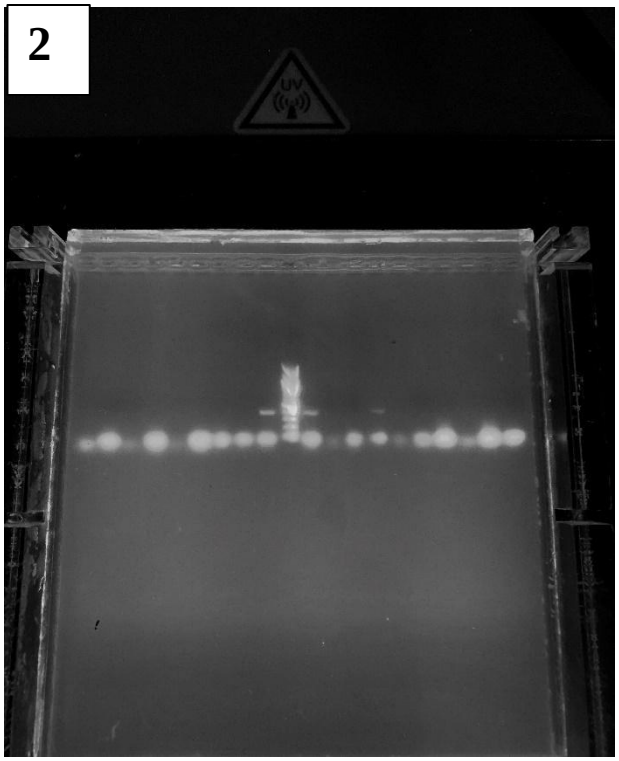


Figure C.1. Results from Gel Electrophoresis of preliminary PCR trial. This was completed to view the differences in base pairs between *E. alberti* (393bp), *E. coli* (212bp), *E. ferguonsii* (757bp), and to make sure that non- *Escherichia* species would not provide a result.



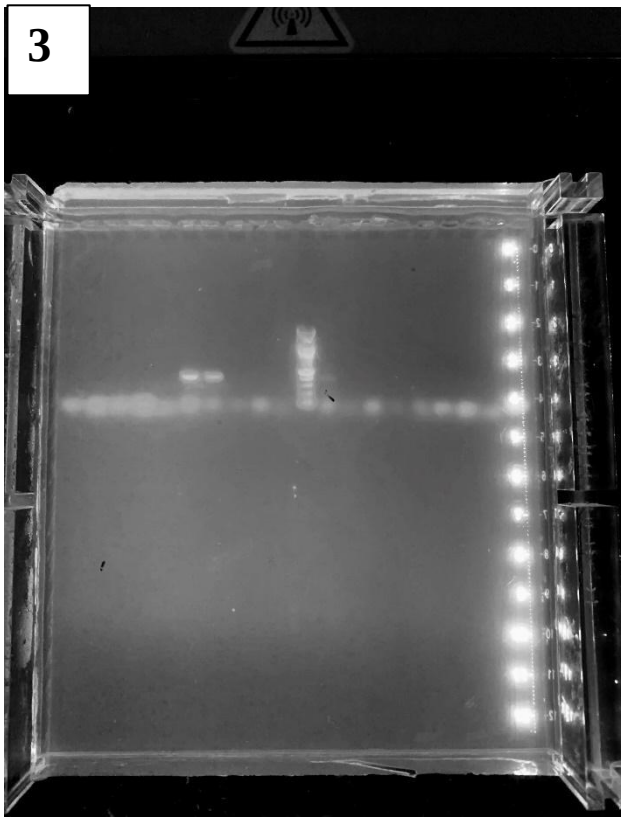
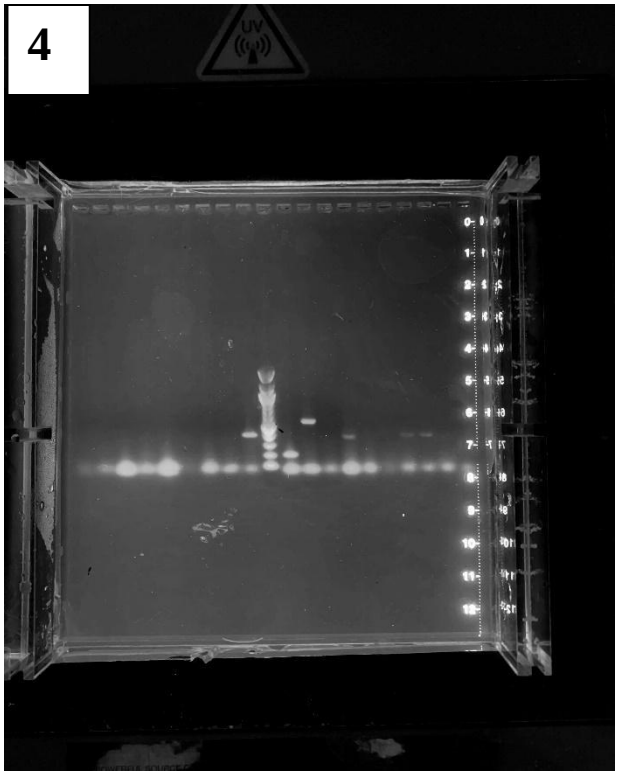


Figure C.2-3. Gel Electrophoresis results showing positive identification of *E. albertii* and *E. coli*. *E. fergusonii* was not identified during PCR trials. Figures also detail issues with identification resulting from primer dimer and a lack of sufficient template DNA.



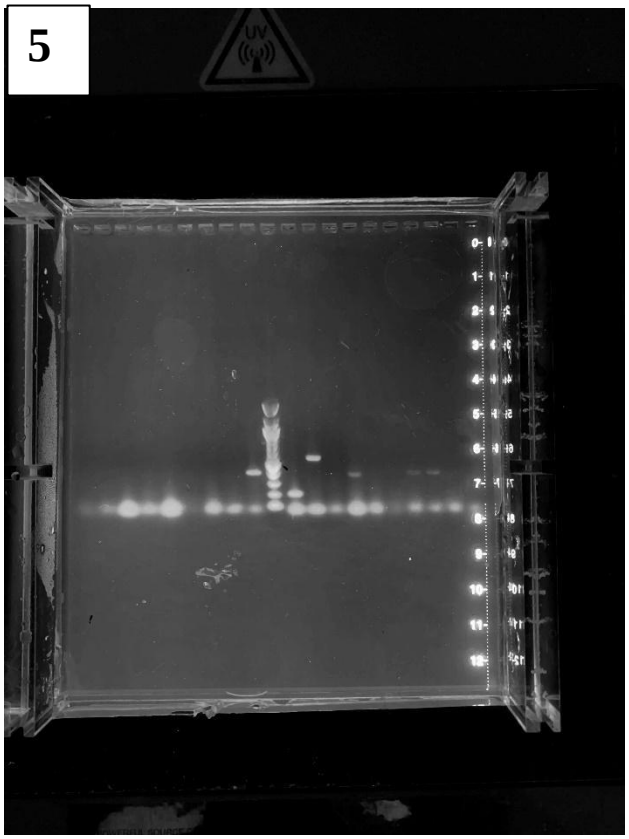


Figure C.4-5. Gel Electrophoresis results showing positive identification of *E. albertii* and *E. coli*. *E. fergusonii* was not identified during PCR trials. Figures also detail issues with identification resulting from primer dimer and a lack of sufficient template DNA.

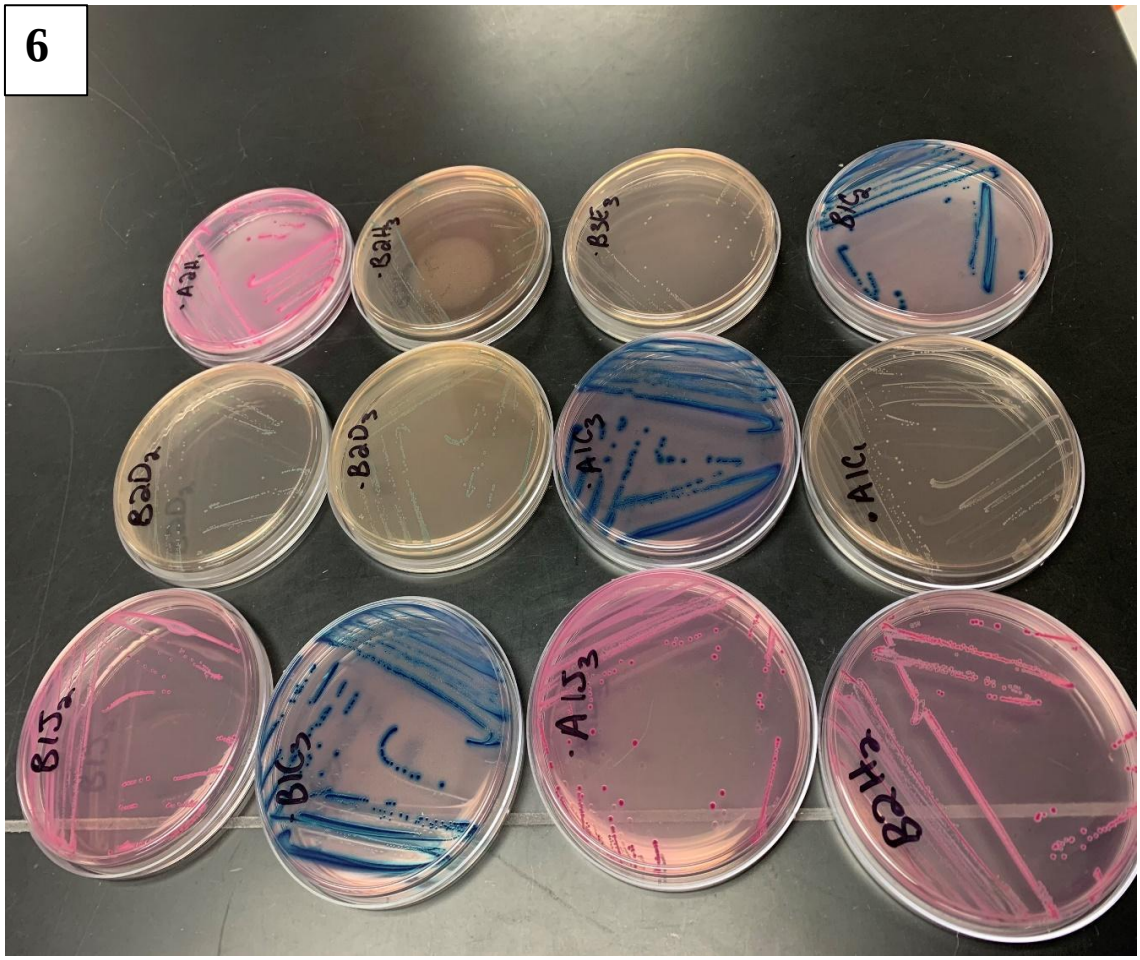
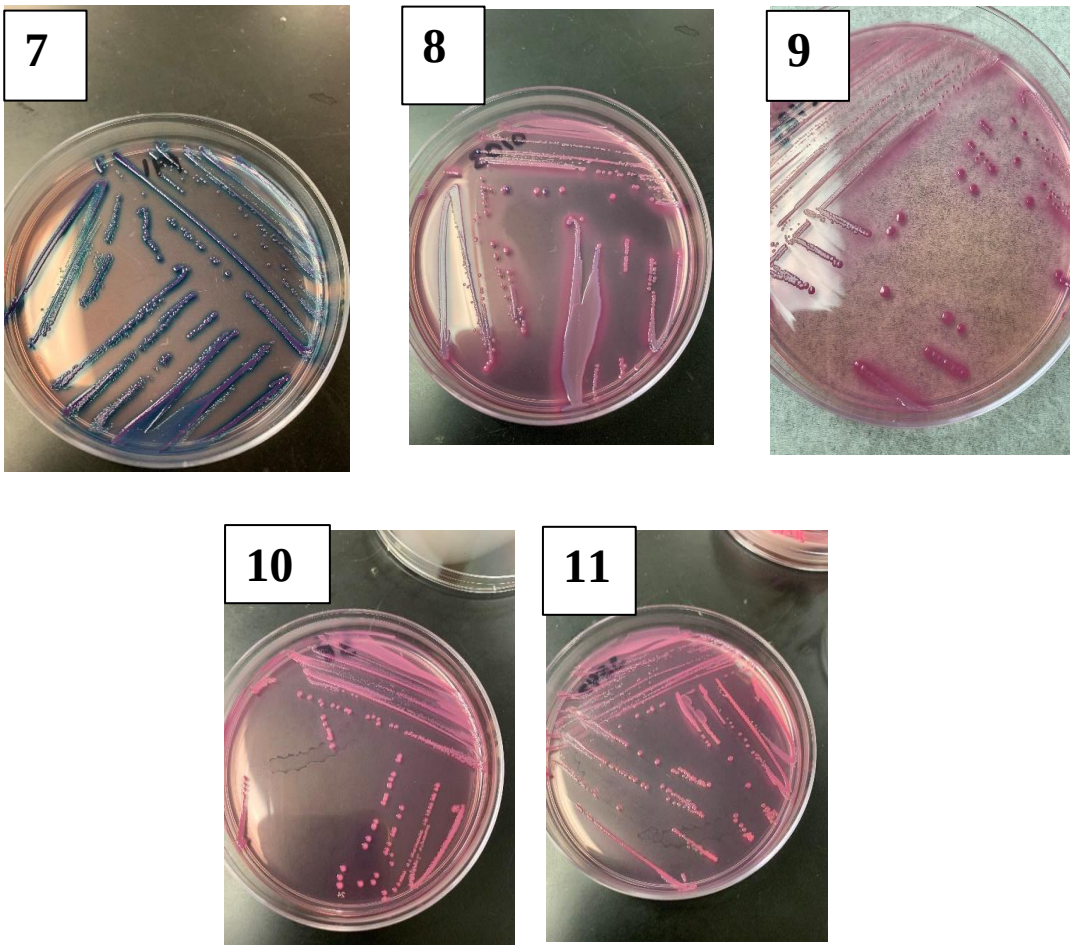
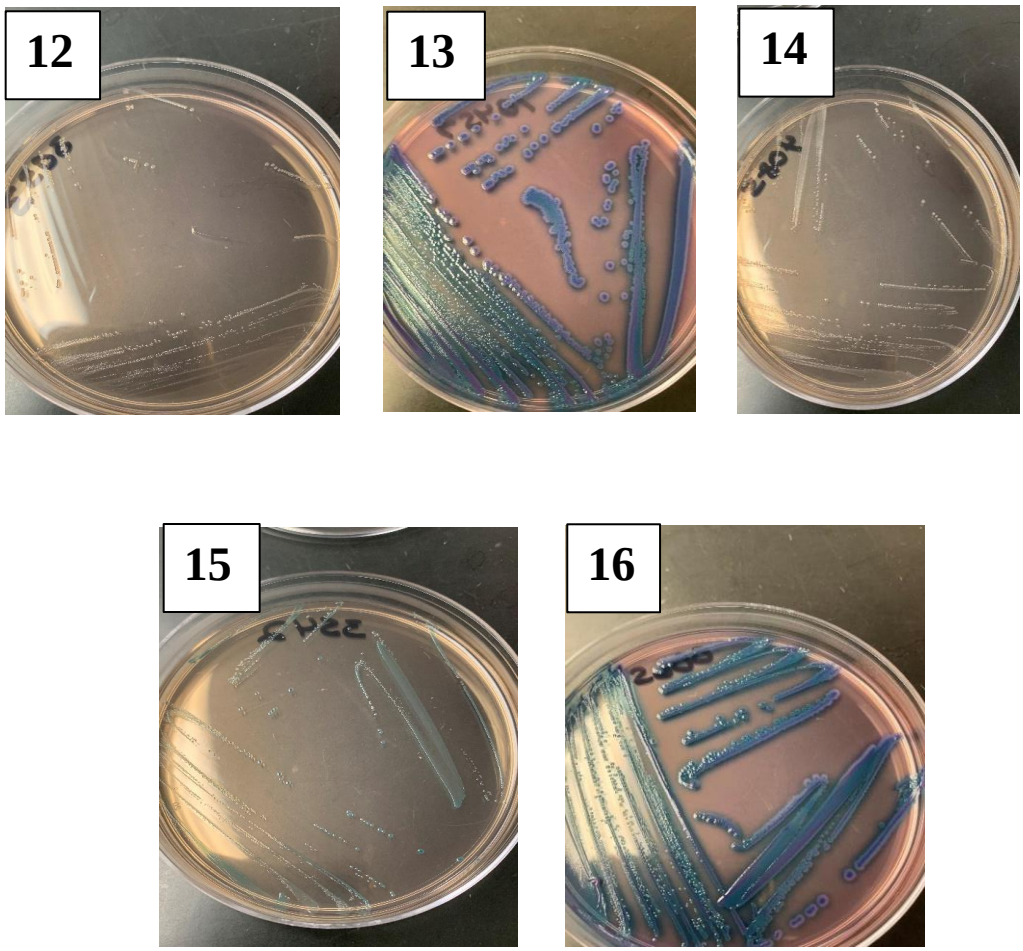


Figure C.6. Results from plating samples contacting a combination of either *E. albertii* and *Salmonella enterica* spp. or *E. albertii* and *E. coli* onto MXgMAC agar. This agar was developed by a TAMU PhD candidate in the Dept. of Food Science and Technology.



Figures C.7-11. Results from plating *E. coli* and *Salmonella enterica* spp. control samples onto MXgMAC agar.



Figures C.12-16. Results from plating *E. albertii* control samples onto MXgMAC agar. Isolates 0065 (P) and 10457 (M), displayed similar colony morphologies to that of *E. coli* P41 (G).

11. APPENDIX D: NON-THESIS PROGRAM ACCOMPLISHMENTS

Reducing Pathogenic *Escherichia coli* Surrogates on Fresh Beef Cuts by Water-Reducing Antimicrobial Interventions

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Keywords**:** Beef safety interventions; water reduction *Salmonella*; STEC; electrostatic spray

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ABSTRACT

Water use for antimicrobial intervention application for beef harvest has come under increased scrutiny in recent years in an effort to enhance water conservation during beef harvest and fabrication. This study was conducted to determine the efficacy of beef safety interventions for reducing surrogates of the Shiga toxin-producing *Escherichia coli* (STEC) on beef cuts while lowering intervention-purposed water use for a Small or Very Small beef establishment. Beef briskets, shoulder/clods, and rounds were inoculated with a gelatin-based slurry containing 6.80±0.28 log CFU/g non-pathogenic *E. coli*. After 30 min of attachment, inoculated cuts were treated by: conventional lactic acid spray (LA; 2.5%, 55°C), lactic acid spray delivered by an electrostatic spray handheld wand (ESS; 2.5%, 55°C), hot water spray (HW; 82°C), recycled hot water spray (RW; 82°C) wherein previously applied hot water was collected, thermally pasteurized to 82°C, or left untreated (CON). 100 mL of each treatment was sprayed onto marked surfaces of inoculated cuts, after which surviving surrogate *E. coli* were enumerated. LA and ESS treatments produced greater reductions (1.0-1.1 log CFU/300 cm^2) versus hot water interventions (0.3-0.5 log CFU/300 cm^2) (p=<0.0001). Recycling of water reduced water losses by no less than 78% on RW-treated beef cuts. Low water beef safety interventions offer Small and Very Small inspected beef establishments opportunities to incrementally reduce water use during intervention application, but not necessarily without loss of pathogen reduction efficacy.

Research Highlights

- Lactic acid interventions reduced pathogen surrogates by 1.0-1.1 log CFU/300 cm^2.
- Hot water recycling reduced water consumption by $\geq$78.7% for all beef cuts.
- Low water-using beef safety interventions may not yield useful pathogen reduction.

As with many U.S. food industry sectors, there is increasing interest in natural resource conservation, especially water, during beef harvest, fabrication, and manufacture. Nevertheless, resource-conserving beef manufacturing practices must be designed to ensure beef safety is not compromised. Consumption of fresh beef products contaminated with microbial pathogens presents a risk to human health. The U.S. Centers for Disease Control and Prevention (CDC) *(13)* reported that from 2014-2019, seven multistate foodborne disease outbreaks occurred, producing 699 cases of illness, 193 hospitalizations and two fatalities resulting from consumption of *Salmonella* or Shiga-toxigenic *Escherichia coli* (STEC)-contaminated beef products, including multiple outbreaks involving ground beef. A recent report of economic costs of foodborne disease for the U.S. meat and poultry industries estimated $8,978 million annual costs across bacterial, viral, and other types of microbial pathogens. Costs of disease in the U.S. for STEC and non-typhoidal salmonellae were estimated at $276.0 and $2,662.2 million annually *(10)*. Batz et al. *(3)* previously estimated *E. coli* O157:H7 human disease involving beef products cost the U.S. approximated $141 million dollars per annum. By comparison, *Salmonella* disease associated with beef was estimated to cost $229 million in the U.S. annually. Reducing foodborne disease incidence by effectively reducing the transmission of human pathogens on beef products should therefore reduce disease burden and associated costs.

Beef carcasses and derived cuts may be cross-contaminated during the processes of harvest, carcass dressing and fabrication, potentially leading to consumer exposure to

microbial food safety hazard(s) *(2, 5, 9)*. The use of antimicrobial interventions, such as the application of chemical disinfectants (e.g., lactic acid), or physical carcass treatments (e.g., hot water sprays) can effectively reduce consumer risk of disease by decontaminating beef carcasses and/or cuts, thereby reducing potential of microbial pathogen transmission to consumers. Multiple opportunities exist for reducing the volume of water utilized during beef harvest and products manufacture, either through reducing the volume of water initially applied for food safety purposes, or through reclamation and recycling of waters used in carcass washes. Ziara et al. *(17)* reported the consumption of water in a medium-sized U.S. beef packing facility; carcass final washes were reported as requiring a high volume of water per animal (253 L per 1,000 kg live animal weight). Results of that study led to implementation of methods, including rain water collection from cattle pens, replacement of inefficient toilets, installation of quick connect nozzles, and installation of a wastewater heat exchanger for use on carcass washes, designed to reduce water and energy consumption. Other researchers reported a near halving of the water applied onto beef carcasses by application of interventions using an electrostatic spray (ESS) cabinet system versus an in-package spray *(12)*. These researchers reported lauric arginate ester (LAE; 1.0%), cetylpyridinium chloride (CPC; 0.4%), and peracetic acid (PAA; 0.025%) reduced STEC counts on beef carcass cuts by 0.6, 0.3, and 0.2 log CFU/carcass cut, respectively; foodborne pathogen reductions were nonetheless lower than those gained from conventional interventions. This indicates the need for further evaluation of such technologies for controlling pathogens on beef carcasses for the sake of reducing risk of STEC transmission and reducing water usage. The purpose of this research project was to compare beef safety interventions designed for use in Small and Very Small beef harvest establishments,

conventional and reduced water-consuming alike, to determine if one intervention was as effective as the other for reducing STEC surrogates on beef surfaces.

Materials and Methods

*Pathogen surrogates preparation***.** *E. coli* biotype I isolates (American Type Culture Collection [ATCC] BAA-1427, BAA-1428, BAA-1429, BAA-1430, BAA-1431) were selected from the Department of Animal Science Food Microbiology Laboratory (FML) culture collection (Texas A&M University, College Station, TX). These organisms were previously identified by the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) as useful for inoculation onto meat products and carcasses for purposes of intervention validation *(14)*. Organisms were revived from cryo-storage (-80°C) by aseptically inoculating sterile 10 mL of tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD) with a bacterial colony, and then incubating at 37°C for 18-24 h. Following an initial revival period, each strain was individually aseptically inoculated into a new tube containing 10 mL sterile TSB, and incubated for 18-24 h at 37°C. Isolates were then aseptically streaked onto slants of tryptic soy agar (TSA; Becton, Dickinson and Co.) for later use in experiments. All slants were incubated for 24 h at 37°C, and then stored at 5°C until required for use.

E. coli *cocktail preparation for meat inoculation***.** To prepare a cocktail of the pathogen surrogate organisms, isolates were aseptically scraped from TSA slants into 10.0 mL volumes of sterile TSB, followed by incubation at 37°C for 18 h to achieve stationary phase cells (Figure S1). Following 10.0 mL of each culture was aseptically transferred into a sterile 50.0 mL conical Falcon tube (Corning Sciences, Tewksbury, MA), vortexed, and

then centrifugation at 3,500 x *g* for 15 min at 25°C. Following centrifugation, the supernatant was discarded, the pellet was reconstituted in 50.0 mL sterile 0.1% peptone diluent and centrifuged again. Following the second centrifugation, the pellet was resuspended in 50 mL to form a cocktail.

Inoculum slurry preparation and validation. A gelatin-based slurry was developed to deliver inoculum onto beef cut sample surfaces in a manner intended to simulate fecal material cross-contamination, and was prepared in similar fashion as has been reported previously with minor modification *(8, 16)*. Briefly, the slurry was prepared by dissolving 100 g food-grade, unflavored gelatin powder in 1.0 L of 0.1% (w/v) sterile peptone diluent heated to 80°C. Next, the hot gelatin solution was added to 4.0 L sterile 0.1% peptone water heated to 100°C and stirred for 5.0 min. The gelatin slurry was then covered with aluminum foil and stored at 25°C for at least 12 h before the addition of the cocktail inoculum. Using a magnetic stirrer hot plate, the 50 mL cocktail was added to the slurry and mixed for 5 min to ensure uniform dispersion of inoculum prior to being loaded in a compressed air sprayer to deliver the inoculum to beef cut surfaces.

A preliminary experiment was designed to test researchers' efficiency at inoculating beef cut surfaces. Three sets of chilled carcass cuts (brisket, shoulder, and round) were purchased from a local beef products retailer, transported to the FML, and warmed to approximately 38°C to simulate surface temperature conditions for a non-chilled beef carcass. After reaching the desired surface temperature, sample cuts were inoculated with the *E. coli* gelatin slurry using a compressed air sprayer (Ortho Heavy Duty Sprayer, The Fountain Group Inc., New York Mills, NY) delivering 90 mL of inoculum in 18 sec. After 30 min of ambient holding of inoculated cuts for microbial attachment, a sponge sampler

(3M™ Sponge-Sticks, 3M™, Minneapolis, MN) was used to recover attached *E. coli* surrogates from a 100 cm^2 template-marked section from the surface of the inoculated beef cut. The sponge stick was hydrated in 90 ml peptone diluent, excess diluent was aseptically pressed out into the pouch, the sponge was placed on the inoculated surface, and swabbed (horizontally, vertically, and diagonally each five times) across the marked surface. Following sampling, the sponge was re-inserted into the pouch, closed and then transported to the FML. Once arriving at the FML, sample sponges in pouches were hand-massaged for 1.0 min to release cells into the diluent, and serial dilutions prepared to enumerate surviving surrogate *E. coli*. *E. coli* were enumerated on 3M™ Petrifilm™ E. coli/Coliform Count Plates and petrifilms were then incubated at 37°C for 48 h prior to colony enumeration.

Warming of beef carcass cuts to simulate un-chilled carcass surfaces. Ten commodity trimmed beef bottom rounds (IMPS 171), briskets (IMPS 119), and shoulder clods (IMPS 114) were purchased from the local meat vendor used in inoculation efficiency testing. Vacuum-packaged carcass cuts were stored in a holding cooler at 0.6°C for up to three days before use. In order to be warmed to near physiological temperature (approx. 38°C), carcass cuts were placed in a commercial oven (Alkar-RapidPak, Inc., Lodi, WI). In order to retain product moisture and soften subcutaneous fat, dry bulb and wet bulb temperatures were steadily increased each hour over a period of four hours (Table S1). Carcass cuts were heated in packaging to allow for moisture retention during warming. Initial weights of each carcass cuts were taken prior to and after heating to assess purge loss after targeted internal and surface temperatures were attained. Carcass cuts were placed on racks in the oven in their original vacuum packaging. Two internal thermocouple probes were inserted into the largest carcass cuts at the geometric center and approximately one

inch from the surface to verify cuts achieved the typical near-physiological temperature of a beef carcass after slaughter. Once the carcass cuts reached the temperature target (approx. 38°C), they were removed from the oven, weighed in order to determine the impact of warming on moisture loss as a signal that meat cut surfaces deviated from moisture conditions on fresh non-chilled carcass surfaces, and further prepared for sampling.

Antimicrobial intervention application to E. coli-*inoculated beef cut surfaces.* Heated carcass cuts were placed onto plastic trays; packaging was aseptically removed, and beef cuts were then inoculated with the gelatin slurry as described above. After sample inoculation and attachment, samples were treated with one of four antimicrobial treatments: a conventional warmed lactic acid spray (LA; 2.5%, 55°C), a lactic acid spray delivered using an ESS handheld wand sprayer (ESS; 2.5%, 55°C) with a model XT-3 device (Electrostatic Spraying Systems, Inc., Watkinsville, GA; electrical current set at 110 V with air pressure 30.0 psi and tank pressure 12.0 psi), a conventional hot water spray (HW; 82°C) , a recycled hot water spray (RW; 82°C), or were left uninoculated and untreated to function as a control (CON). For the LA, HW, and RW treatments, the antimicrobial treatment was applied via a handheld compressed air sprayer (Ortho Heavy Duty Sprayer, The Fountain Group Inc.). In order to standardize the volume of intervention fluid applied to each cut, a graduated cylinder was used to collect sprayed antimicrobial solution and a timer used to determine the time needed to deliver 100 mL of intervention fluid to each of three 100 cm^2 template-marked surfaces for each sample cut. 100 mL of intervention was selected from preliminary testing wherein 100 mL application resulted in cut surfaces being visually drenched in intervention fluid. Thereafter, samples were treated by intervention fluid for the required time period to deliver approximately 100 mL of each treatment to a sample cut.

Surviving surrogates were collected using three 3M™ Sponge-Sticks pre-moistened with 10 mL of sterile buffered peptone water. Each sponge-stick was swabbed (horizontally, vertically, and diagonally each five times) across one of three different 100 cm^2 marked surfaces to recover as many surviving cells as possible following antimicrobial treatment.

For the RW-treated carcass cuts, residual water that dripped off HW-treated samples was captured in a sterile basin below the treatment chamber. The volume was recorded, loaded into a metallic pan, and then reheated to at least 82°C before being used in the RW treatment *(15)*. Recycled water was routinely mixed with heated fresh water in order to prepare a 4.0 L volume of water for RW treatment for adequate function of the hand-spray device pump. Two 20.0 mL samples of recycled pasteurized water were regularly collected to verify that *E. coli* surrogates recovered from HW treatment did not survive the pasteurization process. Volumes of water collected from HW treatments were subtracted from the total water applied to sample cuts during HW treatment; investigators thereafter calculated reductions in losses of water used in the RW treatment.

For all beef cut samples and treatments, sponges were replaced in their individual pouches and transported to the FML. The sponge hydration fluids from three swabs for a single carcass cut sample were aseptically composited into a 50 mL conical vial for assay. Surviving cells serially diluted, enumerated onto 3M™ E. coli/Coliform Count Plates, and incubated at 37°C for 48 h per manufacturer guidance. Colony counts were recorded as CFU/300 cm^2.

Experimental design and statistical analysis of data. For antimicrobial treatment experiments, the experiment was set up as a randomized complete block design for purposes of treatment assignments to carcass cuts, wherein sample carcass cuts possessed equal

chances of being assigned to one treatment or another. Two complete replicates were completed, each with three independent identically handled samples per cut type and treatment combination (N=6). Treatments were applied to inoculated samples in a pre-designed sequence for the purpose of ensuring HW-treated samples were followed by RW-treated samples. This was done in order to facilitate water capture from HW treatment of carcass cuts for subsequent use in RW treatment. Microbiological data was log-transformed and analyzed using the Standard Least Squares procedure in JMP Pro v15.0 (SAS Institute, Inc., Cary, NC) to complete two-way ANOVA to determine the impact of the main effects of antimicrobial treatment, beef carcass cuts type, and their interaction on resulting numbers of surviving *E. coli* surrogates. Means were separated using Tukey's Honestly Significant Differences (HSD) test at significance level p=0.05.

Results and Discussion

Efficiency of E. coli *cocktail attachment to beef cuts using gelatin-based slurry.* The mean inoculum prepared for application to beef carcass cuts was 6.8±0.3 log CFU/g of slurry. On surfaces of beef rounds, shoulder clods, and briskets, mean numbers of attaching *E. coli* were 4.96±0.26, 5.52±0.11, and 5.92±1.44 log CFU/100 cm^2, respectively, which were not observed to differ greatly by cut and indicated researchers were able to consistently inoculate beef cuts. Though the use of previously refrigerated beef carcass cuts that were subsequently warmed prior to inoculation and testing may have impacted the variability in results obtained on briskets, previous research on O157 and non-O157 STEC attachment to beef does not suggest that warming of cold meat for purposes of inoculation is highly influential in resulting microbiological responses to antimicrobial treatment *(6)*.

Beef cut warming and purge loss by cut. For all beef cuts, warming in the oven resulted in some purge loss upon opening of warmed cuts, resulting in a deviation of meat surface moisture characteristics from the typical condition of harvested animal carcass surfaces (Table S2). Rounds exhibited the highest mean purge of 8.79±8.98%, whereas briskets exhibited the least purge loss (2.60±2.47%).

Reduction of water loss between HW and RW treatment. The mean volumes of water recovered from carcass cuts subjected to the HW treatment and later used in the RW treatment were 370.0±14.1 mL, 315.0±134.0 mL, and 316.7±246.6 mL from shoulder clods, briskets, and rounds, respectively. This resulted in water loss reductions from 78.7% to near 100% during RW treatment versus the HW treatment (Table 1).

Reduction in E. coli *surrogates on beef cuts by antimicrobial treatment.* Statistical analysis of microbiological data from experiments applying antimicrobial treatments to inoculated beef cuts did not return a statistically significant interaction of the main model effects of beef cut type and antimicrobial treatment (p=0.687). Nevertheless, beef cut type and antimicrobial treatment effects each impacted *E. coli* surrogates survival. Figure 1 depicts means of surviving *E. coli* surrogates on treated beef surfaces by carcass cut. *E. coli* organisms were lower on briskets (4.4 log CFU/300 cm^2) versus rounds (4.8 log CFU/300 cm^2) and shoulder clods (4.8 log CFU/300 cm^2), which did not statistically differ from one another (p=0.0004).

E. coli survivor counts also differed by antimicrobial treatment (p=<0.0001). Lactic acid-using antimicrobial treatments (LA, ESS) produced greater reductions in numbers of surviving *E. coli* compared to hot water-using treatments and the control. Additionally, ESS- and LA-specific mean *E. coli* survivor counts did not statistically differ from one another,

likely due to the standardization of applied volume of 2.5% lactic acid for each treatment. Hudson et al. *(7),* using an ESS device identical to the one used in the current study, reported 4.5% lactic acid (500 mL, 55°C) produced a 3.3 log CFU/cm^2 reduction in numbers of O157 and non-O157 STEC on beef outside rounds, while ESS application of 4.5% lactic acid (125 mL, 55°C) produced only a 1.2 log CFU/cm^2 reduction. In contrast to the current study, reducing the volume of applied antimicrobial via ESS resulted in a significant decrease in observed pathogen surrogate reduction.

Hot water (HW) and RW treatments each produced small reductions in the numbers of *E. coli* (~0.3 log CFU/300 cm^2) compared to the CON (Figure 2). These treatments did not statistically differ from one another or the CON with regards to numbers of surviving *E. coli* pathogen surrogates. No *E. coli* were detected from water samples collected from thermally pasteurized waters that were prepared for use in the RW treatment, confirming that water captured from the HW treatment and then pasteurized did not serve to transfer viable *E. coli* onto RW-treated beef cuts. Signorini et al. *(11)* reported similar differences in reductions of generic *E. coli* on beef carcasses following automated hot water (82-87°C, 1.5 bar) treatment, where counts of survivors decreased by only 0.6 log CFU/400 cm^2. Others have previously reported greater reductions in small beef harvesting establishments. Algino et al. *(1)* previously reported a 1.3 $\log_{10}$ CFU/cm^2 reduction in generic *E. coli* on carcass surfaces following use of a 66°C water treatment applied using low pressure nozzle applicator but did not report volume of water applied.

Standardizing the volume of lactic acid intervention fluid applied for both the conventional LA and ESS intervention resulted in non-differing reductions in the numbers of STEC surrogates. This finding indicates that ESS technologies can reduce the volume of

water applied to a beef carcass or cut but may consequently result in lower observed reductions of contaminating microbes, including human pathogens. ESS-applied interventions may require higher antimicrobial concentration in applied interventions to maintain desired pathogen reduction outcomes. Data presented herein demonstrate opportunities for Small and Very Small beef harvesting facilities to improve the conservation of water during antimicrobial intervention application. However, facility personnel must critically evaluate and balance the capabilities of tested interventions to reduce water use without compromising beef safety protection outcomes.

Acknowledgments

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References

1. Algino, R. J., S. C. Ingham, and J. Zhu. 2007. Survey of antimicrobial effects of beef carcass intervention treatments in very small state-inspected slaughter plants. *Journal of Food Science*. 72:M173-M179.

2. Arthur, T. M., J. M. Bosilevac, D. M. Brichta-Harhay, N. Kalchayanand, D. A. King, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2008. Source tracking of *Escherichia coli* O157:H7 and *Salmonella* contamination in the lairage environment at commercial U.S. beef processing plants and identification of an effective intervention. *Journal of Food Protection*. 71:1752-1760.

3. Batz, M. B., S. Hoffmann, and J. Morris, J.G. 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *Journal of Food Protection*. 75:1278-1291.

4. Ericsson, C. D., H. L. DuPont, J. J. Mathewson, M. S. West, P. C. Johnson, and J. A. Bitsura. 1990. Treatment of traveler's diarrhea with sulfamethoxazole and trimethoprim and loperamide. *Jama*. 263:257-61.

5. Feng, P., S. Delannoy, D. W. Lacher, J. M. Bosilevac, and P. Fach. 2017. Characterization and virulence potential of serogroup O113 Shiga toxin-producing *Escherichia coli* strains isolated from beef and cattle in the United States. *Journal of Food Protection*. 80:383-391.

6. Frenzel, M. A. 2017. Evaluation of antimicrobial interventions applied during further processing of raw beef products to reduce pathgoen contamination. *In*, Department of Animal Science, vol. Ph.D.: Animal Science. Texas A&M University, College Station, TX.

7. Hudson, J. C., T. N. Tolen, K. R. Kirsch, G. Acuff, T. M. Taylor, L. M. Lucia, and A. Castillo. 2019. Comparison of antimicrobial treatments applied via conventional or handheld electrostatic spray to reduce Shiga toxin-producing *Escherichia coli* on chilled beef outside rounds. *Journal of Food Protection*. 82:862-868.

8. Laster, B. A., K. B. Harris, L. M. Lucia, A. Castillo, and J. W. Savell. 2012. Efficacy of trimming chilled beef during fabrication to control *Escherichia coli* O157:H7 surrogates on subsequent subprimals. *Meat Science*. 90:420-425.

9. Laufer, A. S., J. Grass, K. Holt, J. M. Whichard, P. M. Griffin, and L. H. Gould. 2014. Outbreaks of *Salmonella* infections attributed to beef - United States, 1973-2011. *Epidemiology and Infection*:1-11.

10. Scharff, R. L. 2020. Food attribution and economic cost estimates for meat- and poultry-related illnesses. *Journal of Food Protection*. 83:959-967.

11. Signorini, M., M. Costa, D. Teitelbaum, V. Restovich, H. Brasesco, D. García, V. Superno, S. Petroli, M. Bruzzone, V. Arduini, M. Vanzini, A. Sucari, G. Suberbie, T. Maricel, R. Rodríguez, and G. A. Leotta. 2018. Evaluation of decontamination efficacy of commonly used interventions for beef carcasses against Shiga toxin-producing *Escherichia coli*. *Meat Science*. 142:44-51.

12. Stella, J. M., J. B. Luchansky, K. Miller, B. A. Shoyer, L. E. Shane, L. McGeary, M. Osoria, L. J. Stahler, N. J. Sevart, R. K. Phebus, H. Thippareddi, and A. C. S. Porto-Fett. 2017. Use of an electrostatic spraying system or the sprayed lethality in container method to

deliver antimicrobial agents onto the surface of beef subprimals to control Shiga toxin-producing *Escherichia coli. Journal of Food Protection*. 80:1393-1400.

13. U.S. Centers for Disease Control and Prevention. Date, 2020, List of selected multistate foodborne outbreak investigations. Available at: https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html. Accessed June 1, 2020.

14. USDA-FSIS. Date, 2010, Use of non-pathogenic *Escherichia coli* (*E. coli*) cultures as surrogate indicator organisms in validation studies. Available at: http://askfsis.custhelp.com/app/answers/detail/a_id/1392/~/use-of-non-pathogenic-escherichia-coli-(e.-coli)-cultures-as-surrogate. Accessed 3 June 2020.

15. USDA-FSIS. Date, 2017, *Salmonella* compliance guidelines for small and very small meat and poultry establishments that produce ready-to-eat (RTE) products and revised appendix A, July 2017. Accessed 10 April 2019.

16. Villarreal-Silva, M., D. P. Genho, I. Ilhak, L. M. Lucia, J. S. Dickson, K. B. Gehring, J. W. Savell, and A. Castillo. 2016. Tracing surrogates for enteric pathogens inoculated on hide through the beef harvesting process. *Journal of Food Protection*. 79:1860-1867.

17. Ziara, R. M. M., S. Li, B. I. Dvorak, and J. Subbiah. 2016. Water and energy use of antimicrobial interventions in a mid-size beef packing plant. *Applied Engineering in Agriculture*. 32:873-879.

18. Ziegler, F., J. V. Spencer, and W. J. Stadelman. 1954. A Rapid Method for Determining Spoilage in Fresh Poultry Meat. *Poultry Science*. 33:1253-1255.

Figures

Figure 1: Least squares means of surviving Escherichia coli *pathogen surrogates on warmed beef cuts as a function of beef cut type (*p*=0.0004).* Symbols for briskets and shoulder clods depict means from two experimental replicates, each with three independent, identically treated samples (*N*=6), plus or minus the 95% confidence interval (CI). Means not sharing a letter appearing above the mean (A, B) differ at *p*=0.05 by Tukey's Honestly Significant Differences test. Pooled standard error=0.10.

Figure 2: Least squares means of surviving Escherichia coli *pathogen surrogates on warmed beef cuts as a function of antimicrobial treatment (*p*=<0.0001).* Symbols depict means from two experimental replicates, each with three independent, identically treated samples (*N*=6), plus or minus the 95% confidence interval (CI). Means not sharing a letter appearing above the mean (A, B) differ at *p*=0.05 by Tukey's Honestly Significant Differences test. Pooled standard error=0.12. LA: lactic acid spray (55°C, 2.5%); ESS: lactic acid (55°C, 2.5%) applied by electrostatic sprayer wand device; HW: hot water spray (82°C); RW: recycled water using HW-dripped off water pasteurized to 82°C prior to mixing with fresh water prior to application; CON: *E. coli*-inoculated, non-treated control.

Tables

Table 1: Mean water recovery from hot water (HW)-treated beef cuts used during recycled water (RW) treatments and estimated water loss reductions by cut type.

Beef Carcass Cut	*Mean Water Recovery (mL)*[a]	*Water Use Per Replicate (mL)*[b]	*Water Loss Reduction (%)*[c]
Brisket	145±49	200	45.0
Shoulder/Clod	198±11	200	97.5
Round	317±247	200	100

[a] Values report mean±standard deviation of water recovered per sample over two identical replicates (*N*=6) for each beef carcass cut from HW treatment prior to pasteurization and re-use in RW treatment.

[b] Reports the cumulative volume of water applied per replicate to all samples within the sub-primal type.

[c] Calculated as: (Water Use Per Replicate - Mean Water Recovery) x100% = Water Loss %. 100% - Water Loss % = Water Loss Reduction %.