EVALUATING THE IMPACT OF ANTIBIOTIC RESISTANCE ON LACTIC ACID CROSS TOLERANCE AND PERSISTENCE IN SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* ON BEEF

by

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(Under the Direction of Govindaraj Dev Kumar)

ABSTRACT

Beef and beef products have been implicated in several outbreaks involving Shiga-toxin producing *E. coli* (STEC). In 2012, six additional STEC besides the commonly implicated *E. coli* O157: H7 were added to the list of adulterants on beef. An additional complication to the challenge of STEC on beef is the continued isolation of bacteria expressing resistance to at least one antibiotic, some of which have been linked to food borne outbreaks. Several interventions including chemical decontamination are used in the beef industry but, the continued outbreaks and isolation of bacteria on beef allude to the need for more research and improved decontamination technologies. Bacteria exposed to sublethal concentrations of antimicrobials can develop resistance and potential cross-resistance or tolerance to other antimicrobials. Cross-tolerance in bacteria has been evaluated in some studies however none has evaluated antibiotic-resistance (ABR) associated cross tolerance to industrial sanitizers. The goal of the current study

was to evaluate cross-tolerance in ABR STEC to sanitizers used in the beef industry. Resistance to ampicillin and streptomycin commonly expressed in ABR STEC isolates were developed in *E. coli* O157: H7 and the mutant strains were evaluated for phenotypic and genetic changes. Further, ABR profiles were developed in non O157: H7 STEC and the strains were evaluated for cross-tolerance to lactic acid (LA) and peroxyacetic acid (PAA). The impact of amino acids rich media on the efficacy of LA and PAA was also evaluated. It was found that ABR in *E. coli* O157: H7 H1730 increased lag phase duration in the bacteria and improved tolerance to extreme acid stress which was linked to efflux pumps. In the next study, it was found that biofilm formation was also improved in ABR bacteria and genetic mutations potentially contributing towards cross-tolerance were described. Amino acids in beef exudates were observed to significantly increase the bactericidal concentration of PAA for all bacterial strains evaluated. Finally, a novel combination of pelargonic acid and lactic acid was developed and evaluated as an alternative antimicrobial treatment. The results highlight the risks associated with antibiotic associated sanitizer cross-tolerance in STEC and proposes an alternative approach to chemical decontamination.

INDEX WORDS: Beef, Shiga-toxin producing *E. coli*, antibiotic-resistance, antimicrobial cross-tolerance, lactic acid, peroxyacetic acid, pelargonic acid

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DEDICATION

I would like to dedicate this work to my mother, father, my siblings, and my fiancée, Chiamaka Enendu. None of this work would be possible without your continued love, patience, and support. Thank you.

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CHAPTER 1

INTRODUCTION

1.1 Shiga-toxin producing *E. coli* on beef

Shiga toxigenic *Escherichia* coli (STEC) has been responsible for numerous foodborne illness outbreaks in the United States (9). Between 1982 and 2002, ground beef was the most implicated food vehicle for *E. coli* O157: H7 transmission (25). Outbreaks from contaminated beef resulted in over 1000 cases of illnesses and over 200 hospitalizations between 2006 and 2020 (8). In 2019, more than 62,000 pounds of whole cuts of beef (steaks, roasts, and ribs) were recalled due to potential *E. coli* O157: H7 contamination (28). Other serogroups of Shiga-toxin producing *E. coli* (the big six) have also resulted in food borne outbreaks on beef. In 2010, ground beef contaminated by *E. coli* O26 caused an outbreak in Maine leading to a recall of ground beef products in the state (26). There were 2006 cases of infection associated with the big six STEC between 2000 – 2010 and within this time, the number of reported infections increased from an incidence of 0.12 per 100,000 population in 2000 to 0.95 per 100,000 in 2010 (17). Cattle are a significant reservoir for Shiga-toxin producing *E. coli* (18, 23). Infection of cattle can occur from animal feed, potentially contaminated water from different sources, and improperly composted animal manure (27). At the processing level, during slaughter and evisceration, carcass surfaces could get contaminated with foodborne pathogens that are part of the commensal microflora of cattle. Additionally, beef carcass trims that are primarily utilized for ground beef preparation frequently get contaminated, leading to potential contamination of the final product (15). To mitigate these risks, the use of steam/hot-water and antimicrobial washes

containing organic acids on beef carcasses is a common practice (1, 13). More recently, the beef trim wash has also gained interest as an added intervention because worker hands and tools more readily recontaminate beef trims used in ground beef production (19). Despite several mitigation strategies in place, beef-associated recalls and outbreaks have continued, resulting in significant economic and health burdens.

1.2 **Risk and development of antibiotic-resistance and antimicrobial cross-tolerance**

Antibiotic-resistant *E. coli* O157:H7 are routinely isolated from beef. In 2015, multidrug-resistant *E. coli* was isolated from 20% of ground beef and 41% of beef cow ceca (24). A surveillance study of retail meat conducted between 2016 to 2017 reported that 16 *E. coli* isolates were recovered from 14 of 85 ground beef samples, and 2.3% contained genes conferring resistance to Ampicillin (30). The beef production process involves multiple stages. Cattle are typically moved from cow-calf systems (permanent herd, for the production of beef cattle) to backgrounding (post-weaning) and to feedlot /finishing operations before transport to a slaughterhouse for processing (6). At all these stages, antimicrobials are used as prophylaxis/metaphylaxis to prevent or resolve endemic infectious diseases (6). At post-production after slaughter, antimicrobials like lactic acid, citric acid, and acetic acid are applied to surfaces of whole carcasses to control foodborne pathogenic bacteria like *E.coli* and *Salmonella* ((29). Antimicrobials are also used to decontaminate beef processing equipment like beef grinders which can be vehicles for bacterial transfer because bacteria can attach firmly to grinder surfaces (14). The continuous but inevitable use of antimicrobials along the continuum of beef production can result in the development of antimicrobial resistance in pathogens. This phenomenon has been extensively reviewed (2, 6, 12) and may partly explain the recovery of bacteria on beef grinders and surfaces of beef carcass despite antimicrobial interventions (14).

The development of resistance to biocides could potentially contribute towards the cross-resistance of *E. coli* to other biocides and antibiotics (16). It was shown that *E. coli* O157: H7 adapted to low-level Triclosan exhibited cross-resistance to chloramphenicol, tetracycline, amoxicillin, trimethoprim, benzalkonium chloride, and chlorohexidine (4, 5).

1.3 Research gap, current challenge, and potential alternative

The potential for antibiotic-biocide cross-tolerance exists; however, no study has shown a link between antibiotic resistance in STEC and cross-tolerance to acid-based biocides. Understanding the role of antibiotic resistance in fortifying stress response in STEC might be essential in understanding risk factors that could lead to the pathogen's survival on beef. Common organic acids like lactic acid, citric acid, and acetic acid for carcass decontamination in the beef industry have been studied and observed to have some efficacy against STEC (29). However, concentrations below 4% acid do not significantly reduce the bacteria count even when combined with a high-temperature hurdle (3, 21) and practical concentrations used in the Industry could be as low as 1 %. Also, the presence of amino acids, for example on beef, could contribute to the survival of STEC exposed to acid-based sanitizers. Acid resistance systems that utilize the amino acids glutamate, glutamine, arginine, lysine, and serine to consume protons within the intracellular space of cells during acid stress have been characterized (7, 11, 20, 22). The costs associated with the use of consistently high concentrations of organic acids combined with the development of tolerance to these sanitizers highlights the potential for mitigation strategies that could resolve tolerance development in STEC to acid-based sanitizer treatments. Pelargonic acid, a short-chained fatty acid with 9 carbon atoms, might provide better antimicrobial activity than the organic acids alone when used synergistically with common organic acids. This fatty acid has been demonstrated to disrupt the bacterial cell membrane of

Salmonella serotypes Newport. It is bactericidal towards *S*. Newport, *S*. Oranienburg, and *S*. Typhimurium at low concentrations of 31.25 mM (10). The potential for a synergistic antimicrobial activity between pelargonic acid and currently used biocides such as lactic acid exists. The combination of low pH with membrane disruption could result in an intervention measure with improved efficacy against STEC.

1.4 Project objectives

The goal of this dissertation was to evaluate the influence of antibiotic-resistance on antimicrobial cross-tolerance in Shiga-toxin producing *E. coli*. Phenotypic and genetic changes in bacteria with resistance profiles to the common antibiotics ampicillin and streptomycin were evaluated and an alternative antimicrobial, combining two antimicrobial technologies was evaluated against highly resistant or tolerant strains. The specific objectives were:

1. **To determine the influence of antibiotic-resistance on the phenotypic and genetic characteristics of *E. coli* O157:H7 (Chapters 3 and 4).** Information on genetic mutations, changes in growth rates and biofilm formation from the acquisition of antibiotic-resistance were presented in these chapters.
2. **To evaluate the influence of antibiotic-resistance in Shiga-toxin producing *E. coli* on cross-tolerance to lactic acid and peroxyacetic acid (Chapters 3, 4, 5, 6).** There are no studies yet linking antibiotic-resistance in Shiga-toxin producing *E. coli* (STEC) with cross-tolerance to lactic acid or peroxyacetic acid. These chapters provide this information.
3. **To evaluate the impact of Nutrients and antibiotic resistance on peroxyacetic acid tolerance (Chapter 6)**

   The presence of amino acids in media may influence acid tolerance. Acid tolerance in the presence of amino acids rich media could also vary between antibiotic resistant and

antibiotic susceptible bacteria. This chapter provides information on the variations in peroxyacetic acid tolerance between antibiotic resistant and antibiotic susceptible STEC in the presence of amino acids.

4. **To evaluate the efficacy of pelargonic acid fortified lactic acid against STEC (Chapter 7**). Alternative interventions, especially those that combine multiple antimicrobial technologies are needed to combat bacterial contamination and the development of antimicrobial tolerance. In this chapter, we presented a novel combination of pelargonic acid and lactic acid with improved efficacy against STEC.

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CHAPTER 2

LITERATURE REVIEW

2.1 Pathogenicity and important characteristics of Shiga-toxin producing *E. coli*

Shiga-toxin producing *Escherichia coli* (STEC) are non-sporulating, Gram negative bacillus (rod), facultative anaerobes that harbor and express the genes for Shiga-toxins type 1 (Stx-1) and 2 (Stx-2) (59). *E. coli* O157:H7 is perhaps the most virulent STEC frequently associated with outbreaks of human infections and was first recognized as a foodborne pathogen in 1982 during the investigation of two outbreaks of hemorrhagic colitis linked to the consumption of undercooked ground beef (94). The big six STEC also known to cause infections include *E. coli* O26, O45, O103, O111, O121 and O145 (98)

It was estimated that the number of annual illnesses in the United States caused by STEC was 231,157, with *E. coli* O157: H7 responsible for 40.3 % of these illnesses while the big six STEC serogroups were responsible for 59.7 % (57). STEC evolved from their non-pathogenic counterparts and acquired genes from *Shigella dysenteriae* encoding for the extracellular cytotoxin Stx through transduction (98). The Stx cytotoxins or verotoxins (Stx-1 and Stx-2) were so named for their toxic effect *in vitro* on African green monkey kidney (Vero cell lines) and have similar mechanisms of action. They target the 28S subunit of the ribosomal RNA, inhibiting the synthesis of proteins and leading to cell death (42).

Shiga toxins are virulence factors that belong to the AB5 family of protein toxins (8). They have an enzymatically active A moiety and a nontoxic B moiety that binds to cellular receptors (8). The B moiety or subunit is a nonglycosylated homopentameric protein that binds

to glycolipid globotriaosylaceramide (Gb3) on the surface of host cells and delivers the active A unit into the cell (37).

Shiga toxin 2 (Stx-2) is a relatively stable toxin. In one study, it was observed that Stx-2 was not inactivated by temperature/time conditions of 63 °C for 30 min, 72 ºC for 15 s or 89 °C for 1 s during milk pasteurization but required treatment at 100 °C for 5 min for inactivation (90). In another study, pasteurization conditions for apple juice (63 °C for 30 min, 72 °C for 15 s or 89 °C for 1 s) did not inactivate Stx-2, but all toxin activity was lost after exposure to 100 °C for 5 min (91). A study evaluating the effect of microwave heating on the stability of Stx in milk showed that 165 kJ microwave energy or thermal heating did not destroy the biological effect of Stx-2, but exposure to 198 kJ or heating at 95 °C for 5 min reduced Stx-2 activity (92).

The detection of Stx in food is one way to determine contamination by STEC. Several techniques are used for the detection of Stx. In a 2012 study, highly sensitive enzyme linked immunosorbent Assay (ELISA) and immuno-polymerase chain reaction (immune-PCR) assays capable of detecting 1 and 0.01 pg/ml of Stx 2a in milk were developed using the monoclonal antibody pair Stx2-1 and Stx2-2 (58). Commercial ELISA kits have also been developed. One study in 2015 evaluating the sensitivity of three commercial ELISA kits: Premier EHEC test (Meridian Bioscience Inc., Cincinnati, OH), the ProSpecT Shiga toxin *E. coli* (STEC) Microplate Assay (Remel Inc., Lenexa, KS), and the Ridascreen Verotoxin Enzyme Immunoassay (r-Biopharm AG, Darmstadt, Germany) showed that the specificities of the tests were comparable and two of the kits detected toxins in 67 of 70 serotyped isolates (110). A lateral flow assay (LFA) was developed in 2015 to provide rapid and sensitive detection of Stx directly from STEC culture supernatants. The LFA had a limit of detection of 0.1 ng/ml for Stx-2a (34).

Shiga toxin production in STEC can be influenced differentially by different classes of antibiotics. Exposure of STEC to subinhibitory levels of antibiotics that target DNA synthesis such as ciprofloxacin can induce Stx production but exposure to subinhibitory levels of antibiotics that target the cell wall, ribosome or RNA polymerase such as ampicillin, do not induce Stx production and could reduce production in some cases (78). In one study, chloramphenicol, levofloxacin and streptomycin increased Stx-2 gene expression by a thousand times more than the housekeeping genes (33). In another study, ciprofloxacin increased the total concentration of Stx-2 by ~60 fold after exposure for 6 h despite a > 10,000-fold reduction in recoverable colony-forming units (117). In the same study, fosfomycin had no effect on toxin production (117).

STEC illness could be mild, self-limiting gastroenteritis but could also involve the development of hemorrhagic colitis (bloody diarrhea) found in 90 % of patients and hemolytic uremic syndrome (HUS) seen in 5-15 % of patients (98) . HUS is typically characterized by acute kidney failure, microangiopathic hemolytic anemia (damage of small blood vessels with destruction of red blood cells), and thrombocytopenia (decrease in platelets) (98).

There are 8 pathovars of *E. coli* broadly classified as either diarrhoeagenic *E. coli* or extraintestinal *E. coli.* Diarrhoeagenic *E. coli* include 6 pathovars: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) while extraintestinal *E. coli* include 2 pathovars: uropathogenic *E. coli* and neonatal meningitis *E. coli* (NMEC) (39). STEC are EHEC pathovars that possess 16 potential fimbria-like operons and a type IV pilus called the hemorrhagic coli pilus involved in adherence and biofilm formation. STEC attaches intimately to host cells (intestinal epithelial cells) through interactions between intimin and the translocated

intimin receptor (Tir). Tir cytoskeleton-coupling proteins mediate the rearrangement of actin necessary for pedestal formation and STEC injects several effectors including the Shiga-toxin or verotoxin into the host cell to manipulate host processes (39). Cattle and other ruminants are natural reservoirs of STEC and the infectious dose for STEC O157: H7 is considered to be as low as 100 organisms (98).

Treatment of those infected with STEC diarrhea consists of fluid replacement, careful monitoring of kidney function without antibiotic therapy and supportive care. *E. coli* O157:H7 possesses high resistance to different antibiotics because of its effective multidrug efflux pumps and outer membrane that acts as a barrier (79). Also, treatment of infection with antibiotics such as ciprofloxacin, streptomycin and other antibiotics that target DNA synthesis could exacerbate it's clinical outcome as administration of antibiotics has been associated with onset of HUS due to bacterial lyses and release of Shiga-toxin or induction of Stx bacteriophages and subsequent production of toxin (98).

Commensal and zoonotic bacteria, such as *E. coli*, are carriers of transmissible resistance genes and resistance profiles to antibiotics have been observed in *E. coli* O157:H7 (109). Antibiotics commonly used in human medicine are of similar class or mechanism of action, as those used in food-producing animals (1) (Table 2.2) thus development of resistance in one population could potentially affect the other. STEC are a serious public health threat, and the potential spread of antimicrobial resistance genes constitute an even greater threat.

2.2 Outbreaks of *E. coli* O157: H7 on beef

There have been several outbreaks and recalls involving beef and beef products in the United States. Table 2.1 shows some select outbreaks on beef and beef products between 2007

and 2016. According to the Center for Disease Control and Prevention (CDC.) National Outbreak Reporting System (NORS), 84 outbreaks involving beef and beef products were reported between 2006 and 2018 (19). These cases resulted in 1,146 cases of illnesses, 326 hospitalizations, and 6 deaths (19). Of these reported cases of outbreaks, 17 percent have been from *E. coli* O157: H7. Shiga-toxigenic *E. coli* has been reported to cause about 73,000 illnesses annually in the United States; In a review of outbreaks between 1982 and 2002, food was reported to be the predominant transmission route for *E.coli* O157: H7, accounting for 52 percent of 350 outbreaks recorded within that time (89). Beef and beef products were the most common food vehicles and were implicated in 46 percent of all cases (89). Outbreaks result in substantial financial losses to the beef industry. In the 2018 outbreak on Ground beef, approximately 6,937,195 pounds of various raw, non-intact beef was recalled (105).

2.3 Outbreaks and prevalence of the big six Shiga-toxin producing *E. coli*

There are currently 6 non-O157 STEC recognized as adulterants in beef (107) including *E. coli* O26, O103, O111, O145, O45, and O121. In 2019, an outbreak in the United States from the consumption of ground beef caused by *E. coli* O103 resulted in 209 cases of illness in 10 states leading to 29 hospitalizations (31). *E. coli* O26 was implicated in a 2018 outbreak which led to 18 reported cases of infection, 1 case of hemolytic Uremic syndrome and 1 death (30). Other meat products including Northfork Bison tainted by *E. coli* O121 and *E. coli* O103 have also been reported (32).

The big six STEC have been implicated in several outbreaks on other food products. In a study reviewing food borne outbreaks between 1971-2010 involving the big six, it was reported that 10 out of 12 outbreaks could be classified into commodities including dairy (n =3), game meat (n =2), beef (n=1), pork (n=1), leafy vegetables (n=2) and fruits or nuts (n=1) (Luna-Gierke

et al., 2014). In another study, Shiga-like toxins linked to non O157 Shiga-toxin producing *E. coli* were found on beef (23 %), veal (63 %), pork (18 %), chicken (12 %), turkey (7 %), lamb (48 %), fish (10 %) and shellfish (10 %) (96). Commercial ground beef (n = 4,133) was sampled in one study for the prevalence of non-O157 Shiga-toxin producing *E. coli* strains and it was reported that 338 unique STEC isolates were reported from 300 samples yielding isolates of STEC. In the same study, 0.24 % of the 4,133 samples contained isolates with *eae, subA* and *nle* genes that may be considered a significant food safety threat (10). In 2014, PFP enterprises recalled about 15, 865 pounds of beef products because of contamination by the big six STEC. The recalled products included 10.5 pounds of beef outside skirt steak, 15 pound boxes of preseasoned beef for fajita, 40 pound boxes of Southwest style beef skirts and 20 pound boxes of Patterson food processors beef skirt (70). One of the largest outbreaks from non O157 Shiga toxin producing *E. coli* occurred in 2011 which affected people in 12 European countries, sickened more than 4,000 people and killed 49 (9).

The non O157 STEC have been detected in several animal species including cattle, sheep, goats, buffaloes, guanaco, deer and elk, swine, rabbits, dogs, and shellfish (65). Cattle are identified as the primary reservoir of non O157 STEC and are the most important source of human infection (65). Various countries have reported wide ranges of prevalence of non O157 STEC in feces from dairy (0.4-7.4 %) and beef (2.1-70.1 %) (65). In one report, a total of 26 different serotypes were detected from organic and conventional dairy farms in Minnesota (35). There were 2006 reported cases of non O157 STEC between 2000 – 2010 and infections increased from an incidence of 0.12 per 100,000 population in 2000 to 0.95 per 100,000 in 2010 (56).

2.4 Antibiotic resistant bacteria on beef

Antibiotic-resistant bacteria are a public health challenge. According to the CDC, at least 2.8 million people get an antibiotic-resistant infection annually in the United States, and 35,000 die from these infections (28). According to a Center for Science in the Public Interest (CSPI) white paper, between 1973 to 2009, there were 35 foodborne outbreaks in which bacteria identified were resistant to at least one antibiotic and these outbreaks were common in dairy products (34 %) and ground beef (26 %) (46). Pathogenic *E. coli* with antibiotic-resistance profiles were identified in 5 outbreaks within this time, leading to 923 illnesses. The most common antibiotic-resistance profiles identified from were tetracycline, streptomycin and ampicillin (46). Resistance in bacteria can be induced by long-term exposure to sub-lethal levels of antimicrobials (83). Food animals are often exposed to antimicrobials. Ionophores and sulfonamides are used to prevent coccidiosis in poultry, penicillin, amoxicillin, and other antibiotics administered to cattle to treat various infections and improve feed efficiency (77). In agricultural production and food processing, antimicrobials are used as pesticides, sanitizers, and disinfectants (83). Bacteria carrying resistant genes persist and are widespread in the food chain, and can be directly transmitted to humans through farm animals, consumption of raw or inadequately cooked food, and cross-contamination between contaminated food and other foods (Fig 2.4) (6, 83).

The National Antimicrobial Resistance Monitoring System (NARMS) monitors bacterial antimicrobial-resistance to drug classes generally considered first or second-line treatments in humans. According to the World Health Organization (WHO), antimicrobial drugs (antibiotics) are grouped into three based on their availability and use to treat infections from non-human sources: critically important, highly important, and important (114). The critically important

antimicrobial drugs include ampicillin and streptomycin, the highly important include chloramphenicol and mecillinam, and the important include bacitracin (114). Nearly 80 % of retail meat products (beef, chicken, and turkey) were reported to contain bacteria resistant to most of these antimicrobial groups according to the 2015 integrative NARMS report, and 62 % of Ground beef products had these bacteria (81). Several prevalence studies also report antibiotic-resistant *E. coli* O157: H7 on meat. In one study, *E. coli* O157: H7 from raw meat samples - poultry and beef - had the highest resistance to ampicillin (100 %), tetracycline (83.33 %), and gentamycin (83.33 %) (76); in another study, *E. coli* O157: H7 isolated from dairy cows, salami, cull dairy cow feces, ground beef, bulk tank milk, lettuce, and bovine feces were resistant to aztreonam (99.2 %), cefaclor (96.9 %), ampicillin (93 %), cephalothin (90.7 %), cinoxacin (87.6 %), nalidixic acid (86.1 %), sulfisoxazole (62 %), nitrofurantoin (57.7 %), kanamycin (37.2 %), gentamycin (26.4 %), carbenicillin (21.7 %), cefotaxime (10.9 %), cefmetazole (14.7 %), ciprofloxacin (12.4 %), fosfomycin (10.9 %), cefuroxime (10.9 %), cefmetazole (7 %), streptomycin (7 %), ceftriaxone (6.2 %), tetracycline (3.1 %), norfloxacin (3.9 %), tobramycin (3.1 %), amikacin (2.3 %), trimethoprim (1.6 %) and moxalactam (1.6 %)(100).

Pathogenic *E. coli* and *Salmonella* spp. have been the predominant cause of foodborne outbreaks on beef products. Between 2006 to 2019, beef products contaminated by *Salmonella* or pathogenic *E. coli* accounted for 918 cases of foodborne outbreak infections resulting in 293 hospitalizations, 10 deaths, and the economic loss of $ 1,969,009,360 from medical costs, premature deaths, and productivity loss (29, 103). Ground beef products accounted for over 90 % of these recalls. The highest case count was recorded in 2018 when 403 persons were infected after consuming ground beef contaminated by *Salmonella* Newport. Contamination by *E. coli* was more prevalent in reported outbreaks, and the most associated strain was *E. coli* O157: H7

(29). Concurrently, the National Antimicrobial Resistance Monitoring System (NARMS) has reported annual isolation of multidrug-resistant strains of *E. coli* O157: H7 and *Salmonella* from both pathogen reduction/Hazard Analysis Critical Control Point (PR/HACCP) testing and retail ground beef (81). Since the inception of NARMS in 1996, there has been a decline in the number of multidrug-resistant bacteria isolates; however, the reported figures are still high. In 2015, 21 % HACCP and 5.6 % beef isolates of *Salmonella* were multidrug-resistant, while 20 % of retail ground beef and 41 % beef cow ceca isolates of *E.coli* were multidrug-resistant (81). Evidence of the link between antimicrobial use and the emergence of antimicrobial-resistant bacteria strains has been extensively reviewed and reported by Bennani (6).

Another challenge is the potential cross-resistance of bacteria to different groups of antimicrobials (41). This phenomenon has been reported for antibiotic cross-resistance to other antibiotics and biocide cross-resistance to other biocides and antibiotics. In a 2003 study, it was observed that *E. coli* O157: H7 developed high resistance to triclosan after two sublethal exposures and developed decreased susceptibility to chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim as well as a number of biocides (11). The same researchers compared cross-resistance in *E. coli* K-12 and *E. coli* O55 to *E. coli* O157: H7 and found that *E. coli* K-12 adapted to triclosan developed cross-resistance to chloramphenicol while *E. coli* O55 was cross-resistant to trimethoprim. *E. coli* O157: H7 showed cross-resistance to a wider range of antimicrobials and the researchers concluded that levels of cross-resistance in bacteria may be strain specific (12). In another study, *E. coli* adapted to quaternary ammonium compounds (QAC) through gradual exposure to increasing concentrations of the antimicrobials developed reduced susceptibility to QAC and antibiotics (ampicillin, ceftazidime, chloramphenicol, cefotaxime, nalidixic acid and ciprofloxacin) as well as to phenicol compounds (99). Multiple

antibiotic resistant (Mar) mutants of *E. coli* exhibited cross-resistance to norfloxacin and other fluoroquinolones (36). A study investigating cross-resistance in *E. coli* after adaptation to benzalkonium chloride (BC) showed that the MIC of BC was increased from 25 to 150 μg/ml after 24 passages in gradually higher concentrations of BC. In the same study, cells adapted to BC developed cross-resistance chloramphenicol, but also, adaptation of cells to chloramphenicol resulted in a threefold increase in the MIC of BC from 25 to 70 μg/ml (69).

2.5 Use and prevalence of approved antibiotics in food-producing animals

Antibiotics are used extensively in food animal production. According to the FDA., 25,624,385 Kg of medically important antimicrobial drugs were sold and distributed annually for use in food-producing animals in the United States between 2016 to 2019 (52). The breakdown of sale and distribution by antimicrobial drug class are reported in Fig 2.1. The drug classes with the highest reported sales were tetracyclines, penicillin, and aminoglycosides. When we consider these sale figures by species-specific estimates, sales were the highest in cattle. Between 2016 and 2019, 10,986,046 Kg of antimicrobial drugs were sold and distributed for cattle production (52). These sales estimates are reported in Fig 2.2. The drug classes predominantly used in cattle production are aminoglycosides, cephalosporins, penicillins, tetracyclines, sulfas, and lincosamides (52).

2.6 Development of antibiotic resistance and potential antibiotic-biocide cross-tolerance

Antimicrobials used in agriculture could include drugs such as antibiotics, antifungals and antiprotozoals that target particular microbial pathogens as well as sanitizers such as chlorine, lactic acid, peracetic acid that are used to reduce cross-contamination (6). Antimicrobial agents exert their inhibitory activities through different mechanisms. Antibiotics

generally function by selectively interfering with cellular protein and nucleic acid synthesis at specific target sites (55). Biocides like lactic acid or peracetic acid have been reported to result in bacterial cell damage through the destruction of cell membrane layer structure, perturbation of the cytoplasmic membrane, and leakage of cell protein (85, 106). The development of resistance to antibiotics has been reported for over half a century (2) and is currently a matter of critical concern to public health. Further, it is not currently established if antibiotic resistant strains of bacteria have higher tolerances to sanitizers resulting in increased fitness and survivability (88).

Generally, bacterial resistance to antimicrobials has been reported to develop through: drug target alteration, bacterial outer membrane impermeability, enzymatic modification or destruction, and efflux systems (85, 88). Different adaptation mechanisms in bacteria are listed in table 1.2. Cross-resistance or tolerance typically occurs because antibiotics and biocides mostly exert their inhibitory effects through similar mechanisms. For example, in *E. coli* adaptation to Triclosan, the up-regulation of endogenous multidrug efflux systems leads to elevated resistance to chloramphenicol, tetracycline, amoxicillin, trimethoprim, benzalkonium chloride, and chlorohexidine (88).

2.7 Transmission of antimicrobial resistant bacteria to food animals

The overuse and misuse of antibiotics have been linked to the development of antibiotic-resistant bacteria in food animals (6, 49, 77, 83, 116). Antibiotic-resistant bacteria can be disseminated in the environment through animal waste into water reserves. Environmental dissemination of antibiotic-resistant bacteria in the environment is shown in Fig 2.4. There is no definitive information on environmental contamination with antibiotic-resistant Shiga toxigenic *E. coli* O157: H7 but levels of *E. coli* in the feces of infected food animals can give an estimate. One study reported that calves experimentally infected with *E. coli* O157: H7 shed between 4 log

and 8 log CFU/g of bacteria for 7-20 weeks (38). In another study, it was reported that antibiotic resistance genes were released continuously into the environment through wastewater plant effluents (75). These resistance genes, which could include resistance plasmids and conjugative transposons, can be acquired by other bacteria (7). In the farm environment, antibiotic-resistant bacteria can infect food animals through the water reserves (ponds, dams, or wells), contaminated feed and feed troughs, contaminated farm equipment, ground, and pasture. The spread of antimicrobial-resistant bacteria can also occur between farm environments through infected carrier wildlife vectors, contaminated feedstuff, and humans wearing contaminated clothing (77). A study evaluating the occurrence of antibiotic resistant bacteria in vegetable, aquaculture, and caged animals (VAC) which is an integrated recycling farm system in northern Vietnam reported a high frequency of sulfamethoxazole bacteria (2.14 – 94.44 %) and high concentrations of sulfamethoxazole (612-4330 ng/l), erythromycin (154-2246 ng/l) and clarithromycin (2.8 – 778 ng/ml) in the city canal. In another study, tetracycline resistance genes (*tet*(B), *tet*(C), *tet*(O) and *tet*(W))were found in wastewater samples from a cattle operations, soil, and fecal floor (115). An investigation of antimicrobial resistant bacteria in 23 samples of commercial broiler feed and 66 samples of raw feeding materials revealed the presence of *E. coli* resistant to ampicillin, tetracycline, and streptomycin in 22.9 %, 27.6 % and 19.0 % of feed ingredient isolates and 22.4 %, 41.4 % and 17.0 % of poultry feed isolates (40).

2.8 Resistance, tolerance, and persistence

Resistance in bacteria to an antimicrobial is defined as 'the inherited ability of microorganisms to grow at high concentrations of an antimicrobial irrespective of the duration of treatment (13). Resistance in bacteria is quantifiable and is done by measuring the Minimum Inhibitory Concentration (MIC) (Fig 2.5). For example, in one study, the MIC of ampicillin

against several strains of Shigatoxigenic *E. coli* isolated from patients with diarrhea was determined to be between 7.812 – 15.62 µg/mL (80). In another study, the MIC for a range of antibiotics against bovine isolates of *E. coli* was determined; MIC for ampicillin was ≥ 128 µg/mL, ciprofloxacin ranged from 0.015-0.03 µg/mL, and gentamicin ranged from 0.5 - 1 µg/mL (87). The MIC of an antimicrobial is 'the minimum concentration of the antimicrobial required to prevent net growth of the bacteria (13).

Unlike resistance, tolerance is used when describing bacteria's ability to survive bactericidal concentrations of antimicrobials – that is, concentrations that exceed the MIC (Fig 2.5) (13). Tolerance is not fully understood, but Brauner et al. propose the Minimum Duration of Killing (MDK) as an excellent quantifiable metric for tolerance (13). MDK is defined as 'the typical duration of antimicrobial treatment required to kill a given proportion of the bacterial population at concentrations that far exceed the MIC' and is based on the time-kill curve – time to kill 99 percent of the bacterial population (13). They argue that tolerant bacteria will have a higher MDK than non-tolerant bacteria. In one study on hospital isolates of *Enterococcus faecium* obtained between 1997 - 2015, it was shown that isolates obtained post-2010 were tenfold more tolerant to alcohol killing than older isolates – this correlated with increased alcohol use in hospitals (86). Persistence occurs only in a subpopulation of bacteria that are not killed by antimicrobial treatments, which effectively kills the majority of the bacterial population. The presence of a bimodal or multimodal time-kill curve is used to detect persistence (13).

2.9 Interventions used in beef processing

Several interventions are applied to combat microbial contamination on beef and beef products. Throughout the process of beef production, microbial contaminants can be introduced into the edible parts from the gastrointestinal tract, the hide, workers and the environment (111).

In this section, two of the common antimicrobials used in beef carcass decontamination relevant to the work covered by this dissertation are discussed and a third antimicrobial that could show promise as an alternative to existing treatments is highlighted.

2.9.1 *Lactic acid*

Lactic acid is an organic acid produced by the fermentation of natural food sources such as dextrose, sucrose, or starch. It occurs naturally in many food products and is used in almost every food industry segment as an acidulant and pH regulator (104). Lactic acid is generally recognized as safe (GRAS) at 21CFR184.1061 as an antimicrobial agent, flavoring agent, curing, pickling agent, and solvent. It is used as a pathogen inhibitor for processed meat at an approved level of up to 5 percent: 2-5 % solution on beef sub-primal and trimmings, 2-2.8 % solution for beef heads and tongues, and 5 % for livestock carcass before fabrication (111).

The antibacterial action of lactic acid is suggested to occur due to its ability to penetrate the cytoplasmic membrane of pathogens in its undissociated form; this leads to the disruption of transmembrane proton motive force and reduction of intracellular pH. In a study by Wang (106), *E. coli*, *Salmonella,* and *L. monocytogenes* were observed to leak proteins at a rate of 11.76 µg/mL, 11.36 µg/mL, and 16.29 µg/mL after exposure to lactic acid for 6 h using a micro protein assay. Lactic acid is a potent outer membrane disintegrating agent - at a pH of 3.6 to 4 between 40 % and 60 % of its molecules are present in the undissociated form - that can cause injury for gram-negative bacteria and sensitize bacteria to detergents and lysozyme. Damage to cells' outer membrane by lactic acid enables other compounds' antimicrobial activity against gram-negative bacteria (3).

Several studies have evaluated the antimicrobial efficacy of lactic acid on a beef carcass. In a study by Woolthuis and Smulders (113), 1.25 % (vol/vol) lactic acid spray was found to reduce *Enterobacteriaceae* counts on 24h postmortem calf carcass from 1.8 log CFU/m^2 to below their limit of detection (< 1.3 log CFU/m^2). Better results were obtained by Castillo (17), who observed a 5.2 log CFU/cm^2 reduction in *E. coli* O157: H7 on a hot beef carcass, treated with a prechill water wash and 250 ml of 2 % lactic acid for 15 s, compared to only a prechill water wash treatment. When combined with a post-chill treatment of 500 ml 4 % lactic acid solution applied onto the outside rounds of the beef carcass for 30 s, an additional 2 to 2.4 log CFU/cm^2 reduction was observed. This same group evaluated the efficacy of a warm 4 % lactic acid solution on a chilled in-plant beef carcass (brisket, clod, and neck regions) and found that *E. coli* O157: H7 was reduced to 0 % from an initial 7.5 – 30 % *E. coli* O157: H7 positive samples (18). In another study by Gill, 2 % and 4 % lactic acid reduced *E.coli* O157: H7 at the distal surfaces of beef by ≥ 2 log CFU/100 cm^2 when sampled 5 min and 60 min after treatment respectively (54). It is important to note that the highest antimicrobial efficacy is consistently observed when 4 % of lactic acid is used for decontamination (43, 54). Approved concentrations of lactic acid for use in beef decontamination are presented in table 2.5.

2.9.2 *Peracetic acid*

Peracetic acid is the peroxide of acetic acid and is a clear, colorless liquid with a strong, pungent acetic acid odor (68). It is available in equilibrium with acetic acid and hydrogen peroxide with commercial solutions, typically at concentrations of 5-15 % (74). The acid is prepared by mixing the corresponding carboxylic acid and hydrogen peroxide in a reaction called per hydrolysis (74).

$$R - COOH + H_2O_2 \longleftrightarrow R - COOOH + H_2O$$

Peracetic acid is a potent antimicrobial used in the food industry to disinfect clean-in-place processes, pasteurizers, fresh produce, and other industrial processes (74, 84). The acetic acid degradation product of peracetic acid is regarded as safe (GRAS) for direct use as a food additive in baked goods, dairy product analogs, meat products, condiments, oils, cheeses, relishes, and sauces (21 CFR 184.1005). It is also directly used at recommended concentrations of 100 ppm on fruits, vegetables, nuts, cereal grains, spices (40 CFR 180.1196), 100 – 200 ppm on food contact articles and food-processing equipment (21 CFR 178.1010(c)(25), 21 CFR 178.1010(b)(30)), 200 – 315 ppm on dairy processing equipment (21 CFR 178.1010(c)(33), 21 CFR 178.1010(b)(38)), and not more than 80 ppm on lye peeling of fruits and vegetables that are not raw agricultural commodities (21 CFR 173.315(a)(2)).

Peracetic acid functions by the formation of highly oxidative radical species (reactive oxygen species), which result in specific damage to biomolecules: Protein denaturation, oxidation of sulfhydryl, disulfide, and double bonds in proteins, inactivation of catalase enzyme, and adverse reactions of DNA. base molecules (68, 74). However, the antimicrobial activity is reduced if the pH is alkaline or neutral because peracids are negatively charged under these conditions. Peracetic acid has been shown to have a range of activity against several microorganisms, including the foodborne pathogens *Salmonella* and *E.coli* O157: H7 (51, 112), on meat grinders (reductions in bacterial counts of 0.05 log CFU/cm^2 to 0.3 log CFU/cm^2) and fruit surfaces (5-log reduction when used at 2 times its recommended concentration), but, no significant activity was observed on a beef carcass notwithstanding the temperature or concentration (pre and post-chill) (67). Application of peracetic acid to hot beef carcass surfaces caused a 0.7 log CFU/cm^2 reduction in both *E. coli* O157: H7 and *S.* Typhimurium (67). In a study evaluating the inactivation of STEC on fresh beef, it was observed that peracetic acid

reduced the bacterial population by 0.21 log CFU/g (from an initial concentration of 6.82 log CFU/g to 6.61 log CFU/g) (14). Approved concentrations of peracetic acid used for beef decontamination are shown in table 2.5.

2.9.3 *Pelargonic acid*

Pelargonic acid, also known as Nonanoic acid, is a naturally occurring clear to a yellowish oily liquid that is insoluble in water but soluble in alcohol, ether, and organic solvents (95). It is a 9-Carbon straight-chain saturated fatty acid found naturally in several plants (82). It has been used as a herbicide and approved by the Food and Drug Administration (FDA) for use as a food additive (82). Table 2.3 shows different food applications for pelargonic acid and permissible concentrations.

Although previously reported as an antimicrobial fatty acid with antifungal properties, studies done by (45) showed its antimicrobial activity against *Salmonella* Newport, Javiana, and Typhimurium. They evaluated its antimicrobial activity against other antimicrobial fatty acids – lauric acid, myristic, palmitic, margaric, stearic, and oleic acid – and found that it had the lowest inhibitory concentration of 31.25 mM compared to a minimum of 62.5 mM for the other fatty acids.

Antimicrobial fatty acids like Pelargonic acid exhibit broad-spectrum antimicrobial activity. Generally, they solubilize bacterial cell membranes leading to the release of large sections of various membrane proteins and lipids and interference with electron transport and oxidative phosphorylation (44).

2.9 Factors affecting antimicrobial efficacy on beef

Antimicrobials applied on the surface of food products like beef during decontamination could be affected by several factors such as temperature, exposure time, type of chemical and concentration, spray pressure, method of application, stage in the process of production and the presence of high organic load or nutrients. Several studies evaluating antimicrobial efficacy during decontamination combine two or more of these factors (17, 43, 50, 54, 63, 102). Although all factor combinations highlighted play an important role in antimicrobial efficacy during decontamination, the interaction between pathogenic bacteria and organic matter or nutrients on the surface of food products during antimicrobial treatment require some consideration. In one study evaluating the effect of exposure time and organic matter on the efficacy of antimicrobial compounds against STEC, it was reported that the presence of beef purge (organic matter) significantly affected the activity of PAA and hypobromous acid (BR). In aqueous solutions with organic matter, PAA and BR reduced the population of STEC by at least 5.0 log CFU/ml regardless of exposure time however in the presence of beef purge the effect of PAA was dependent on the exposure time, with reductions of about 1-2 log CFU/ml at the lowest exposure time (15 s) while BR was ineffective against STEC regardless of the exposure time (63). In another study, 0.02 % peroxyacetic acid and acidified sodium chlorite solutions had little effect on the numbers of aerobes, *E. coli*, and coliforms on meat from plants (54). Organic matter present on the surface of beef could interact with certain antimicrobials. For example, PAA acts as a protein denaturant, and in one study, PAA showed a higher decay rate during treatment of solution containing casein (48) Also, certain nutrients like amino acids present in high amounts on beef surfaces and beef run off could be used by bacteria to survive acid based antimicrobial

treatment through acid resistance pathways. The process of acid resistance in STEC has been extensively studied (16, 62, 66).

2.10 Acid resistance systems

Acid resistance systems in bacteria protect the cells from extreme acid stress (pH 2.5 or less). There are three main acid resistance systems in *E. coli*. The first is a glucose repressed oxidative system with an unclear mechanism of action while the other two systems with clearly defined mechanisms utilize the amino acids glutamate and arginine (53). In both the glutamate and arginine dependent systems, protons within cells are consumed during the decarboxylation of glutamate or arginine to gamma amino butyric acid and agmatine respectively. The end products of the decarboxylation reaction are transported out of the cell and exchanged for new substrates (16). Other amino acids besides glutamate and arginine have also been reported to play roles in acid resistance (Table 2.4). A study in 2007 showed that in acidic environments (pH 5.8) and lysine-rich environment, *E. coli* induces the expression of the *cadBA* operon catalyzing the decarboxylation of lysine to cadaverine (101). The decarboxylation of lysine to cadaverine results in the consumption of intracellular protons within acid challenged cells (101). The role of lysine in acid resistance was also investigated by (47) in a study that showed that increasing concentrations of lysine to more 0.5 mmol l^{-1} enhanced the recovery rate of *E. coli* O157: H7 to10 % (47). Researchers investigating the role of glutamine (one of the most abundant food-borne free amino acids) in acid resistance observed that the amino acid provides resistance to acid stress (pH 2.5) through an enzymatic release of ammonia (71). In the study, glutamine was converted to glutamate by an acid-activated glutaminase *YbaS* and the resulting ammonia released in the reaction were quickly protonated to ammonium ions. This system however worked best in combination with the glutamate dependent system (71). Recently, the amino acid

serine was identified to also play a role in acid resistance through a deamination system (108). In the 2022 study, serine was transported into the cell during acid stress and deaminated by deaminases (SdaA and SdaB) to produce pyruvate. The deamination reaction produced ammonia which as shown with the glutamine dependent system is protonated to ammonium ions with a resulting increase in the cytoplasmic pH (108).

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Table 2.1: Foodborne outbreaks involving *E. coli* O157: H7 on beef

Year	Vehicle	Case count	Hospitalizations	Death	Reference
2016	Beef products	11	7	0	(27)
2014	Ground beef	12	7	0	(26)
2011	Lebanon Bologna	14	3	0	(25)
2010	Beef	21	9	0	(24)
2009	Ground beef	49	31	2	(22, 23)
2008	Ground beef	49	27	0	(21)
2007	Ground beef patties	40	21	0	(20)
Total		196	105	2	

Table 2.2: Bacterial mechanisms of resistance to biocides (97)

Mechanism	Nature	Level of susceptibility to other biocides*	Cross-resistance
Permeability	Intrinsic (acquired)	No	Yes
Efflux	Intrinsic/acquired	Reduced	Yes
Degradation	Intrinsic/acquired	Reduced	No
Mutation (target site)	Acquired	Reduced	No**
Phenotypic change	Following exposure	Reduced	Yes
Induction (stress response)	Following exposure	Variable	Yes

*Level of susceptibility to other biocides is defined according to the concentration of the biocide

**Mutation in target site does not occur with other biocides, but cross-resistance does occur with specific antibiotics

Table 2.3: Reported use of Pelargonic acid in the Food industry by the Flavor and Extract Manufacturers' Association (82)

Food Category	Usual (ppm)	Max (ppm)
Meat Products	6.50	13.1
Frozen dairy	2.14	9.38
Fats, oils	1.00	10.00
Alcoholic Beverages	1.00	3.00
Soft Candy	1.57	4.99
Baked goods	10.04	23.54
Gelatins, puddings	1.91	5.73
Nonalcoholic beverages	1.57	4.99

Table 2.4: Reports on amino acids linked directly or indirectly to acid resistance

Amino acid	Study overview	Reference
Threonine	Serine/Threonine protein kinase in *Streptococcus mutans*	(60)
Lysine	*E. coli* interaction with lysine permease LysP	(101)
Ornithine	Ornithine decarboxylase system	(4, 64)
Glutamate	Glutamate decarboxylases and acid resistance system 2 (AR2) in *E. coli*	(5, 15)
Glutamine	Glutamine dependent acid resistance in *E. coli* through release of ammonia	(71)
Serine	Serine deamination as a new acid resistance mechanism in *E. coli*	(108)
Histidine	Acid sensing histidine kinase	(61)
Arginine	Arginine dependent acid-resistant in *E. coli*	(5, 47, 62, 93)
Tyrosine	Tyrosine decarboxylation operon in a putative acid resistance locus in *Lactobacillus brevis*	(72)

Table 2.5: Approved concentrations of peroxyacetic acid and lactic acid used for beef decontamination **a.** approved concentrations of peroxyacetic acid used for beef decontamination **b.** approved concentrations of lactic acid used for beef decontamination

a.

Typical concentrations of peroxyacetic acid used for meat application				
Intervention Point	**Typical Conc. PAA**	**Max. Conc. Allowed**	**Typical Contact Time**	**ref**
Carcass Wash (Spray)	200-400 ppm	1200 ppm	10-30 s	FCN 1738
Head Wash (Spray)	200-400 ppm	1200 ppm	10-30 s	FCN 1738
Hot Box (Spray)	20-50 ppm	1200 ppm	5-15 s	FCN 1738
Primals/cuts (Dip/Spray)	200-400 ppm	1200 ppm	10-30 s	FCN 1738
Tenderizers (Spray)	200-400 ppm	1200 ppm	10-20 s	FCN 1738

*FCN: FDA food contact notification *https://envirotech.com/wp-content/uploads/2017/04/Choosing-a-PAA-conc-Meat-and-Poultry.pdf*

b.

Typical concentrations of lactic acid for meat application		
Intervention Point	**Approved Conc.**	**ref.**
Livestock carcasses prior fabrication	5 % v/v	(111)
Beef sub-primals and trimmings	2-5 % v/v	(111)
Beef heads and tongues	2-2.8 % v/v	(111)

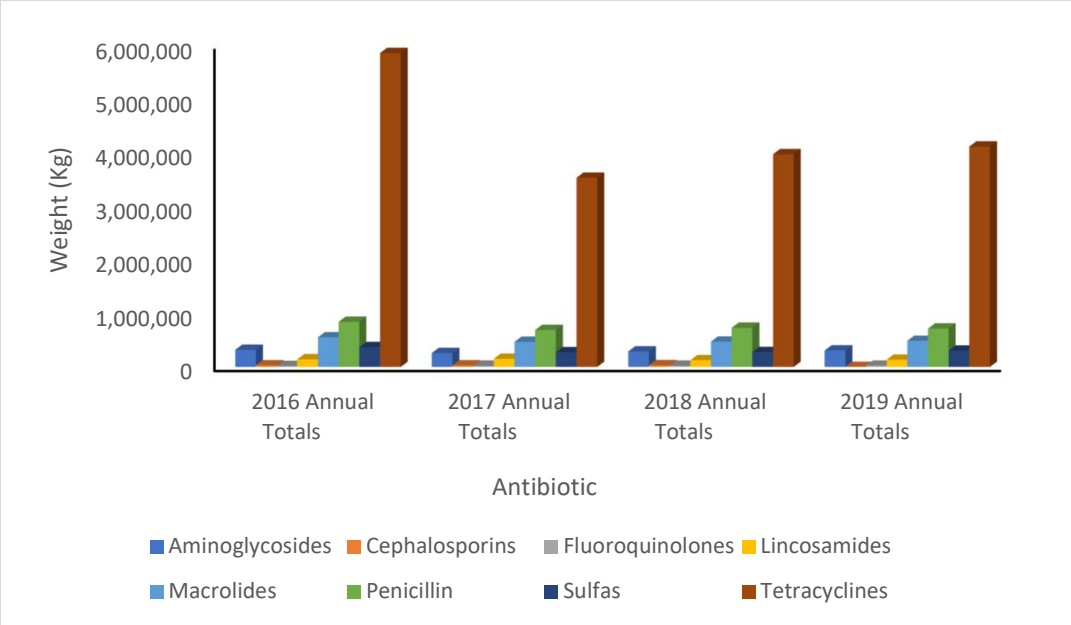

Fig 2.1: Sales and distribution data for medically important antibiotics approved for use in food-producing animals in the United States by antibiotic class (52)

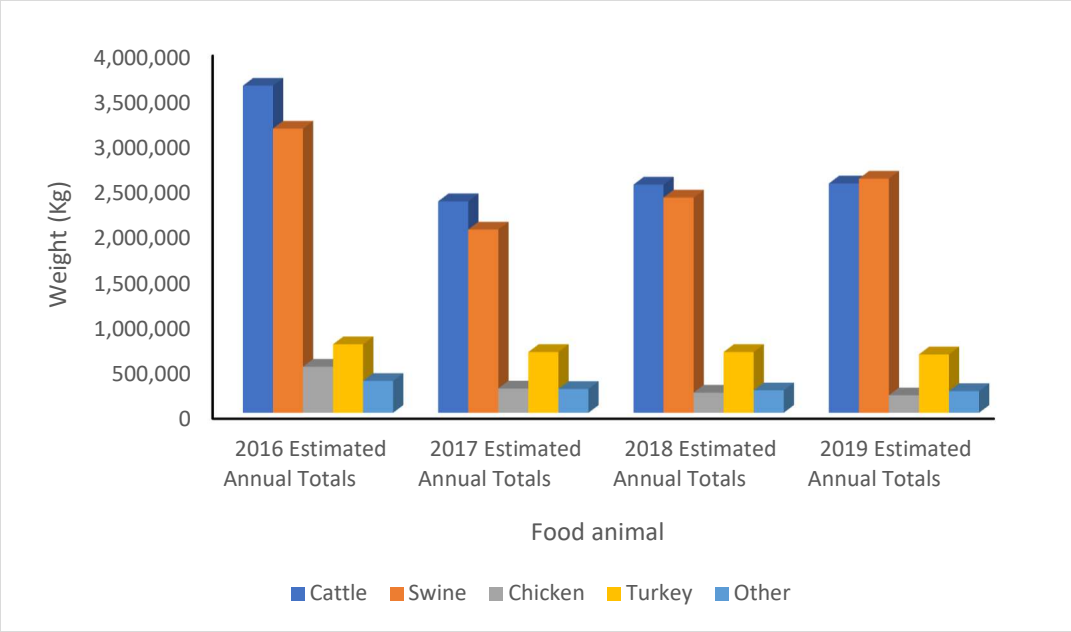

Fig 2.2: Sales and distribution data for medically important antibiotics approved for use in food-producing animals in the United States by food animal (52)

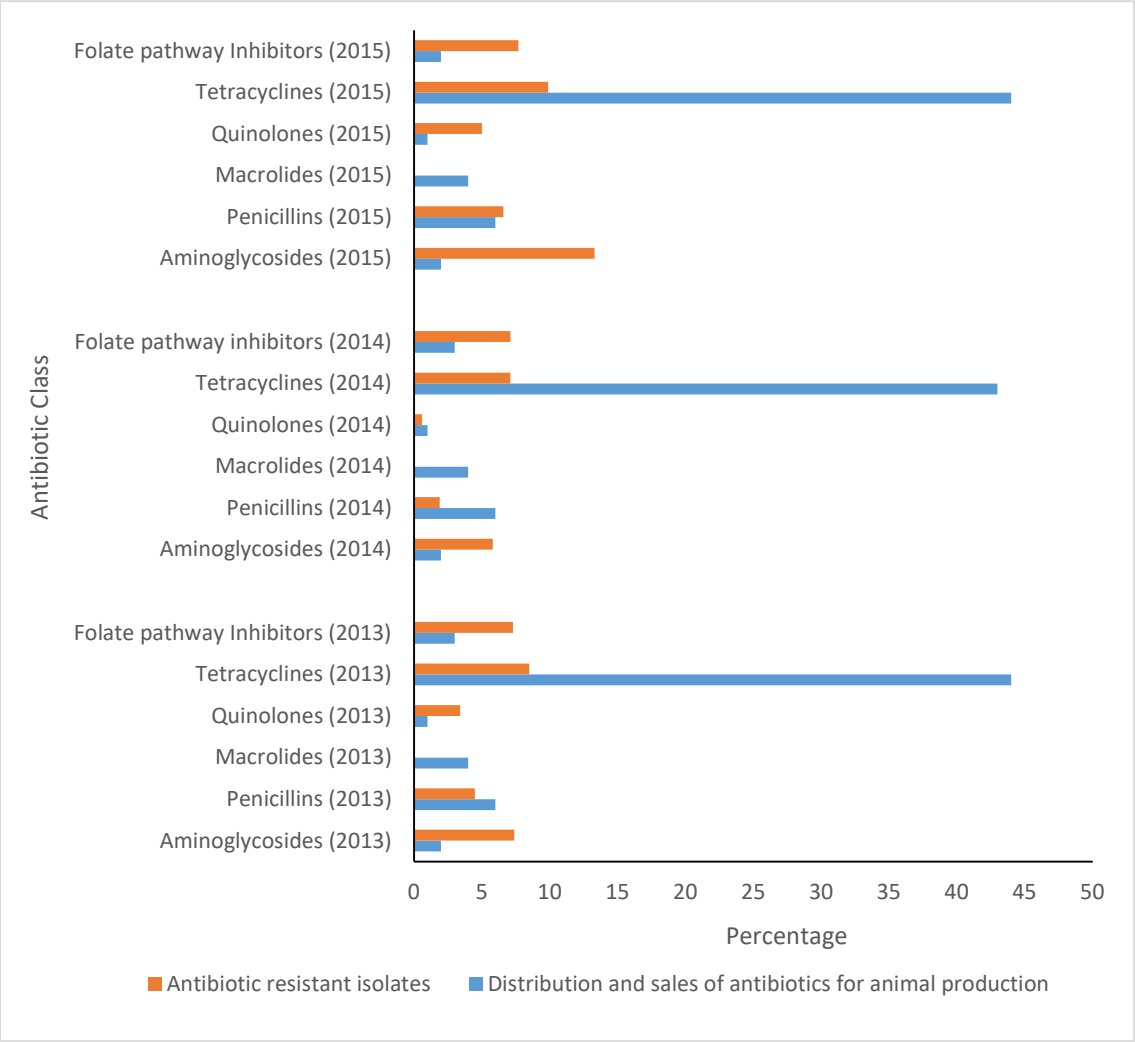

Fig 2.3: Distribution of antibiotics for use in food producing animals and percent resistant *E. coli* O157 isolates between 2013 - 2015

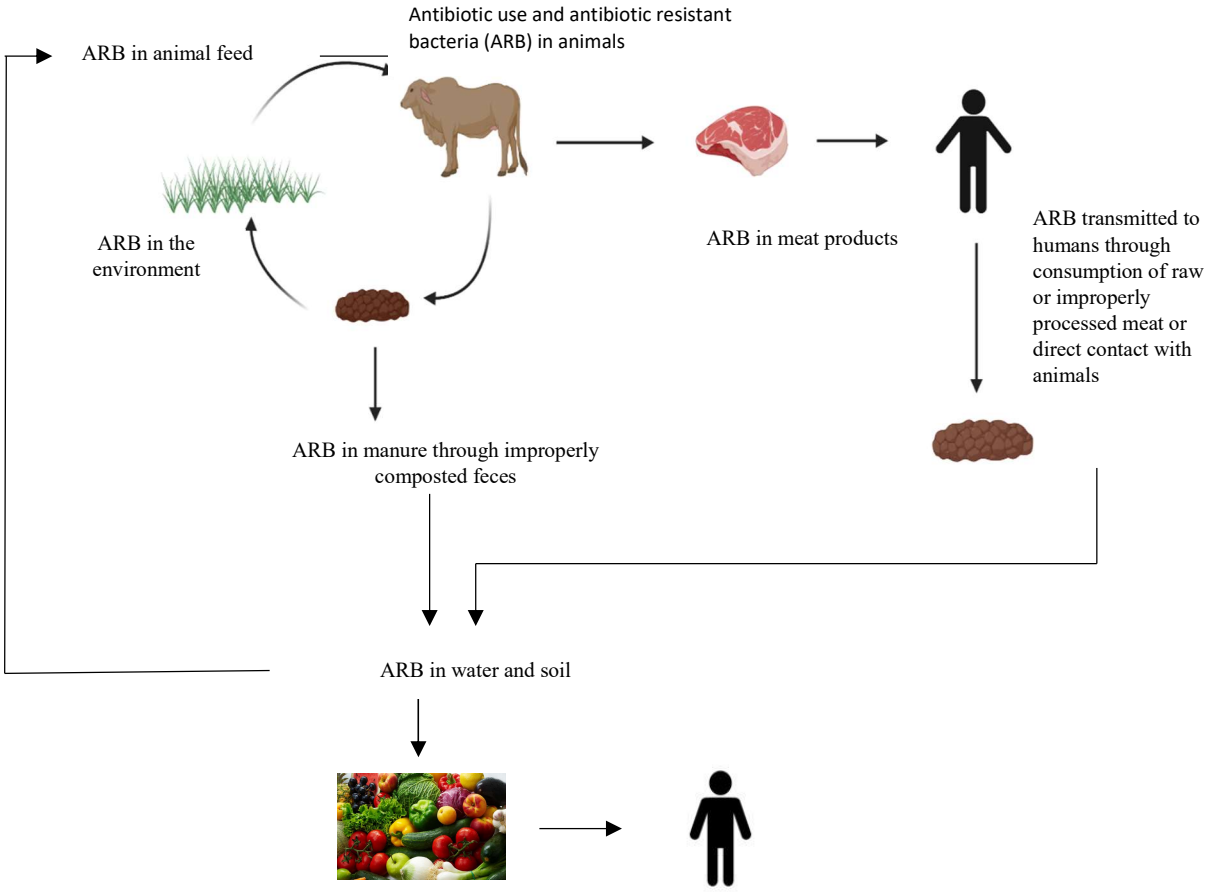

Fig 2.4: Dissemination of antibiotic resistant bacteria in the environment

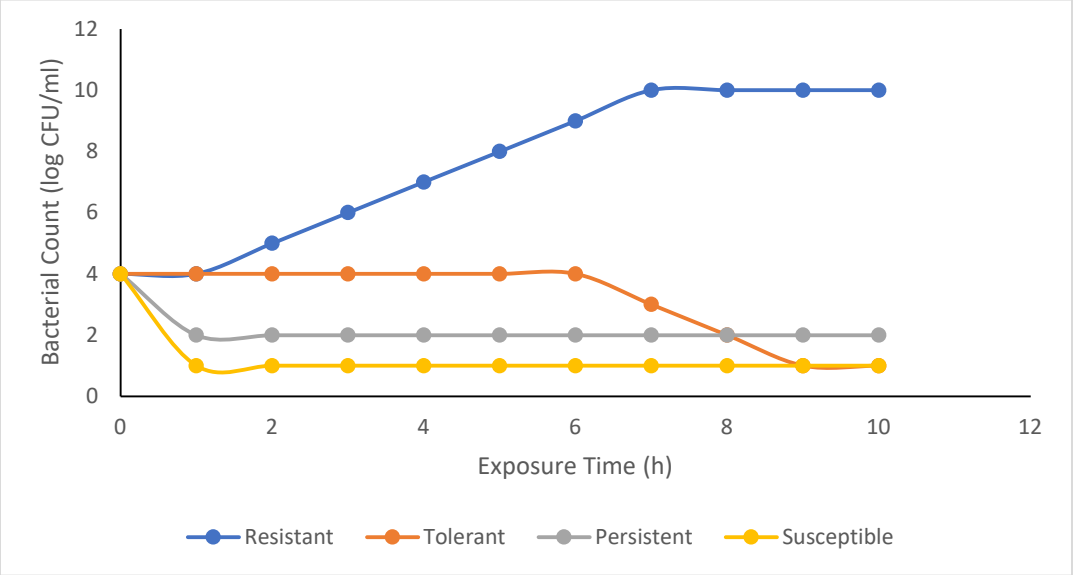

Fig 2.5: Differences in antimicrobial stress response between susceptible, resistant, tolerant and persistent bacteria.

CHAPTER 3

ANTIBIOTIC RESISTANCE INFLUENCES GROWTH RATES AND CROSS-TOLERANCE TO LACTIC ACID IN *E. COLI* O157: H7 H1730[1]

[1]Oguadinma, I.C., Mishra A., Juneja V.K., and Dev Kumar G. 2022. *Foodborne Pathogens and Disease*. Reprinted here with permission of the publisher.

Abstract

Escherichia coli O157:H7 contaminated beef has been implicated in numerous food-borne outbreaks. Contamination occurs despite the use of antimicrobial interventions such as lactic acid. Also, resistance to antibiotics such as ampicillin and streptomycin among isolates has been frequently reported. The influence of antibiotic resistance on growth rates and cross-tolerance of lettuce isolate- *E. coli* O157:H7 H1730 to lactic acid was evaluated. Antibiotic resistant strain variants were generated by conferring resistance to either ampicillin (amp C) or streptomycin (strep C) or both ampicillin and streptomycin (amp C strep C) through incremental exposure to the antibiotics. Ampicillin resistance was also conferred by plasmid transformation to generate strains amp P and amp P strep C. The MIC of lactic acid on all the strains evaluated was 0.375 % v/v. The lag phase duration of all strains except *E. coli* O157:H7 amp P strep C increased with increasing concentration of lactic acid. Strains amp P strep C, and amp C were most tolerant to 5 % lactic acid with declines in cell population of 2.86 and 2.56 log CFU/ml respectively ($p < 0.05$). Strain amp P strep C was the most tolerant when evaluated by the Live/dead viability assay. The addition of the efflux pump inhibitor carbonyl cyanide m-chlorophenyl hydrazone (CCCP) with 2.5 % lactic acid resulted in a significant increase in sensitivity in the NR wildtype and amp C strain resulting in a 6.62 and 6.65 log CFU/ml reduction respectively while the highly tolerant amp P strep C had a 2.90 log CFU/ml decrease. Tolerance to lactic acid was significantly influenced by both antibiotic resistance profile of the strain and lactic acid concentration. The results from this study indicate that *E. coli* O157:H7 strains with certain antibiotic resistance profiles might be more tolerant to lactic acid.

3.1 Introduction

Shiga toxigenic *Escherichia coli* (STEC) has been implicated in outbreaks and recalls associated with produce and beef (16). The pathogen was responsible for 30 multistate beef outbreaks and 30 multistate lettuce outbreaks between 2006 and 2020 (14).

2.8 million people get ABR bacterial infections annually in the United States, and 35,000 die from these infections (15). Resistance in bacteria can be induced by long-term exposure to sub-lethal levels of antimicrobials (35) resulting from their usage to treat infections and improve feed efficiency (32). Bacteria carrying ABR can be directly transmitted to humans through farm animals, consumption of raw or inadequately cooked food, and cross-contamination (10, 35). While over 265000 cases of foodborne illnesses occur each year due to STEC, the prevalence of antibiotic resistance (ABR) in STEC might be underrepresented.

Resistance to critically important antibiotics such as ampicillin and streptomycin has been reported in *E. coli* O157:H7 isolated from cattle and beef (9, 26, 31). A study of 118 *E. coli* O157:H7 isolates indicated that cattle isolates had the highest resistance to antibiotics (Meng et al., 1998). Resistance to streptomycin-sulfisoxazole-tetracycline (AMR-SSuT) was the most common ABR pattern observed among the *E. coli* O157:H7 isolates from the study (33). *E. coli* O157: H7 from raw meat samples - poultry and beef - had the highest resistance to ampicillin (100 %) (31) and streptomycin (7 %) (36). In 2015, 20 % of retail ground beef and 41 % beef cow ceca isolates of *E. coli* were multidrug-resistant (34). ABR microorganisms can have higher tolerance to antimicrobial agents such as sanitizers by efflux pump associated antimicrobial exporters (2).

Lactic acid is a commonly used sanitizer during beef production (8, 13, 38) and is applied at concentrations ranging from 2-5 % on beef sub-primal and trimmings, 2-2.8 % for beef heads and

tongues, and 5 % for livestock carcass before fabrication (38). The antibacterial action of lactic acid occurs due to its ability to penetrate the cytoplasmic membrane of pathogens in its undissociated form, resulting in the disruption of transmembrane proton motive force and reduction of intracellular pH (1). The presence of *E. coli* O157:H7 in beef could occur because of the pathogens ability to adapt to acid stress through acid resistance systems (AR) (22, 30) and efflux pumps (17). Evaluating the role of antibiotic resistance associated cross-tolerance to lactic acid in *E. coli* O157:H7 could help highlight a potential loophole for ABR *E. coli* O157:H7 survival on beef carcasses.

The objective of this study was to evaluate the tolerance of ampicillin and streptomycin resistant variants of *E. coli* O157:H7 H1730 to lactic acid. Changes in tolerance and survival upon lactic acid exposure was evaluated and compared between the antibiotic non-resistant parent strain of *E. coli* O157:H7 H1730 and the antibiotic resistant variants. The results from this study are intended to help understand the risks of antibiotic associated lactic acid cross-tolerance in *E. coli* O157:H7.

3.2 Materials and Methods

3.2.1 Bacterial strain

The strain of *E. coli* O157:H7 used in this study was a human isolate from a lettuce outbreak *E. coli* O157:H7 H1730 - obtained from the Center for Food Safety, University of Georgia Griffin campus Culture collection. The bacterial strain was confirmed using the API 20E identification system (apiweb^{TM}, bioMe'rieux Inc, USA) and whole-genome sequencing. The isolate was revived from frozen storage by transferring to Tryptic Soy Broth (TSB, Neogen, Lansing, MI) and incubating at 37 °C for 24 h. The bacterial strain was evaluated for antibiotic resistance by growing

cells on Tryptic Soy Agar (TSA; Neogen Lansing, MI) containing 100 µg/ml of streptomycin, and ampicillin before the development of the antibiotic-resistant strain variants.

3.2.2 Development of antibiotic-resistant variants

3.2.2.1 *Chromosomal resistance*

Chromosomal resistance to streptomycin and ampicillin was developed according to the method described by Li et al. (29) with modifications. Briefly, 900 µl of TSB amended with 10 µg/ml of antibiotic was inoculated with 100 µl of the non-resistant wildtype strain to obtain a final concentration of 5 log CFU/ml. Upon observation of turbidity, the culture was used to inoculate a fresh batch of TSB containing a 10 µg/ml increment in antibiotic concentration than the previous one. This was repeated until a resistance to 100 µg/ml of ampicillin and streptomycin was achieved. Antibiotic adapted strains retained resistance to the antibiotics without presence of selective pressure.

3.2.2.2 *Transformation by electroporation*

Transformation of cells to take up a green fluorescence ampicillin resistance plasmid (GFP amp Plasmid) was performed following the method described by (18) with minor modifications. Briefly, competent cells were prepared by inoculating 45 ml of Tryptic soy broth (TSB) with 1 ml of an overnight culture of *E. coli* O157:H7 H1730. The culture was incubated at 37 ℃ for 4 h to achieve an optical density of 0.8 following which the cells were placed in ice for 15 min. The culture was then centrifuged at 1400 g for 10 min to pellet the cells, and the supernatant discarded. The pelleted cells were washed three times with 15 % ice-cold glycerol and stored at −80 ℃ until use. The cells were transformed using the host range plasmid pGFPuv (36). Electroporation conditions applied were 2.5 kV,25 µF, and 400 Ω using the Gene Pulser II system (Bio-Rad,

Hercules CA). Colonies of transformed cells expressed fluorescence upon excitation with UV light (365 nm) and were resistant to 100 µg/ml ampicillin.

3.2.3 Determination of the Minimum Inhibitory Concentration (MIC) of Lactic acid

The minimum inhibitory concentration (MIC) of lactic acid (LA, L-lactic acid, Xena International Inc., Illinois) for the antibiotic-resistant and non-resistant bacterial strains was determined using a 96 well plate broth dilution method described by Kumar et al. (19) with some modifications. Briefly, LA stock solutions were serially diluted in 96 well plates -180 µl in each well – and inoculated with 20 µl of 5 log CFU/ml of bacteria. Serial dilutions were performed from initial LA concentrations of 5 % and 3 % to obtain LA concentrations of 2.5 %, 1.5 %, 1.25 %, 0.75 %, 0.62 %, 0.37 %, 0.31 %. 0.18 %, 0.15 %, 0.09 %, 0.07 %, 0.04 %, 0.03 % and 0.02 % v/v. The 96-well plates (Costar® 96 Well Flat Bottom, Corning LifeSciences Inc. ME, USA) were incubated for 24 h at 37 ℃, and the growth kinetics were observed using the Bio-Tek Cytation 3 image reader (BioTek Instruments, Inc. USA). Conditions in the Bio-Tek Cytation 3 image reader were set as follows: the total runtime was set at 24 h with read intervals of 30 min, the shaker was set to an orbital shake every 10 s at a frequency of 283 cycles per minute (cpm) (3mm), the read speed was set to Normal with a delay of 100 msec and the optical density was read at an absorbance of 600 nm. Un-inoculated blanks of TSB were used as a control for this experiment.

3.2.4 Evaluation of Growth rates

The growth rates for the different bacterial strains in TSB and the subminimum concentration of LA were evaluated using the turbidimetric technique. The experiment was conducted in a 96 well microplate by inoculating 20 µl of 6.76±0.71 log CFU/ml of bacteria to 180 µl of media. Growth rates were observed for 24 h at 37 ℃ using the Bio-Tek Cytation 3 image reader (BioTek

Instruments, Inc. USA). Conditions in the bio-Tek citation 3 image reader were set as previously described. Three biological and three technical replicates were performed.

3.2.5 Mathematical modeling for bacterial growth

The modified Gompertz model (24) modified by (6) was fitted to the growth curve of these bacterial strains using MATLAB software (version R2021a, The MathWorks, Inc. Natick, MA). The model can be described by the following equation (25), where *N* is the bacterial population at a given time, N_0 is the initial bacterial population, $O.D._{min}$ is the lowest O.D. value above the detection threshold, *A* is the logarithmic increase of bacterial population, *L* is the lag time, μ is the maximum growth rate, and *t* is time:

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

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

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

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

3.2.6 LA Tolerance

Overnight cultures of *E. coli* O157:H7 H1730 grown either on Tryptic Soy Agar (TSA; Neogen, Lansing MI), TSA + 100 µg/ml of ampicillin, TSA + 100 µg/ml of streptomycin, and TSA + 100 µg/ml of streptomycin and ampicillin were used to prepare an 8 log CFU/ml ~ OD_{600nm} 0.2

bacterial inoculum in 1x Phosphate Buffered Saline (PBS; VWR chemicals LLC, Solon, Ohio). The bacterial strains were exposed to 2.5 % and 5 % v/v LA for 30 s as follows: 1 ml of the suspended culture was transferred to 9 ml of media containing either 2.5 % or 5 % LA for the exposure time. Activity of LA on exposed bacteria was neutralized by transferring cells to Dey-Engley neutralizing broth (DE neutralizing broth; Hardy diagnostics, Santa Maria, USA) before serial dilutions in 1x PBS. Exposed cells were enumerated on TSA or TSA with appropriate antibiotics and left to incubate for 24 h at 37 ℃ before the enumeration of colonies.

3.2.7 Viability assay

The live/dead assay was performed on lactic acid-stressed cells. Live/dead assays use a combination of SYTO 9 (Green fluorescent nucleic acid stain for intact bacterial membranes) and propidium iodide (red fluorescent nucleic acid stain for compromised cell membranes) to quantitatively distinguish live and dead bacteria. The assay was performed following instructions on the commercial Invitrogen™ LIVE/DEAD *Bac*Light™ bacterial viability kit L13152 (ThermoFisher Scientific, NYSCE: TMO, USA) with some modifications for this study. Briefly, bacterial cells were prepared by suspending overnight cell cultures grown on TSA in 10 ml of 1X PBS and adjusting the optical density OD_{600nm} to 0.2 ~ 8 log CFU/ml. Cell cultures were exposed to 5 % v/v LA by transferring 100 µl of the bacterial cell suspension to 900 µl of acid for 30 s. Cultures were centrifuged at 16,300 x g for 2 min using the Corning LSE™ high speed microcentrifuge (Corning life Sciences, NY, USA) to obtain pellets, and the supernatant discarded. The bacterial cells were washed twice using sterile deionized water (SDW) and re-suspended in 1 ml SDW. **A** 100 µl aliquot of the bacterial cell suspension was mixed with 100 µl of a 2X working solution of the LIVE/DEAD *Bac*Light staining reagent (SYTO 9: Propidium iodide) in a black opaque 96-well microplate (ThermoScientific flat bottom Microfluor ® 2, ThermoScientific,

NYSCE: TMO, USA). The mixture was incubated at room temperature in the dark for 15 min and the fluorescence intensity was read using the BioTek Cytation microplate image reader (BioTek Instruments, Inc. USA). The excitation and emission wavelengths were set as follows: 485 nm/530 nm for the green stain (SYTO 9) and 485 nm/630 nm for the red stain (Propidium iodide). The LIVE/DEAD (G/R) ratio was calculated by dividing the fluorescence intensity value of SYTO 9 by the intensity value of Propidium iodide. Percent of live cells after acid exposure was calculated by dividing the G/R ratios of the acid exposed cells by the G/R ratios of non-acid exposed cells (controls) and multiplying by 100.

3.2.8 *Efflux pumps in antimicrobial cross-tolerance*

The efflux pump inhibitor Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP) was used to investigate the possible role of efflux pumps in bacterial tolerance to LA. Bacterial strains were exposed to 2.5 % LA as the highest clarity in sensitivity among strains to the antimicrobial were observed in previous experiments. A CCCP stock solution of 1 mg/ml was prepared in Dimethyl Sulfoxide (DMSO; Sigma Chemical CO, St. Louis, MO) and was added to TSB amended with 2.5 % LA to obtain a final CCCP concentration of 10 μg/ml. Bacterial strains NR, amp C and amp P strep C were added to TSB+2.5 % LA and TSB+2.5 % LA+ CCCP to obtain a final concentration of 7.47±0.17 log CFU/ml. Exposure of the cells was sustained for a duration of 30 s, following which the cells were enumerated using methods previously described. Three biological and three technical replicates of each experiment was performed.

3.2.9 Statistical Analysis

Three biological and three technical replicates of all experiments were. Significant differences between the parameters were compared using the one-way analysis of variance (ANOVA) and

means were compared using the Tukey's Honestly Significant Difference (HSD) test at a 0.05 significance level. Statistical analysis was conducted with the JMP statistical software (SAS Institute Inc, USA). Plate counts from the LA exposure study was converted to log CFU/ml and counts with 0 were assigned a value of 10 CFU/ml based on the lowest limit of detection – 10 CFU/ml or 1 log CFU/ml. Differences were considered significant at $P < 0.05$ level of probability.

3.3 Results

3.3.1 *Minimum Inhibitory Concentration (MIC) and growth parameters of E. coli O157:H7*

The type of strain and concentration of LA significantly affected growth parameters ($p < 0.05$). A concentration of 0.375 % LA v/v inhibited the growth of all the strains that were tested. Differences in antibiotic resistance profiles did not affect the MIC ($p > 0.05$). Growth of the strains in the presence of sub-MIC concentrations of LA (Fig. 3.1 and 3.2)- (0.0625 %, 0.125 % and 0.25 % v/v) resulted in significant differences in growth parameters such as lag phase duration (λ) and maximum specific growth rate (μ_{max}) among the strains ($p < 0.05$). A strong positive correlation between an increase in the lag phase duration and concentration of LA in the medium was observed in strains NR (r=0.77), amp P (r=0.79), strep C (r=0.84), amp C strep C (r=0.89) and amp C (r=0.95). Although a negative correlation was calculated between LA concentration and lag phase duration for strain – amp P strep C (r= -0.18), the correlation was small, and the lag phase durations were not significantly different for different LA concentrations. Only strains strep C (r=0.01) and amp P strep C (r=0.22) demonstrated a positive correlation between maximum specific growth rate (μ_{max}) and LA concentration. In the presence of 0.0625 % and 0.125 % lactic acid, strain amp P strep C had the longest lag phase duration but in presence of 0.25 % LA the longest lag phase duration was observed in strain strep C.

3.3.2 *Evaluation of strain tolerance to LA*

The pH of TSB amended with 2.5 % v/v LA was 2.76±0.001. The decline in population among *E. coli* O157:H7 strains exposed to 2.5 % v/v LA (Fig. 3.3) differed significantly ($p < 0.05$). The, NR and amp C strep C strains had the highest population decline of 2.74±0.61 log CFU/ml and 3.54±0.82 log CFU/ml, respectively post exposure to 2.5 % v/v LA ($p < 0.05$). Strains amp C and amp P strep C had the least population decline of 0.50±0.20 log CFU/ml and 0.17±0.16 log CFU/ml, respectively ($p < 0.05$) after exposure to 2.5 % v/v lactic acid. Post exposure to 5 % v/v lactic acid, strains-strep C, NR, and amp P had the highest decreases in population of 6.22±0.04 log CFU/ml, 5.92±0.07 log CFU/ml and 5.80±0.09 log CFU/ml, respectively ($p < 0.05$). The pH of TSB amended with 5 % v/v LA was 2.50±0.02. The highest tolerance to the 5 % v/v LA was observed in strain amp C followed by amp P strep C and amp C strep C with population declines of 2.56±0.21 log CFU/ml ($P < 0.05$), 2.86±0.08 and 3.54±0.82, respectively.

3.3.3 *Viability of* **E. coli** *O157: H7 after exposure to lactic acid*

The percentage of live cells present in the samples after exposure to 5 % v/v LA for 30 s was evaluated (Fig. 3.4). Strains amp P strep C and amp C had the highest percent live cells of 94.00 ± 14.88 % and 76.99 ± 8.91 respectively ($p < 0.05$). The percent live cells observed in strains amp P (54.78 ± 12.53 %), NR (44.01 ± 6.51 %), strep C (43.10 ± 2.82 %) did not differ significantly from amp C strep C (37.34 ± 0.89 %) which had the least percent live cells ($p > 0.05$).

3.3.4 *Role of efflux pumps in antimicrobial cross-tolerance*

In presence of CCCP all three strains demonstrated a higher susceptibility to 2.5 % LA (Fig. 3.5) ($p \leq 0.05$). Exposure to 2.5 % LA resulted in bacterial population decreases of 0.63±0.15, 0.39±0.17 and 3.44±0.40 log CFU/ml in strains amp C, amp P strep C and NR ($p \leq 0.05$)

respectively. The synergistic tests performed with 2.5 % LA+ CCCP resulted in significant decreases among all three strains. Both amp C and NR populations decreased to below the limit of detection while amp P strep C decreased by 2.90±0.48 log CFU/ml ($p \leq 0.05$).

3.4 Discussion

The food supply chain has been identified as a potential source of antibiotic-resistant bacterial strains and genes (7, 34). STEC have been commonly associated with cattle and produce fertilized or irrigated with cattle manure or farm runoffs respectively (20, 27). The use of medically important antibiotics to treat diseases and to promote growth in cattle have resulted in antibiotic-resistant strains of *E. coli* O157:H7 being isolated from cattle and ground beef. The isolation of *E. coli* O157:H7 resistant to Ampicillin and streptomycin in isolates with multidrug resistance to streptomycin, sulfonamide, and tetracycline (AMR-SSuT) (7, 23). The isolation of these pathogens from beef even after the use of antimicrobial interventions (13, 23) alludes to the potential of AMR associated LA-cross tolerance..

Tolerance can be defined as the ability to sustain increased durations of exposure to an antimicrobial (11, 28). The result from this study indicates that no differences among the strains were observed in their ability to resist and grow in presence of LA while significant differences were observed among the strains in their tolerance to LA.

Growth parameters of the strains were compared at sub-MIC concentrations of the lactic acid. Among all the strains evaluated, strep C had the longest lag phase duration for all three sub-MIC concentrations of LA tested and the lowest maximum specific growth rate (μ_{max}) when grown in the presence of 0.25 % lactic acid. The highest decrease in population after exposure to 5 % LA v/v among all the strains tested was also in strep C. Strains amp C, amp P strep C, and amp C strep

C were the most tolerant to 5 % LA exposure for a duration of 30 s. Extended lag phase durations have been observed in cells that develop tolerance to an antibiotic (21) but this phenomenon was not observed during cross-tolerance to antimicrobial sanitizers such as LA in *E. coli* O157:H7.

MDK_{99} is defined as (the minimum duration for killing 99 % of cells and is an important parameter for detecting tolerance). Both culture-based and viability staining were used to study the susceptibility of the strains to lactic acid. While a 99 % decrease (2 log) was observed in all the strains that were exposed to 5 % LA, viability staining indicated the presence of viable cells after all the treatments. The differences in plate counts and viability assays could have occurred due to a transition to a stationary phase by the cells during exposure to lactic acid, resulting in reduced growth on the plating media (21). Strains with higher tolerance to LA were resistant to ampicillin and streptomycin. LA efflux pump activity in bacteria can result in tolerance to sanitizers (3, 17). The use of CCCP to inhibit the energy of efflux pumps (4, 5) resulted in a significantly higher susceptibility to 2.5 % LA in NR, amp C. Strain amp P strep C had higher tolerance to the synergistic exposure to LA and CCCP than the other two strains. Overexpression of efflux pumps might contribute to increased tolerance to antimicrobials in certain strains. More work is required to elucidate the mechanism of LA tolerance in strain amp P strep C.

3.5 Conclusion

The result from this study highlights the risks associated with antibiotic associated sanitizer cross tolerance in bacterial foodborne pathogens.

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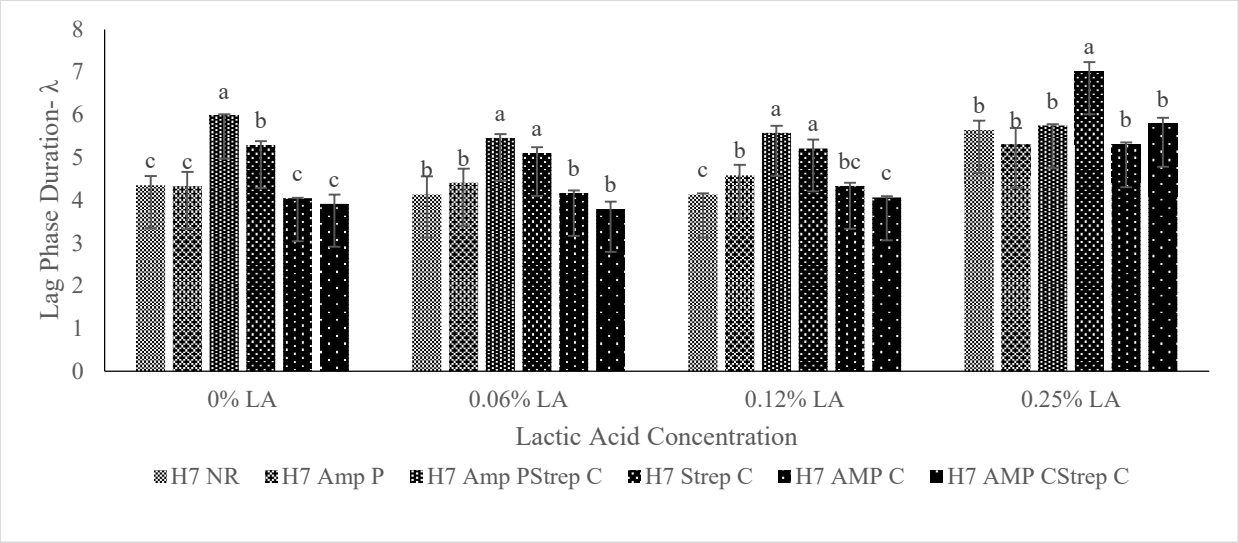
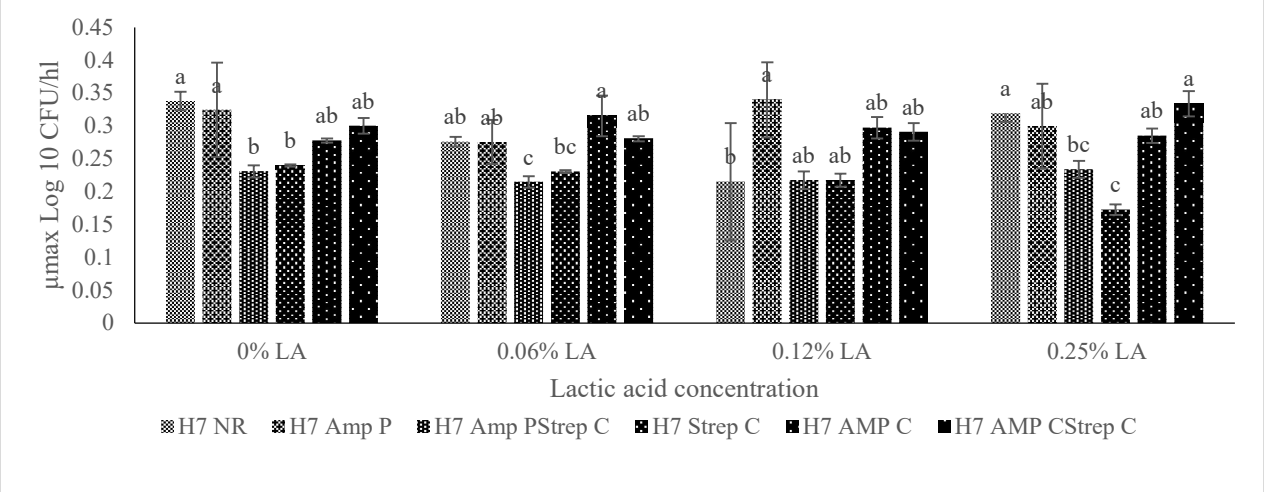
Table 3.1*:* List of *E. coli* O157: H7 H1730 strain variants used in this study.

Bacterial Strain	**Mode of resistance**	**Strain name for this study**
***Escherichia coli* O157: H7**	No resistance (parent strain)	H7 NR
***E. coli* O157: H7 ampicillin (Amp) resistant**	Plasmid	H7amp P
***E. coli* O157: H7 amp resistant**	Chromosome	H7amp C
***E. coli* O157: H7 streptomycin (strep) resistant**	Chromosome	H7strep C
***E. coli* O157: H7 amp and strep resistant**	Plasmid* and chromosome	H7 amp P strep C
***E. coli* O157: H7 amp and strep resistant**	Chromosome	H7amp C strep C

a


Lag Phase Duration- λ
0% LA
0.06% LA
0.12% LA
0.25% LA
Lactic Acid Concentration
H7 NR
H7 Amp P
H7 Amp PStrep C
H7 Strep C
H7 AMP C
H7 AMP CStrep C

b


μmax Log 10 CFU/hl
0% LA
0.06% LA
0.12% LA
0.25% LA
Lactic acid concentration
H7 NR
H7 Amp P
H7 Amp PStrep C
H7 Strep C
H7 AMP C
H7 AMP CStrep C

c

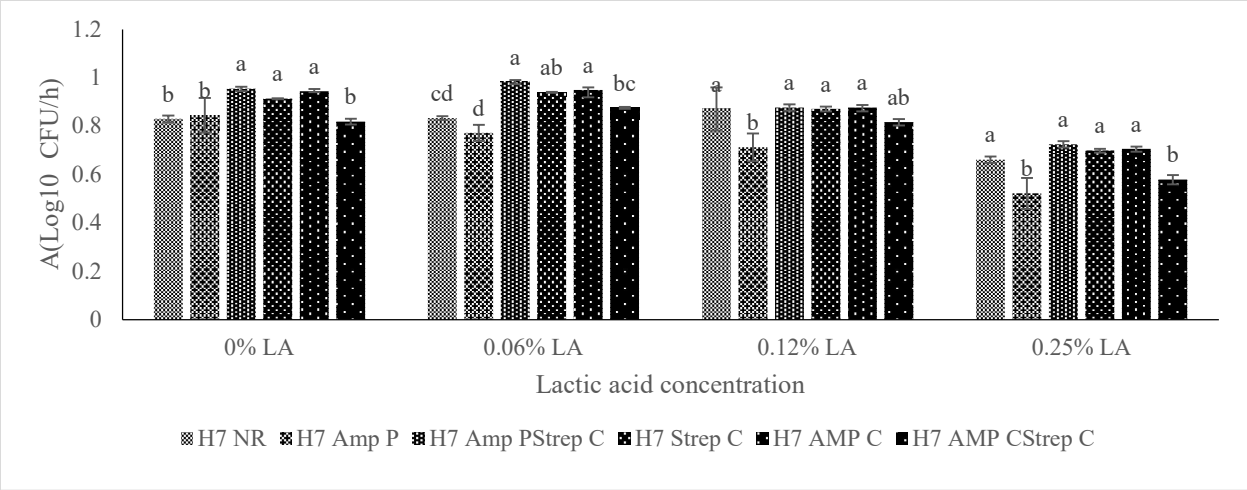
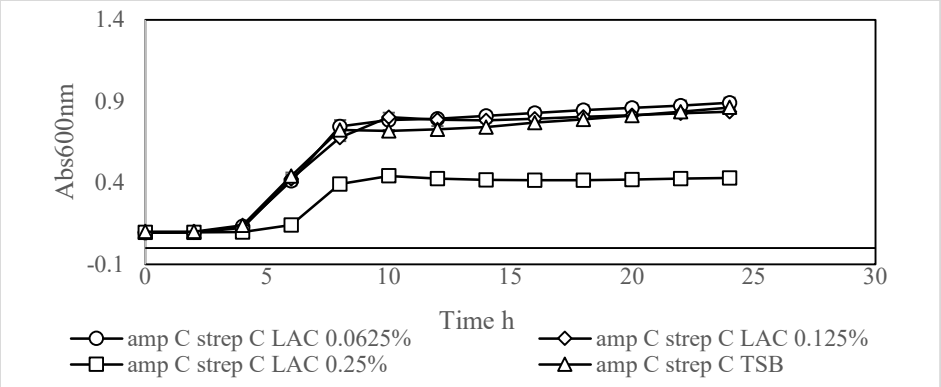
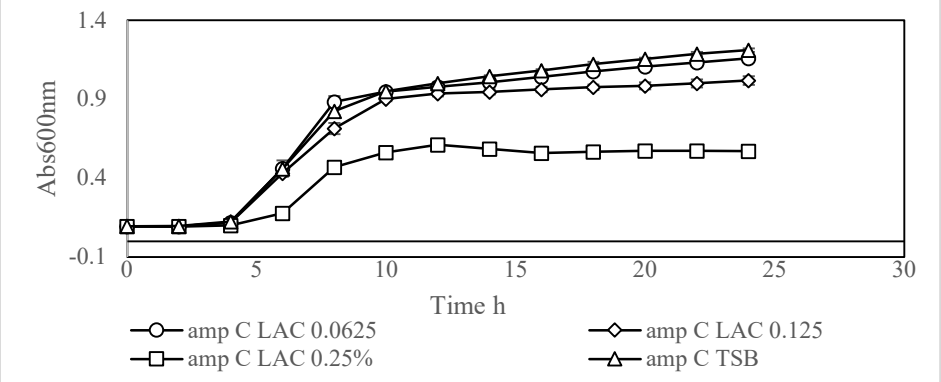


Fig 3.1. Comparison of growth parameters a. Lag phase duration (L) b. maximum growth rate (μ_{max}) c. change in bacterial population (A) among bacterial strains grown in TSB supplemented with 0 %, 0.0625 %, 0.125 % and 0.25 % LA. Significant differences among growth parameters among different strains have been denoted by different alphabet.

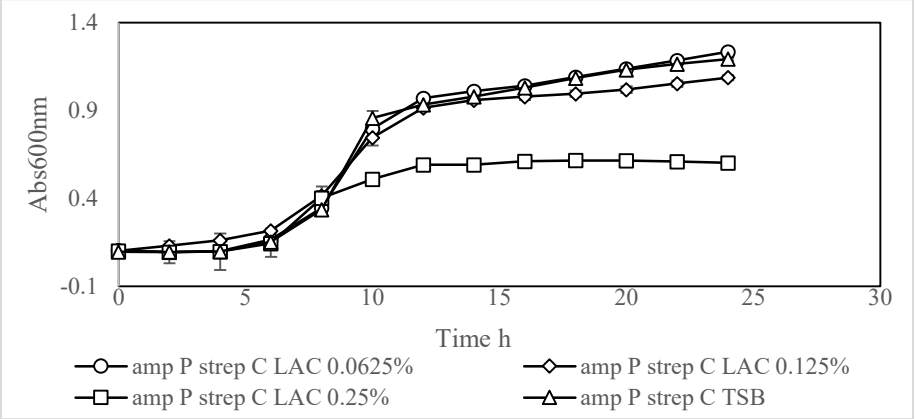
a.


1.4
0.9
0.4
-0.1
Abs600nm
0
5
10
15
20
25
30
Time h
amp C strep C LAC 0.0625%
amp C strep C LAC 0.125%
amp C strep C LAC 0.25%
amp C strep C TSB

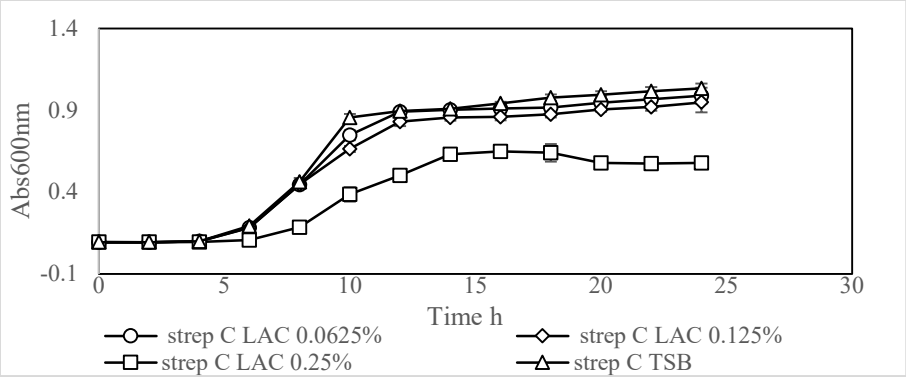
b.


1.4
0.9
0.4
-0.1
Abs600nm
0
5
10
15
20
25
30
Time h
amp C LAC 0.0625
amp C LAC 0.125
amp C LAC 0.25%
amp C TSB

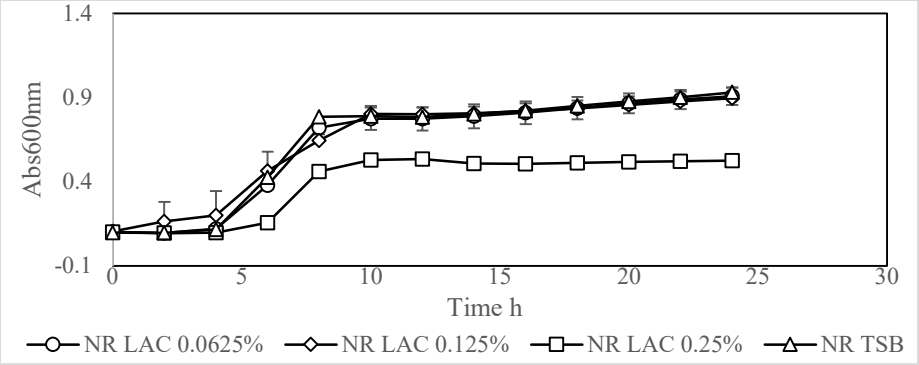
C.


1.4
0.9
0.4
-0.1
Abs600nm
0
5
10
15
20
25
30
Time h
amp P strep C LAC 0.0625%
amp P strep C LAC 0.125%
amp P strep C LAC 0.25%
amp P strep C TSB

d.



e.



f.

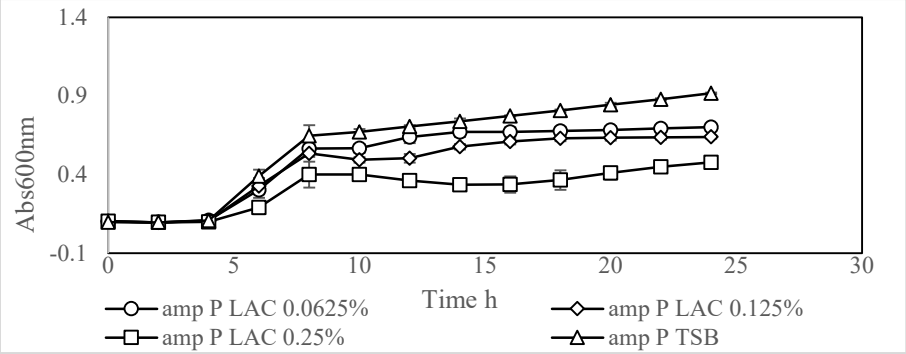


Fig 3.2: Growth of *E. coli* O157: H7 H1730 strains a. H7 amp C strep C b. H7 amp C c.H7 amp P strep C d.H7 Strep C e. H7 NR f. H7 amp P at the Sub-MIC concentrations of Lactic acid (0.25 %, 0.125 % and 0.0625 % in TSB).

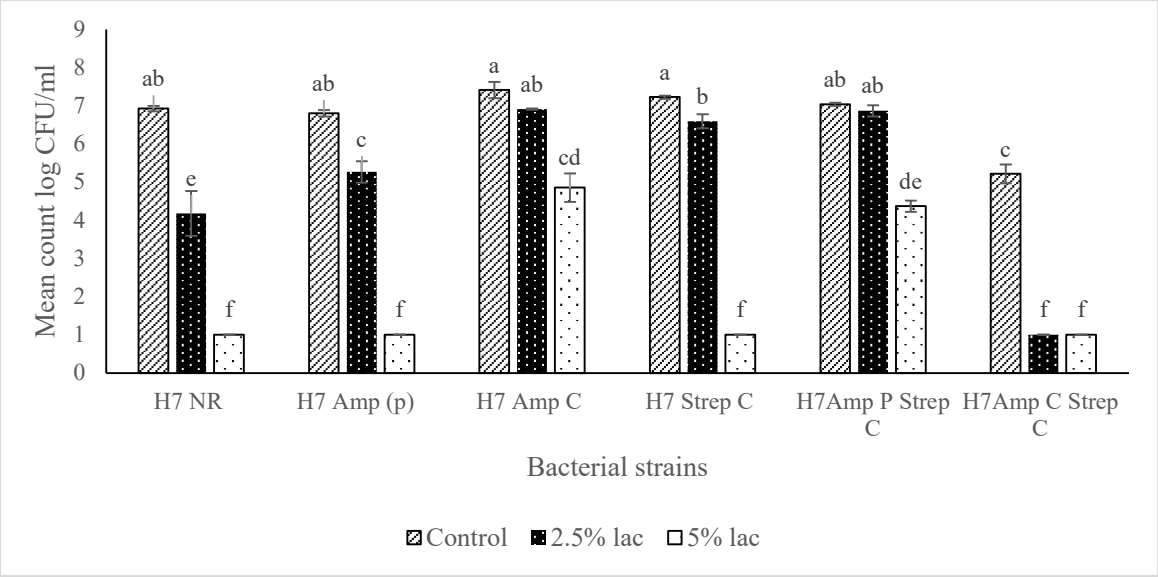


Fig 3.3: Comparison of survival among *E. coli* O157:H7 H1730 strains after exposure to 2.5 % and 5 % v/v lactic acid for 30 s. Significant differences in survival have been denoted by different alphabet.

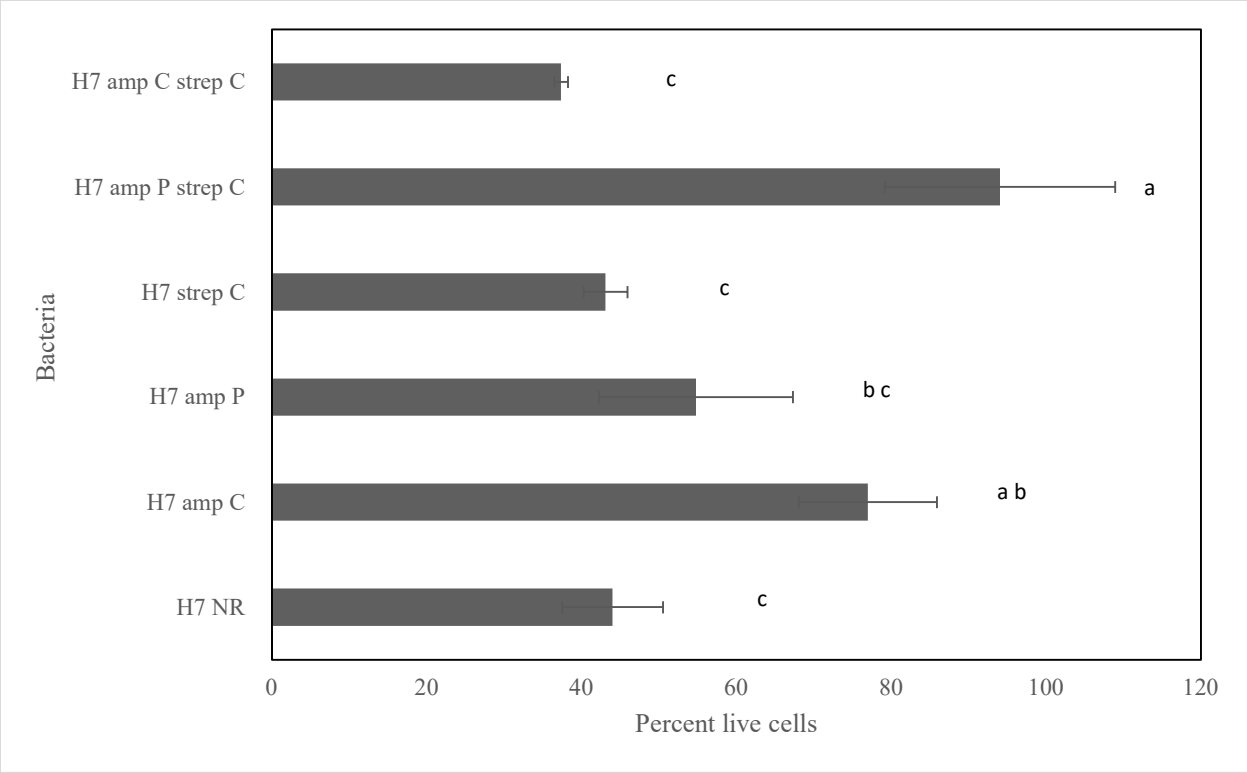


Fig 3.4: Percent live cells among strains after exposure to 5 % v/v lactic acid for 30 s Cells with significant differences in Live cells after treatment are denoted by different alphabet.

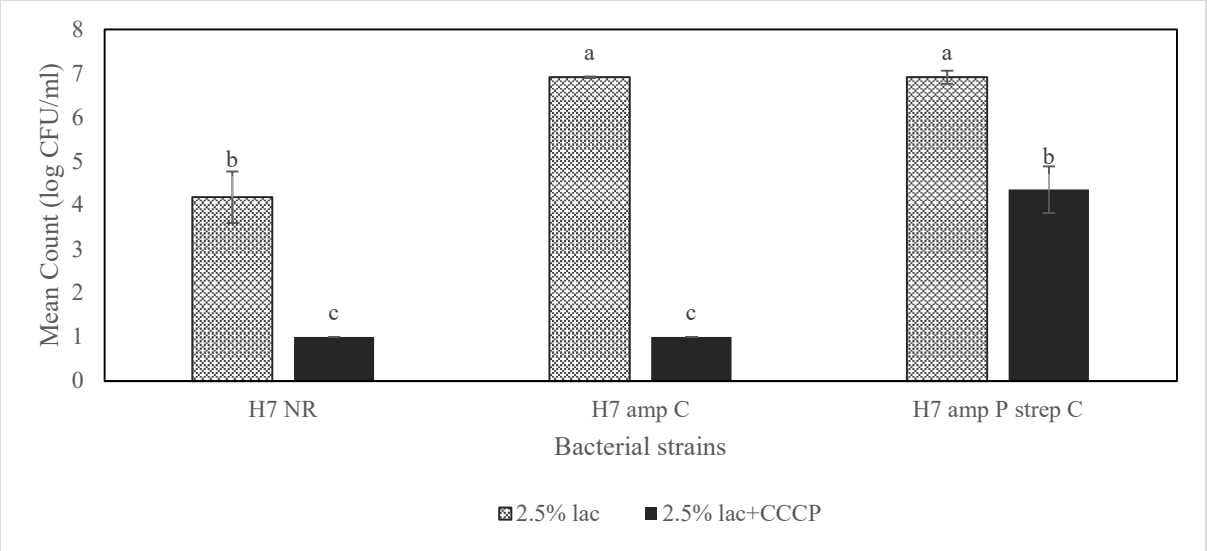


Fig 3.5: Comparison between acid tolerance of strains H7 NR, H7 amp C and H7 amp P strep C with and without the presence of CCCP.

CHAPTER 4

CHARACTERIZATION OF CHANGES IN LACTIC ACID TOLERANCE AND BIOFILM FORMATION AMONG ANTIBIOTIC RESISTANT VARIANTS OF *E. COLI* O157: H7 H1730[2]

[2]Oguadinma, I.C., Olszewska, M. A., den Bakker, H. C., Mishra A., and Dev Kumar G. 2022. Submitted to *Applied and Environmental Microbiology*.

Abstract

The cross tolerance of antibiotic resistant Shiga toxin producing *Escherichia coli* (STEC) to lactic acid (LA), a sanitizer commonly used to sanitize beef carcasses was evaluated. STEC are usual causatives of foodborne illnesses and outbreaks and are frequently isolated from beef. Resistance to critically important antibiotic such as ampicillin and streptomycin has been observed in STEC isolates from beef and dairy. In this study, the influence of antibiotic resistance in *E. coli* O157: H7 H1730 on cross tolerance to LA and biofilm formation was evaluated. Further, changes in biofilm formation were also determined among the parent strain and antibiotic resistant variants. Strain variants resistant to ampicillin (amp C) and streptomycin (strep C) or both (amp C strep C) were developed through incremental exposure to the antibiotics. Resistance to ampicillin was also conferred by plasmid transformation to generate strains amp P and amp P strep C. Biofilm formation in 1:10 TSB was evaluated by the crystal violet (CV) assay and confocal laser scanning microscopy (CLSM). *E. coli* O157: H7 H1730 amp P strep C (H7 amp P strep C) formed the most biofilm biomass of OD_{570nm}1.12±0.16 compared to biomass of OD_{570nm} 0.74±0.11 and 0.73±0.10 in strains H7 amp P and H7 NR ($P \leq 0.05$) by CV assay. CLSM showed variation between the maximum thickness, average thickness and biovolume of biofilms formed by the different strain variants. Strains H7 amp C and H7 amp P strep C were the most tolerant to 2.5 % v/v LA with reductions of 0.55±0.28 log CFU/ml and 0.63±0.17 log CFU/ml respectively in bacterial population. Strain amp C survived exposure to 5 % v/v LA. Antibiotic resistance in the bacterial strains resulted in single nucleotide substitutions at 24 chromosomal positions. Certain ABR profiles in *E. coli* may improve biofilm formation and increase tolerance to LA.

4.1 Introduction

Antibiotic resistance (ABR) is a growing public health threat. According to the CDC, ABR bacteria are responsible for at least 2.8 million cases of annual infections in the United States and 35,000 deaths (6). ABR bacteria are widespread in the food chain and can be transmitted to humans through consumption of raw or inadequately cooked food, cross-contamination, or farm animals (3, 24). Increased cross tolerance to sanitizers dues to efflux pump activity or biofilm formation might result from genetic changes associated with antibiotic resistance in bacteria. The risk of antibiotic resistance associated sanitizer cross-tolerance in STEC is a food safety and public health concern.

Shiga toxin-producing *E. coli* can attach to and grow on food processing environments, equipment, and food contact surfaces under appropriate conditions to form biofilms (37). The recalcitrance of biofilm cells on sanitized surfaces harboring biofilms makes biofilm formation a challenge for the food industry (18, 19). Also, bacteria in biofilms are more tolerant to sanitizers than planktonic cells.

Several factors contribute to the regulation of biofilm formation in bacteria. Some of these include environmental factors such as pH, nutrient availability, temperature and other microbial characteristics such as interaction between different bacterial populations and metabolic activity (25). Recently, efflux pumps have been identified for their role in regulating biofilm formation in bacteria. One study on *E.coli* reported that the transport genes mdtF and isrA belonging to the RND and ABC superfamilies were expressed at significantly higher levels during biofilm growth (27). In another study, the expression of yihN, an MFS-encoded efflux gene, was 2-fold greater in *E. coli* K12 during biofilm formation (2). Other studies also reported up-regulation of efflux pumps genes that directly or indirectly contribute to biofilm formation in *E. coli* (15, 17, 21, 34)

Efflux pumps are known for mediating ABR and antimicrobial cross-tolerance in bacteria. They are over-expressed in several antibiotic-resistant bacterial strains and can promote recalcitrance (36, 38, 39). For example, the genes Y14748 (*aadA2*) and Z18955 (bla_{CARB-2}) conferred resistance to streptomycin and ampicillin in *Salmonella typhimurium* DT104 (8) and *Mar* or *acrAB* mutants of *E.coli* were less susceptible to fluroquinolones (10) and Triclosan (22). In a previous study, it was observed that resistance to ampicillin and streptomycin in *E. coli* O157: H7 H1730 compared to the parent strain resulted in changes in the bacterial growth rates and cross-tolerance to 2.5 % and 5 % lactic acid after exposure for 30 s. Lactic acid is a common antimicrobial used to prevent microbial contaminants on beef carcass and is approved for use at concentrations of 2-5 % v/v (41). The potential for better biofilm formation or cross-tolerance to antimicrobials in antibiotic resistant bacteria is a potential food safety challenge.

Therefore, this study objective was to explore the influence of antibiotic resistance on biofilm formation and extreme lactic acid stress in *E. coli* O157:H7 H1730. Genetic mutations in tolerant bacterial strain variants were also identified. Results from this study are intended to help understand the risk of the antibiotic-associated increase in biofilm formation and lactic acid cross tolerance in *E. coli* O157: H7.

4.2 Materials and Methods

4.2.1 Bacterial strain

The strain of *E. coli* O157:H7 used in this study was a human isolate from a lettuce outbreak -*E. coli* O157:H7 H1730 - obtained from the Center for Food Safety, University of Georgia Griffin campus Culture collection. The isolate was revived from frozen storage by transferring to Tryptic Soy Broth (TSB, Neogen, Lansing, MI) and incubating at 37 °C for 24 h. The bacterial strain was

evaluated for antibiotic resistance by growing cells on Tryptic Soy Agar (TSA; Neogen Lansing, MI) containing 100 µg/ml of streptomycin, and ampicillin before the development of the antibiotic-resistant strain variants.

4.2.2 Development of antibiotic-resistant variants

4.2.2.1 *Chromosomal resistance*

Chromosomal resistance to streptomycin and ampicillin was developed by sequentially transferring the bacterial strain to increasing concentrations of the antibiotics in increments of 10 µg. This was achieved in a batch system type set up where 100 µl of culture from a lower antibiotic concentration was transferred to fresh media containing a higher antibiotic concentration until adaptation to 100 µg/ml of antibiotic was achieved. Bacterial strains with antibiotic resistance were isolated by streaking on TSA supplemented with either 100 µg/ml of streptomycin or ampicillin.

4.2.2.2 *Transformation by electroporation*

Transformation of cells to take up a green fluorescence ampicillin resistance plasmid (GFP amp plasmid) was performed following the method described by Kumar et al. (16) with minor modifications. Briefly, competent bacterial cells were prepared as follows: 45 ml of Tryptic soy broth (TSB) was inoculated with 1 ml of an overnight culture of *E. coli* O157:H7 H1730. The culture was incubated at 37 ℃ for 4 h to achieve an optical density of 0.8. When the optical density (OD_{600}) reached 0.8, the cells were placed in ice for 15 min. The culture was then centrifuged at 1400 g for 10 min to pellet the cells, and the supernatant discarded. The pelleted cells were washed three times with 15 % ice-cold glycerol and stored at −80 ℃ until use. Transformation of the cells to incorporate a plasmid was done by electroporation using the host range plasmid pGFPuv.

Electroporation conditions applied were 2.5 kV,25 µF, and 400 Ω using the Gene Pulser II system (Bio-Rad, Hercules CA). The generated strain variants are listed in Table 4.1.

4.2.3 Evaluation of Cross-tolerance to lactic acid

Overnight cultures of *E. coli* O157:H7 H1730 grown either on Tryptic Soy Agar (TSA; Neogen, Lansing MI), TSA + 100 µg/ml of ampicillin, TSA + 100 µg/ml of streptomycin, or TSA + 100 µg/ml of streptomycin and ampicillin were used to prepare an 8 log CFU/ml ~ OD_{600nm} 0.2 bacterial inoculum in 1x PBS. The bacterial strains were exposed to 1 %, 2.5 % and 5 % v/v lactic acid for 5 min as follows: 1 ml of the suspended culture was transferred to 9 ml of media containing either 1 %, 2.5 % or 5 % lactic acid for the exposure time. Activity of lactic acid on exposed bacteria was neutralized by transferring cells to Dey-Engley neutralizing broth (DE neutralizing broth; Hardy diagnostics, Santa Maria, USA) before serial dilutions in 1x PBS. Exposed cells were enumerated on TSA or TSA with appropriate antibiotics and left to incubate for 24 h at 37 ℃ before the enumeration of colonies.

4.2.4 Viability assay

Live/dead assay uses a combination of SYTO 9 (Green fluorescent nucleic acid stain for intact bacterial membranes) and propidium iodide (red fluorescent nucleic acid stain for compromised cell membranes) to distinguish live and dead bacteria quantitatively. The assay was performed following instructions on the commercial Invitrogen™ LIVE/DEAD *Bac*Light™ bacterial viability kit L13152 (ThermoFisher Scientific, NYSCE: TMO, USA) with some modifications for this study. Briefly, bacterial cells were prepared by suspending overnight cell cultures grown on TSA in 10 ml of 1X PBS and adjusting the optical density OD_{600nm} to 0.2 ~ 8 log CFU/ml. Cell cultures were exposed to 5 % v/v LA by transferring 100 µl of the bacterial cell suspension to 900

µl of acid for 300 s. Cultures were centrifuged at 16,300 x g for 2 min using the Corning LSE™ high-speed microcentrifuge (Corning life Sciences, NY, USA) to obtain pellets, and the supernatant was discarded. The bacterial cells were washed twice using sterile deionized water (SDW) and re-suspended in 1 ml SDW. A 100 µl aliquot of the bacterial cell suspension was mixed with 100 µl of a 2X working solution of the LIVE/DEAD *Bac*Light staining reagent (SYTO 9: Propidium iodide) in a black opaque 96-well microplate (ThermoScientific flat bottom Microfluor ® 2, ThermoScientific, NYSCE: TMO, USA). The mixture was incubated at room temperature in the dark for 15 min and the fluorescence intensity was read using the BioTek Cytation microplate image reader (BioTek Instruments, Inc. USA). The excitation and emission wavelengths were set as follows: 485 nm/530 nm for the green stain (SYTO 9) and 485 nm/630 nm for the red stain (Propidium iodide). The LIVE/DEAD (G/R) ratio was calculated by dividing the fluorescence intensity value of SYTO 9 by the intensity value of Propidium iodide. Percent of live cells after acid exposure was calculated by dividing the G/R ratios of the acid exposed cells by the G/R ratios of non-acid exposed cells (controls) and multiplying by 100.

4.2.5 Biofilm formation using CV assay

Overnight cultures of each bacterial strain grown on TSA, TSA + ampicillin, TSA + streptomycin or TSA + ampicillin + streptomycin for 24 h at 37 ºC were transferred to 1x PBS and serially diluted to obtain concentrations of 6 log CFU/ml. From each culture, 20 µL was transferred into 96-well microplates (Costar® 96 Well Flat Bottom, Corning LifeSciences Inc. ME, USA) loaded with 180 µL of 1:10 TSB and biofilms were developed at 37 °C for 24 h. Planktonic cells were washed away by a novel steam-based technique described by (32) and biofilms were quantified using the microplate colorimetric crystal Violet assay described by (5). Briefly, washed microplates were dried in an oven at 45 °C for 4 h and left overnight at room temperature (25 °C).

In each well, 100 µl of 0.1 % crystal violet dye was added and the plates were left to stand for 15 min at room temperature. The plates were washed thoroughly using 1x Phosphate Buffered Saline (PBS; VWR chemicals LLC, Solon, Ohio), and then 100 µl of 33 % acetic acid was added to extract the dye. Optical density was read at 570 nm using a microtiter plate reader (Bio-tek Cytation 3 imaging reader, Biotek Instruments, USA).

4.2.6 Enumeration of bacteria in biofilms

Overnight cultures of each bacterial strain grown on TSA, TSA + ampicillin, or TSA + ampicillin + streptomycin for 24 h at 37 °C were transferred to 1x PBS and serially diluted to obtain concentrations of 6 log CFU/ml. From each culture, 20 µL was transferred into 96-well microplates loaded with 180 µL of 1:10 TSB or SDW and biofilms were developed at either 25 °C or 37 °C for 24 h. The supernatant was removed, and planktonic cells were washed away with 200 µl of sterile 1X PBS using a micropipette. Each well was loaded with 200 µl of 1X PBS, sealed with a sterile breathable sealing film (sterile sealing films; Axygen) and sonicated at 42 KHz ± 6 % (FS30H Ultrasonic Cleaner, Fisher Scientific) at 20 °C for 30 s to dislodge biofilms. Enumeration was done using a droplet plate method where colony forming units (CFU) of cells within the perimeter of the drop were used to determine bacterial populations from serially diluted samples (12). Colonies were counted after 24 h incubation at 37 °C. Uninoculated blanks were used as controls and there were three biological and three technical experimental replicates.

4.2.7 Biofilm formation on chamber slides and CLSM

The inocula of the EC were added to 8-well chamber slides (Nunc™ II; Lab-Tek™; Thermo Fisher Scientific, Waltham, MA) at 400 µL per well and let to form biofilms at 37 °C for 24 h. Following this, the chambers were rinsed twice with NaCl (8.5 g L^{-1}) and refilled with NaCl containing 5 µM

Syto®9 (LIVE/DEAD BacLight™ viability kit, Molecular Probes, Life Technologies, Eugene, OR), a cell permeant green fluorescent DNA label. The slides were then kept in the dark at room temperature for 15 min to enable the fluorescent labelling of the bacteria. The solution was then removed, and the chambers were washed three times with NaCl. Before chambers were detached from the slides the formalin solution (Sigma, St. Louis, MO) was applied and let to fix the mature biofilms for 30 min. After this, coverslips were placed onto the slides and BacLight™ mounting oil (Molecular Probes) was used to seal their corners, and lastly nail polish was used to seal the slides. The slides were left overnight at 4 °C and observed the day after. Images were acquired with a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss Microscopy, Thornwood, NY). All biofilms were scanned using water-immersion objective lens (Zeiss, 40 × C Pan-Apochromat, NA 1.3) with a 488-nm argon laser and a 561-nm diode-pumped solid-state laser. The fluorescence was recorded within the range from 500 to 600 nm to collect green fluorescence. Up to ten stacks of horizontal plane images (260 × 260 µm) with a z-step of 0.4 µm were acquired for each chamber at its different areas. Serial images were captured and processed by Zeiss Zen 2.3 software (Carl Zeiss). Quantitative structural parameters were then extracted from confocal image series with COMSTAT 2, an image analysis software (www.comstat.dk) developed and described by Heydorn et al. (13) and Vorregaard (33). To describe biofilms under study we proceeded with maximum thickness and surface to volume ratio (SVR) for biofilm architecture studies. This experiment was repeated twice using independent bacterial cultures.

4.2.8 Preparation of genomic DNA

The ZymoBIOMICS DNA micro prep kit (Zymo Research Corp., Irvine, CA, USA) was used to extract genomic DNA from 1 mL of overnight TSB cultures inoculated from the freezer stocks. The DNA was extracted following the manufacturer's instructions. DNA purity was assessed from

the A_{280}/A_{260} ratio measured using the BioTek Cytation 3 Image reader (BioTek Instruments, Inc., Winookski, VT.).

4.2.9 Sequencing of DNA

ABR strains with significant alterations in sanitizer tolerance or biofilm formation were sequenced. Whole genome sequencing was done on the Illumina MiSeq platform at the Microbial Genome Sequencing Center (MiGS, Pittsburgh, PA); all strains were sequenced in duplicate to account for any artifacts that the sequencing procedure or downstream analyses may introduce. Libraries were created using the Illumina Nextera library kits and sequencing consisted of 150 basepair paired end reads with a targeted minimum sequence depth of 50X. Low coverage Oxford Nanopore long read sequencing and genome assembly as described in Wick et al. (40) was used to generate closed genomes of the parental strains. To infer candidate mutations responsible for the observed changes in phenotype, sequenced mutant strains were interrogated for SNP and insertion/deletion mutations using a graph-based method (Mccortex (33) as implemented in mcOutbryk (https://github.com/hcdenbakker/mcOutbryk) and a read mapping (BWA-mem ref) and variant caller (FreeBayes ref) approach, using the closed parental genomes as references. The closed parental genomes were annotated using Prokka (26) and the mutations found in the mutant strains were annotated using SNPeff (9).

4.2.10 Statistical Analysis

All experiments were conducted in triplicates. Plate counts from the lactic acid exposure study were converted to log CFU/ml and counts with 0 were assigned a value of 10 CFU/ml based on the lowest limit of detection – 10 CFU/ml or 1 log CFU/ml. Analysis was done using one way ANOVA and means were compared using the Tukey's HSD test at a 0.05 significance level with

the JMP Pro 15 (SAS Institute, Cary, NC, USA) statistical software. Analyses of biofilms with CLSM (analysis of variance – ANOVA) were performed using Statistica software ver. 13.1 (StatSoft Inc., Tulsa, OK) and means from the CV assay were compared using the Tukey's HSD test with JMP pro-15. Differences were considered significant at $P < 0.05$ level of probability.

4.3 Results

4.3.1 Cross-tolerance to extreme acid stress

The reduction in bacterial population after exposure to 1 %, 2.5 % v/v and 5 % v/v lactic acid for 5 min was evaluated (Fig. 4.1). All bacterial strains evaluated survived exposure to 1 % v/v LA ($P > 0.05$). The average reduction in bacterial population at 1 % v/v LA was 6.44±1.14 log CFU/ml. Bacterial strain variants H7 amp P strep C, H7 amp C and H7 strep C showed the least reductions of 0.55±0.28 log CFU/ml, 0.63±0.17 log CFU/ml and 1.08±0.21 log CFU/ml respectively after exposure to 2.5 % v/v lactic acid and were significantly different from other strain variants assayed ($P<0.05$). The highest decline in the bacterial population of 5.92±0.07 log CFU/ml was observed in H7 NR.

All bacterial strains evaluated reduced in population to below the limit of detection when exposed to 5 % v/v lactic acid, with one exception, strain variant amp C showed survival at this concentration with a decline of 3.33±0.05 log CFU/ml.

4.3.2 Cell viability

Cell viability was calculated and presented as the percent of live cells in the sample after exposure to 5 % lactic acid (Fig. 4.2). The highest percentage 99.8±0.32 % of live cells was observed in amp C, which was significantly different from the percentages observed for strains H7 strep C, H7 amp P strep C, H7 amp C strep C and H7 NR ($P \leq 0.05$). The percentage of live cells 21.54±7.72 %

observed in H7 NR was significantly lower ($P \leq 0.05$) than the percentage for the antibiotic resistant mutants.

4.3.3 Biofilm formation in bacterial strains

4.3.3.1 *Biofilm formation by CV assay*

The bacterial strain variants with multidrug resistance to ampicillin and streptomycin formed better biofilms in 1:10 TSB than the non-antibiotic resistant strain (Fig. 4.3). Strain H7 amp P strep C was observed to form the highest biofilm biomass of OD_{570nm} 1.12±0.16, which was higher than biofilm biomass formed by strains H7 amp P and H7 NR ($P \leq 0.05$). The least biofilm formation of OD_{570nm} 0.74±0.11 and 0.73±0.10 was observed in strains H7 amp P and H7 NR respectively ($P > 0.05$).

4.3.3.2 *Enumeration of bacteria in biofilm*

Three strains (H7 NR, H7 amp C and H7 amp P strep C) were used for enumeration. Biofilms formed by bacterial strains suspended in 1:10 TSB or SDW at 25 °C or 37 °C were enumerated after incubation for 24 h (Fig. 4.4). Bacterial counts differed significantly between cells suspended in 1:10 TSB and SDW ($P \leq 0.05$) but counts were not significantly impacted by incubation temperature. The mean bacterial count observed in 1:10 TSB was 5.68±0.42 log CFU/ml while the mean count in SDW was 1.64±1.03 log CFU/ml ($P \leq 0.05$). The highest bacterial count of 5.96±0.57 log CFU/ml for biofilms developed in 1:10 TSB was observed in strain H7 amp P strep C at 37 °C and the lowest count of 5.26±0.37 log CFU/ml was observed in strain H7 amp C at 25 °C ($P > 0.05$). In SDW, the highest count of 2.31±1.59 log CFU/ml was observed in strain H7 amp P strep C at 25 °C and the least count of 1.00±0.00 log CFU/ml was observed in strains H7 NR and H7 amp C both at 37 °C ($P > 0.05$)

4.3.3.3 *Biofilm formation by CLSM*

The maximum thickness, average thickness, and average volume of colonies at substratum (biovolume) were evaluated for all the bacterial strains (Fig. 4.5). The highest maximum biofilm thickness of 46.25±4.43 µm and 42.66±16.87 µm were observed in strains H7 amp P strep C and H7 NR respectively. The least maximum thickness of 15.60±3.36 µm was observed in strain H7 strep C ($P \leq 0.05$). Comparison of the bacteria by average thickness showed that strains H7 amp P, H7 amp P strep C and H7 NR had the highest average thickness of 29.93±2.92 µm, 26.17±4.36 µm and 25.26±9.95 µm respectively ($P \leq 0.05$). The least average thickness of 11.88±2.99 µm was also observed in strain H7 strep C. Strains H7 amp P and H7 amp P strep C had the highest observed biovolumes of 1.50×10^6 μm^3 and 1.47×10^6 μm^3 respectively ($P \leq 0.05$) while H7 amp C and H7 strep C had the lowest biovolumes of 7.21×10^5 μm^3 and 5.11×10^5 μm^3 respectively.

4.3.4 In silico analysis

While we did not find any deletion or insertion related mutations in the mutants, there were several single nucleotide substitution differences between the parental and mutant strains (Table 4.2). We found nucleotide substitutions at 24 chromosomal positions. Sixteen nucleotide substitutions were found either in intergenic regions or were synonymous mutations, while 8 caused non-synonymous mutations in putative protein coding regions. We did not find a shared pattern of substitutions among the ampicillin resistant mutants, but all streptomycin resistant mutants had one nucleotide substitution in common: a non-synonymous substitution in the *rps*L gene. Additionally, a subset streptomycin resistant mutants had a second non-synonymous substitution in the *rpsL* gene. Bacterial strain H7 amp P strep C contained unique non-synonymous substitutions at the *rps*L gene and GTPase HflX. The *rpsL* gene encodes 30S ribosomal protein S12, a protein that has been well characterized in the past for its involvement in streptomycin resistance (30). HflX has also been

studied and suggested to be an important component in the functional coupling between cotranslational quality control and stress-induced translation reprogramming (42).

4.4 Discussion

Biofilm formation is a challenge in the food industry because bacterial cells present in biofilms are more tolerant to the effects of commercial sanitizers. Biofilms are able to develop in sites that harbor detritus in food processing plants, for example holes and crevices in flooring materials and polyvinyl chloride conveyor belts (18). Efflux pumps are highly active in bacterial biofilms, but efflux pumps also contribute towards bacterial tolerance to antimicrobials. Efflux pumps were observed to be upregulated in bacteria with fluoroquinolone and multidrug resistance (31). In a previous study, it was observed that *E. coli* O157: H7 H1730 with resistance to ampicillin and streptomycin survived exposure to 2.5 % v/v and 5 % v/v lactic acid (LA) compared to the non-antibiotic resistant parent strain. This improved tolerance was attributed to efflux pumps after cells became more sensitive to LA exposure in the presence of the efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP) (23). In the current study, the potential for antibiotic resistance associated improvement in biofilm formation and tolerance to acid stress was evaluated.

A diverse range of efflux pumps from different families play roles in biofilm formation by *E. coli*. A study conducted in 2010 reported that 22 mutant strains of *E. coli* that lacked the genes *acrD*, *acrE*, *mdtE* (RND superfamily), *emrD*, *emrK* (MFS superfamily) and *emrE* (SMR superfamily) exhibited extremely low biofilm formation compared to the wild type strain (20). Further, the genes *acrD* promoted resistance to aminoglycosides such as streptomycin (28), *acrE* was upregulated with increasing concentrations of levofloxacin and ofloxacin (7), and *emrD* was involved in the transport of amphipathic molecules, and resistance to several antimicrobials such as linezolid, tetraphenylphosphonium chloride, rifampicin, erythromycin and minocycline (1, 29).

In the present study, strains with antibiotic resistance exhibited several single nucleotide substitutions at 24 chromosomal positions including nucleotide substitutions in the *acrD* gene. Also, strain H7 amp P strep C expressed substitutions in *acr*D, *rps*L (unique for resistance to streptomycin) and GTPase Hflx (important for stress response in *E. coli*).

Bacterial strain H7 amp P strep C exhibited improved biofilm formation when compared to the non-antibiotic parent strain (H7 NR) by CV assay and CLSM. Although enumeration of cells in biofilm did not indicate any significant differences among the bacterial strains, higher cell counts after biofilm formation was observed in H7 amp P strep C. To our knowledge, no study has linked the presence of antibiotic resistance in *E. coli* to the development of improved biofilm forming phenotypes. Further, strains H7 amp C and strains amp P strep C developed cross-tolerance to exposure to 2.5 % and 5 % v/v LA for 300 s compared H7 NR. Tolerance is the ability to sustain increased duration of exposure to an antimicrobial (4). Results from this study are consistent with findings from a previous study where *E. coli* O157: H7 H1730 with resistance to ampicillin and streptomycin became more tolerant to LA after 30 s exposure (23). Comparison between the susceptibility of the bacterial strains by viability staining also indicated improved tolerance in the antibiotic resistant strain variants compared to the non-resistant parent strain. The observed difference between the survival of strains by plate counts and viability assay may be attributed to transition to a stationary phase by cells during LA exposure. This transition results in reduced growth on plating media (11).

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Table 4.1: List of *E. coli* O157: H7 H1730 strain variants used in this study.

Bacterial Strain	**Mode of resistance**	**Strain name for this study**
***Escherichia coli* O157: H7**	No resistance (parent strain)	H7 NR
***E. coli* O157: H7 ampicillin (Amp) resistant**	Plasmid	H7amp P
***E. coli* O157: H7 amp resistant**	Chromosome	H7amp C
***E. coli* O157: H7 streptomycin (strep) resistant**	Chromosome	H7strep C
***E. coli* O157: H7 amp and strep resistant**	Plasmid* and chromosome	H7 amp P strep C
***E. coli* O157: H7 amp and strep resistant**	Chromosome	H7amp C strep C

Table 4.2: Single nucleotide polymorphisms (SNPs) in antibiotic resistant *E. coli* O157: H7 H1730 compared to the parent strain. **a.** SNPs at intergenic regions **b**. non-synonymous substitutions **c**. Stop lost **d.** Synonymous substitutions. DNA nucleotide abbreviations: Thymine (T), Adenine (A), Cytosine (C) and Guanine (G).

a.

	SNPs at Intergenic regions								
	lac operon					intergenic region			
Bacteria	120574	120595	120601	120612	120635	575673	128133	291880	374289
H7 NR	T	C	G	G	G	G	G	A	C
H7 amp C	T	C	G	G	G	G	C	G	C
H7 amp P strep C	C	A	A	C	A	G	C	G	C
H7 amp P	C	A	A	C	A	G	C	G	C
H7 strep C	T	C	G	G	G	G	C	G	C
H7 amp C strep C	T	C	G	G	G	G	C	G	C

b.

	Non-synonymous substitutions						
Bacteria	rne (Ribonuclease E)	small hypothetical protein	Sucrose permease	rpsL (30S ribosomal protein S12)		Cell division protein DamX	GTPase HflX
	1196453	2652169	2916296	3908341	3908470	3942835	4930649
H7 NR	G	G	A	C	T	G	C
H7 amp C	G	T	G	C	T	T	C
H7 amp P strep C	G	T	G	A	C	T	A
H7 amp P	G	G	G	C	T	T	C
H7 strep C	G	T	G	A	T	T	C
H7 amp C strep C	G	G	G	A	T	T	C

c.

	Stop lost
	3' prime end IS3 family transposase IS2
Bacteria	1281756
H7 NR	A
H7 amp C	G
H7 amp P strep C	G
H7 amp P	G
H7 strep C	G
H7 amp C strep C	G

d.

	Synonymous substitutions						
Bacteria	3' end of lacI 120685	5' end hypothetical protein 1322042	Inner membrane transport protein YnfM 2009004	3 ' end D-galactose-binding periplasmic protein 2689884	Phospho transferase RcsD 2764453	5 ' end of Multidrug efflux pump subunit AcrD 3017891	hypothetical protein 3958242
H7 NR	T	G	T	G	T	C	G
H7 amp C	T	G	C	A	T	C	A
H7 amp P strep C	C	G	C	G	G	T	G
H7 amp P	C	G	C	G	T	C	G
H7 strep C	T	G	C	G	T	T	G
H7 amp C strep C	T	G	C	G	T	T	G

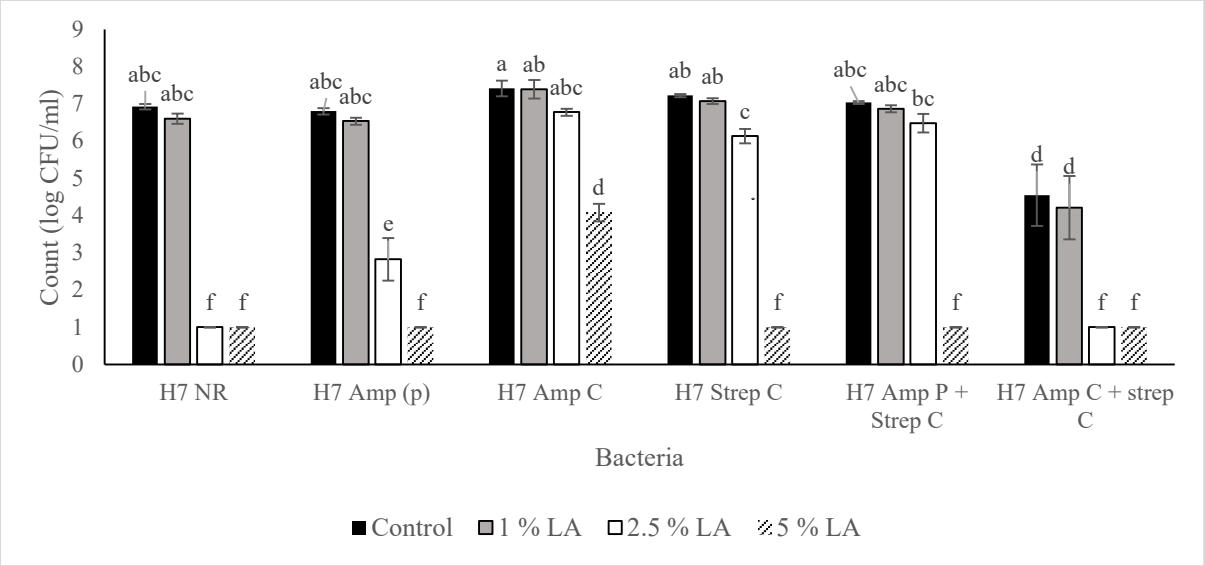

Fig 4.1: Mean count of *E. coli* O157:H7 H1730 (log CFU/ml) after exposure to 1 %, 2.5 % and 5 % v/v lactic acid for 300 s

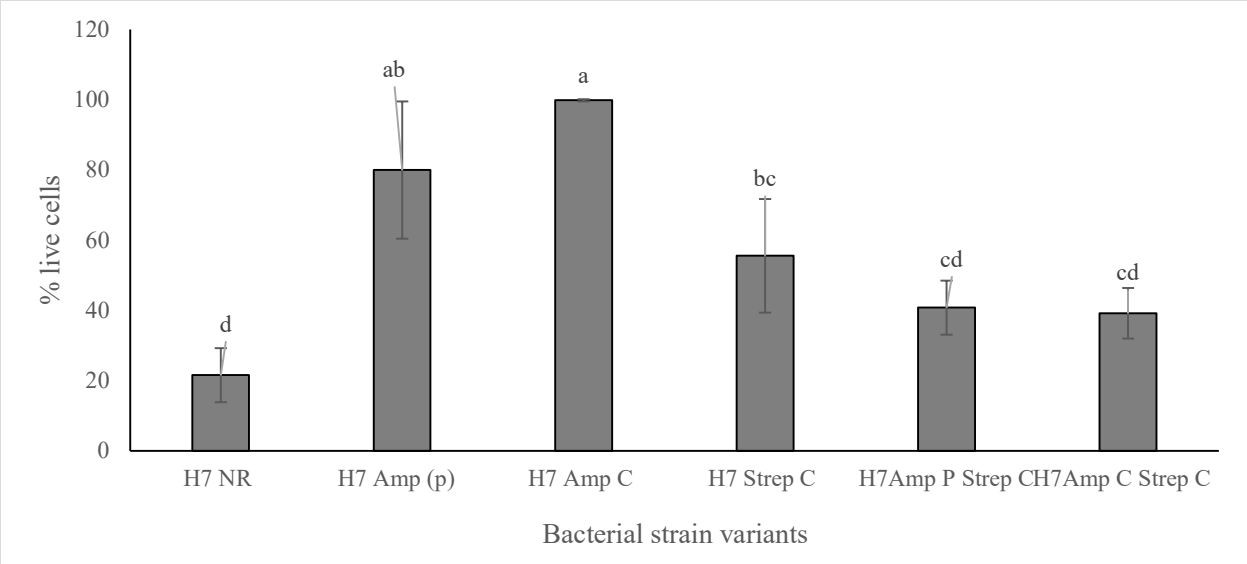

Fig 4.2: Percent live cells after exposure to 5 % v/v lactic acid for 300 s

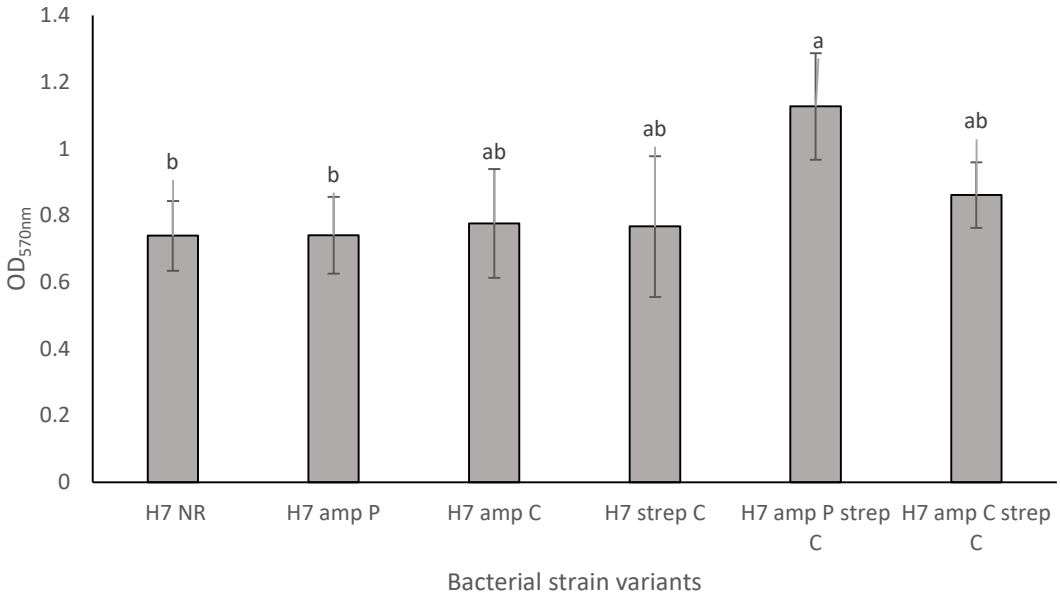

Fig 4.3: Biofilm Formation in *E. coli* O157: H7 H1730 by Crystal Violet assay

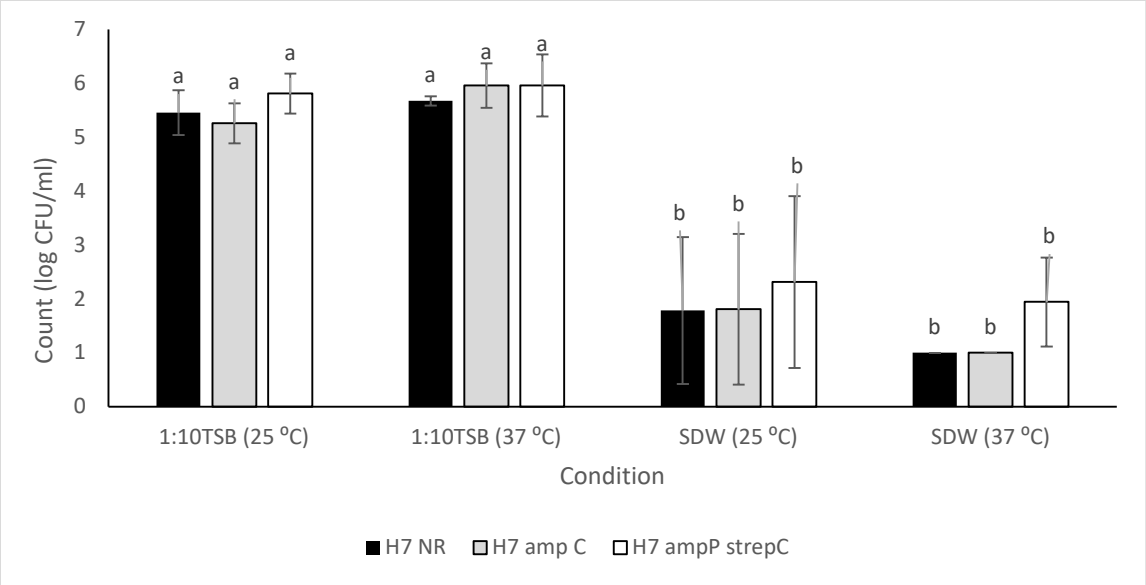

Fig 4.4: Enumeration of bacteria in biofilms developed in 1:10 TSB and SDW at 25 °C and 37 °C

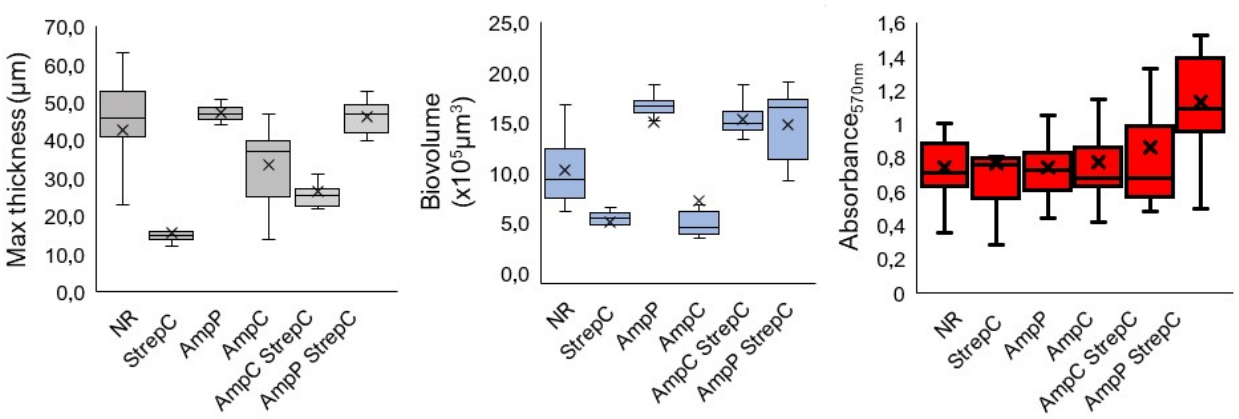

Fig 4.5: Biofilm Formation in *E. coli* O157: H7 H1730 by CLSM

CHAPTER 5

COMPARISON BETWEEN THE TOLERANCE OF ANTIBIOTIC RESISTANT O157:H7 AND NON O157: H7 SHIGA-TOXIN PRODUCING *E. COLI* TO LACTIC ACID[3]

[3]Oguadinma, I.C., Mishra A., and Dev Kumar G. 2022. To be submitted to *Journal of Food Protection*.

Abstract

The occurrence of antibiotic resistant (ABR) bacteria in the food chain is a growing public health challenge. ABR associated cross-tolerance to sanitizers in Shiga-toxin producing *E. coli* O157: H7 could potentially contribute towards cross-tolerance to lactic acid (LA). In this study the impact of ABR on growth parameters and tolerance to LA in O157: H7 and non-O157: H7 STEC serogroups were evaluated and compared. Resistance to ampicillin and streptomycin were developed in *E. coli* O157: H7 H1730, O157: H7 43895, O121: H19 and O26: H11. ABR profiles were developed chromosomally through incremental exposure to ampicillin (amp C) and by transformation using a plasmid that conferred resistance to ampicillin to generate amp P strep C. The minimum inhibitory concentration (MIC) of LA for all strains evaluated was 0.375 % v/v. Increasing LA concentrations at 0.0625 % v/v, 0.125 % v/v, and 0.25 % v/v (subMIC) correlated positively with the lag phase duration, and negatively with the maximum growth rate and change in population density for all strains evaluated except O157: H7 amp P strep C. Strains O121 NR (non-ABR), O121 amp C, O121 amp P strep C, O157: H7 H1730 amp C and O157: H7 H1730 amp P strep C were not inactivated after exposure to 1 % and 2.5 % v/v LA for 300 s. No recovery of cells was observed after the strains were exposed to 5 % v/v LA for 300 s. ABR strains O157: H7 H1730 amp C and O157: H7 H1730 amp P strep C demonstrated a high tolerance to LA ($P \leq 0.05$). The results indicate that ABR in some STEC serogroups may improve tolerance to LA.

5.1 Introduction

Shiga-toxin producing *E. coli* (STEC) are non-sporulating, Gram negative bacillus expressing the genes for Shiga-toxins type 1 and 2. STEC is a major food borne pathogen worldwide. Illnesses resulting from STEC infection could be a mild, self-limiting gastroenteritis but could also involve the development of hemorrhagic colitis (bloody diarrhea) found in 90 % of patients and hemolytic uremic syndrome (HUS) seen in 5-15 % of patient (27). HUS is typically characterized by acute kidney failure, microangiopathic hemolytic anemia (damage of small blood vessels with destruction of red blood cells), and thrombocytopenia (decrease in platelets) (27).

E. coli O157: H7 is the most common STEC serogroup responsible for 40.3 % of domestically acquired illnesses in the United States but the big six STEC serogroups are also prevalent and have resulted in 59.7 % cases of illnesses in the US (16). Approximately 75 % of non-O157: H7 serogroups isolated from cases of illnesses in the United States between 1983 – 2002 were STEC O26, O45, O103, O111, O121, and O145 (18). The first outbreak of *E. coli* O157: H7 occurred on beef in 1982 (26). *E. coli* O157: H7 became the first microbial agent considered as an adulterant in ground beef in the United States soon after a multistate Pacific Northwest outbreak of *E. coli* O157:H7 in 1992-1993 which led to recalls of ground beef and beef trimmings contaminated with the pathogen (15, 24). The six most prevalent non-O157: H7 STEC serogroups were recently added to the list of adulterants on beef (15).

In recent years, there has been a growing challenge of antimicrobial resistant bacteria in the food chain as bacteria can develop resistance to antibiotics and other antimicrobials used in food production (25). Approximately 2.8 million people get antibiotic resistant (ABR) bacterial infections annually in the United States, and 35,000 die from these infections (8). Long-term

exposure to antibiotics and other antimicrobials at sub-lethal concentrations can induce the development of resistance in bacteria (25). Furthermore, resistance to one antimicrobial group can result in cross-tolerance to other antimicrobials (14). Ampicillin and streptomycin are two common ABR profiles identified in STEC isolates and in a previous study, the presence of resistance to ampicillin and streptomycin in *E. coli* O157: H7 improved tolerance of the bacteria to lactic acid (LA) (23).

Lactic acid is a commonly used sanitizer during beef (3, 7, 32) applied at concentrations ranging from 2-5 % on beef carcass (33). LA disrupts transmembrane proton motive force and reduces intracellular pH of bacteria by penetrating the cytoplasmic membrane of cells in its undissociated form. (1). The continued foodborne outbreaks associated with *E. coli* O157:H7 on beef despite consistent use of sanitizers could be occurring because of the pathogens ability to adapt to acid stress through acid resistance systems (AR) (11, 22) and efflux pumps (9). Tolerance of ABR *E. coli* O157: H7 to LA was linked to efflux pumps on the cells (23). Investigating the role of ABR in sanitizer tolerance is important to identify possible loopholes in STEC survival on beef carcass.

The objective of this study was to evaluate cross-tolerance to LA in Shiga-toxin producing *E. coli* O157: H7 H1730, O157: H7 43895, O121: H19 and O26: H11 with resistance to ampicillin and streptomycin. The impact of ABR on growth parameters of the cells and tolerance to sub-lethal concentrations of LA were also investigated.

5.2 Materials and Methods

5.2.1 Bacterial strain

Four serogroups of Shiga toxin-producing *E. coli* were used in this study. *E. coli* O157: H7 H1730 was a human isolate from a lettuce outbreak, *E. coli* O157: H7 (ATCC 43895) was from a 1982 ground beef outbreak, and *E. coli* O121: H19 (strain TW08980) and *E. coli* O26: H11 (strain 3012-03) were from a 2016 Missouri flour outbreak. All strains were obtained from the Center for Food Safety, University of Georgia Griffin campus Culture collection. Isolates were revived from frozen storage by transferring to Tryptic Soy Broth (TSB, Neogen, Lansing, MI) and incubating at 37 ℃ for 24 h. The bacterial strains were evaluated for antibiotic resistance by growing cells on Tryptic Soy Agar (TSA; Neogen Lansing, MI) containing 100 µg/ml of streptomycin, and ampicillin before the development of the antibiotic-resistant strain variants (Table 5.1).

5.2.2 Development of ampicillin and streptomycin adapted strains

5.2.2.1 *Chromosomal resistance*

Chromosomal resistance to streptomycin and ampicillin was developed by sequentially transferring the bacterial strains into increasing concentrations of the antibiotics. Briefly, 100 µl of culture from a lower antibiotic concentration was transferred to fresh media containing 900 µl a higher antibiotic concentration. This process was repeated with a 10 µg/ml increase in the antibiotic concentration for each transfer until adaptation to 100 µg/ml of antibiotic was achieved. Bacterial strains with antibiotic resistance were isolated by streaking on TSA supplemented with either 100 µg/ml of streptomycin or ampicillin.

5.2.2.2 *Transformation by electroporation*

Transformation of cells to take up a green fluorescence ampicillin resistance plasmid (GFP amp plasmid) was performed following the method described by Kumar et al. (20) with minor modifications. Briefly, competent cells were prepared as follows: 45 ml of Tryptic soy broth (TSB) was inoculated with 1 ml of overnight cultures of the bacterial strains. Cultures were incubated at 37 ℃ for 4 h to achieve an optical density (OD_{600nm}) of 0.8 and then placed in ice for 15 min. The cultures were then centrifuged at 1400 g for 10 min to pellet the cells, and the supernatant discarded. The pelleted cells were washed three times with 15 % ice-cold glycerol and stored at −20 ℃ until use. The cells were transformed using the host range plasmid pGFPuv (28). Electroporation conditions applied were 2.5 kV, 25 μF, and 400 Ω using the

5.2.3 Preparation of bacterial inoculum

Stock cultures of the bacterial strains were prepared by streaking each strain to TSA or TSA + 100 μg/ml of antibiotics (TSA + Amp, TSA + Amp + Strep) and incubating at 37 °C for 24 h. Colonies from overnight cultures were scraped from the plates with a sterile loop and suspended in phosphate-buffered saline (1x PBS; VWR International, Radnor, PA). The bacterial population was adjusted to 6 log CFU/ml.

5.2.4 Determination of the minimum inhibitory concentration (MIC) of LA

The minimum inhibitory concentration (MIC) of lactic acid (LA, L-lactic acid, Xena International Inc., Illinois) for the antibiotic-resistant and non-resistant bacterial strains was determined using a 96 well plate broth dilution method described by Kumar et al. (10) with some modifications. Briefly, LA stock solutions were serially diluted in 96 well plates -180 μl in each well – and inoculated with 20 μl of 5 log CFU/ml of bacteria. Serial dilutions were performed

from initial LA concentrations of 5% and 3% to obtain LA concentrations of 2.5 %, 1.5 %, 1.25 %, 0.75 %, 0.62 %, 0.37 %, 0.31 %. 0.18 %, 0.15 %, 0.09 %, 0.07 %, 0.04 %, 0.03 % and 0.02 % v/v. The 96-well plates (Costar® 96 Well Flat Bottom, Corning LifeSciences Inc. ME, USA) were incubated for 24 h at 37 ℃, and the growth kinetics were observed using the Bio-Tek Cytation 3 image reader (BioTek Instruments, Inc. USA). Conditions in the Bio-Tek Cytation 3 image reader were set as follows: the total runtime was set at 24 h with read intervals of 30 min, the shaker was set to an orbital shake every 10 s at a frequency of 283 cpm (3mm), the read speed was set to Normal with a delay of 100 ms and the optical density was read at an absorbance of 600 nm. Un-inoculated blanks of TSB were used as a control for this experiment.

5.2.5 Evaluation of bacterial growth rates at sub-lethal concentrations of LA

The growth rates for the different bacterial strains in TSB and the subminimum concentration of LA were evaluated using the turbidimetric technique. The experiment was conducted in a 96 well microplate by inoculating 20 µl of 6.76±0.71 log CFU/ml of bacteria to 180 µl of media. Growth rates were observed for 24 h at 37 ℃ using the Bio-Tek Cytation 3 image reader (BioTek Instruments, Inc. USA). Conditions in the Bio-Tek Cytation 3 image reader were set as described previously. Three biological and three technical replicates were performed, and strains exposed to TSB without the antimicrobial stress were used as controls.

5.2.6 Mathematical modeling for bacterial growth

The modified Gompertz model (13) modified by (2) was fitted to the growth curve of these bacterial strains using MATLAB software (version R2021a, The MathWorks, Inc. Natick, MA). The model can be described by the following equation (17), where N is the bacterial population at a given time, N_0 is the initial bacterial population, $O.D_{min}$ is the lowest O.D. value above the

detection threshold, A is the logarithmic increase of bacterial population, L is the lag time, μ is the maximum growth rate, and t is time:

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

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

The growth parameters assessed were change in bacterial population in log CFU/ml (A), lag phase duration in hours (L), and maximum growth rate in log CFU/h (μ_{max}).

5.2.7 Bactericidal concentration of LA

A commercial food grade LA (88 % v/v) was obtained from Xena Inc (Xena International Inc; Polo IL, USA) and LA solutions were prepared in TSB at concentrations of 0.5 %, 1 %, 1.5 %, 2 %, 2.5 % and 5 % v/v. The pH of LA was determined using a pH meter (Oakton pH 510 Benchtop Meter, Oakton Instruments, Vernon Hills, IL, USA) with a sensitivity of 0.01 and 2-point calibration. The concentration at which LA could prevent survival and regrowth of the STEC strains was considered as the bactericidal concentration. The bactericidal concentration of LA for all the bacterial strains was evaluated as follows: Each 900 µl of 0.5 % v/v, 1 % v/v, 1.5 % v/v, 2 % v/v, 2.5 % v/v and 5 % v/v LA was inoculated with 100 µl of the bacteria for 300 s. Solution was centrifuged immediately after exposure for 1 min at 13000 g using a Corning high speed microcentrifuge (Corning Inc., Corning, NY). The supernatant was discarded, and pellets were resuspended in 1 ml sterile deionized water (SDW). From the resuspended solution, 100 µl was transferred to 100 µl of 2x TSB in 96-well plates (Costar® 96 Well Flat Bottom, Corning Life Sciences Inc. ME, USA) and incubated for 24 h at 37 °C. The plates were observed for

turbidity to determine survival and regrowth after incubation by determining the OD_{600nm} using the BioTek Cytation.

5.2.8 Reduction in bacterial population upon exposure to 2.5 % and 5 % v/v LA

Overnight cultures of the bacterial strains grown either on TSA or TSA + 100 µg/ml of ampicillin, and TSA + 100 µg/ml of streptomycin and ampicillin were used to prepare 8 log CFU/ml of bacterial inoculum in 1x PBS. From the suspended culture, 100 µl was transferred to 900 µl of TSB containing 2.5 % or 5 % lactic acid for 30 s and 300 s. The solution was centrifuged immediately after exposure for 1 min at 13000 g using the Corning LSE high speed microcentrifuge. The supernatant was discarded, and the pellets were resuspended in 1 ml 1x PBS. Enumeration of bacteria was done using the droplet plate method where colony forming units (CFU) of cells within the perimeter of drop were used for enumerating bacterial populations from serially diluted samples. The number of colonies formed were counted after incubation for 24 h at 37 °C. The limit of detection for the assay was 10 cells or 1.00 log CFU/ml.

5.2.9 Statistical analysis

All experiments were conducted in three biological and three technical replicates. Differences between the bactericidal concentration of LA and decline in population of all bacterial strains evaluated after acid exposure were compared using the one-way analysis of variance (ANOVA). Significant differences between the means were compared using the Tukey's Honestly Significant Difference (HSD) test at a 0.05 significance level, using JMP statistical software (SAS Institute Inc, USA).

5.3 Results

5.3.2 Growth parameters of bacterial strains at sublethal concentrations of lactic acid

Differences in antibiotic resistance (ABR) profile did not affect the MIC of LA which was determined to be 0.375 % v/v for all bacterial strains evaluated ($P > 0.05$). Growth parameters of O157: H7 STEC (Fig. 5.1a, 5.1c, 5.1e) and O121: H19 and O26: H11 (Fig. 5.1b, 5.1d, 5.1f) at 0.0625 % v/v, 0.125 % v/v and 0.25 % v/v LA were evaluated to describe differences between the bacterial strains due to the acquisition of ABR. As observed, the type of strain and concentration of LA affected the growth parameters ($P \leq 0.05$). Generally, LA concentration of 0.25 % v/v significantly increased the average lag phase duration (L) by 1.15 h, decreased the average maximum growth rate (μmax) by 0.08 log CFU/h, and decreased the average change in bacterial population (A) by 0.3 log CFU/ml ($P \leq 0.05$).

The L, μmax and A for bacterial strain O157: H7 43895 was not significantly different for both the parent strain and the antibiotic resistant variants across all 3 sublethal LA concentration ($P > 0.05$) except at the μmax for O157: H7 43895 amp C in 0.0625 % v/v LA which was significantly different from the other variants ($P \leq 0.05$). The μmax for O157: H7 43895 amp C at 0.0625 % v/v LA was 0.41±0.03 log CFU/h compared to 0.32±0.01 log CFU/h for O157: H7 43895 NR and 0.28±0.03 log CFU/h for O157: H7 43895 amp P strep C. Growth parameters in the no-LA control was not significantly different for all the strains ($P > 0.05$).

The same pattern was observed for strain O121: H19 at sublethal concentrations of LA. However, significant differences were observed at the L and A of cells exposed to 0.0625 % v/v LA. The longest L of 1.81±0.05 h at 0.0625 % v/v LA was observed for O121 NR ($P \leq 0.05$) and the highest A of 0.66±0.01 log CFU/ml was observed for O121 amp P strep C ($P \leq 0.05$). In the

controls, the shortest L of 1.96±.0.06 h was observed for O121 NR and the highest μmax of 0.31±0.01 log CFU/h was observed for O121 amp C ($P \leq 0.05$).

The difference between variants of strain O26: H11 was observed at 0.25 % v/v LA. The μmax of 0.14±0.01 log CFU/h for O26 amp P strep C observed at 0.25 % v/v LA was significantly different from the μmax of 0.19±0.01 log CFU/h observed for O26 NR ($P < 0.05$). In the control, O26 NR had the shortest L of 0.95±0.06 h ($P \leq 0.05$). The A of 0.65±0.01 observed for O26 NR in the control was significantly different from the A of 0.58±0.01 observed for O26 amp P strep C ($P \leq 0.05$).

Bacterial strain O157:H7 H1730 amp P strep C had the longest L of 5.97±0.04 h, 5.45±0.08 h, 5.58±0.17 h and 5.74±0.03 h in the control, 0.0625 % v/v LA, 0.125 % LA and 0.25 % LA respectively compared to all other strain variants evaluated ($P \leq 0.05$). At 0.0625 % v/v LA, the L of 5.45±0.08 h for O157: H7 H1730 amp P strep C was significantly different from L for O157: H7 H1730 amp C and O157: H7 H1730 NR ($P \leq 0.05$). The μmax of 0.21±0.01 log CFU/h for O157: H7 H1730 amp P strep C was the smallest at this concentration ($P \leq 0.05$) but O157: H1730 NR had the smallest A of 0.83±0.04 log CFU/ml compared to the other O157: H7 H1730 strain variants ($P \leq 0.05$). O157: H7 H1730 amp P strep C had the longest L of 5.58±0.17 h at 0.125 % v/v LA and the smallest μmax of 0.23±0.01 log CFU/h at 0.25 % v/v LA compared to the other O157: H7 H1730 strain variants ($P \leq 0.05$).

Increase in the LA concentration of the medium correlated positively with the (L) and negatively with the (μmax) and (A) for all bacterial strains except O157:H7 H1730 amp P strep C that showed a negative correlation with L ($r = -0.18$) and positive correlation with μmax ($r = 0.21$) (Table 5.4).

5.3.3 Bactericidal concentration of LA and variations between the bacterial strain variants

The bacterial strains were grouped into 3 (Tolerant, moderately susceptible, and highly susceptible) according to the bactericidal concentrations of LA observed. Tolerant strains in this study were inhibited by 5 % v/v LA, moderately susceptible strains were inhibited by 2 – 2.5 % v/v LA and highly susceptible strains by 1 – 1.5 % v/v LA (Table 5.3).

The bactericidal concentrations between the variants of O157: H7 43895 were not significantly different ($P > 0.05$) but, in O157: H7 H1730, bactericidal concentration of 1.00±0.00 % v/v LA observed in the parent antibiotic susceptible strain was significantly different from the bactericidal concentrations of 5.00±0.00 % v/v LA observed for O157: H7 H1730 amp C and O157: H7 H1730 amp P strep C ($P \leq 0.05$). The bactericidal concentrations for the ABR strains of O157: H7 H1730 were higher than the bactericidal concentrations for all strain variants of O157: H7 43895 ($P \leq 0.05$)

The bactericidal concentrations for all three variants of strain O121 was 5.00±0.00 % v/v LA ($P > 0.05$) and the bactericidal concentrations for O26 NR, O26 amp C and O26 amp P strep C were 2.00±0.86 % v/v, 2.00±0.86 % v/v, and 2.50±0.00 % v/v respectively ($P > 0.05$). Bactericidal concentrations for all strain variants of O121 were higher than the bactericidal concentrations for all strain variants of O26 ($P \leq 0.05$) (Fig 5.2).

5.3.4 Reduction in the population of bacterial strains exposed to LA

The pH of media amended with LA were 2.76±0.00 for 2.5 % v/v and 2.5±0.02 for 5 % v/v. Survival of the bacterial strains in LA were significantly impacted by both LA concentration (2.5 % and 5 %) and exposure time (30 s and 300 s) ($P \leq 0.05$). The highest population decline of 2.74± 0.61 log CFU/ml was observed in O157: H7 H1730 NR and the least population decline of

0.17±0.16 was observed in O157: H7 H1730 amp P strep C post exposure to 2.5 % v/v LA for 30 s. After exposure to 2.5 % v/v LA for 300 s, O157: H7 H1730 NR and O26 amp P strep C showed the highest population declines of 5.92±0.07 and 4.92±1.05 log CFU/ml respectively ($P \leq 0.05$). The most tolerant strains at 2.5 % v/v LA for 300 s were O157: H7 H1730 amp C 0.63±0.17 log CFU/ml and O157: H7 H1730 amp P strep C 0.55±0.29 log CFU/ml. The highest tolerance in 5 % v/v LA after 30 s exposure was observed in O157: H7 H1730 amp P strep C and O157: H7 H1730 amp C with 2.66±0.14 and 2.56±0.21 log CFU/ml decline in bacterial population respectively ($P \leq 0.05$). All bacterial strains except O157: H7 amp C declined in population to below the limit of detection after exposure to 5 % v/v LA for 300 s (Fig 5.3).

5.4 Discussion

The overuse of antimicrobials in the food chain promotes the development of antimicrobial resistant bacterial strains (4). In cattle production, antibiotics are used to treat diseases and promote growth in animals but long term exposure of bacteria to sub-lethal concentrations of antibiotics could exert selective pressure, favoring isolates with resistance genes or inducing cross-resistance to unrelated antibiotics (6, 25). In this study, resistance to ampicillin and streptomycin or both were induced in O157: H7 and non O157 STEC exposed to sublethal concentrations of each antibiotic over time.

The MIC of LA was the same for both the O157: H7 and the big six serogroups evaluated indicating that the presence of an ABR profile does not impact resistance to LA in STEC. Tolerance, which is defined as the ability of bacteria to sustain increased duration of exposure to an antimicrobial (5) might be impacted. In a previous study, the presence of resistance to ampicillin and streptomycin in O157: H7 H1730 affected cross-tolerance to LA (23). In this study, improved tolerance of O157: H7 H7130 with ABR profiles was also observed. Different

bacterial serotypes might however vary in response to ABR associated cross-tolerance. ABR in the other strain variants evaluated did not significantly improve or diminish observed tolerance to LA.

Tolerance to LA due to ABR may be attributed to the activity of efflux pumps. In a previous study where *E. coli* O157: H7 H1730 with multidrug resistance to ampicillin and streptomycin was more tolerant to LA than the non ABR strain variant, the addition of the efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP) resulted in increased sensitivity to LA (23). Efflux pumps modulate the activity of a large number of antibiotics and are transport proteins involved in the extrusion of toxic substrates from the internal to the external environment of cells (29, 30, 31). Efflux pumps have a broad substrate range and bacteria with antibiotic resistance over express these pumps (29). Expression of efflux pumps could also potentially vary between serogroups of *E. coli* and requires further exploration. In this study, all three variants of *E. coli* O121 showed comparable levels of LA tolerance by bactericidal concentration to O157: H7 H1730 amp C and O157: H7 H1730 amp P strep C.

Cells that develop ABR were reported to spend an extended time in the lag phase (12). Results from this study indicate that the impact of ABR on lag phase duration might be serogroup dependent as an extension in the lag phase duration due to ABR was only observed in strains O157: H7 H1730, O121: H19 and O26: H11. In the presence of LA at subMIC, lag phase was significantly extended when bacterial strains were exposed to concentrations greater than 0.0625 % v/v LA for all the bacterial strains except O157: H7 H1730 with multidrug resistance. The longest lag phase durations for all the bacterial strains were observed when the cells were exposed to 0.25 % v/v LA.

Previous studies have reported variations in acid resistance between STEC serotypes. Among the serotypes evaluated in this study, previous studies have reported O121 to be the most acid resistant (19, 21). In this study, all strain variants of O121 had bactericidal concentrations of 5 % v/v LA which was higher than the bactericidal concentrations observed for the other strains but comparable to the highly acid tolerant O157: H7 H1730 amp C and O157: H1730 amp P strep C.

5.5 Conclusion

The highest tolerance to LA was observed in strains O121 NR, O121 amp C, O121 amp P strep C, O157: H7 H1730 amp C and O157: H7 H1730 amp P strep C confirming the high acid tolerance of O121 but also indicating that ABR in some STEC strains could improve acid tolerance.

The results highlight the impact of antibiotic resistance on growth parameters in STEC and the risk of antibiotic associated tolerance to sanitizers.

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Table 5.1: List of Shiga toxin producing *E. coli* strain variants used in this study**.**

Bacterial Strain	**Mode of resistance**	**Strain name for this study**
***Escherichia coli* O157:H7 H1730**	No resistance (parent strain)	O157:H7 H1730 NR
***E. coli* O157:H7 H1730 amp resistant**	Chromosome	O157:H7 H1730 amp C
***E. coli* O157:H7 H1730 amp and strep resistant**	Plasmid* and chromosome	O157:H7 H1730 amp P strep C
***E. coli* O157:H7 43895**	No resistance (Parent strain)	O157:H7 43895 NR
***E. coli* O157:H7 43895 amp resistant**	Chromosome	O157:H7 43895-amp C
***E. coli* O157:H7 43895 amp and strep resistant**	Plasmid and chromosome	O157:H7 43895-amp P strep C
***E. coli* O26:H11**	No resistance (parent strain)	O26:H11 NR
***E. coli* O26:H11 amp resistant**	Chromosome	O26:H11 amp C
***E. coli* O26:H11 amp and strep resistant**	Plasmid and chromosome	O26:H11 amp P strep C
***E. coli* O121:H19**	No resistance (parent strain)	O121:H19 NR
***E. coli* O121:H19 amp resistant**	Chromosome	O121:H19 amp C
***E. coli* O121:H19 amp and strep resistant**	Plasmid and chromosome	O121:H19 amp P strep C

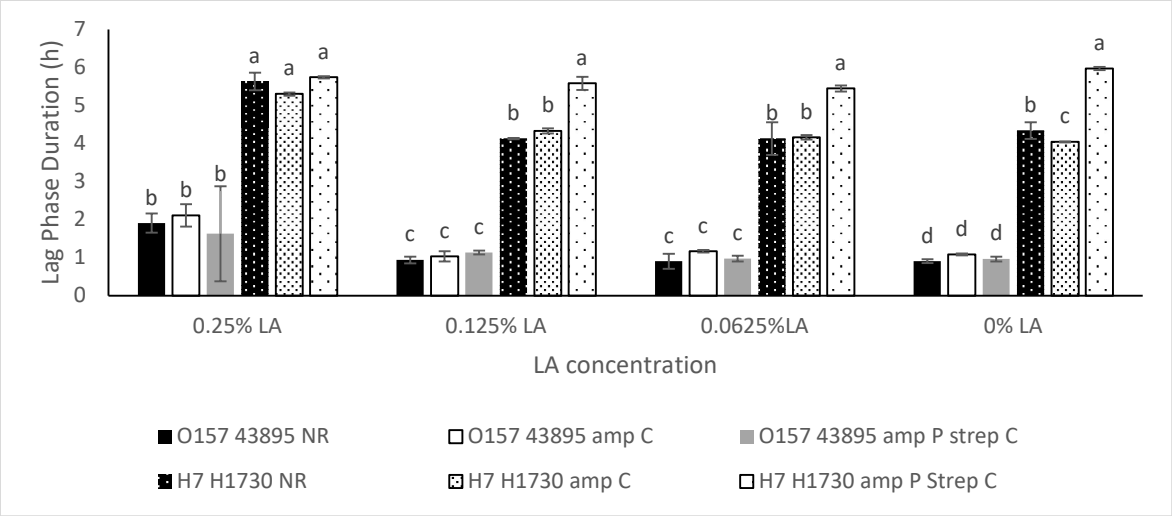
Table 5.2: Correlation between LA concentrations at subMIC and growth parameters of STEC

Bacteria	L (h)	μmax (log CFU/h)	A (log CFU/ml)
O157:H7 43895 NR	0.84	-0.48	-0.94
O157:H7 43895 amp C	0.82	-0.78	-0.92
O157:H7 43895 amp P strep C	0.45	-0.56	-0.97
O157:H7 H1730 NR	0.76	-0.07	-0.72
O157:H7 H1730 amp C	0.95	-0.05	-0.93
O157:H7 H1730 amp P strep C	-0.18	0.21	-0.93
O121:H19 NR	0.85	-0.43	-0.96
O121:H19 amp C	0.87	-0.81	-0.95
O121:H19 amp P strep C	0.82	-0.71	-0.95
O26:H11 NR	0.92	-0.39	-0.96
O26:H11 amp C	0.91	-0.66	0.94
O26:H11 amp P strep C	0.8	-0.65	-0.91

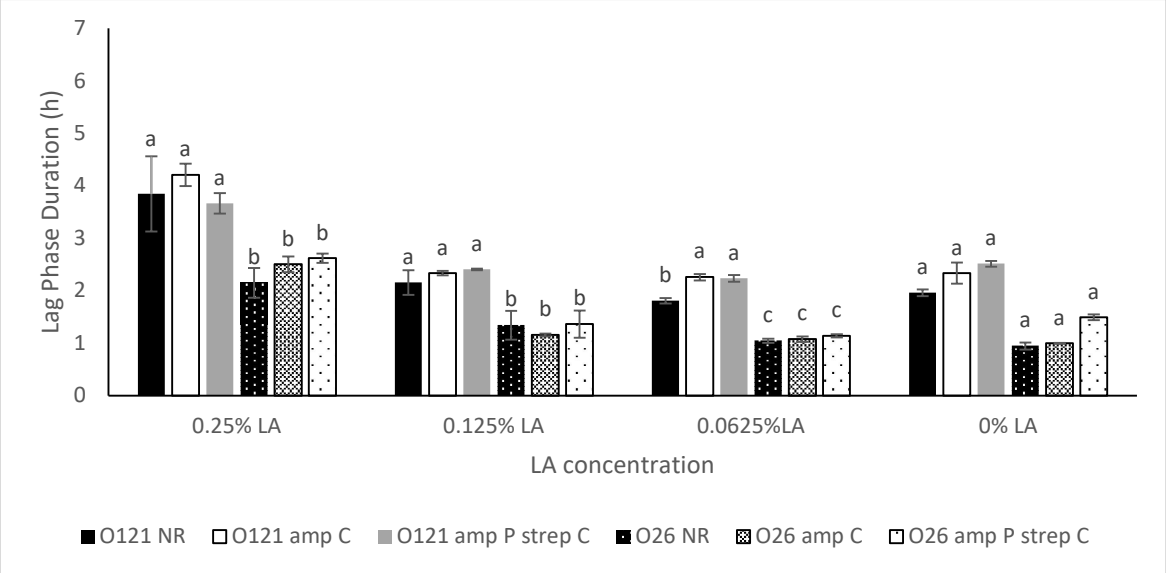
Table 5.3: Classification of the bacterial strains according to bactericidal concentrations of LA

Bacteria	Classification	Bactericidal concentration (% v/v)
	Tolerant (5 % v/v)	
O121:H19 NR		5.00±0.00
O121:H19 Amp c		5.00±0.00
O121:H19 Amp P Strep C		5.00±0.00
O157:H7 H1730 Amp C		5.00±0.00
O157:H7 H1730 Amp P strep C		5.00±0.00
	Moderately susceptible (2-2.5 % v/v)	
O26:H11 Amp P Strep C		2.50±0.00
O26:H11 Amp c		2.00±0.86
O26:H11 NR		2.00±0.86
	Highly susceptible (1-1.5 % v/v)	
O157:H7 43895 Amp P strep C		1.50±0.86
O157:H7 43895 NR		1.50±0.86
O157:H7 43895 Amp c		1.00±0.00
O157:H7 H1730 NR		1.00±0.00

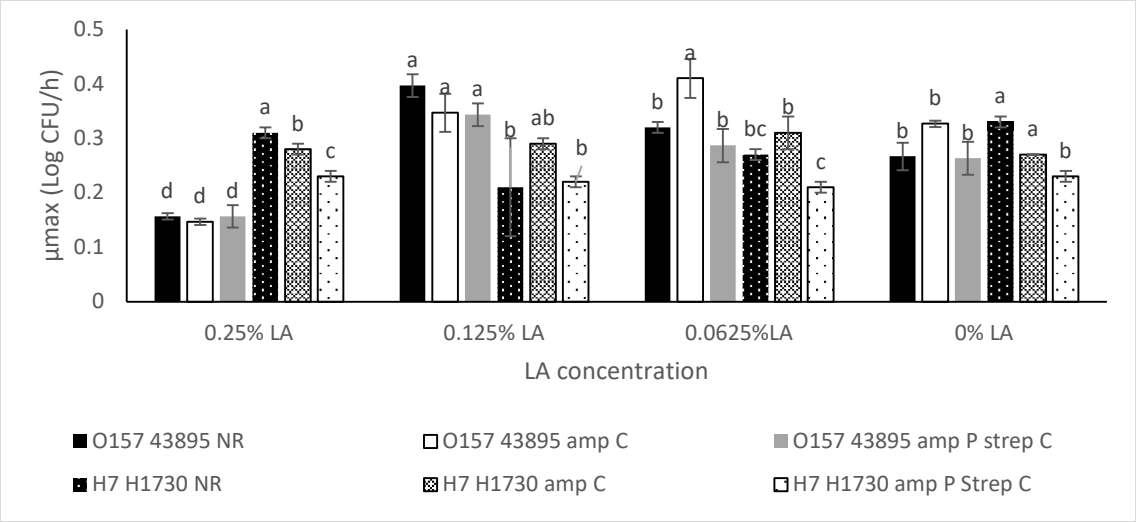
a.


Lag Phase Duration (h)
0.25% LA
0.125% LA
0.0625%LA
0% LA
LA concentration
O157 43895 NR
O157 43895 amp C
O157 43895 amp P strep C
H7 H1730 NR
H7 H1730 amp C
H7 H1730 amp P Strep C

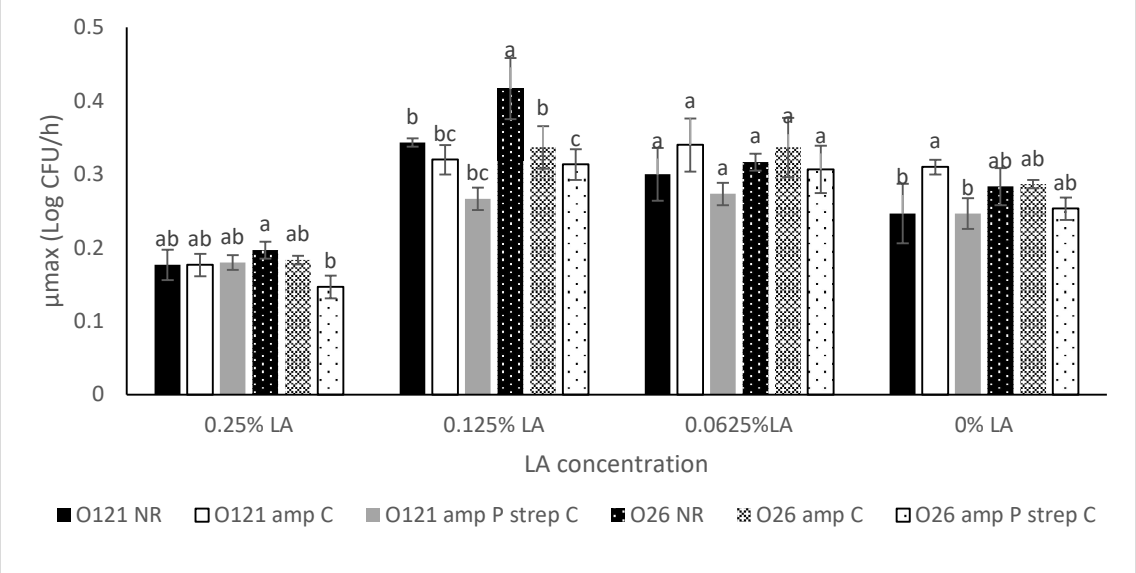
b.


Lag Phase Duration (h)
0.25% LA
0.125% LA
0.0625%LA
0% LA
LA concentration
O121 NR
O121 amp C
O121 amp P strep C
O26 NR
O26 amp C
O26 amp P strep C

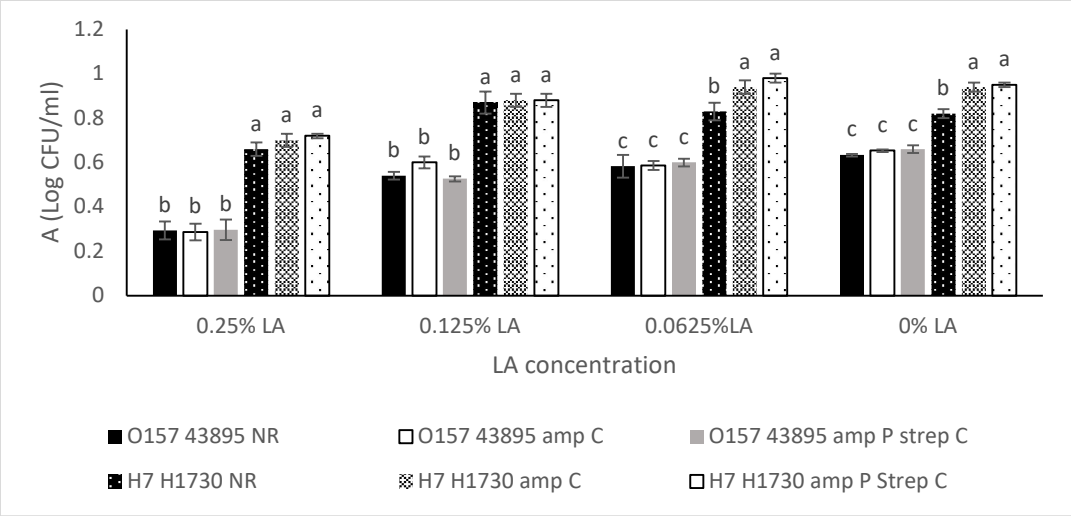
c.


µmax (Log CFU/h)
0.5
0.4
0.3
0.2
0.1
0
0.25% LA
0.125% LA
0.0625%LA
0% LA
LA concentration
O157 43895 NR
O157 43895 amp C
O157 43895 amp P strep C
H7 H1730 NR
H7 H1730 amp C
H7 H1730 amp P Strep C

d.


µmax (Log CFU/h)
0.5
0.4
0.3
0.2
0.1
0
0.25% LA
0.125% LA
0.0625%LA
0% LA
LA concentration
O121 NR
O121 amp C
O121 amp P strep C
O26 NR
O26 amp C
O26 amp P strep C

e.



f.

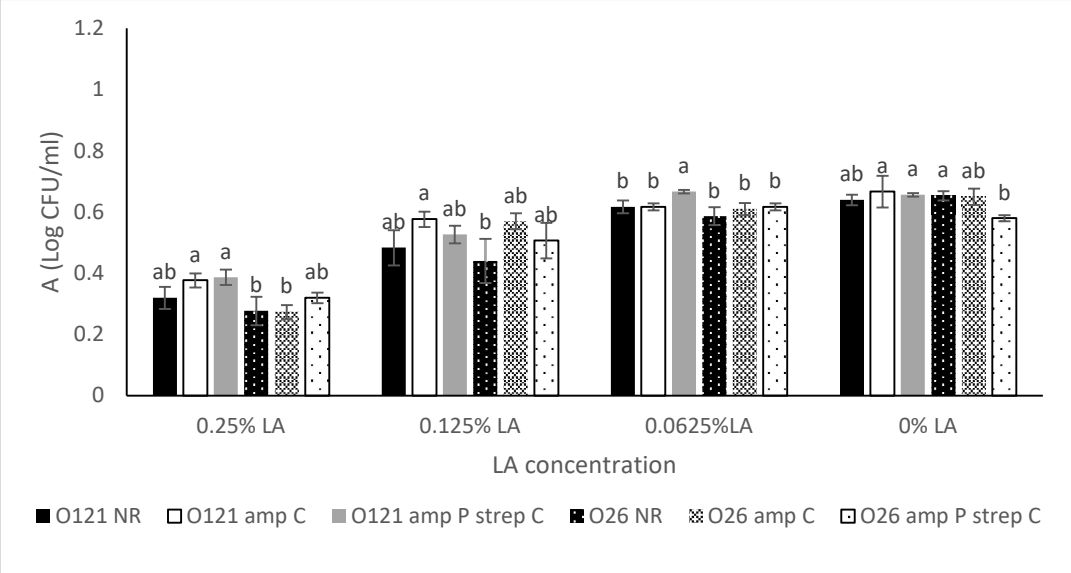


Fig 5.1: Comparison of growth parameters: Lag phase duration (L), maximum growth rate (µmax) and change in bacterial population for bacterial strains grown in TSB supplemented with 0.0625 %, 0.125 % and 0.25 % lactic acid (A). **a**. L for O157: H7 STEC **b**. L for non O157 STEC **c**. µmax for O157: H7 STEC **d.** µmax for O157: H7 non STEC **e.** A for O157: H7 STEC **f.** A for non O157: H7

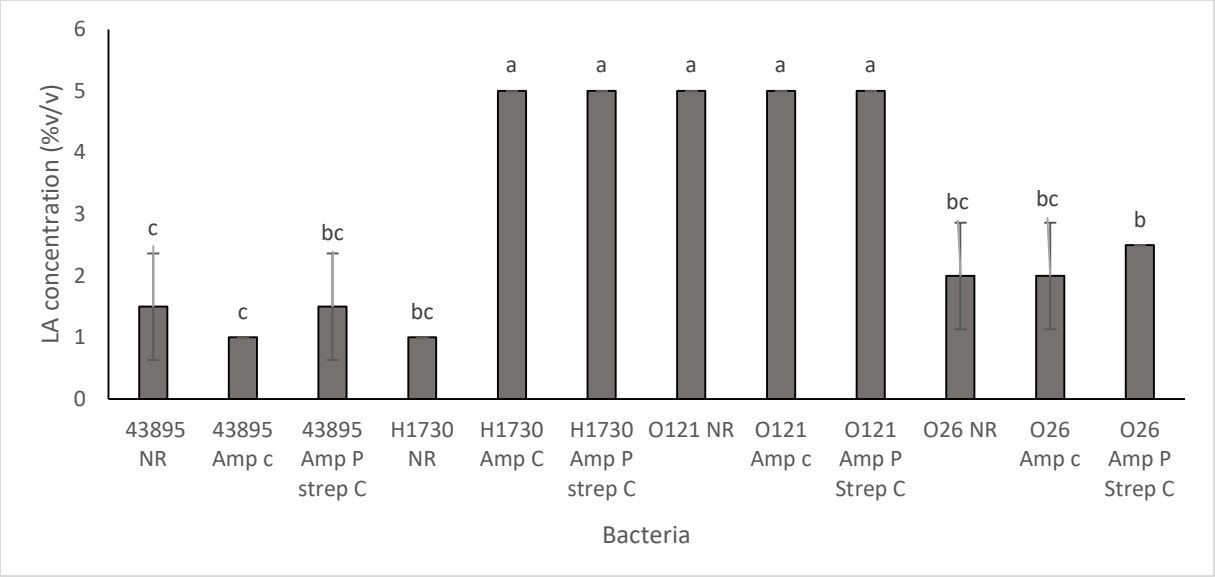
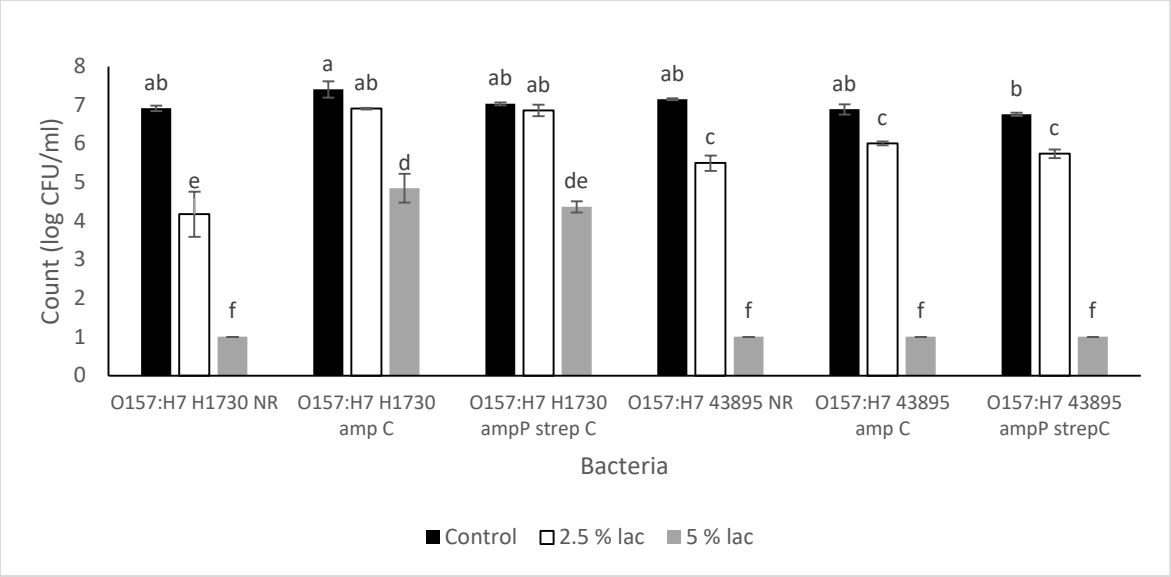
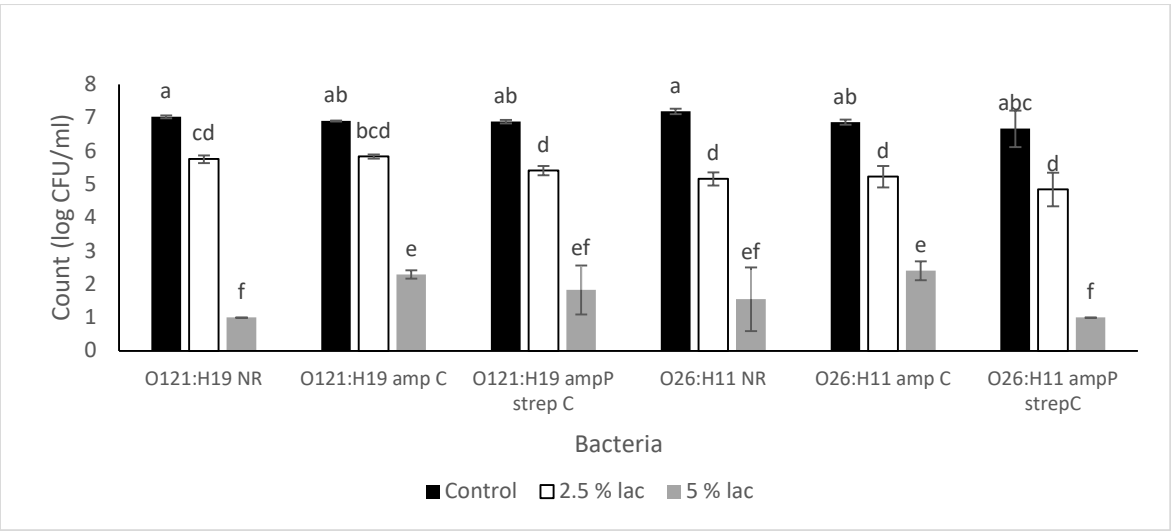


Fig 5.2: Bactericidal concentrations of LA for the different strain variants

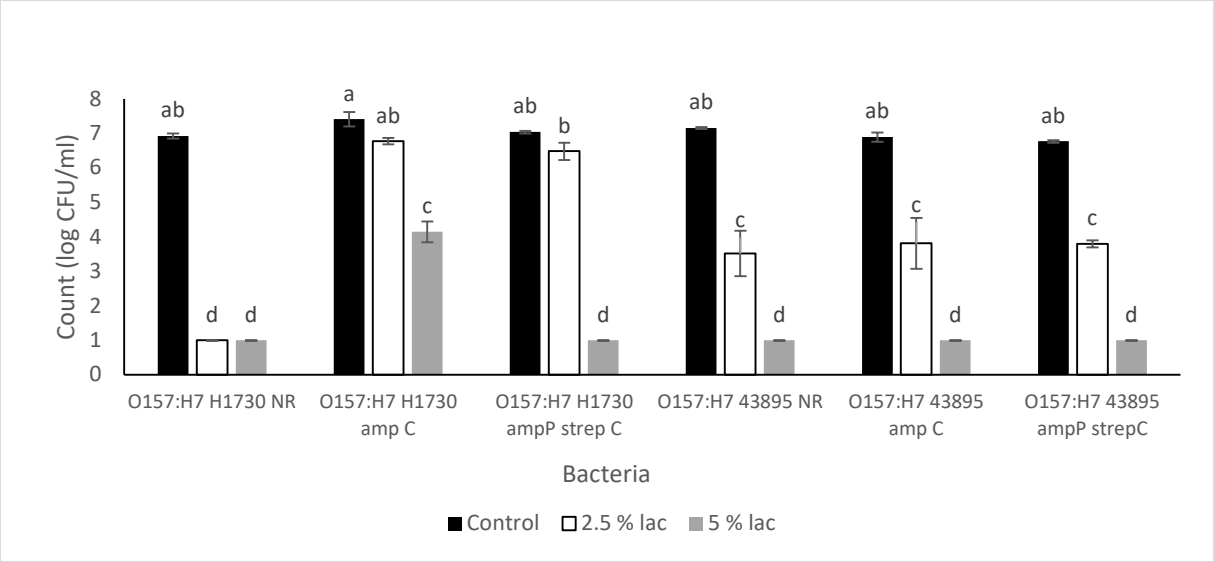
a.


Count (log CFU/ml)
0
1
2
3
4
5
6
7
8
ab
e
f
a
ab
d
ab
ab
de
ab
c
f
ab
c
f
b
c
f
O157:H7 H1730 NR
O157:H7 H1730 amp C
O157:H7 H1730 ampP strep C
O157:H7 43895 NR
O157:H7 43895 amp C
O157:H7 43895 ampP strepC
Bacteria
Control
2.5 % lac
5 % lac

b.


Count (log CFU/ml)
0
1
2
3
4
5
6
7
8
a
cd
f
ab
bcd
e
ab
d
ef
a
d
ef
ab
d
e
abc
d
f
O121:H19 NR
O121:H19 amp C
O121:H19 ampP strep C
O26:H11 NR
O26:H11 amp C
O26:H11 ampP strepC
Bacteria
Control
2.5 % lac
5 % lac

c.



d.

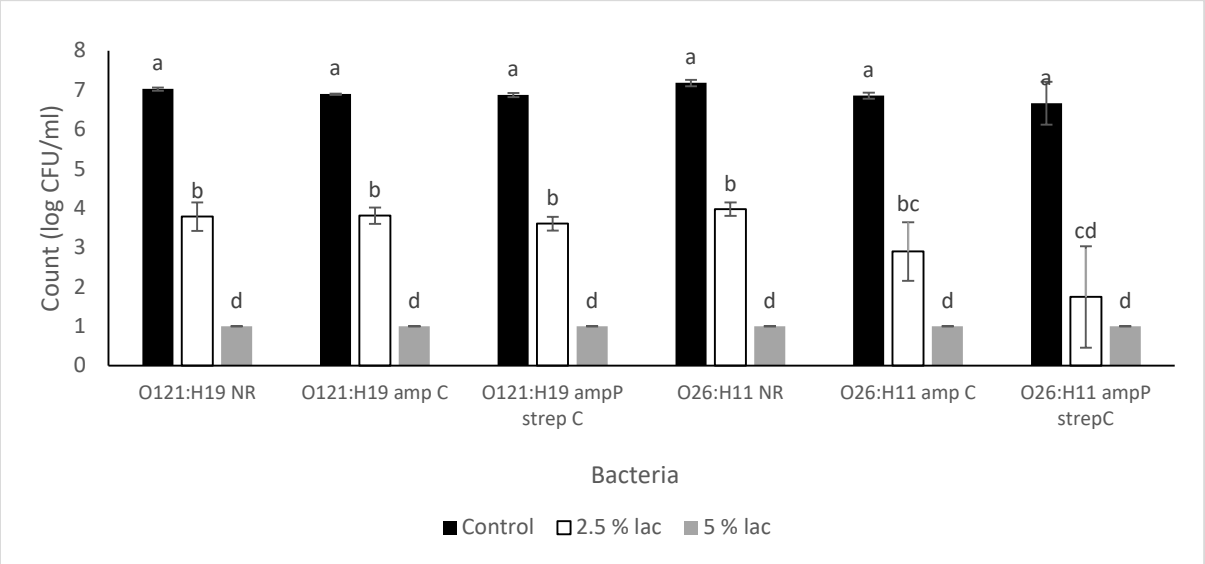


Fig 5.3: Survival of STEC exposed to 2.5 % and 5 % LA **a.** O157 STEC exposed to LA for 30 s **b.** O121 and O26 STEC exposed to LA for 30 s **c.** O157 STEC exposed to LA for 300 s **d**. O121 and O26 STEC exposed to LA for 300 s.

CHAPTER 6

INFLUENCE OF ANTIBIOTIC-RESISTANCE AND EXUDATE ON PEROXYACETIC ACID TOLERANCE IN O157 AND NON-O157 SHIGA TOXIN PRODUCING *E. COLI*[4]

[4]Oguadinma, I.C., Mishra, A., Suh, J. H., Singh, M., and Dev Kumar G. 2022. Submitted to *International Journal of Food Microbiology.*

Abstract

Shiga toxin producing *Escherichia coli* (STEC) continues to cause foodborne outbreaks associated with beef and beef products despite consistent use of antimicrobial interventions. In this study, the influence of antibiotic resistance (ABR) in *E. coli* O157:H7 H1730, O157:H7 43895, O121:H19 and O26:H11 on tolerance to peroxyacetic acid (PAA) was evaluated. Further, bactericidal concentrations of PAA in the presence of nutrient rich media (Tryptic Soy Broth, TSB and beef exudates) and nutrient deficient media (Sterile Deionized Water, SDW and Phosphate Buffered Saline, PBS) were evaluated for all bacterial strains. Antibiotic resistance to ampicillin (amp C), or ampicillin and streptomycin (amp P strep C) was generated in each bacterial strain through incremental exposure to the antibiotics or by plasmid transformation (n=12 total strains). The mean bactericidal concentrations of PAA were higher ($P \leq 0.05$) in nutrient rich media (205.55±31.11 in beef exudate and 195.83±25.00 ppm in TSB) than in nutrient deficient media (57.91±11.97 ppm in SDW and 56.66±9.56 ppm in PBS). Strain O157: H7 amp P strep C was the most tolerant to PAA ($P \leq 0.05$). At 200 ppm in nutrient rich media and 60 ppm in nutrient deficient media, all bacterial strains declined in population to below the limit of detection. Analysis of the beef exudates indicated the presence of diverse amino acids that have been associated with acid tolerance. The results from this study indicate that beef exudates could contribute to acid tolerance and suggest that some STEC bacterial strains with certain ABR profiles might be more tolerant to PAA.

6.1 Introduction

Shiga toxin producing *E. coli* (STEC) resulted in 508 outbreaks, 8,540 illnesses, 1,167 hospitalizations, and 21 deaths between 2015 to 2020 according to reports by the Centers for Disease Control and Prevention National Outbreak Reporting System (6). Beef was linked to 318 of these outbreaks, resulting in 4,244 illnesses and 9 deaths (6). Currently, *E. coli* O157:H7 and six non-O157 STEC (O26, O103, O111, O145, O45, O121) are recognized as adulterants in beef (31).

Microorganisms originating from dust, dirt and fecal matter associated with hide can be introduced to meat during slaughter/dressing procedures and fabrication or further processing (7). Sanitizers are commonly employed to reduce the microbial burden of carcasses (26) and Peroxyacetic acid (PAA) is a common antimicrobial intervention used for decontaminating beef and is used at an approved concentration of 200 ppm during the washing, rinsing, and cooling of beef carcasses (13, 31).

The development of tolerance to sanitizers by foodborne pathogens could result in the abatement of treatment efficacy (31). Environmental conditions such as organic matter or genetic adaptations due to mutations in foodborne pathogens can contribute towards diminished effectiveness of sanitizers against target pathogens (2). Amino acids present on beef could also influence the efficacy of acid treatment by contributing towards acid tolerance (21, 11, 14). *E. coli* can utilize glutamate, arginine, and lysine to protect cells from acid stress by consuming intracellular protons during inducible decarboxylation reactions (17, 27). Further, the influence of these systems on acid tolerance in *E. coli* could be exacerbated by the role of efflux pumps in acid tolerance systems and the upregulation of these pumps in antibiotic-resistant bacteria (10, 16, 32). Mutations in the efflux pump AcrAB-TolC increased tolerance to a diverse set of

antibiotics including β-lactams, chloramphenicol, rifampicin, tetracyclines and quinolones in *E. coli* (16). Additionally, TolC is also required for the expression of glutamate decarboxylase, a key component of the glutamate-dependent acid-resistance system (10). Previous work has indicated that the acquisition of antibiotic resistance in *E. coli* O157:H7 H1730 resulted increases tolerance to lactic acid, a commonly used sanitizer in beef processing (25).

Meat exudates, defined as the aqueous solution, runoff or natural juice from fresh meat, are rich in amino acids from proteins and their degradation byproducts (5). Meat exudates are suggested to be representative of the metabolic profile of the whole meat sample (5). Analysis of exudates from beef show a rich mix of fatty acids, nucleotides and amino acids such as glutamate, arginine, lysine, glutamine, serine (5).

Therefore, the objective of the current study was to evaluate and compare the influence of antibiotic resistance in O157 and non O157 STEC on tolerance to PAA. Further, the impact of nutrient rich environments simulated by Tryptic Soy Broth (TSB) and beef exudates and nutrient deficient environment simulated by Sterile Deionized Water (SDW) and Phosphate Buffered Saline (PBS) on acid tolerance was evaluated. Presence of amino acids that have been known to contribute towards acid tolerance were also determined in beef exudates. Understanding factors that can contribute towards increased tolerance to PAA in STEC is critical as it is a commonly used sanitizer for food products and contact surfaces that might harbor pathogens.

6.2 Materials and Methods

6.2.1 Bacterial strain

Four serogroups of Shiga toxin-producing *E. coli* were used in this study: *E. coli* O157:H7 H1730 was a human isolate from a lettuce outbreak, *E. coli* O157:H7 (ATCC 43895) was from a

1982 ground beef outbreak, and *E. coli* O121:H19 (strain TW08980) and *E. coli* O26:H11 (strain 3012-03) were from a 2016 Missouri flour outbreak. All strains were obtained from the Center for Food Safety, University of Georgia Griffin campus culture collection. Isolates were revived from frozen storage by transferring to TSB (Neogen, Lansing, MI) and incubating at 37 ℃ for 24 h. The bacterial strains were evaluated for antibiotic resistance by growing cells on Tryptic Soy Agar (TSA; Neogen Lansing, MI) containing 100 µg/ml of streptomycin, and ampicillin before the development of the antibiotic-resistant strain variants (Table 6.1).

6.2.2 Development of ampicillin and streptomycin adapted strains

6.2.2.1 *Chromosomal resistance*

Chromosomal resistance to streptomycin and ampicillin was developed by sequentially transferring the bacterial strains into increasing concentrations of the antibiotics. Briefly, 100 µl of culture from a lower antibiotic concentration was transferred to fresh media containing 900 µl of a higher antibiotic concentration. This process was repeated with a 10 µg/ml increase in the antibiotic concentration for each transfer until adaptation to 100 µg/ml of antibiotic was achieved. Bacterial strains with antibiotic resistance were isolated by streaking on TSA supplemented with either 100 µg/ml of streptomycin or ampicillin.

6.2.2.1 *Transformation by electroporation*

Transformation of cells to take up a green fluorescence ampicillin resistance plasmid (GFP amp plasmid) was performed following the method described by Kumar et al. (20) with minor modifications. Briefly, competent cells were prepared as follows: 45ml of Tryptic Soy Broth (TSB) was inoculated with 1ml of overnight cultures of the bacterial strains. Cultures were incubated at 37 ℃ for 4 h to achieve an optical density (OD_{600nm}) of 0.8 and then placed in ice

for 15 min. The cultures were then centrifuged at 1,400 g for 10 min to pellet the cells, and the supernatant discarded. The pelleted cells were washed three times with 15 % ice-cold glycerol and stored at −20 ℃ until use. The cells were transformed using the host range plasmid pGFPuv (SnapGene, 2021). Electroporation conditions applied were 2.5 kV, 25 μF, and 400 Ω using the Gene Pulser II system (Bio-Rad, Hercules CA).

6.2.3 Gas chromatography-mass spectrometry-based metabolomics approach for characterization of amino acids in beef exudates

6.2.3.1 *Collection of beef exudates*

Beef exudates were the pooled runoff from 9 different freshly slaughtered beef carcasses after water washing and before acid wash/chilling obtained from the University of Georgia Meat Science and Technology Center. Samples were collected in 50 ml sterile tubes (Corning Inc., Corning, NY), stored at 4 °C immediately after collection/during transport and stored at -20 °C before use. For antimicrobial study, exudates were filter sterilized using a 500 ml Filter Unit with a 0.45 μm aPES membrane (Thermo Fisher Scientific, Pittsburgh, PA) prior to use.

6.2.3.2 *Analysis of amino acid composition in beef exudates*

The beef exudate samples were frozen at -80 °C for 3 h and then freeze dried overnight**.** Once dry each beef exudate sample was transferred to a Gas Chromatography (GC) vial using MeOH**.** The beef exudate samples were evaporated in the oven at approximately 70 °C**.** After evaporation, 40 μl of pyridine was added, and the preparation was mixed thoroughly while warming to help dissolve the solids into solution**.** After complete dissolution, 100 μl of BSTFA (N, O-Bis(trimethylsilyl)trifluoroacetamide) + TMS (trimethylsilyl) (99:1) was added to each sample**.** The vials were crimp capped and allowed to react overnight in the oven at approximately

70 °C to enable the derivatization reaction to occur**.** The process was repeated by adding another 100 µl of BSTFA + TMS (99:1) to each sample**.** Vials were crimp capped and left in the oven again at approximately 70 °C for about 7 h until complete derivatization occurred as evidenced by a formation of yellow coloration. Each sample was transferred into a GC vial insert and kept for analysis on the GC-MS. GC-MS analysis was performed according to previous methods (18, 23) with a slight modification. The composition (identification) of amino acids was confirmed using authentic standards, including threonine, lysine, ornithine, carnitine, glutamate, glutamine, serine, carnosine, asparagine, histidine, glutathione, anserine, methionine, tryptophan, arginine, and tyrosine (total 16 target amino acids). Each standard was prepared for GC-MS analysis, as described above (BSTFA derivatization).

6.2.4 Preparation of bacterial inoculum

Stock cultures of the bacterial strains were prepared by streaking each strain to TSA or TSA + 100 µg/ml of antibiotics (TSA + Amp, TSA + Amp + Strep) and incubating at 37 °C for 24 h. Colonies from overnight cultures were scraped from the plates with a sterile loop and suspended in PBS (VWR International, Radnor, PA). The bacterial population was adjusted to 6.45±0.11 log CFU/ml.

6.2.5 Bactericidal concentrations of PAA in nutrient rich and nutrient deficient media

In this study, the bactericidal concentration of PAA was the concentration that prevented survival and regrowth of bacterial strains. Beef exudate and TSB were used to evaluate bactericidal concentrations of PAA in nutrient rich media and SDW and PBS were used for evaluation in nutrient deficient media. PAA (15 % or 15000 ppm) was obtained from Lab Alley, LLC (Lab Alley; Spicewood TX, USA). Solutions of PAA in TSB or beef exudate were prepared to

achieve concentrations ranging from 100 to 400 ppm with 50 ppm increments. In SDW and PBS, PAA concentrations ranged from 15 to 105 ppm with 15 ppm increments. Each 900 µl of TSB or beef exudate + PAA solution was inoculated with 100 µl of the bacteria for 300 s. Solution was centrifuged immediately after exposure for 1 min at 13,000 g using a Corning high-speed microcentrifuge (Corning Inc., Corning, NY). The supernatant was discarded, and pellets were resuspended in 1 ml SDW. From the suspended solution, 100 µl was transferred to 100 µl of 2x TSB in 96-well plates (Costar® 96 Well Flat Bottom, Corning Life Sciences Inc. ME, USA) and incubated for 24 h at 37 °C. The plates were observed for turbidity to determine survival and regrowth after incubation by determining the optical density (OD_{600nm}).

6.2.6 Reduction in bacterial population after exposure to PAA

Overnight cultures of the bacterial strains grown either on TSA or TSA + 100 µg/ml of ampicillin, and TSA + 100 µg/ml of streptomycin and ampicillin were used to prepare 8 log CFU/ml of bacterial inoculum in 1× PBS. From the suspended culture, 100 µl was transferred to 900 µl of nutrient rich media containing 200 ppm of PAA or nutrient deficient media containing 60 ppm of PAA for 300 s. The solution was centrifuged immediately after exposure for 1 min at 13,000 g using the Corning high speed microcentrifuge. The supernatant was discarded, and the pellets were resuspended in 1 ml 1× PBS. Enumeration of bacteria was done using the droplet plate method where colony forming units (CFU) of cells within the perimeter of drop were used for enumerating bacterial populations from serially diluted samples. The number of colonies formed were counted after incubation for 24 h at 37 °C. The limit of detection for the assay was 50 CFU/ml or 1.69 log CFU/ml.

6.2.7 Statistical analysis

All experiments were conducted in triplicates. Significant differences between the bactericidal concentrations of PAA in the media were compared using the one-way analysis of variance (ANOVA) and means were compared using the Tukey's Honest Significant Difference (HSD) test at a 0.05 significance level with the JMP statistical software (SAS Institute Inc, USA). Principal component analysis (PCA) was conducted on SIMCA-P+ ver. 15.0 (Umetrics, Umeå, Sweden) to distinguish different groups (SDW, PBS, beef exudate), based on the organic load (amino acid profiles). A heatmap based on canonical-correlation analysis (CCA) between amino acids and the inhibitory concentration of PAA with Pearson's correlation coefficient ($-1 < \text{value} < +1$) was generated using R software (Ver. 4.2.1) with *CCA* and *mixOmics* packages.

6.3 Results

6.3.1 Analysis of amino acid composition in beef exudates

Of the 16 target amino acids, 11 amino acids (threonine, lysine, ornithine, carnitine, glutamate, serine, asparagine, glutathione, methionine, tryptophan, and tyrosine) were found in the beef exudate samples. Threonine, lysine, carnitine, glutamate, serine, and asparagine were most commonly detected in beef exudates collected for this study (> 85 % of the exudate samples). TSB consists of the digest of soybean and casein, with most amino acids (including lysine and glutamate) described above. Based on the identified amino acid composition, PCA clearly distinguished the beef exudate group (amino acid rich) from SDW and PBS groups (amino acid deficient media), explained by 53.5 % of the total variation on the PC1 axis (Fig. 6.1).

6.3.2 Effect of nutrient rich and nutrient deficient media on the bactericidal concentration of PAA

The bactericidal concentration of PAA for all bacterial strains evaluated was significantly higher in nutrient rich media compared to the nutrient deficient media ($P \leq 0.05$) (Fig 6.2). The mean bactericidal concentrations in beef exudate and TSB were 205.55±31.11 ppm and 195.83±25 ppm respectively while the mean bactericidal concentrations in SDW and PBS were 57.91±11.97 ppm and 56.66±9.56 ppm respectively. The most tolerant bacterial strain after PAA exposure in beef exudates and TSB was O157:H7 H1730 amp P strep C with mean bactericidal concentrations of 283.00±57.73 ppm in beef exudate and 250.00±0.00 ppm in TSB ($P \leq 0.05$). The bactericidal concentrations observed for the other strains ranged from 183.33 ppm to 200 ppm and were not significantly different ($P > 0.05$).

In SDW and PBS, the bactericidal concentrations of 80.00±8.66 ppm and 75.00±15.00 ppm respectively was also observed in O157:H7 H1730 amp P strep C ($P \leq 0.05$) which was the most tolerant strain. The least tolerant strain was O157:H7 H1730 NR exposed in SDW with bactericidal concentration of 35.00±8.66 ppm ($P \leq 0.05$).

6.3.3 Variations in response to PAA between the antibiotic-resistant and non-resistant strains

Each bacterial serotype and their antibiotic-resistant variants were compared for variations in response to PAA in both nutrient rich and nutrient deficient media. For O157:H7 43895 in nutrient rich media, the mean bactericidal concentrations did not differ significantly between the non-antibiotic and antibiotic-resistant variants ($P > 0.05$) however the highest inhibitory concentration of 200.00±0.00 ppm was observed in O157:H7 43895 amp C exposed in beef

exudate. In nutrient deficient media, the non-antibiotic resistant strain O157:H7 43895-NR exposed to PAA in PBS was the most tolerant with bactericidal concentration of 60.00±0.00 ppm although this did not vary significantly from the other strain variants ($P > 0.05$).

For O121 in nutrient rich media, there were no significant differences in bactericidal concentrations of PAA ($P > 0.05$) for all strain variants evaluated but bacterial strains exposed in TSB all showed lower bactericidal concentration values of 183.33 ppm compared to 200.00 ppm for strains exposed in beef exudates. The bacterial strains exposed in nutrient deficient media were inhibited at 60.00±0.00 ppm.

A different pattern was observed in O157:H7 H1730. In the presence of nutrient, O157:H7 H1730 amp P strep C was the most tolerant with bactericidal concentrations of 283.33±57.73 ppm and 250.00±0.00 ppm respectively in beef exudate and TSB ($P \leq 0.05$) while the least tolerant strain was O157: H7 H1730 NR exposed to PAA in TSB with bactericidal concentration of 183.33±28.86 ppm ($P \leq 0.05$). The bactericidal concentrations in nutrient deficient media ranged from 35.00±8.66 ppm in O157: H7 H1730 NR to 80.00±8.66 ppm in O157: H7 H1730 amp P strep C ($P > 0.05$).

For O26 exposed to PAA in nutrient rich media, there were no differences in the bactericidal concentrations between the strain variants ($P > 0.05$). All the bacterial strains were inhibited by 200 ppm of PAA. In nutrient deficient media, O26 amp C exposed in SDW was the most tolerant, with bactericidal concentration of 65.00±8.66 ppm compared to O26 amp P strep C exposed in PBS which was the least tolerant with bactericidal concentration of 45.00±0.00 ppm ($P \leq 0.05$).

6.3.4 Antimicrobial efficacy of PAA

The reduction in bacterial population after exposure to 200 ppm PAA in TSB and beef exudate and 60 ppm PAA in SDW and PBS was evaluated (Fig 6.3). All bacterial strains declined in population to below the limit of detection after exposure to PAA at the evaluated concentrations in nutrient rich and nutrient deficient media ($P > 0.05$). The average reduction in bacterial population was 6.42±0.52 log CFU/ml in both nutrient rich and nutrient deficient media at the PAA concentrations evaluated.

6.4. Discussion

E. coli can develop tolerance to acid-based sanitizers through acid resistance systems. Amino acids such as glutamate, arginine and lysine are involved in acid resistance systems in *E. coli* (27, 28). Glutamate can provide tolerance to both acid and oxidative stress (1, 28). In the current study, amino acid profiles of beef exudates were determined and the tolerance of STEC to PAA in amino acid rich environments (beef exudate and TSB) were evaluated. Similar to the beef exudate, TSB is rich in amino acids such as lysine and glutamine due to the addition of casein and soy peptone (9).

As shown in Fig. 6.1B, a heatmap from CCA between 16 target amino acids and the inhibitory concentration of PAA proved that the amino acids (threonine, lysine, ornithine, carnitine, glutamate, serine, asparagine, glutathione, methionine, tryptophan, and tyrosine) observed in beef exudates have a strong positive correlation with the inhibitory concentration of PAA.

Amino acids such as glutamate and lysine can contribute towards tolerance of *E. coli* to acid treatment via acid resistance systems (11, 14, 21).The glutamate dependent acid resistance system is perhaps one of the most characterized in *E. coli* O157:H7 (1, 11, 14, 22, 27).

Glutamate is present in TSB and was detected in all beef exudate samples analyzed in the present study. The glutamate dependent acid resistance system is induced at stationary phase of bacterial growth, and like most amino acid dependent acid resistance systems, intracellular protons during acid stress in cells are consumed during decarboxylation of the amino acid (28). This system has also been reported to protect *E. coli* O157:H7 against oxidative stress. In one report, the presence of glutamate protected *E. coli* O157:H7 from hydrogen peroxide-induced oxidative stress during a pH 2.5 acid challenge (1). PAA is composed of hydrogen peroxide and acetic acid. In the present study, higher mean PAA bactericidal concentrations of 205±31.11 ppm in beef exudate and 195.83± 25.00 ppm in TSB compared to 57.91±11.97 ppm in SDW and 56.66±9.56 ppm in PBS were observed, indicating a possible role of amino acids in tolerance. This result aligns with observations made by Kalchayanand et al. (19) on the effect of beef purge on antimicrobial efficacy. Although factors such as interaction between PAA and the proteins (protein denaturation and PAA decay in the presence of organic compounds) (12) may be considered, the presence of amino acids such as glutamate could be a strong contributor towards the higher inhibitory concentrations observed, through acid resistance systems in bacteria.

In this study, resistance to ampicillin and streptomycin or both were induced in O157 and non-O157 STEC by exposure to sublethal concentrations of each antibiotic over time. Several antibiotic resistant strains were evaluated for cross-tolerance to PAA. Antibiotic resistance among foodborne pathogens is a serious public health concern (2). In cattle production, antibiotics are used to treat diseases and promote growth in animals but long term exposure of bacteria to sub-lethal concentrations of antibiotics could exert selective pressure, favoring isolates with resistance genes or inducing cross-resistance to unrelated antibiotics (4, 26). The consistent isolation of STEC with multidrug resistance to different groups of antibiotics

including ampicillin, streptomycin, sulfonamide, and tetracycline despite the use of antimicrobial interventions alludes to the potential of ABR associated cross-tolerance to antimicrobials. Tolerance is the ability of bacteria to sustain increased duration of exposure to an antimicrobial (3). Some reports suggest a fitness cost associated with the acquisition of some antibiotic resistance profiles, although chromosomal resistance, at least in vitro, may be cost neutral (29). In this study, the presence of an antibiotic-resistance resulted in the increased tolerance to PAA in only *E. coli* O157:H7 H1730 amp P strep C.

Difference in stress tolerance to PAA was observed among the strains evaluated in this study. Bacteria exposed to stressors could transition into a state of low metabolic activity where they cannot be cultured on routine media but remain viable. In one study, it was observed that bacteria exposed to weak acids transitioned into the VBNC state but were resuscitated when exposed to nutrient rich media (8). In another study, bacteria in VBNC state due to sunlight exposure were resuscitated by growth in TSB (24). The transition of stressed cells into a viable but non culturable (VBNC) state after exposure to PAA requires further exploration.

6.5 Conclusion

The presence of amino acids in beef carcass exudate could protect bacteria from both acid and oxidative stress. In this study, bacterial strains were more tolerant to PAA in amino acid rich media compared to amino acid deficient media. One ABR strain demonstrated significantly higher tolerance to PAA. The results in this study highlights the importance of several mechanisms of adaptation to PAA in STEC such as amino acid metabolism and antibiotic resistance.

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Table 6.1: List of Shiga toxin producing *E. coli* strain variants used in this study.

Bacterial Strain	**Mode of resistance**	**Strain name for this study**
***Escherichia coli* O157:H7 H1730**	No resistance (parent strain)	O157:H7 H1730 NR
***E. coli* O157:H7 H1730 amp resistant**	Chromosome	O157:H7 H1730 amp C
***E. coli* O157:H7 H1730 amp and strep resistant**	Plasmid* and chromosome	O157:H7 H1730 amp P strep C
***E. coli* O157:H7 43895**	No resistance (Parent strain)	O157:H7 43895 NR
***E. coli* O157:H7 43895 amp resistant**	Chromosome	O157:H7 43895-amp C
***E. coli* O157:H7 43895 amp and strep resistant**	Plasmid and chromosome	O157:H7 43895-amp P strep C
***E. coli* O26:H11**	No resistance (parent strain)	O26:H11 NR
***E. coli* O26:H11 amp resistant**	Chromosome	O26:H11 amp C
***E. coli* O26:H11 amp and strep resistant**	Plasmid and chromosome	O26:H11 amp P strep C
***E. coli* O121:H19**	No resistance (parent strain)	O121:H19 NR
***E. coli* O121:H19 amp resistant**	Chromosome	O121:H19 amp C
***E. coli* O121:H19 amp and strep resistant**	Plasmid and chromosome	O121:H19 amp P strep C

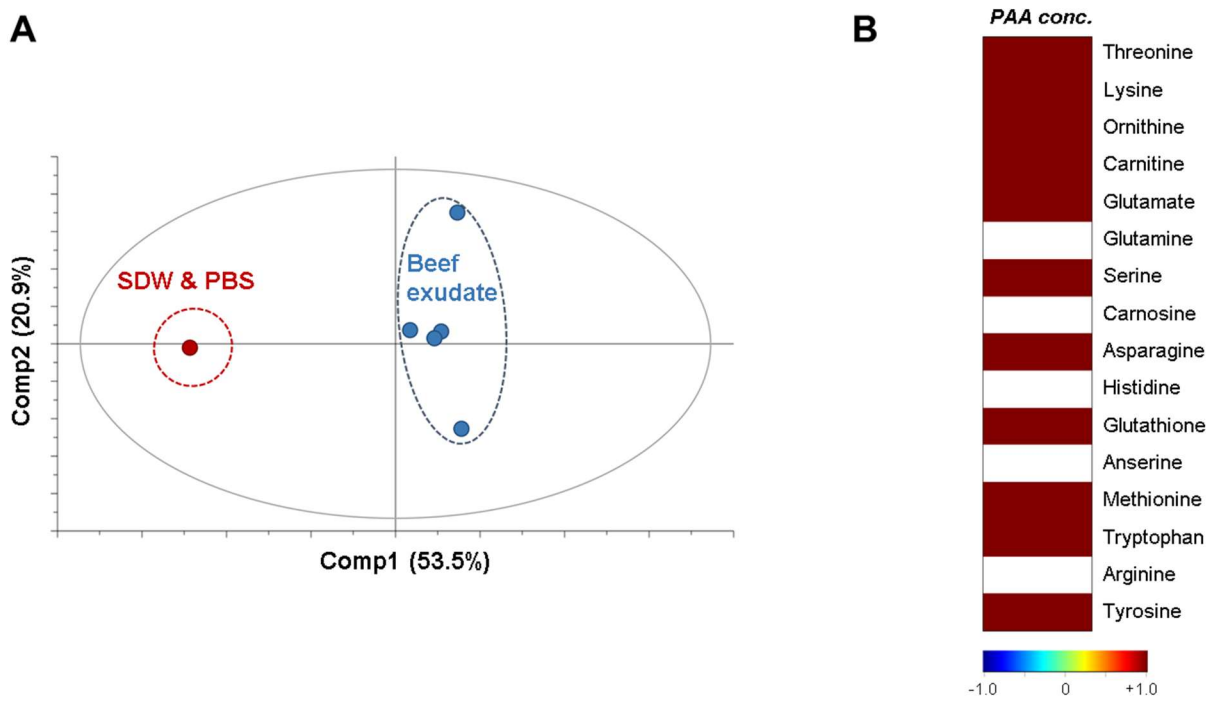
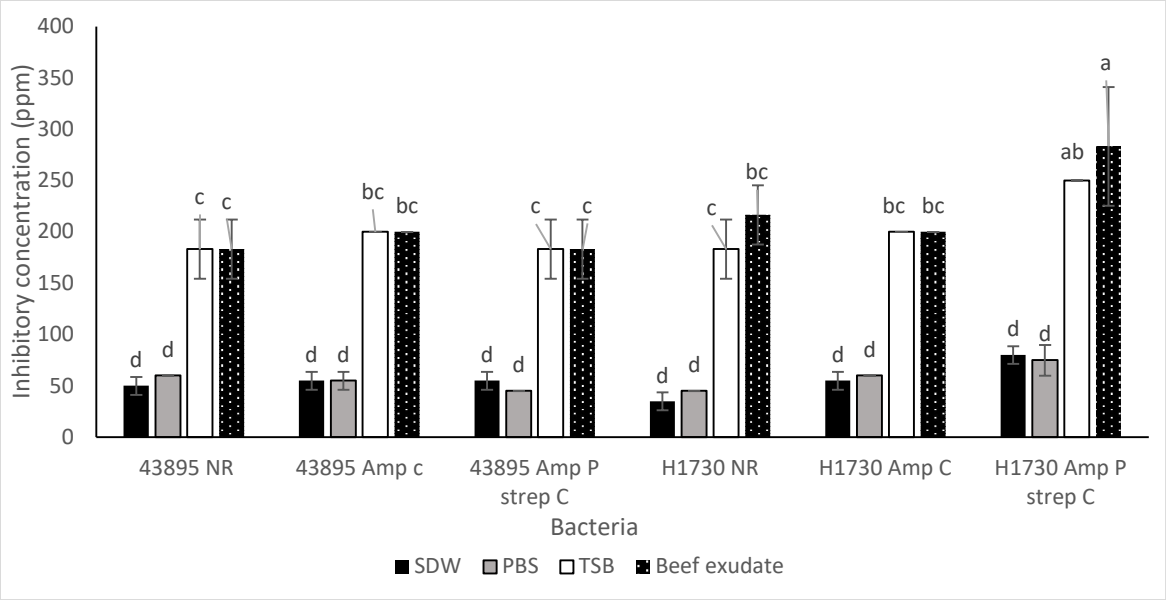


Fig 6.1 (A) PCA score scatter plot of beef exudate (blue) and SDW & PBS (red) groups, based on the organic load (amino acid composition), and (B) heatmap showing positive (red) and negative (blue) correlations between amino acids and the inhibitory concentration of PAA (white denotes null correlations due to the absence of amino acids in the samples).

a.



b.

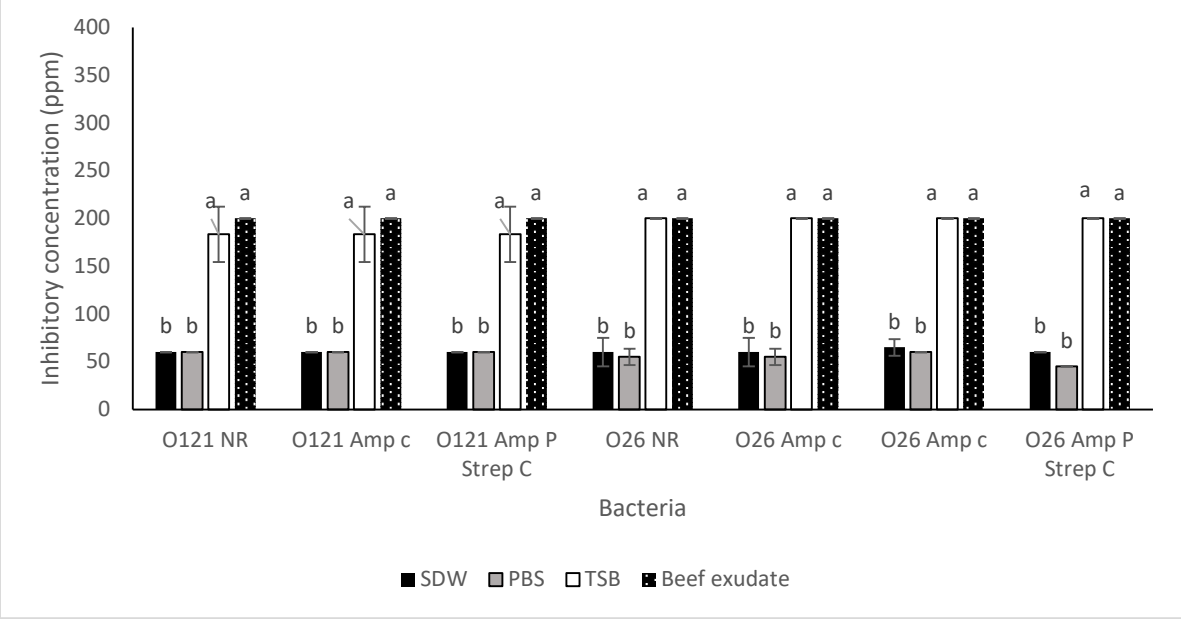
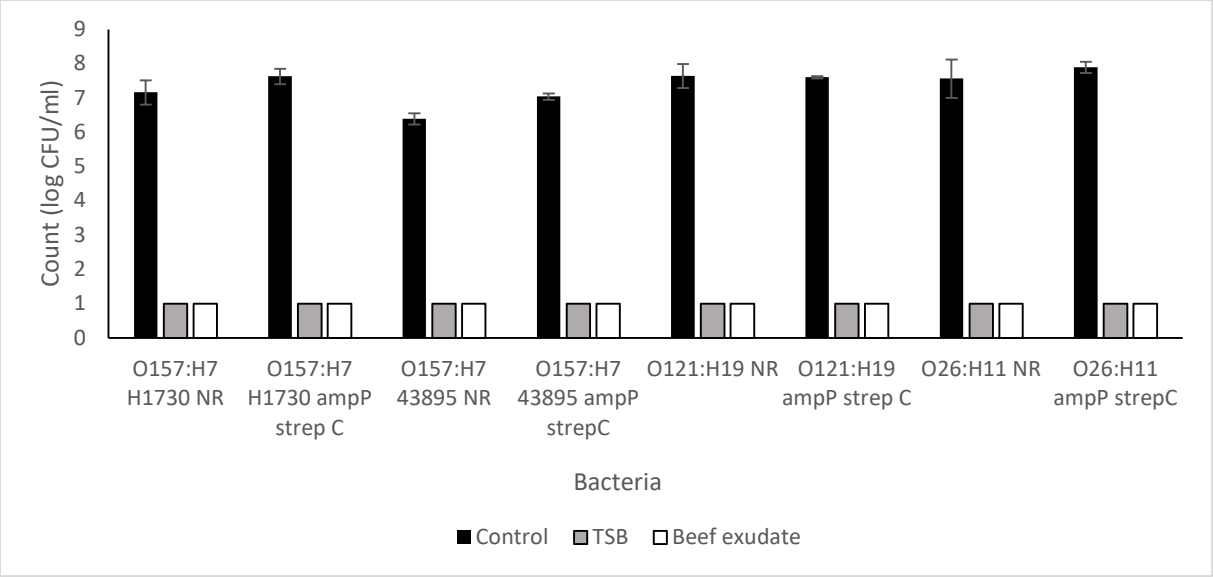


Fig 6.2: Bactericidal concentrations of PAA in amino acid rich and amino acid deficient media **a.** O157 STEC exposed to PAA **b.** Non O157 STEC exposed to PAA.

a.



b.

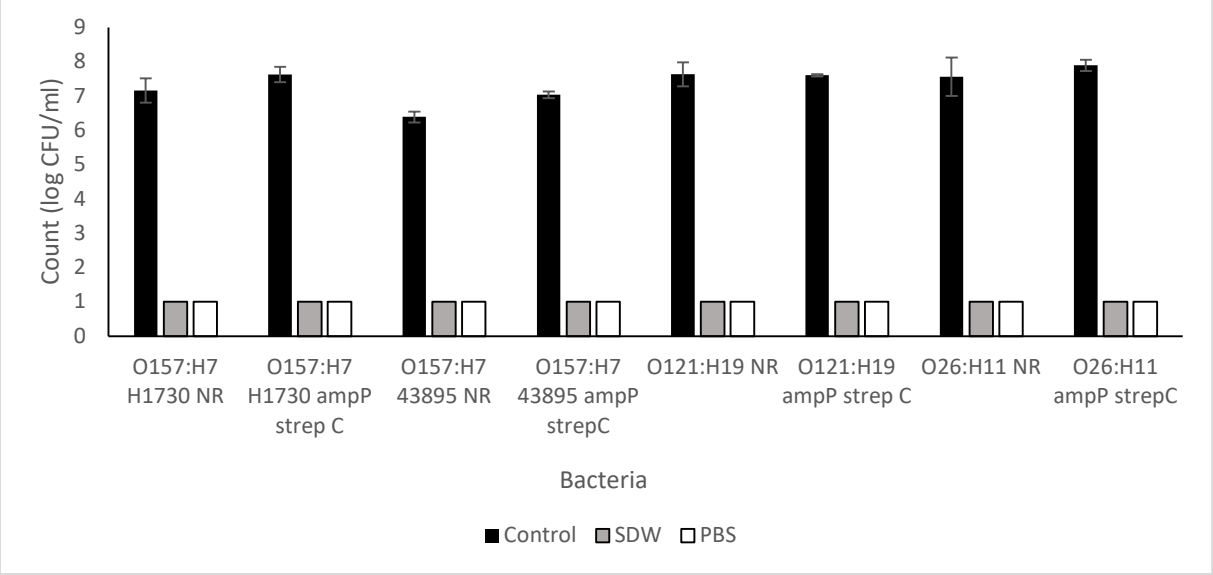


Fig 6.3: Decline in bacterial population exposed to PAA for 300 s **a.** Exposure to 200 ppm PAA in amino acid rich media **b.** Exposure to 60 ppm PAA in amino acid deficient media.

CHAPTER 7

A NOVEL COMBINATION OF PELARGONIC ACID AND LACTIC ACID AS AN ANTIMICROBIAL INTERVENTION

[1]Oguadinma, I.C., and Dev Kumar G. 2022. To be submitted to *Frontiers in Microbiology*

Abstract

A novel combination of pelargonic acid (PA) and lactic acid (LA) was evaluated for its efficacy as an alternative antimicrobial against Shiga toxin-producing *Escherichia coli* on beef. The bacterial strain used in this study was *E. coli* O157: H7 H1730 and its ampicillin and streptomycin resistant variants demonstrated from previous studies to be highly acid tolerant (O157: H7 H1730 NR, O157: H7 H1730 amp C and O157: H7 H1730 amp P strep C). Blends of PA and 1 % v/v LA were prepared at final concentrations of 0.125 mM PA + 1 % v/v LA, 0.25 mM PA + 1 % v/v LA, 0.5 mM PA + 1 % v/v LA and 1 mM + 1 % v/v LA and reduction in bacterial population when exposed to these blends for 300 s in Tryptic Soy Broth (TSB) or beef exudate were compared to 1 % v/v LA, 10 mM PA, 20 mM PA and 31.25 mM PA. Also, shifts in the pH of blended solutions were compared to the pH of 1 % LA, 2.5 % LA and 5 % LA. Exposure of bacterial strains to 1 % v/v LA, 0.125 mM PA + 1 % v/v LA or 10 mM PA did not significantly reduce the bacterial population ($P > 0.05$) however, exposure to 20 mM PA, 31.25 mM PA and the PA+LA blends except 0.125 mM PA + 1 % LA resulted in a decline of > 4 log CFU/ml in bacterial population ($P < 0.05$). The pH of 1 % v/v LA was 3.37±0.02 and the addition of PA at test concentrations did not significantly affect pH of the solution ($P > 0.05$). Filament formation was observed in sub-populations of bacteria exposed to 10 mM pel. The results from this study describe the synergistic effect of a novel PA and LA combination and provides some evidence for its efficacy as an antimicrobial treatment.

7.1 Introduction

Several antimicrobial interventions are used in the decontamination of beef carcass and beef products. Post-harvest chemical antimicrobial interventions on beef carcass include lactic acid, peroxyacetic acid, acidified sodium chlorite, lactoferrin, ozone, electrolyzed water, calcium hypochlorite, chlorine dioxide, and anhydrous ammonia (10, 24). Lactic acid (LA) is one of the most used antimicrobial interventions in the beef industry. It is generally regarded as safe (GRAS) 21CFR184.1061, does not have an impact on product quality at recommended concentrations and leaves no residues on treated surfaces (10, 19). LA is approved for use at concentrations of 2-5 % solution on beef sub-primal and trimmings, 2-2.8 % solution for beef heads and tongues, and 5 % for livestock carcass before fabrication (24) although practical applications could be as low as 1 % LA. Currently, physical interventions such as hot water treatment or steam pasteurization and chemical interventions such as antimicrobial solutions are the most effective interventions used in U.S. beef plants (6, 7, 10, 14, 17). Despite the use of antimicrobial interventions, food borne outbreaks on beef continue to persist, alluding to the need for improved antimicrobial technologies and treatments.

Multiple hurdles and combination treatments have been explored and determined to offer potential food safety benefits (8, 9). Some of the reported combination treatments include combinations of multiple wash steps, LA combined with various temperatures, LA combined with hot water sprays, LA combined with hot air and LA blended with citric acid (8, 9, 18). Another approach to multiple hurdle treatments could be the combination of antimicrobials with different but synergistic mechanisms of action, for example, a blend of organic acids like LA and antimicrobial fatty acids like pelargonic acid (PA). LA penetrates the cytoplasmic membrane of cells in its undissociated form resulting in reduced intracellular pH and disruption of

transmembrane proton motive force (1) while antimicrobial fatty acids like PA disrupt bacterial membrane lipids and alter membrane fluidity (5). Moreover, PA is regarded as safe (GRAS) and occurs naturally as esters in the essential oil of *pelargonium* spp.

The objective of this study was therefore to evaluate the efficacy of a novel blend of PA and LA at different concentrations against STEC. The goal of this study was to describe the synergistic antimicrobial activity of PA and LA and provide some evidence for its potential use as an alternative antimicrobial treatment.

7.2 Materials and methods

The most tolerant STEC strains to LA and PAA (O157: H7 H1730 amp P strep C and O157: H7 H1730 amp C) from the previous chapters of this dissertation and their antibiotic susceptible strain variant (O157: H7 H1730 NR) were used to evaluate the efficacy of LA fortified with PA. Details of the bacterial strains and development of antibiotic resistance to ampicillin and streptomycin are repeated here.

7.2.1 Bacterial strain

The strain of *E. coli* O157:H7 used in this study was the human isolate from a lettuce outbreak -*E. coli* O157:H7 H1730 - obtained from the Center for Food Safety, University of Georgia Griffin campus Culture collection. The isolate was revived from frozen storage by transferring to Tryptic Soy Broth (TSB, Neogen, Lansing, MI) and incubating at 37 °C for 24 h. The bacterial strain was evaluated for antibiotic resistance by growing cells on Tryptic Soy Agar (TSA; Neogen Lansing, MI) containing 100 µg/ml of streptomycin, and ampicillin before the development of the antibiotic-resistant strain variants.

7.2.2 Development of antibiotic-resistant variants

7.2.2.1 *Chromosomal resistance*

Chromosomal resistance to streptomycin and ampicillin was developed by inoculating 900 µl of TSB amended with 10 µg/ml of antibiotic with 100 µl of the non-resistant wildtype strain to obtain a final concentration of 5 log CFU/ml. Upon observation of turbidity, the culture was used to inoculate a fresh batch of TSB containing a 10 µg/ml increment in antibiotic concentration than the previous one. This was repeated until a resistance to 100 µg/ml of ampicillin and streptomycin was achieved. Antibiotic adapted strains retained resistance to the antibiotics without presence of selective pressure.

7.2.2.2 *Transformation by electroporation*

Transformation of cells to take up a green fluorescence ampicillin resistance plasmid (GFP amp Plasmid) was performed following the method described by Dev Kumar et al. (4) with minor modifications. Briefly, competent cells were prepared by inoculating 45 ml of Tryptic soy broth (TSB) with 1 ml of an overnight culture of *E. coli* O157:H7 H1730. The culture was incubated at 37 ℃ for 4 h to achieve an optical density of 0.8 following which the cells were placed in ice for 15 min. The culture was then centrifuged at 1400 g for 10 min to pellet the cells, and the supernatant discarded. The pelleted cells were washed three times with 15 % ice-cold glycerol and stored at −80 ℃ until use. The cells were transformed using the host range plasmid pGFPuv (SnapGene, 2021). Electroporation conditions applied were 2.5 kV, 25 µF, and 400 Ω using the Gene Pulser II system (Bio-Rad, Hercules CA). Colonies of transformed cells expressed fluorescence upon excitation with UV light (365 nm) and were resistant to 100 µg/ml ampicillin.

7.2.3 Preparation of LA and LA+PA

A 1 M stock solution of PA (Pelargonic acid, Acros Organics, New Jersey, USA) was prepared by adding 1.58 g of PA to 10 ml of Sterile Deionized Water (SDW) with 0.1 % w/v saponin from quillaja bark (Sigma-Aldrich, St. Louis, MO). The mixture was homogenized using a vortexer (Vortex-2 Genie, Scientific Industries, Bohemi, NY, USA) at maximum speed of 10 (3200 rpm) for 1 min to form an emulsion. LA was prepared from an 88 % commercial FCC grade stock solution ((LA, L-lactic acid, Xena International Inc., Illinois). Dilution of LA and PA from the stock solution to achieve the required concentrations, and preparation of PA + LA (1 %) test solutions were calculated by $C_1V_1=C_2V_2$: To prepare 0.125 mM PA + 1 % LA at a final volume of 10 ml, 1.25 µl of PA from the 1 M PA stock solution was transferred to 9.9975 ml 1 % LA using a micropipette. The mixture was agitated using the vortexer at maximum speed of 10 (3200 rpm) for 1 min. The same calculation was used to prepare other PA+LA solutions (0.25 mM PA + 1 % LA, 0.5 mM PA + 1 % LA, and 1mM PA + 1 % LA), PA solutions (10 mM PA, 20 mM PA and 31.25 mM PA) and LA solutions (1 % v/v, 2.5 % v/v and 5 % v/v).

7.2.4 Measurement of pH

The pH of each freshly prepared LA and PA+LA solution was measured to determine possible changes in pH due to the addition of PA. The pH measurement was done using a pH meter (Oakton pH 510 Benchtop Meter, Oakton Instruments, Vernon Hills, IL, USA) with a sensitivity of 0.01 and 2-point calibration.

7.2.5 Preparation of inoculum

The bacterial strains were prepared from stock cultures by streaking each to TSA or TSA + 100 µg/ml of antibiotics (TSA + Amp, TSA + Amp + Strep) and incubating at 37 °C for 24 h. Colonies

from overnight cultures were scraped from the plates with a sterile loop and suspended in phosphate-buffered saline (1x PBS; VWR International, Radnor, PA).

7.2.6 Exposure of bacterial strains to pelargonic acid

Overnight cultures of the bacterial strains grown either on TSA or TSA + 100 µg/ml of ampicillin, and TSA + 100 µg/ml of streptomycin and ampicillin were used to prepare 8.13±0.30 log CFU/ml of bacterial inoculum in 1x PBS. From the suspended culture, 100 µl was transferred to 900 µl of TSB containing PA for 300 s. The solution was centrifuged immediately after exposure for 1 min at 13000 g using the Corning LSE high speed microcentrifuge (Corning Inc., Corning, NY). The supernatant was discarded, and the pellets were resuspended in 1 ml 1x PBS. Enumeration of bacteria was done using the droplet plate method where colony forming units (CFU) of cells within the perimeter of drop were used for enumerating bacterial populations from serially diluted samples. The number of colonies formed were counted after incubation for 24 h at 37 °C. The limit of detection for the assay was 10 cells or 1.00 log CFU/ml.

7.2.7 Exposure of bacterial strains to pelargonic acid + lactic acid

Bacterial strains were prepared and suspended in 1x PBS as described above to achieve a concentration of 8.13±0.30 log CFU/ml. From the suspended culture, 100 µl was transferred to 900 µl of TSB or beef exudate containing PA + LA blends for 300 s. The solution was centrifuged immediately after exposure for 1 min at 13000 g using the Corning LSE high speed microcentrifuge (Corning Inc., Corning, NY). The supernatant was discarded, and the pellets were resuspended in 1 ml 1x PBS. Enumeration of bacteria was done using the droplet plate method where colony forming units (CFU) of cells within the perimeter of drop were used for enumerating bacterial populations from serially diluted samples. The number of colonies formed were counted

after incubation for 24 h at 37 °C. The limit of detection for the assay was 10 cells or 1.00 log CFU/ml.

7.2.8 Microscopy

The morphology of cells exposed to sublethal concentration of pel (10 mM) for 12 h were evaluated through phase contrast microscopy (Nikon Eclipse Ci-L; Nikon Corp., Japan). Bacterial cells exposed to acid stress were used to prepare wet mount preparations of 20 µl, and cells were studied using a Nikon 100X Plan Apo objective (Nikon Eclipse 50i; Nikon Corp., Japan) with the Nikon Elements software and a scale of 5 µm to spot bacterial filaments. Direct counts of filamentous cells (F) and regular sized cells (R) were determined. The average counts of F and R were used to determine the F/R ratio. The experiment was conducted in triplicate (3 fields per slide). Each field had between 50 to 200 cells.

7.2.9 Statistical Analysis

All experiments were conducted in triplicates. Significant differences between the pH of test solutions and reduction of bacterial population after exposure to PA or PA+LA were compared using the one-way analysis of variance (ANOVA). Means were compared using the Tukey's Honestly Significant Difference (HSD) test at a 0.05 significance level, using JMP statistical software (SAS Institute Inc, USA).

7.3 Results

7.3.1 pH of test solutions

The pH of 1 M PA was determined to be 3.55±0.18 in a previous study on the efficacy of PA against *Salmonella* (Kumar, Solval, Mishra, & Macarisin, 2020). This pH value was used as a benchmark for the pH of 10 mM, 20 mM and 31.25 mM PA used in the present study. The pH of

all 3 LA concentrations were significantly different ($P \leq 0.05$). The pH of 1 % v/v LA, 2.5 % v/v LA and 5 % v/v LA were 3.37±0.02, 2.76±0.00 and 2.50±0.02 respectively. The addition of 0.25 mM PA to 1 % LA did not significantly change the pH ($P > 0.05$) however, significant changes were observed when 0.5 mM and 1mM PA were added ($P \leq 0.05$). The pH of 0.5 mM PA + 1 % LA and 1 mM + 1 % LA were 3.17±0.01 and 3.16±0.01 respectively. The pH levels observed in these two test solutions differed from those observed in 1 % LA by 0.2 and 0.21 respectively (Fig 7.1).

7.3.2 Efficacy of antimicrobial solutions

The 10 mM PA and 1 % LA solutions were the least effective at reducing bacterial population after exposure (Fig 7.2) and the decline in bacterial population observed at these concentrations were not significantly different from the control ($P > 0.05$). The average decline in bacterial population in 10 mM PA was 0.16±0.30 log CFU/ml and the average decline in 1 % v/v LA was 0.39±0.49 log CFU/ml. The bacterial strains evaluated survived exposure to 20 mM PA however, there were variations in the population decline observed. The least tolerant strain to 20 mM PA exposure was O157: H7 H1730 NR with a reduction of 4.56±0.78 log CFU/ml in population. The most tolerant strain with a population decline of 2.76±0.28 log CFU/ml was O157: H7 H1730 amp C. Decline in bacterial population observed in O157: H7 H1730 NR was significantly different from the decline observed in O157: H7 H1730 amp C ($P \leq 0.05$). PA at 31.25 mM concentration was the most effective against the bacterial strains evaluated. There were no significant differences between the decline in bacterial population at 31.25 mM PA ($P > 0.05$). However, the average declines in bacterial population observed were 5.11±0.44 log CFU/ml, 5.07±1.24 log CFU/ml and 4.13±0.14 log CFU/ml for O157: H7 H1730 amp P strep C, O157: H7 H1730 NR and O157: H7 H1730 amp C respectively.

7.3.3 Synergistic effect of PA and LA

LA at 1 % v/v was ineffective against the bacterial strains evaluated and much higher concentration of PA (31.25 mM) was required to significantly reduce the bacterial population. Therefore, the synergistic effect of PA and LA was evaluated. A combination of 10 mM PA and 1 % LA reduced the population of all bacterial strains evaluated to below the limit of detection hence the lowest concentrations of PA combined with 1 % v/v LA required to reduce bacterial populations to below the limit of detection were explored in both TSB and beef exudate (Fig. 7.3 and Fig. 7.4). The most effective solutions were (1 mM PA + 1 % LA) and (0.5 mM PA + 1 % LA) which reduced the bacterial population to below the limit of detection. The observed mean reduction in the bacterial population post exposure to 1 mM PA + 1 % LA and 0.5 mM PA + 1% LA were both 6.87±0.28 log CFU/ml.

At 0.25 mM PA + 1 % LA, the population of all bacterial strains were reduced to below the limit of detection except for O157: H7 NR which had a decline of 4.70±0.11 log CFU/ml ($P \leq 0.05$). The decline in bacterial populations at 0.125 mM PA + 1 % v/v LA was not significantly different for the strains evaluated ($P > 0.05$). The average decline in bacterial population at 0.125 mM PA + 1 % v/v LA was 1.90±0.28 log CFU/ml.

7.3.4 Morphology of cells at sublethal concentration of Pel

Bacterial strains O157: H7 amp C and O157: H7 amp P strep C exposed to sublethal concentration of PA (10 mM) for 12 h formed filamentous cells that ranged from 6.29 to 152.03 µm in length following magnification of cells under dark-field illumination (Fig 7.4). The F/R ratio was not significantly different for the bacterial strains evaluated and the mean F/R ratio was 1.53±0.64 ($P > 0.05$). Longer filaments were however observed in strain H7 amp P strep C. The

longest filament observed for this strain was 152.03 µm in length. The longest filament observed in amp C was 25.68 µm. Few filaments were observed for O157: H7 NR however floccular biofilms were predominantly observed after exposure to 10 mM PA for 12 h.

7.4 Discussion

PA and LA exert their antimicrobial activities through different mechanisms. PA interferes with electron transport and oxidative phosphorylation by solubilizing bacterial cell membranes (3) while LA penetrates the cytoplasmic membrane of cells in its undissociated form leading to reduction of intracellular pH and disruption of transmembrane proton motive force (22). In this study, the pH of LA significantly reduced from 3.37±0.02 at 1 % v/v concentration to 2.76±0.00 at 2.5 % v/v and 2.50±0.02 at 5 % v/v concentration while PA at 1 M (≈ 15 %) concentration was 3.55±0.18. Addition of 0.25 mM PA to 1 % LA did not significantly change the pH of the solution and although concentrations of 0.5 mM PA and 1 mM PA in 1 % LA resulted in a significant change, the actual change in values were small (from 3.37±0.02 for 1 % LA to 3.17±0.01 and 3.16±0.01 respectively). These values support the non pH dependent mechanism of PA.

Disruption of bacterial cell membranes can be lethal to cell survival as several survival machinery including efflux pumps involved in acid resistance systems described in previous chapters of this dissertation are housed on the membrane (21). Combining PA, a cell membrane solubilizer with LA could mean more efficient delivery of LA into the intracellular space of the cells and higher reduction in intracellular pH/disruption of transmembrane proton motive force. In this study, exposure to 1 % LA or 10 mM PA did not significantly reduce the bacterial population of any of the strains tested. However, combining as low as 0.5 mM PA with 1 % LA resulted in complete reduction in bacterial population to below the limit of detection. PA is used

in the food industry as a food additive at concentrations ranging from 1.91 ppm in Gelatins and puddings to 6.50 ppm in meat products (15). A challenge with the application of PA as an antimicrobial on food commodities is its impact on quality. PA significantly reduced the population of *Salmonella* and *E. coli* O157: H7 on tomatoes and lettuce at high concentrations of 30 mM and 50 mM, however, the skin strength and firmness of tomatoes treated with PA significantly reduced after 1 day of storage and the color and skin elasticity of lettuce were significantly impacted by the PA treatment (2, 23). A combination of PA and LA at concentrations presented in this report may be a better antimicrobial alternative than PA at 30 mM/50mM or LA at 1 % v/v concentration with no significant impact on quality. Future studies will need to evaluate the impact of PA+LA as an antimicrobial on different food commodities.

It is important to use lethal concentrations of antimicrobials due to the development of bacterial tolerance to antimicrobials at sublethal concentrations. One survival strategy in bacteria exposed to antimicrobial stress is the formation of filaments. Filaments are transient structures that consist of individual cells that are attached due to incomplete cell division and are formed in response to stressors such as membrane active antimicrobials (4, 5). In one study, it was observed that exposure of *Salmonella* to PA in TSB and M9 media amended with 20 mM PA resulted in the formation of transient multinucleated elongated filaments which were released in a time dependent manner (5). In another study, *E. coli* and *Salmonella* formed filamentous cells > 5µm after exposure to sunlight for 180 min (16). In this study, exposure of *E. coli* to 10 mM PA for 300 s induced filamentation and at least in one strain, the longest filament observed was 152.03 µm.

7.5 Conclusion

The results from this study highlight the synergistic activity of PA+LA and its potential as an alternative antimicrobial treatment.

7.6 References

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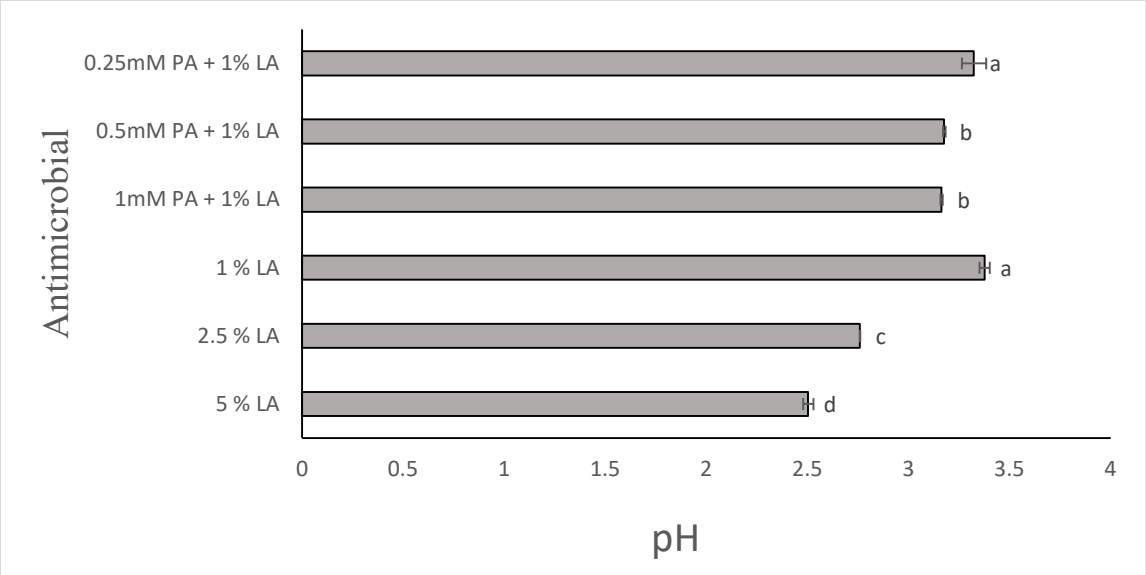


Fig 7.1 pH of 1 %, 2.5 %, 5 % v/v lactic acid and pelargonic acid + lactic acid blends

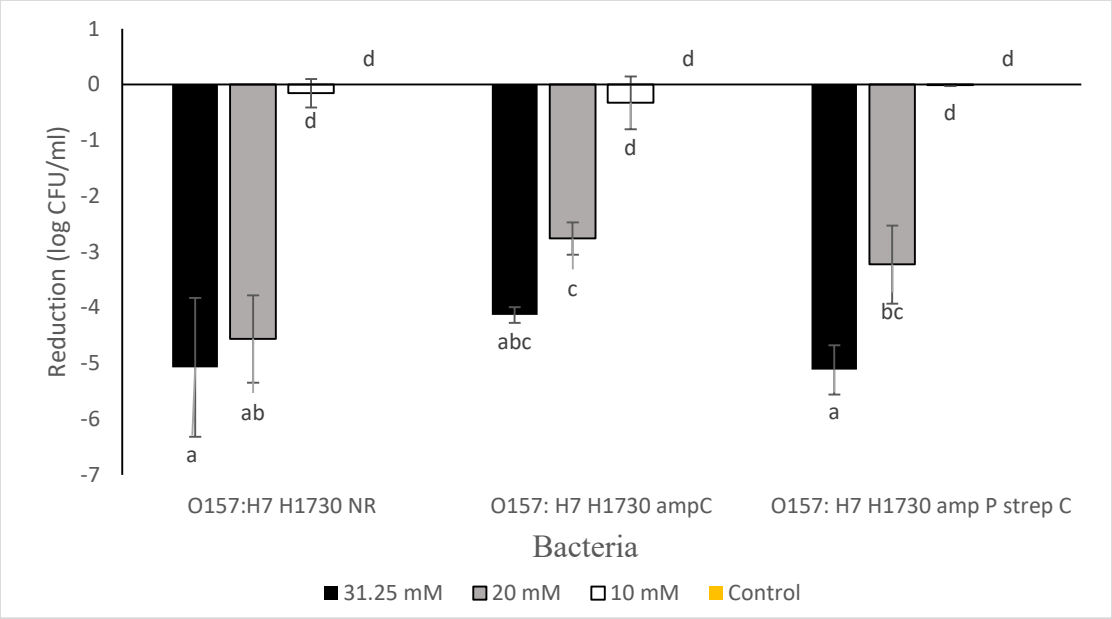


Fig 7.2 Reduction in population bacterial strains exposed to 10 mM, 20 mM, and 31.25 mM pelargonic acid for 300 s at room temperature (25 °C)

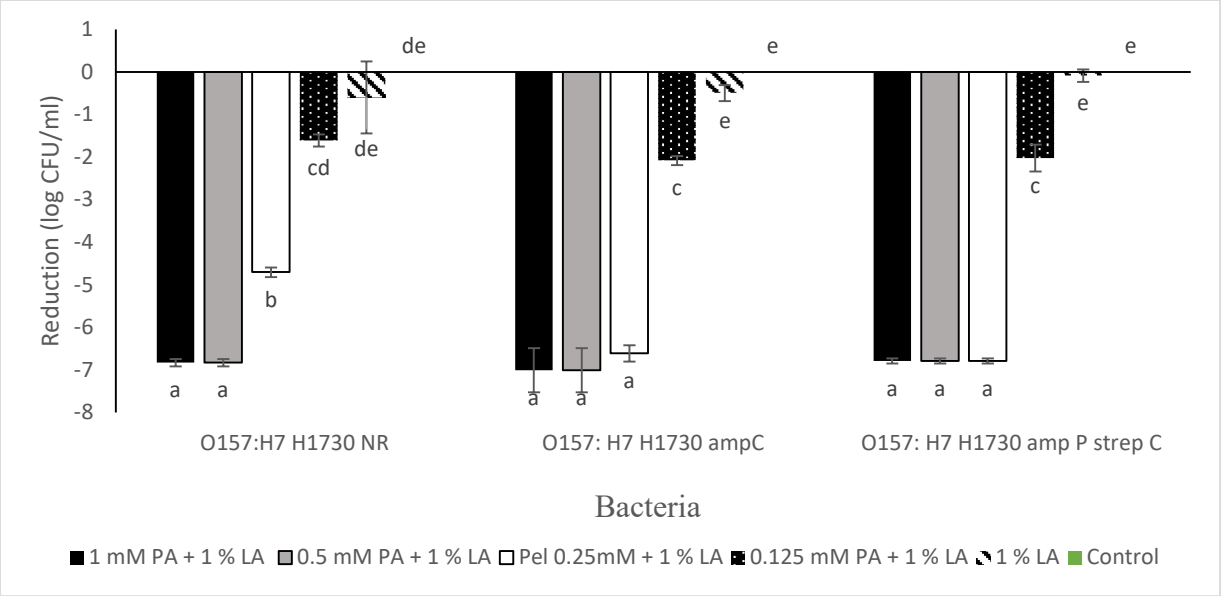


Fig 7.3 Reduction in population of bacterial strains exposed to pelargonic acid + lactic acid blends in TSB for 300 s at room temperature (25 °C)

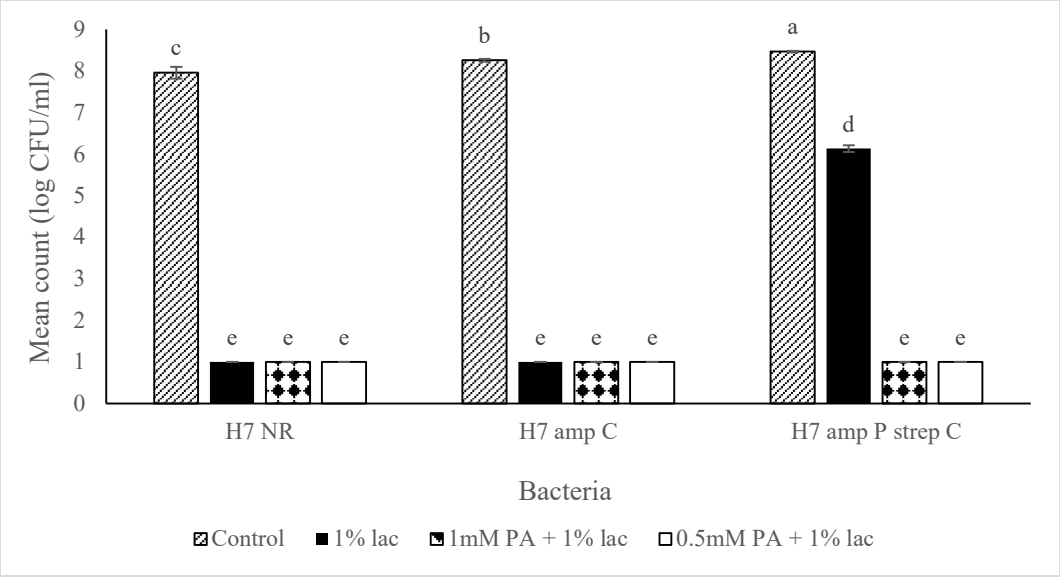
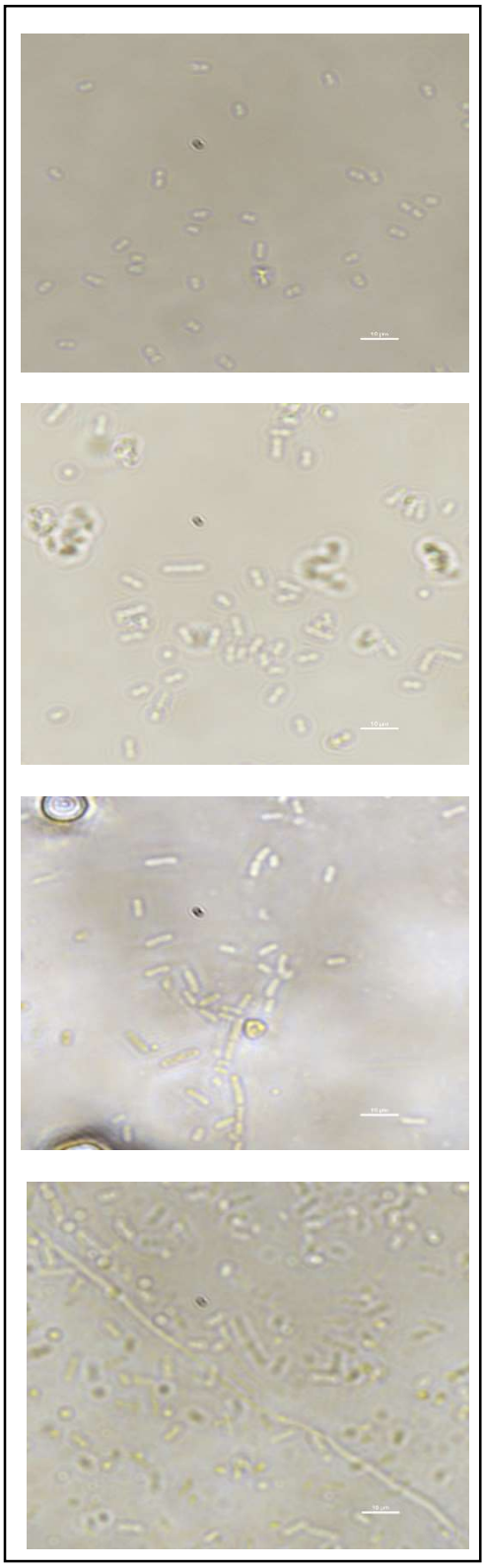


Fig 7.4 Survival of bacterial strains exposed to pelargonic acid + lactic acid blends in beef exudate for 300 s at room temperature (25 °C)



TSB Control

O157: H7 H1730 NR exposed to 10 mM PA

O157: H7 H1730 amp C exposed to 10 mM PA

O157: H7 H1730 amp C strep C exposed to 10 mM PA

Fig 7.5: Formation of filamentous cells by bacterial strains exposed to 10 mM PA for 12 h

CHAPTER 8

SUMMARY AND CONCLUSION

In Chapter 3, the risk of antibiotic-resistance associated cross-tolerance to lactic acid was evaluated in Shiga toxin producing *E. coli* O157: H7 H1730 isolated from a lettuce outbreak. Although cross tolerance has been investigated in several studies, they have mainly focused on antibiotic resistance associated cross tolerance to other antibiotics or biocide resistance associated cross-tolerance to antibiotics. To our knowledge, this was the first study linking the acquisition of antibiotic resistance (ampicillin and streptomycin) to cross-tolerance to biocides (lactic acid). Information on the impact of antibiotic-resistance on the growth parameters of the bacterial strains were provided in this chapter and a possible role of efflux pumps in cross-tolerance was described.

A description of the possible impact of antibiotic-resistance on biofilm formation and tolerance to extreme acid stress in *E. coli* O157: H7 H1730 was presented in chapter 4. Genetic mutations resulting from the acquisition of antibiotic resistance, especially mutations associated with efflux pumps were presented. The results described in this chapter showed that antibiotic resistance could improve biofilm formation in this strain and influence cross-tolerance to extreme acid stress. The results for tolerance to acid stress in this chapter agreed with those from the previous chapter where the antibiotic resistant strains were the most tolerance to acid exposure.

In Chapters 5 and 6, antibiotic-resistance associated cross-tolerance to lactic acid and peroxyacetic acid was described for both O157: H7 Shiga toxin producing *E. coli* (STEC) and non O157: H7 STEC. Also, the impact of organic load, especially amino acids found on beef carcass, on the tolerance of STEC to acid stress was described. The results from this study clearly showed that the presence of organic load significantly impacts the inhibitory concentration of the sanitizers. Variations in response of the bacterial strains and their antibiotic-resistant variants to acid stress alludes to strain difference to acid stress and antibiotic-resistance associated cross tolerance to acid exposure.

A novel antimicrobial treatment combining pelargonic acid and lactic acid was developed and described in Chapter 7. The efficacy of this treatment combination was evaluated on the most tolerant bacterial strains from the previous chapters. The results in this chapter highlighted the synergistic effect of pelargonic acid and lactic acid and its potential as an alternative antimicrobial treatment. The combination of the two antimicrobial technologies performed significantly better than the individual antimicrobials and could be a promising alternative for current treatments.

This dissertation provided information on antibiotic-associated cross-tolerance to sanitizers. Although, most of the studies conducted in this work was laboratory based and utilized laboratory developed bacterial strains, the results obtained from these studies provide some additional insights on the risk of antibiotic resistance in STEC. A future direction for studies on antibiotic associated cross-tolerance may need to use bacterial strain isolates from outbreaks or clinical cases of infection expressing antibiotic resistance profiles. It is also important to pay close attention to the techniques used for such analysis. For example, classical techniques for antimicrobial studies use the minimum inhibitory concentration (MIC) or

minimum bactericidal concentration (MBC) which mostly involve the exposure of bacteria to the antimicrobial for 24 h before assaying for color change, turbidity, or counts. A better idea of differences in response to antimicrobial exposure may be gleaned by considering the MDK_{99} (the minimum duration for killing 99 % of cells). This can be achieved by short time exposure (relevant to intended use or industrial application; in this dissertation, 30 s and 300s exposure times were used) to the antimicrobial followed by removal of the antimicrobial stress either with the use of a neutralizing solution, or wash steps and subsequent assay.

Finally, a novel antimicrobial was developed and evaluated for its efficacy against STEC. This novel combination of pelargonic acid and lactic acid showed tremendous promise as an alternative antimicrobial treatment against STEC however, further studies evaluating its performance on different food matrices will be needed to ascertain its full efficacy compared to existing antimicrobial treatments.