Reducing *Escherichia coli* O157:H7 in agriculture: interventions for cattle and romaine lettuce

by

Joshua Martin Maher

B.S., Iowa State University, 2013
M.S., Iowa State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences & Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2020

Abstract

The foodborne pathogen *Escherichia coli* O157:H7 represents a significant public health threat, with cattle representing the primary reservoir for this bacteria. Outbreaks of *E. coli* O157:H7 in beef carry a storied past; however, recently romaine lettuce has also been implicated in numerous outbreaks in the United States, sickening hundreds and dramatically altering consumer's perspective on the safety of this commodity. This dissertation focuses on novel interventions for the control of *E. coli* O157:H7 in agriculture, emphasizing cattle and fresh-cut romaine lettuce. The first pre-harvest food safety intervention evaluated the administration of *Megasphaera elsdenii* (ME)—a lactic acid-utilizing probiotic—and monitored the influence on *E. coli* O157:H7 shedding in cattle. Inclusion of ME in cattle finishing diets did not reduce the prevalence of *E. coli* O157:H7 compared to cattle fed control diets (P=0.5012), yet the ME x time interaction was significant (P=0.0323), indicating prevalence varied based upon dose and sampling period. ME reduced the odds of *E. coli* O157:H7 prevalence by 50% during sampling period 1 (P=0.0921) and increased the odds by 23% during sampling period 2 (P=0.6130). A similar pre-harvest food safety study included Enogen Feed Corn® (EFC)—which features a genetically enhanced α-amylase enzyme trait—fed as steam-flaked corn and silage to investigate the potential impact on *E. coli* O157:H7 shedding in cattle. A 43.3% reduction in the odds of *E. coli* O157:H7 prevalence in cattle fed diets containing EFC versus a control corn diet was observed, with possible mechanisms for this reduction requiring further investigation. Finally, the need for effective postharvest produce wash interventions for the control of *E. coli* O157:H7 in fresh-cut romaine lettuce was addressed, with a twofold purpose: 1) assessment of sodium bisulfate (SBS) and peroxyacetic acid (PAA) effect on produce quality compared to chlorine, water, and unwashed control treatments, and 2) evaluation of potential *E. coli* O157:H7

reductions using a blend of SBS and PAA as a postharvest wash intervention. Quality data—including overall visual quality, browning and discoloration, phytotoxicity, and color analyses—indicated all treatments were acceptable for fresh-cut romaine lettuce, with these data informing treatment concentration selection in the subsequent inoculation study. When applied to inoculated product, all treatments achieved significant reductions of *E. coli* O157:H7 in comparison to the unwashed control. SBS+PAA produced the greatest reduction (2.3 CFU/g), followed by SBS (2.2 CFU/g), and PAA (1.9 CFU/g); however, these reductions were statistically similar to one another ($P>0.05$). Following the scientific presentation of each food safety technology (probiotics, genetically enhanced animal feed, and postharvest wash water interventions), approval considerations were provided with respect to the United States (U.S.) and European Union (EU) regulatory frameworks. Careful analysis of the similarities and differences between U.S. and EU policy was provided, addressing: 1) regulatory definitions, 2) the approval process, and 3) risk assessment vs. the precautionary principle. This dissertation celebrates the promise of novel food safety technologies for the control of *E. coli* O157:H7 in agriculture, while capturing the scientific, regulatory, and approval-related issues surrounding them.

Reducing *Escherichia coli* O157:H7 in agriculture: interventions for cattle and romaine lettuce

by

Joshua Martin Maher

B.S., Iowa State University, 2013
M.S., Iowa State University, 2014

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences & Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2020

Approved by:

Major Professor
Sara E. Gragg

Copyright

Abstract

The foodborne pathogen *Escherichia coli* O157:H7 represents a significant public health threat, with cattle representing the primary reservoir for this bacteria. Outbreaks of *E. coli* O157:H7 in beef carry a storied past; however, recently romaine lettuce has also been implicated in numerous outbreaks in the United States, sickening hundreds and dramatically altering consumer's perspective on the safety of this commodity. This dissertation focuses on novel interventions for the control of *E. coli* O157:H7 in agriculture, emphasizing cattle and fresh-cut romaine lettuce. The first pre-harvest food safety intervention evaluated the administration of *Megasphaera elsdenii* (ME)—a lactic acid-utilizing probiotic—and monitored the influence on *E. coli* O157:H7 shedding in cattle. Inclusion of ME in cattle finishing diets did not reduce the prevalence of *E. coli* O157:H7 compared to cattle fed control diets (P=0.5012), yet the ME x time interaction was significant (P=0.0323), indicating prevalence varied based upon dose and sampling period. ME reduced the odds of *E. coli* O157:H7 prevalence by 50% during sampling period 1 (P=0.0921) and increased the odds by 23% during sampling period 2 (P=0.6130). A similar pre-harvest food safety study included Enogen Feed Corn® (EFC)—which features a genetically enhanced α-amylase enzyme trait—fed as steam-flaked corn and silage to investigate the potential impact on *E. coli* O157:H7 shedding in cattle. A 43.3% reduction in the odds of *E. coli* O157:H7 prevalence in cattle fed diets containing EFC versus a control corn diet was observed, with possible mechanisms for this reduction requiring further investigation. Finally, the need for effective postharvest produce wash interventions for the control of *E. coli* O157:H7 in fresh-cut romaine lettuce was addressed, with a twofold purpose: 1) assessment of sodium bisulfate (SBS) and peroxyacetic acid (PAA) effect on produce quality compared to chlorine, water, and unwashed control treatments, and 2) evaluation of potential *E. coli* O157:H7

reductions using a blend of SBS and PAA as a postharvest wash intervention. Quality data—including overall visual quality, browning and discoloration, phytotoxicity, and color analyses—indicated all treatments were acceptable for fresh-cut romaine lettuce, with these data informing treatment concentration selection in the subsequent inoculation study. When applied to inoculated product, all treatments achieved significant reductions of *E. coli* O157:H7 in comparison to the unwashed control. SBS+PAA produced the greatest reduction (2.3 CFU/g), followed by SBS (2.2 CFU/g), and PAA (1.9 CFU/g); however, these reductions were statistically similar to one another ($P>0.05$). Following the scientific presentation of each food safety technology (probiotics, genetically enhanced animal feed, and postharvest wash water interventions), approval considerations were provided with respect to the United States (U.S.) and European Union (EU) regulatory frameworks. Careful analysis of the similarities and differences between U.S. and EU policy was provided, addressing: 1) regulatory definitions, 2) the approval process, and 3) risk assessment vs. the precautionary principle. This dissertation celebrates the promise of novel food safety technologies for the control of *E. coli* O157:H7 in agriculture, while capturing the scientific, regulatory, and approval-related issues surrounding them.

Table of Contents

List of Figures

List of Tables

Acknowledgements

"From everyone who has been given much, much will be required; and to whom they entrusted much, of him they will ask all the more." - Luke 12:48

To my advisor Dr. Sara Gragg, you have been a tremendous mentor and friend. You provided the guidance and support I needed during this adventure and I have learned so much from your example. Your attention to detail, clear thinking, and utter selflessness provide me with a model to measure my own professional, academic, and personal lives. I am truly grateful you took me on as a PhD student in Food Science, with little prior food science experience, and gave me an enriching and challenging experience, one which I could not have done without you believing in me.

To my committee, Drs. Londa Nwadike, Jim Drouillard, Eleni Pliakoni, Justin Kastner and Sara Gragg, I am grateful for the guidance and support you have provided me. Drs Nwadike and Pliakoni, I sincerely appreciate your encouragement and counsel. Thank you, Dr. Drouillard for the opportunities at the Beef Cattle Research Center and for challenging me to achieve my potential. Dr. Kastner, I appreciate your constant inspiration, mentoring, and direction. You have been a tremendous role model for me and an even greater friend. I appreciate each of you as you helped me grow as a student and an individual.

I wish to thank Dr. Brian Lubbers and Dr. Gary Anderson, for bringing me to K-State 6 years ago to work in the Veterinary Diagnostic Lab. Thank you for your guidance and friendship. Drs. Jason Ackleson and Justin Kastner, thank you for taking me to Yale University—where the first PhD in the United States was awarded in 1861—to present some of the concepts within this dissertation to policy leaders and students from across the globe, a truly enriching experience.

My appreciation to Dr Lydia Zeglin for serving as my outside chair, and for the support of Dr. Ching Kang. I have learned a great deal from you all.

My appreciation to the folks at MS Biotec LLC., Syngenta, and Jones-Hamilton Co. for their guidance and funding. Without their contributions this work would not have been possible.

Many thanks to all the students and lab mates which helped me along the way. To Katelynn Stull, Danny Unruh, Lindsay Beardall, Christine Rock, Luke Edmonds, Bennett Uhl, and Brock Brethour, thank you for the countless hours in the lab together. I cherish these memories. To Kelly Gude and Kostas Batziakas thank you for going through this PhD process with me, I appreciate all your support. Thank you to my fellow Food Science students Carla Schwan, Daniel Vega, Nicholas Sevart, Katia Pozuelo, and Karina Desiree and colleagues, Drs. Randy Phebus, Jessie Vipham, Valentina Trinetta, and Jeanette Thurston for the guidance, support, and friendship throughout these years. Thank you to all the students at the Beef Cattle Research Center, especially Vanessa Veloso and Adrian Baker whom I greatly appreciate for welcoming me with open arms.

Thanks to the great people at K-State Olathe, past and present. To my close friends Lauren and Eric Racki, thank you for many years of noon (sometimes later) lunches, helping me process the difficulties and stresses of work, school, and life and always energizing me to accomplish my goals. I treasure these memories and all those to come. Thank you, Nate Scherman, Jacob Jenott, Amy Jenott, Emily Loeb, Kimberly Ramacciotti, Debbie Kirchhoff, Andy Stout, and Drs. Jackie Spears and Paige Adams, and all of those who have been fundamental to my journey. Thank you for accepting an Iowa State graduate as your own, Go Cyclones!

I would like to acknowledge the support and love from my amazing family. To my Mom, you have always been my biggest fan, thank you for your unconditional love. To my Dad, you taught me the value of hard work and dedication, thank you for being my role model. I am truly thankful for the sacrifices you both made for me, providing me with all the tools and opportunities I needed to succeed. To my younger brother Mason, thank you for always being there. You are my closest friend, and I am so thankful for your support. Thank you to my grandparents, Gary (Papa) and Janet (Nana) Morgan and Robert and Barbara Maher for always cheering me on and for being wonderful role-models. To my in-laws, Thomas, Carol, Kathryn and Luke (Bernadette and Maximilian), Jonathan, and Lauren, thank you for your encouragement, love, and support.

Thank you to Abigail, my beautiful wife of 5 years, and our three children, Leah, James, and Grace. I appreciate your support and sacrifice during this challenging time in my career. The joy and laughter you bring me every day has given me strength to do things which I couldn't have done alone. You are my inspiration.

I thank God for the countless blessings He continues to bestow upon me. His overwhelming love, grace, and protection are gifts without which none of this would be possible. May this work bring Him glory.

Dedication

I dedicate this dissertation to my loving wife, Abigail, and three wonderful children, Leah, James, and Grace. You are my joy and inspiration.

"Each of us has a personal responsibility to care for creation, this precious gift which God has entrusted to us. This means, on the one hand, that nature is at our disposal, to enjoy and use properly. Yet it also means that we are not its masters. Stewards, but not masters."

- Pope Francis, *Embracing the Way of Jesus*, 2017

Chapter 1 - Review of Literature

The following literature review aims to provide a broad account of the agriculture forces surrounding the food safety technologies studied. It is not intended to systematically analyze results of previously published literature; rather, this review introduces food safety concepts, provides historical context, and develops a food safety narrative in which the technologies presented in the following chapters reside. The author took this approach to succinctly introduce each of the multi-disciplinary topics covered within this dissertation, all of which address the primary research question: Reducing *Escherichia coli* O157:H7 in Agriculture.

1.1. *Escherichia coli* O157:H7

1.1.1 Introduction

Escherichia coli (*E. coli*) is one of the largest and most diverse groups of bacteria, found in the environment, food, and the gastrointestinal (GI) tract of humans and animals (Centers for Disease Control and Prevention, 2020). Originally characterized by Theodor Escherich in 1885, most *E. coli* strains remain relatively harmless, constituting normal flora in both human and animal GI tracts (Lim et al., 2010). Evolution of this bacteria produced pathogenic strains via the acquisition of virulence factors, specifically through transposons, bacteriophages, and plasmids (Lim et al., 2010). Virulence properties, clinical presentation, pathogenicity mechanisms, and serotype inform the characterization of pathogenic strains of *E. coli* (Matthews et al., 2017), aiding public health responses and microbiological research of this foodborne pathogen. Among the family of pathogenic *E. coli*, lies *E. coli* O157:H7, one of the most severe and dangerous foodborne pathogens, and the focus of this dissertation.

1.1.2. Pathogenicity, Disease, and Important Characteristics

Escherichia coli O157:H7 is a non-sporulating, Gram negative bacillus (rod), with mesophilic, aerobic, and facultative anaerobic growth characteristics (Cortes-Sanchez & Salgado-Cruz, 2017; Kaper & O'Brien, 1998). *E. coli* O157:H7 was first recognized as a foodborne pathogen in 1982 during investigation of two outbreaks of hemorrhagic colitis linked to consumption of undercooked ground beef (Centers for Disease Control (CDC), 1982; J. G. Wells et al., 1983). The ability of *E. coli* O157:H7 to cause foodborne illness is enhanced by its low infective dose, requiring less than 100 cells to cause infection (Riley et al., 1983), shedding light on the ease of person-to-person or waterborne transmission. Presently, 167 O antigens, 53 H antigens, and 74 K antigens—considered when identifying *E. coli* serogroups (O) and serotypes (H)—have been identified, with categorization of 6 main diarrheagenic *E. coli* categories: Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Diffusely Adhering (DAEC), Enteroaggregative (EAEC), and Enterohemorrhagic (EHEC) (Matthews et al., 2017). The O antigen represents an oligosaccharide connected to lipopolysaccharides within the cell wall, while the K and H antigens are capsular and flagellar, respectively (Matthews et al., 2017). Differing from other *E. coli* serotypes, O157:H7 grows at temperatures $\geq 44.5^{o}$C, tolerant to acidic environments, and can survive drying and fermentation, adding to the difficulty of removing this pathogen from food production systems (Matthews et al., 2017).

The pathogenic forms of this bacteria, particularly the "Shiga toxin-producing *E. coli*" or STEC, evolved from their non-pathogenic counterparts, acquiring genes from *Shigella dysenteriae* encoding for the extracellular cytotoxin Stx via transduction (J. L. Smith et al., 2014). Enterohemorrhagic *E. coli* (EHEC) are a subset of STEC, with cattle serving as the

primary reservoir, are pathogenic to humans, and produce the Stx cytotoxin or "verotoxins" (Stx1 and Stx2), named after their *in vitro* toxic effects seen in African green monkey kidney "Vero" cell lines (Cortes-Sanchez & Salgado-Cruz, 2017). The Stx verotoxins contain similar modes of action—with Stx2 eliciting greater toxicity—targeting the 28s ribosomal subunit of RNA (comprised of 60S ribosomal subunits) and generating cell death within intestinal epithelial cells and renal blood vessel endothelial cells (Cortes-Sanchez & Salgado-Cruz, 2017).

The spectrum of *E. coli* O157:H7 illness includes mild, self-limiting gastroenteritis potentially leading to hemorrhagic colitis (bloody diarrhea). *E. coli* O157:H7 infections can progress to development of hemolytic uremic syndrome (HUS), seen in 5-15% of patients and characterized by acute kidney failure, thrombocytopenia (decrease in platelets), and microangiopathic hemolytic anemia) (decrease of small blood vessels and red blood cells) (J. L. Smith et al., 2014). Estimates show that children four years old and younger accounted for almost one-third of the confirmed cases of *E. coli* O157:H7 within the EU (European Centre for Disease Prevention and Control, 2019) while in the U.S., HUS remains the leading cause of acute kidney failure in children (Matthews et al., 2017).

Pathogenic characteristics of *E. coli* O157:H7, which occur prior to the production of the Stx verotoxin, include the bacteria's ability to adhere to the host cell membrane and colonize the large intestine. Attachment and effacing (A/E) mechanisms—encoded on the pathogenicity island known as the locus of enterocyte effacement (LEE island)—reveal how *E. coli* O157:H7 can colonize the intestine, destroying microvilli of the host intestinal mucosa (Cortes-Sanchez & Salgado-Cruz, 2017; Matthews et al., 2017). Simply, the infection process begins with ingestion of *E. coli* O157:H7 contaminated food leading to cell contact with host epithelial cells and initiation of the type III secretion system (T3SS) via protein expression by the LEE island. The

T3SS injects TIR (translocated intimin receptor; *tir* gene) into the epithelial cell to serve as the receptor for intimin. Intimin, which is produced by the *eae* gene within the *E. coli* O157:H7 cell, tightly binds to TIR, promoting strict attachment of the bacterial cell to the host cell. This process breaks down the cellular membrane, producing an A/E lesion in a pedestal formation on the host cell surface. Once this adherence is solidified, the *E. coli* O157:H7 cell begins production of Stx verotoxins and excretes them into the host bloodstream, inhibiting protein synthesis and mediating HUS or other GI or neurological disease conditions (Griffin & Tauxe, 1991; Lim et al., 2010; Matthews et al., 2017; Naylor et al., 2003; J. L. Smith et al., 2014).

Treatment of those infected with *E. coli* O157:H7 is limited to supportive therapy, including rest and rehydration (Gragg, 2014), with new therapeutic approaches such as probiotic and homeopathic therapies under investigation (Rahal et al., 2012). Treatment of *E. coli* O157:H7 infections with antibiotics is not recommended (Bush & Perez, 2020). *E. coli* O157:H7 possesses a protective outer membrane barrier, and multidrug efflux pumps which render common classes of antibiotics obsolete, including macrolides, tetracyclines, and fluoroquinolones (Miryala & Ramaiah, 2019). Antibiotics are not effective at reducing the duration of *E. coli* O157:H7 infection or subsequent health outcomes (Panos et al., 2006), with research showing treatment using β-lactam antibiotics (penicillins or cephalosporins) was associated with the development of HUS (K. E. Smith et al., 2012).

Commensal and zoonotic bacteria, such as *E. coli*, are carriers of transmissible resistance genes, with the potential to transfer from the GI tract of animals to the human gut microbiome (Aarestrup et al., 2002; Corpet, 1988; Linton et al., 1977). An increase of antimicrobial resistance (AMR) in *E. coli* O157:H7 populations has been witnessed in the last few decades (Wilkerson et al., 2004). *E. coli* O157:H7 developed resistance to several antibiotics including

streptomycin, sulfisoxazole, and tetracycline (Lim et al., 2010; Matthews et al., 2017; Srinivasan et al., 2007). Antibiotics commonly used in human medicine are of similar class or mechanism of action, as those used in food-producing animals (Aarestrup et al., 2008), thus development of resistance in one population affects the other. *E. coli* O157:H7 represents an undeniably dangerous public health threat, with additional risks regarding potential multi-species spread of antimicrobial resistance genes.

1.1.3. Impact, Reservoirs, and Implicated Food Products

Among the $15.6 billion estimated cost of foodborne illness per year in the U.S. by the USDA Economic Research Service in 2013, *E. coli* O157:H7 costs an estimated $271 million, with costs including identification of infections, outpatient and inpatient medical expenses, lost wages and productivity losses, and cost of premature deaths (Economic Research Service, 2013). Along with representing a significant economic and financial hardship, *E. coli* O157:H7 related outbreaks have shaken consumer confidence in the safety of implicated products, particularly produce, as produce-related outbreaks become increasingly commonplace (T. Callaway et al., 2009; Centers for Disease Control and Prevention, 2018). Most recently, a 2019 outbreak of *E. coli* O157:H7 in fresh-cut romaine lettuce, the 4th outbreak of this bacteria in romaine lettuce in the last 3 years, resulted in 167 cases, 85 hospitalizations, and 15 cases of Hemolytic Uremic Syndrome (HUS; Centers for Disease Control and Prevention, 2019). *E. coli* O157:H7 is estimated to cause over 63,000 foodborne illnesses annually (Mead et al., 1999; Scallan et al., 2011), with recent produce-related outbreaks revealing a shift in this bacteria's behavior in food production systems (Zangari et al., 2014).

Cattle are recognized as the primary reservoir of *E. coli* O157:H7 (T. Callaway et al., 2009; Chapman et al., 1993; Hancock et al., 1997; C. J. McDaniel et al., 2014) . More

specifically, this foodborne pathogen colonizes the lymphoid follicle-dense mucosa at the terminal rectum in cattle (Naylor et al., 2003). Rates of EHEC carriage have reportedly been as high as 60% in bovine herds, with typical rates between 10-25%. *E. coli* O157:H7 prevalence in U.S. feedlot cattle is lower than dairy cattle (1.6% vs 3.2%), with seasonal variation associated with a greater likelihood of testing positive during the warmer months of the year (Matthews et al., 2017). The relationship between cattle and *E. coli* O157:H7 is well documented (Berry et al., 2017; T. Callaway et al., 2009; Castro et al., 2019; C. J. McDaniel et al., 2014; Whipp et al., 1994), with a more robust discussion of prevalence and influence in cattle found in section 1.3. of this literature review.

Food represents the primary transmission route for *E. coli* O157:H7 (Heiman et al., 2015); however, recreation and drinking water have also been linked to *E. coli* O157:H7 infections (Matthews et al., 2017). The United States Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC), and Department of Agriculture (USDA) surveyed foodborne illness data from 1998-2017 and determined that 26.6% of foodborne *E. coli* O157:H7 illnesses were attributable to beef and 47.7% to vegetable row crops (Interagency Food Safety Analytics Collaboration, 2019). Raw vegetables—including alfalfa sprouts—have frequently been implicated in outbreaks of pathogenic *E. coli*, with increases in leafy vegetable outbreaks in the last 10 years. *E. coli* O157:H7 can persist in soil, water, animal, and produce environments, with the ability to survive in sub-optimal pH, temperature, and acidic conditions (Lim et al., 2010). *E. coli* O157:H7 has the capacity to internalize in live lettuce and spinach plants, and become bound by several aspects of the plant physiology including stems, leaves, and sprouts (Luna-Guevara et al., 2019). Production of exopolysaccharide (EPS) confers *E. coli* O157:H7 with protection against harsh conditions, along with acid and heat tolerance (Wick et

al., 2005). These characteristics, along with other features of produce and leafy green production that influence pathogen persistence (described in detail in section 1.4 of this literature review), likely lend explanation as to why *E. coli* O157:H7 is able to persist within the produce environment (Lim et al., 2010).

1.1.4. United States and European Food Law and Regulation

The persistence of *E. coli* O157:H7 in the food chain presents a unique problem that influences global trade, food security, agriculture, and international legislative cooperation. Within the U.S., food safety policy and regulation underwent a transformation following the 1993 Jack in the Box outbreak, including large scale regulatory, scientific, and public health reform, heavily influencing the comprehensive regulation in place today (Centers for Disease Control and Prevention, 2017; Murano et al., 2018). Following the outbreak, the U.S. Department of Agriculture (USDA) required implementation of a Hazard Analysis and Critical Control Point (HACCP) system within all federally inspected meat and poultry processing plants. This required development of procedures and management practices, creating a system where hazards (physical, chemical, or microbiological) can be identified and controlled, and includes microbial testing throughout slaughter facilities, as well as measures for the prevention and removal of contamination (USDA, 1997). Additionally, the USDA declared *E. coli* O157:H7 as an "adulterant" in raw ground beef in 1994 and in non-intact beef trimmings in 1999 (Murano et al., 2018); therefore, the USDA may seize and condemn a product found to be adulterated or potentially withdrawal federal inspection of the facility, forcing a cease of operations. (Armstrong & Staman, 2018)

Today, food law and regulation in the U.S. is chiefly comprised of "enabling acts" including the *Food Safety Modernization Act* (FSMA; 21 U.S.C. § 301 *et seq*), *Federal Meat*

Inspection Act (FMIA; 21 U.S.C. § 601 *et seq*), *Poultry Products Inspection Act* (PPIA; 21 U.S.C. § 451 *et seq*), and *Egg Products Inspection Act* (EPIA ; 21 U.S.C. § 1031 *et seq*), which give regulatory power over the safety of domestic and imported food to the USDA and FDA (Johnson, 2016; Sanchez, 2015; Unruh, 2018). Similar legislation exists within the EU, primarily The General Food Law (GFL) (EC Regulation 178/2002) adopted by the European Parliament and Commission, entrusting the European Food Safety Authority (EFSA) with authority to complete assessment on all risks associated with the food chain (European Commission, 2016). The GFL is the food safety framework at the Union and nation levels across the European agri-food sector (European Commission, 2016) that establishes the Rapid Alert System for Food and Feed (European Commission, 2018) for the monitoring of bacteria like *E. coli, Salmonella,* and *Listeria* within the food chain. Additionally, EC Regulation 854/2004 outlines specific controls on products of animal origin intended for human consumption, with similar HACCP protocols and audit structure to the U.S. (Regulation (EC) No 854/2004). Overall, food safety infrastructure and regulation within the EU and U.S. protects safe domestic and imported food; yet, these pathogens persist, indicating the need for additional solutions.

1.2. A Brief Note on Antimicrobial Resistance

Analogous to the dangers of *E. coli* O157:H7 in agriculture, AMR is recognized as one of the greatest public health dangers facing human and animal populations (Walker et al., 2009) and, if left unchecked, threatens to reduce or eliminate future lifesaving antimicrobial use within the human and animal population. Urgently, the AMR problem (including multi-drug resistant bacteria), necessitates a multidisciplinary, global response. The World Health Organization (WHO) estimates that 25,000 patients die within the European Union (EU) each year, due to AMR infections (European Centre for Disease Prevention and Control, 2018). In the United

States (U.S.), methicillin-resistant *Staphylococcus aureus* (MRSA) alone kills more Americans each year (~19,000) than HIV/AIDs, Parkinson's, emphysema, and homicide combined (Klevens et al., 2007), constituting a significant public health danger.

The threat of AMR, although established by scientific literature and supported by public health data, often sparks dramatic headlines grabbing the public's attention. Although these notions might represent captivating headlines and nothing more, these scenarios have led governments to successfully recalibrate their AMR policies with particular attention to antibiotic use in food production (George, 2017). The EU developed a formal approach toward combating AMR, including the development of a strategic action plan strengthening surveillance and promoting antibiotic stewardship (Baku, 2011). Additionally, the EU banned antibiotics for growth promotion in 2006, observing lower rates of AMR subsequently (Laxminarayan et al., 2013). In the U.S., the National Action Plan, established in 2015, directed federal agencies to increase their multi-sectorial responses toward AMR (President's Council of Advisors on Science and Technology, 2015). In 2012 the U.S. Food and Drug Administration (FDA) released Guidance for Industry (GFI) #209 containing policy phasing out the use of medically important antimicrobial drugs for use in food-producing animals. GFI #213 followed shortly after in 2013 establishing a timeline to bring therapeutic antimicrobial drugs under veterinary oversight, often requiring a prescription, known as the Veterinary Feed Directive (VFD) (Center for Veterinary Medicine, 2019a, 2019c). Both GFI #209 and 213 changed how antibiotics have been used in the U.S. for decades yet represent key milestones in the fight against AMR. Similarly, the U.S. multi-agency collaboration known as NARMS (National Antimicrobial Resistance Monitoring Systems) is responsible for surveying AMR present in enteric bacteria isolated from retail meat, food animals, and human sources (Karp et al., 2017). NARMS ranks among the largest, and

most comprehensive, frameworks for tracking resistance within human and animal populations, assisting public health officials and promoting stewardship of antibiotic usage (Center for Veterinary Medicine, 2019b).

Generally, AMR refers to a microorganism's ability to evade the drugs designed to destroy them via the acquisition of genes or DNA mutations (Aarestrup et al., 2008). Transmission of AMR genes is not confined to a bacterial species or host (human, animal, environment, etc.) (Leverstein-van Hall et al., 2002; Schaberg & Zervos, 1986). Commensal and zoonotic bacteria potentially carry transmissible resistance genes—including *E. coli* and *Enterocccoccus faecium/faecalis*—with the ability to transfer genes successfully from the GI tract of farm animals to the human gut (Aarestrup et al., 2002; Corpet, 1988; Linton et al., 1977). Routine therapeutic antibiotic use in animal production—and growth promotant use prior to its ban in 2017 (21 CFR § 514 and 558)—can potentially result in increased AMR genes present among GI bacterial species in food animals (Srinivasan et al., 2007). As research continues to uncover the specifics of how AMR manifests itself within human, animal, and environmental populations only one thing is for certain: AMR is increasingly complex, requiring an equally complex solution.

Dr. Robert Redfield, director of the Centers for Disease Control and Prevention (CDC), indicated in the CDC's 2019 report on Antibiotic Resistance Threats in the United States, that we need to "stop relying only on new antibiotics" and "adopt aggressive strategies that keep the germs away and infections from occurring in the first place" (p. vi, Centers for Disease Control and Prevention, 2019a). The AMR problem necessitates research exploring technologies—or "antibiotic alternatives"—that limit dependence on these drugs, with attention to the fields of animal agriculture and pre-harvest food safety. The author presents technologies in the

following chapters which hold food safety promise and present additional weapons to combat public health threats including AMR. If Dr. Redfield's words hold true, novel approaches combating public health threats—including food animal diet modification, probiotic administration, and alternative produce wash interventions—require research and characterization, which are presented in the subsequent chapters.

1.3. Pre-harvest Food Safety in Beef Cattle

Pre-harvest food safety differs from the postharvest phase, in that it is further from the finished food product and must encompass the complex environment of animal agriculture. Pre-harvest represents the beginning of the food production system, focusing on prevention and control of foodborne pathogens, the majority of which are zoonotic (Torrence, 2018). For example, cattle represent the principle reservoir for *E. coli* O157:H7 (Chapman et al., 1993; Hancock et al., 1997; C. J. McDaniel et al., 2014; Renwick et al., 1993; Whipp et al., 1994), with studies showing up to 30% of cattle are asymptomatic carriers (T. Callaway et al., 2006, 2009; Reinstein et al., 2007; Stanford et al., 2005). Pre-harvest food safety research begins with determining the sources of foodborne pathogens in the environment, understanding pathogen transmission to the food animal, studying the disease mechanisms, and measuring the impact on animals, plants, and humans (Torrence, 2018). Establishing critical points along the food production continuum is critical for applying interventions to control foodborne pathogens in animals.

Pre-harvest food safety measures consist of on-farm interventions, which fall into 3 main categories: 1) exposure-reduction, 2) anti-pathogenic, and 3) exclusion (LeJeune & Wetzel, 2007; The PEW Charitable Trusts, 2019). Briefly, exposure-reduction methods target the introduction of foodborne pathogens via workers, insects, and pests, with emphasis on food,

water, and animal hygiene. Anti-pathogenic pre-harvest methods include antimicrobials, vaccines, and immune stimulants, providing direct interaction with the target pathogens. Exclusion or pro-commensal pre-harvest food safety measures, indirectly inhibit pathogens via administration of nonpathogenic organisms that compete for nutrients or an ecological niche, also known as "competitive exclusion" (LeJeune & Wetzel, 2007; McAllister et al., 2011). Pre-harvest measures provide the first step towards controlling food safety hazards from farm-to-fork and are important for reducing foodborne pathogens, such as *E. coli* O157:H7, in beef products.

1.3.1. Introduction to Ruminant Nutrition

Although this dissertation is not focused on ruminant nutrition, a basic understanding of the ruminant nutrition system, specifically for beef cattle, is required prior to discussing potential modifications and pre-harvest interventions. Ruminants have a highly complex GI anatomy and physiology, with a segregated stomach and specialized digestive tract, each providing necessary mechanisms by which this mammalian species accesses energy stored in forage and grains (Jouany, 2006; Watson & Preedy, 2015). These animals typically spend more than one-third of their time grazing, one-third ruminating (chewing cud), and one-third idling; therefore, ruminants spend a significant amount of their lives harvesting forages (Parish et al., 2017). The ruminant four-chamber stomach—rumen, reticulum, omasum, and abomasum—influences the fermentation of feedstuffs by symbiotic microorganisms including bacteria, fungi, yeasts, viruses and protozoa (Enany, 2018). Within the reticulorumen resides microbes, which ferment feedstuffs, breaking down the cell wall of plants and fermenting carbohydrates to produce volatile fatty acids (VFA), microbial proteins, and B vitamins, which the ruminant uses for energy (Parish et al., 2017). The rumen provides an "anaerobic fermentation chamber," where microbes—95% bacteria—breakdown feed into usable energy, impacting the health and

physiology of the animal (Cholewińska et al., 2020). Essential for proper digestion of forages, rumen fermentation occurs prior to the main sites of digestion, with rumen microorganisms fermenting the majority of dietary compounds (Abrão et al., 2014; Chaucheyras-Durand & Durand, 2010). In a sense, ruminants exhibit a mutualistic relationship with the microbes that reside within them; microbial populations initiate break down of feed into usable nutrients while ruminants provide a host environment and constant food supply (Watson & Preedy, 2015).

1.3.2. Feedstuffs and *E. coli* O157:H7 Populations

Within the feedlot segment of U.S. cattle production, animals are routinely fed diets containing high concentrations of grain, thereby optimizing feed efficiency (T. Callaway et al., 2003; National Academies of Sciences, 2016). Corn, the most widely utilized source of grain in U.S. cattle finishing diets (Samuelson et al., 2016), primarily consists of starch, which is a significant ruminant energy source. Alteration of feed has a substantial effect on metabolism, animal health, and the ruminal microbial ecosystem (Deusch et al., 2017; Russell & Rychlik, 2001; Shabat et al., 2016), with a notable impact on *E. coli* O157:H7 shedding in feedlot cattle.

Diet manipulation and its effect on foodborne pathogen shedding in feedlot cattle is well documented within the literature. For example, cattle fed finishing diets with high concentrations of the high protein food source distiller's grains (DGs), demonstrate increased levels of *E. coli* O157:H7 shedding (Chaney et al., 2018), depending upon the percentage of DGs included (Chaney et al., 2018; Jacob et al., 2008; J. E. Wells et al., 2009). Cattle fed steam-flaked grain show reductions in *E. coli* O157:H7, by variable mechanisms (Fox et al., 2007; Loneragan & Brashears, 2005; Ohya et al., 2000; Stephens, Loneragan, Karunasena, et al., 2007a), demonstrating the practicality of pre-harvest food safety strategies. Cattle fed processed high grain diets often have starch escape ruminal degradation, leading to post-ruminal

fermentation in the hindgut (Brake & Swanson, 2018; Huntington, 1997; Nocek & Tamminga, 1991; Owens & Soderlund, 2006; Theurer, 1986). Post-ruminal fermentation yields the production of VFAs, which exhibit an inhibitory effect on *E. coli* shedding in cattle (Diez-Gonzalez et al., 1998; Goepfert & Hicks, 1969; Ohya et al., 2000). Diet manipulations that increase post-ruminal fermentation and favor production of bactericidal VFAs represent a potential pre-harvest food safety intervention for reducing foodborne pathogen shedding (Lowe et al., 2010; Munns et al., 2015).

Feed modification to reduce foodborne pathogens was first demonstrated in 1998 by shifting animal diets containing high grain to a period of hay-based rations, thereby significantly reducing *E. coli* by 10^6 after 5 days (Diez-Gonzalez et al., 1998). Similar research on switching cattle diets from grain to hay suggest dietary manipulations may reduce bovine fecal *E. coli* O157:H7 prevalence; however, the diet change also decreased body weight gain (approximately 1.25lb/head per day) indicating an adverse financial consequence (Keen et al., 1999). The research modelled over 2 decades ago in 1998, demonstrated an important yet undefined link between animal diets and *E. coli* O157:H7 prevalence in cattle, one which scientists still explore today. Current research has characterized novel feed ingredients with potential action against foodborne pathogens. Purple prairie clover and sainfoin—two legume forages—have demonstrated *in vitro* potential against *E. coli* O157:H7 (Liu et al., 2013). Cattle feed supplements containing seaweed, specifically *Ascophyllum nodosum,* decreased the prevalence of *E. coli* O157:H7 in feedlot steers (Braden et al., 2004) with similar effects observed *in vitro* (Y. Wang et al., 2009). Orange peel and pulp—a common citrus byproduct—reduced *E. coli* O157:H7 in the GI tract of ruminants (T. Callaway et al., 2011), with at least a 2 log reduction of *E. coli* O157:H7 and *Salmonella* reported for inoculated rumen fluid (T. Callaway et al., 2008;

Nannapaneni et al., 2008). These mechanisms of action are not well understood but provide important contributions toward establishing pre-harvest food safety as a critical, growing area of research. It is important to note that pre-harvest food safety controls such as feed manipulation cannot be performed without thoughtful consideration of effects on animal performance, health, and efficiency.

1.3.3. Probiotics and Prebiotics

Fasting, diet supplementation, and bacteriophage therapy have all been investigated as potential pre-harvest food safety interventions in feedlot cattle (Thomas & Elliott, 2013). However, probiotic and prebiotic administration to food animals—when together known as symbiotics—is particularly promising. Significant research has been generated throughout the previous three decades on use of these live microbial additives, with specific focus on reducing foodborne pathogens like *E. coli* O157:H7.

Probiotics are widely recognized as nonpathogenic microorganisms that provide health benefits to the host (Fuller, 1992). Probiotics in cattle fall under two broad categories: lactic acid bacteria (LAB) and propionic acid bacteria (PAB). LABs, including *Bifidobacterium* spp. and *Lactobacillus* spp., are common intestinal inhabitants and routinely added to silage (Nagaraja, 2019). Concerns have been raised about the possibility of probiotic organisms providing resistance genes to the resident microbiome, including foodborne pathogens, via horizontal gene transfer (Subirats et al., 2019). Current research has established a framework for screening potential probiotic strains with the goal of mitigating the spread of AMR (Ayala et al., 2019).

Probiotic benefits in ruminants include improved GI health, resistance to disease, and reduced bloating or diarrhea (Enany, 2018). Additional benefits—such as improved efficiency, production, growth, and reduction in foodborne pathogen shedding—require attention (Enany,

2018). Research suggests that favorable modulation of cattle gut microbiota influences nutrient acquisition and immune system development (Seo et al., 2010); however, strain selection and dosing parameters warrant further study (Maldonado-Gómez et al., 2016) in order to fully understand their impact and potential benefits. More specifically, future success of probiotics in livestock warrants characterization of mechanisms, strains, dosing, and specific host benefit (Watson & Preedy, 2015).

Prebiotic supplementation— including addition of nondigestible food ingredients that stimulate the growth of resident bacterial species residing in the host's microbiota—influences fermentation and ruminant health (Gibson & Roberfroid, 1995; Wanapat, 2007; Watson & Preedy, 2015). For decades farmers have fed livestock prebiotics, with health benefits only recently being characterized. In contrast to concerns about probiotics, prebiotic administration does not contribute to AMR gene exposure and is safe for long-term consumption (Montagne et al., 2003). A prebiotic, mannan oligosaccharides (MOS) are used not for the purpose of "feeding" beneficial bacteria, but instead for providing necessary bait for unwanted bacteria to cling to and pass through the digestive tract (Ghazanfar et al., 2017), thereby preventing colonization or infection (Waldroup et al., 2003). Prebiotic substances are readily fermented by beneficial ruminant bacteria and poorly fermented by pathogenic bacteria, with existing supplements including: pectin, guar gum, oat gum, and nondigestible oligosaccharides (Shah, 2000). Novel combinations of probiotic and prebiotic technologies, referred to as symbiotics (Collins & Gibson, 1999; Watson & Preedy, 2015) may aid in colonization of the gut by probiotic bacteria. Prebiotics and probiotics combine to provide benefits to the host, as is demonstrated by increases in GI populations of *Bifidobacteria* following prebiotic supplementation (Crittenden & Playne, 2008; Montagne et al., 2003).

1.3.3.1 Direct-Fed Microbials

Within the U.S., direct fed-microbials (DFM)—a source of live (viable), naturally-occurring microorganisms—are specified probiotics used within commercial agriculture production (M. M. Brashears & Guillen, 2014). DFMs modify the composition and activity of the gut microbiota (Nagaraja, 2019). Historically, DFMs influenced gut health and GI microbiota establishment in young ruminants, with current applications improving fiber digestion, ruminal acidosis prevention, and anti-foodborne pathogen action against foodborne pathogens such as *E. coli* O157:H7 and *Salmonella spp.* (McAllister et al., 2011). DFMs act in a number of ways in cattle, including: 1) changing the microbiota composition, 2) changing metabolic activity of current microbiota, and 3) reducing clinical infections and/or increasing animal performance, all of which support positive health outcomes in ruminants (Seo et al., 2010).

Use of DFMs as a pre-harvest intervention has been shown to be effective, with 4 main strategies: 1) host immune response enhancement, 2) production of antimicrobial compounds, 3) stimulation of the animal immune system, and 4) competitive exclusion (T. R. Callaway et al., 2008). Competitive exclusion refers to the prevention of establishment or proliferation of certain bacteria by introducing beneficial bacteria into an ecological niche (LeJeune & Wetzel, 2007; McAllister et al., 2011). Specifically, the "Nurmi Concept" describes feeding cattle probiotics to influence pre-harvest food safety and reduce pathogens, which is a concept that has been researched for over 50 years (Nurmi & Rantala, 1973). DFM production of antibacterial substances can occur in the rumen lower digestive tract, including bacteriocins, short-chain fatty acids, hydrogen peroxide, and lactic acid (Nagaraja, 2019).

DFMs for use in cattle are often classified as lactic acid bacteria (LAB), and include species of *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Propionibacterium,* and *Bacillus* (Seo et al., 2010). A meta-analysis by Wisener et al. (2015), demonstrated that DFMs fed to cattle reduce the odds of fecal *E. coli* O157:H7 shedding by approximately 54% compared to a placebo or no treatment (Wisener et al., 2015). In cattle, *L. acidophilus* strain NP51 has been studied extensively, and has demonstrated a reduction of *E. coli* O157:H7 shedding in cattle by 48 to 80% (M. Brashears et al., 2003; Stephens, Loneragan, Chichester, et al., 2007; Stephens, Loneragan, Karunasena, et al., 2007b; S. Younts-Dahl et al., 2005; S. M. Younts-Dahl et al., 2004). A probiotic preparation containing *L. acidophilus* and *Streptococcus bovis* reduced *E. coli* O157:H7 shedding via increased production of Gram negative-toxic volatile fatty acids (VFAs), in infected calves (Ohya et al., 2000). Bacteriocins isolated from both non-pathogenic *E. coli* (Schamberger & Diez-Gonzalez, 2004) and LAB (*L. paracasei, S. bovis,* and *L. rhamnosus*) demonstrate anti-*E. coli* O157:H7 effects, yet require further research to characterize (Lee et al., 2002; McAllister et al., 2011; Tsai et al., 2010). Investigations into DFM-influenced immune response in cattle demonstrate potential anti-foodborne pathogen effects within the ruminant hindgut (Davis et al., 2007; Lessard et al., 2009; McAllister et al., 2011; Raabis et al., 2019; Szabó et al., 2009).

1.3.3.2. *Megasphaera elsdenii*

Megasphaera elsdenii (ME), a common DFM, is a lactic acid-utilizing bacterium present in the rumen of cattle (Lianmin Chen et al., 2019). Originally isolated and described as *Peptostreptococcus elsdenii* (Gutierrez et al., 1959), ME reduces lactic acid buildup within the rumen of grain-fed cattle by converting lactic acid to VFAs, the main source of energy for ruminants (L. Chen & Wang, 2016; Lianmin Chen et al., 2016; Weimer & Moen, 2013). *M.*

elsdenii moderates ruminal acidosis (RA; Long et al., 2014; Weimer et al., 2015), a common disorder in feedlot cattle characterized by lactate accumulation within the rumen followed by a drop in the pH of ruminal fluid (Hernández et al., 2014; Kenney et al., 2015; H. Wang et al., 2015).

Research into this DFM has revealed additional applications; notably, orally dosing steers with ME decreases the amount of roughage required during the adaptive step-up period, allowing earlier placement on high-concentrate finishing diets (Miller et al., 2013). Additionally, dairy cattle dosed with ME show increases in milk production (Stevens et al., 2017). ME applications are not limited to cattle, with investigations on potential protective function of dosing newborn piglets with antibiotic-sensitive ME strains, delaying colonization of antibiotic resistant ME strains (Stanton & Humphrey, 2011). DeClerck et al. suggested that dosing cattle with ME favorably alters rumen ecology, promoting papillae growth (DeClerck et al., 2020). Following its original isolation in 1959, the past 70 years of research on this organism have shown substantial potential for the promotion of both rumen health and animal productivity; however, no research exists demonstrating a potential pre-harvest food safety application.

1.4. Produce Safety

1.4.1. Background

The U.S. FDA *Food Safety Modernization Act* (FSMA; 21 U.S.C. § 301 *et seq*) establishes the Produce Safety Rule (PSR), which sets minimum standards for safe growing, harvesting, packing, and holding of fresh fruits and vegetables (FDA, 2020). As the first federal produce regulation of its kind, this rule was not immune to criticism, as the 2013 proposal received more than 36,000 public comments addressing issues and concerns. The PSR evolved to include such considerations from the public, producing evidence-based standards regarding

agricultural water, soil amendments, domesticated and wild animals, as well as worker hygiene and training, all of which represent possible routes of contamination by foodborne pathogens (FDA, 2020; Produce Safety Alliance (PSA), 2017; Yang & Swinburne, 2016). The rule details microbiological water testing requirements for agricultural water used in production and postharvest applications, establishes worker training and hygiene standards, proposes validated composting methods used when applying biological soil amendments of animal origin, and presents cleaning and sanitization protocols for use on postharvest equipment, containers, tools, and packing houses (FDA, 2020; Produce Safety Alliance (PSA), 2017). The PSR shifted focus of produce safety efforts in the U.S. towards prevention rather than reaction in response to outbreaks.

1.4.2. Romaine Lettuce (*Lectuca stiva* L. var *longifolia*) Safety

Romaine lettuce harvest, production, handling, and irrigation all significantly influence the susceptibility of this commodity to contamination events (Kintz et al., 2019). Romaine lettuce lacks a thermal processing step, leading to reliance on good preharvest practices and postharvest interventions to remove foodborne pathogens prior to consumption (Gil et al., 2015; Schuenzel & Harrison, 2002). Additionally, the low infectious dose of *E. coli* O157:H7—less than 100 bacterial cells—increases the possibility of ingesting produce harboring enough pathogen to cause illness (Kintz et al., 2019). Diverse surface morphologies, commodity epidermis abrasions, increases in consumer preferences for fresh produce, changes in production methods, and emergence of pathogens not previously associated with fresh produce, have all contributed to increases in produce-related outbreaks (Burnett & Beuchat, 2001; C. McDaniel & Jadeja, 2019; Meireles et al., 2016). Between 2017 and 2019, *E. coli* O157:H7 leafy green outbreaks resulted in 464 cases, 215 hospitalizations, and 6 deaths (Centers for Disease Control

and Prevention, 2019d). Foodborne illness outbreaks within this commodity are becoming increasingly common, representing a significant public health threat and warranting novel intervention research efforts.

1.4.3. Case Study: 2018 Yuma, Arizona *E. coli* O157:H7 Outbreak

With illnesses reported in 36 states, the 2018 Yuma romaine lettuce outbreak was the largest *E. coli* O157:H7 outbreak in the last 10 years (Figure 1-3; Centers for Disease Control and Prevention, 2018). CDC and FDA prevention measures suggested abstaining from romaine lettuce consumption, and the industry was called to voluntarily withdrawal all romaine lettuce from the market and cease shipping (Beecher, 2019). Referred to as the nation's "salad bowl", Yuma and Southern California produce 90% of the U.S. grown leafy greens sold domestically each year (Beecher, 2019). Optimistically, during a sampling assignment following the 2018 outbreak, FDA found no indication of widespread *Salmonella* and *E. coli* O157:H7 contamination of romaine lettuce stored in commercial coolers and storage facilities in the region (U.S. Food and Drug Administration, 2019). This case study exemplifies the dichotomy of food safety decision-making: implementing both proactive and reactive measures. The outbreak sparked debate amongst the multilayer food safety community: produce growers, regulators, researchers, and ultimately, the consumers. Investigators cited 36 growing fields, 23 farms in Arizona and California, and 7 intermediate shippers where distribution of the contaminated lettuce could have originated (Anglen, 2018). Adding to the confusion, a pinpointed source was never identified. Environmental sampling discovered *E. coli* O157:H7 in irrigation canals but investigation as to how the bacteria could have entered the water is still ongoing (Centers for Disease Control and Prevention, 2018). Unsubstantiated media claims only added more confusion to the investigation, conflating proximity of a down-stream feedlot as "most

likely…the source of the outbreak"(Charles, 2018). This case study illustrates the numerous layers of complexity associated with produce outbreaks. More recently, a 2019 outbreak of *E. coli* O157:H7 in romaine lettuce was identified, resulting in 167 cases and 85 hospitalizations (Centers for Disease Control and Prevention, 2019c), providing more evidence that this type of produce outbreak is becoming ever more common.

1.4.4. Produce Safety Interventions

The produce industry routinely uses chlorine to reduce the risk of foodborne pathogen contamination in fresh-cut romaine lettuce wash water (Feliziani et al., 2016); however, increases in produce outbreaks challenge its efficacy (Centers for Disease Control and Prevention, 2019b; Kintz et al., 2019; C. McDaniel & Jadeja, 2019; Ölmez & Kretzschmar, 2009). Furthermore, the production of toxic disinfection byproducts following the reaction of chlorine with organic matter is concerning (Joshi et al., 2013) and even led to the ban of chlorine-based sanitizers in many countries of the EU (Haute et al., 2013; Meireles et al., 2016).

Peroxyacetic acid (PAA), an organic acid, contains acetic acid and hydrogen peroxide (Kitis, 2004). PAA produces antimicrobial effects against a wide spectrum of microorganisms, including bacteria, viruses, and fungi via the production of reactive oxygen species, which damage lipids and DNA of the cell and denature bacterial proteins, leading to an increase in cell wall permeability (Kitis, 2004; Ölmez & Kretzschmar, 2009; Vandekinderen et al., 2009; Warburton, 2014). Recent research describes reductions of generic *E. coli* on fresh romaine lettuce up to 4.07 log CFU/g after 5 minutes (Pahariya et al., 2019). PAA activity against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* was evaluated against chlorine-based sanitizers, with greater reductions achieved by PAA against *E. coli* O157:H7 than near-neutral electrolyzed water (Singh et al., 2018). PAA demonstrates antimicrobial properties

against *Campylobacter* and *Salmonella* during poultry processing (Ae Kim et al., 2017) and *Listeria innocua* in whole apples (Kim et al., 2018); however, PAA is not an effective stainless steel food processing surface sanitizer (Rossoni & Gaylarde, 2000) or control measure against *E. coli* O157:H7 and *Salmonella* Typhimurium on beef carcass surfaces (King et al., 2005).

Sodium bisulfate (SBS), once dissolved in water, lowers pH (Kim et al., 2018), thereby creating an osmotic effect that stresses bacterial cells and often results in cell death (Li et al., 1997). SBS is a natural food acid with GRAS status (Generally Recognized as Safe; 21 CFR 582.1095) and has been used in combination with PAA as an effective postharvest treatment against *Listeria innocua* on inoculated whole apples (Kim et al., 2018) and *Salmonella* spp. on whole chicken drumsticks (Dittoe et al., 2019). Additionally, the World Health Organization has approved the use of SBS in food and food production with no restrictions (FAO JECFA, 2007). Previously published research has explored the combination of SBS and PAA as an intervention for whole apples, poultry processing, chicken drumsticks, and pet food; however, intervention efficacy from previous research presents a difficult challenge, as reductions were observed using different food matrices, concentrations, and foodborne pathogens. (Bodie et al., 2019, 2019; Dhakal et al., 2019; Kim et al., 2018; Micciche et al., 2019). Therefore, extrapolating previous research to understand how a combination of SBS and PAA would impact foodborne pathogens on leafy greens is a challenge and, thus, should be the focus of future research efforts.

Recently, analysis of intervention efficacy on lettuce, spinach, and other leafy greens has compared both alternative and traditional methodologies. Jung et al. (2017), reported that soaking romaine heads in electrolyzed water yielded significant reductions of *Salmonella enterica*, *Listeria monocytogenes*, and *E. coli* O157:H7 compared to tap water, lactic and phosphoric acid-based sanitizers, and a citric acid-based sanitizer (Jung et al., 2017). Pahariya et

al, compared acidified sodium chlorite and sodium hypochlorite to PAA, with PAA resulting in significant reductions of generic *E. coli* after soaking fresh-cut romaine lettuce (Pahariya et al., 2019). When comparing PAA and chlorine efficacy against *E. coli* O157:H7 in romaine lettuce, Singh et al. (2018), observed a 2.2 and 2.1 log CFU/g reduction, respectively (Singh et al., 2018). Interventions that acidify the washing environment via the use of organic acids (citric, lactic, peroxyacetic, etc.) have demonstrated variable success at reducing microbial populations (Neal et al., 2012). Acidifying antimicrobials are promising; however, resistance to organic acids by Gram-negative species (Sheu & Freese, 1973) warrants investigation into new applications. Non-chemical interventions in produce have also been explored—including ultraviolet light, ozone, and irradiation—with significant efficacy, but also challenges, regarding consumer and industry acceptance (Aslam et al., 2020; Murray et al., 2017).

1.5. Produce Quality

1.5.1. Introduction

The efficacy of food safety interventions used in produce cannot be completely understood without research designed to understand the impact on produce quality. Quality can be defined by a combination of characteristics dependent upon the relationship with the commodity. A conventional definition of quality includes any of the features that make something what it is, or the degree of excellence or superiority (A. A. Kader, 2002; Kyriacou & Rouphael, 2018). To the consumer, high quality produce has a pleasant appearance, firm texture, and offers good flavor, aroma, and nutritional value. More recently, consumer perceptions of quality produce have been linked to terms such as locally grown, fresh, organic, and natural (Canavari & Olson, 2007; Orsini et al., 2016), most of which don't accompany a standardized definition. The organic market has experienced rapid expansion in recent years with a consistent 10-15% annual growth

rate, representing an 80-billion-dollar industry in the United States as of 2014 (IFOAM, 2016); thus, produce with high quality characteristics is in high demand.

A definition of quality to a producer often relates to the commodity's appearance, disease and pest resistance, yield, hardiness, and ability to withstand handling and shipping (A. A. Kader, 2002). The produce retailer's definition of quality is influenced by appearance and shelf life of the commodity. With the evolution of produce trends, consumer preferences, agricultural practices, and technology, quality definitions remain relative to the individual and susceptible to change. The U.S. consistently rates quality of produce as very important by all income and geographic groups, with 99% of consumers rating high quality produce as very important or somewhat important in supermarket selection (Food Marketing Institute, 2004). Similar trends have been observed in the EU with quality in fresh fruits and vegetables being the number-one driver of customer satisfaction (Laubli & Ottink, 2018).

1.5.2. Quality Parameters

Postharvest quality research requires defined attributes that can be objectively measured and combined with subjective measurements, including appearance and taste. Attributes associated with the overall quality of fresh fruits and vegetables include appearance (visual), kinesthetic (feel), flavor (taste and smell), nutritional value, and safety (Barrett et al., 2010; A. A. Kader, 2002). Produce appearance is determined by shape, size, color, and the lack of defects, whereas internal quality is shaped by flavor, texture, and the absence of defects (Musacchi & Serra, 2018). These attributes can be evaluated using objective scales based on instrument measurements, or subjective methods based on human judgment using hedonic scales. Despite limitations, visual quality evaluation using numerical scales with defined attributes of a commodity represents a widely used technique (Shewfelt, 2014, p. 14). This includes the use of

overall visual quality scales (Ma. Cantwell, 2013; A. Kader & Marita, 2010), browning or discoloration scales (M. Cantwell & Suslow, 2002; Rico et al., 2007), and phytotoxicity scales (European and Mediterranean Plant Protection Organization, 2014), assuming objective measurements are taken by an expert judge. Size, color, shape, and gloss can be evaluated within the laboratory as they further influence produce appearance quality. Size measurements can be taken using calipers, scales, and water displacement methods in the laboratory, whereas shape measurements are taken using a ratio of dimensions and comparing against diagrams or models for the commodity (Pliakoni et al., 2019). Color evaluation occurs by evaluating uniformity or intensity, often measured using light reflectance meters or light transmission meters. Color measurement using a food colorimeter provides a means to evaluate visual quality of a commodity along with detection of physical changes in appearance (Barrett et al., 2010; Shewfelt, 2014, p. 14), thus demonstrating a relatively simple and reliable method for evaluating produce quality following postharvest intervention application. In many cases, appearance quality characteristics are used in maturity scales and U.S. grading standards; however, postharvest life of a commodity based on appearance exceeds that of postharvest life based on flavor and texture (Camelo & Nations, 2004; A. A. Kader, 2002). Texture evaluation measures firmness, juiciness, fibrousness, succulence, and other sensory and textural qualities that are commodity specific. Pressure testers, penetrometers, or other firmness instruments evaluate commodity “toughness”, while juiciness, succulence, and other sensory quality evaluation occurs using juice or water extraction, titratable sugar measurement, or organoleptic evaluation via sensory testing (Bartoshuk & Klee, 2013; Camelo & Nations, 2004; A. A. Kader, 2002). Regardless of method, postharvest quality evaluation efforts offer an empirical and reproducible

system for characterizing fresh fruits and vegetables and aid the delivery of high-quality, nutritious produce to the consumer.

1.5.3. Romaine Lettuce and Leafy Green Quality

The demand for fresh-cut produce—defined as any fruit or vegetable or combination which has been altered from its original structure yet remains in a fresh state—experienced a 50% increase in the U.S. in the last decade, representing a multi-billion dollar sector, partly due to convenience and consumer trends (Qadri et al., 2015). The fresh-cut leafy green industry has experienced similar increases, yet the postharvest quality of this commodity is uniquely susceptible to environmental changes, harsh handling and processing, and water loss (Qadri et al., 2015). The vulnerability of this commodity hastens the need for care during handling, postharvest wash intervention application, packing, and shipping. Water comprises 90-95% of the salable weight of fruit and vegetable commodities; therefore, water loss denotes the primary culprit of produce weight loss throughout storage and subsequent quality deterioration. Water loss significantly influences economic aspects of the fresh-cut leafy green industry, as this commodity is sold by weight with any loss of water resulting in a direct loss of income (Fallik, 2014; Kitinoja & Gorny, 1998). Additionally, water loss can lead to decreases in produce quality—including wilting, browning, and decay— and produce grading, consequently losing economic value. For example, most commodities become unsalable after losing 3-10% of their weight via water loss thus indicating the importance of slowing transpiration rates (Bartz & Brecht, 2002; Nicola & Fontana, 2014) and minimizing water loss throughout the postharvest handling of fresh-cut leafy greens to safeguard quality. Leafy greens indicate a uniquely vulnerable sector of the produce market, heavily influenced by foodborne illness outbreaks (section 1.4.3.) and quality deterioration.

1.5.4. The Food Quality and Food Safety Relationship

Postharvest handling and sanitation are essential for reducing the amount of produce-associated foodborne illness, believed to be the primary source of contamination (Warriner et al., 2003). Additional microbial contamination sources include growing conditions, soil content, irrigation and postharvest water source, harvest equipment, animal intrusion and manure (Produce Safety Alliance, 2017). When discussing food safety interventions to control foodborne pathogen contamination on produce, quality cannot be ignored. Research on interventions focus on their efficacy in controlling foodborne pathogen contamination; however, a need exists to investigate both produce quality and produce safety simultaneously (Ramos et al., 2013) as these aspects are not mutually exclusive (Francis et al., 2012).

One example of this relationship includes the evaluation of phytotoxicity effects on fresh fruits and vegetables. Phytotoxicity, also known as plant injury, is the inhibition of plant growth, delay in germination, or other adverse effects in response to a specific compound (phytotoxin) (Baumgarten & Spiegel, 2004; Penn State Extension, 2011). This occurs in various fruit and vegetable varieties, with tremendous variation in how the phytotoxic effects are manifested (European and Mediterranean Plant Protection Organization, 2014). Current research focuses on pesticides, fertilizers, and soil amendments (Kebrom et al., 2019) with little emphasis on chemicals used in food safety interventions. Monitoring phytotoxic effects is warranted when evaluating produce interventions, as high concentrations of these chemicals may produce discoloration, deformation, yellowing, and necrosis (Baumgarten & Spiegel, 2004).

Within the literature, several microbial produce interventions, depending on concentration and duration of application, have shown deleterious effects on produce quality (Alexandre et al., 2012; Guan et al., 2010; Karaca & Velioglu, 2007; Ramos et al., 2013).

Produce interventions including chlorine dioxide generate significant pathogen reductions in produce yet demonstrate negative effects on sensory quality. More specifically, 12 ppm of chlorine dioxide applied to *E. coli* O157:H7 inoculated apples resulted in a >5 log reduction (Du et al., 2003), while 5 ppm reduced *Salmonella* inoculated cantaloupes by 3 logs following 10 minutes of exposure (Mahmoud et al., 2008). Further research on chlorine dioxide shows this treatment, following application to cantaloupes, negatively affects sensory quality (Kaur et al., 2015). Conversely, several treatments, including acidified sodium chlorite, hot-water surface pasteurization, and irradiation have shown little to no observable changes in produce quality (Fan et al., 2006; López-Gálvez et al., 2010; Nei et al., 2009; Sapers, 2014). Gaseous ozone, hot water, and their combination was shown to control *E. coli* O157:H7 in cantaloupes without producing adverse produce quality effects, with melons maintaining initial texture and aroma following intervention application (Selma et al., 2008). Similarly, ozone added to spinach inoculated with *E. coli* O157:H7 yielded a >3 log reduction, without mention of deleterious effects on quality or whether or not quality data was gathered (Yesil et al., 2017). Intervention efficacy and impact on produce quality varies with research on specific pathogen, intervention, and commodity combinations.

Examples of research evaluating both produce safety and quality are limited within the literature. Alexandre et al., (2012) assessed hydrogen peroxide solution efficacy against *Listeria monocytogenes* on red bell peppers, strawberries, and watercress. A treatment containing 5% hydrogen peroxide provided the largest microbial reductions along with the largest negative impact on produce quality, as revealed by color, sensory, and anthocyanin content analyses (Alexandre et al., 2012). The study investigated multiple concentrations of hydrogen peroxide; therefore, investigators recommended the concentration which produced significant

antimicrobial effects without sacrificing produce quality, suggesting a treatment containing 1% hydrogen peroxide. Guan et al. (2010), evaluated sodium acid sulfate, levulinic acid, and sodium dodecyl sulfate against *E. coli* O157:H7 on romaine lettuce. The interventions and their combinations yielded *E. coli* O157:H7 reductions less than 1 log and produced significant quality deterioration of the fresh-cut lettuce, following texture, browning, sogginess, and overall visual quality analyses (Guan et al., 2010). Without evaluating both produce quality and produce safety implications of produce interventions, industry recommendations cannot be made, in agreement with the author's previous assertion that research must include investigation into both areas.

Fruits and vegetables, particularly fresh-cut, represent the fastest growing industry of the produce sector (Nicola & Fontana, 2014). Fresh-cut products signify living plant tissue, susceptible to foodborne pathogens and quality deterioration, both of which are heavily influenced by cultivation, harvest, handling, storage, processing, shipping, retail, and consumer preparation. The globalization of fresh-cut produce increases susceptibility to these pressures and reinforces the need for an approach incorporating both safety and quality (Nicola & Fontana, 2014). Success of the fresh-cut produce sector relies on collaboration between food safety and plant pathology communities through interdisciplinary research (Fallik, 2014).

1.6. References

Aarestrup, F. M., Butaye, P., & Witte, W. (2002). Nonhuman Reservoirs of *Enterococci*. *The Enterococci*, 55–99. https://doi.org/10.1128/9781555817923.ch2

Aarestrup, F. M., Wegener, H. C., & Collignon, P. (2008). Resistance in bacteria of the food chain: Epidemiology and control strategies. *Expert Review of Anti-Infective Therapy; London*, *6*(5), 733–750. http://dx.doi.org/10.1586/14787210.6.5.733

Abrão, F. O., Duarte, E. R., Freitas, C. E. S., Vieira, E. A., Geraseev, L. C., da Silva-Hughes, A. F., Rosa, C. A., & Rodrigues, N. M. (2014). Characterization of fungi from ruminal fluid of beef cattle with different ages and raised in tropical lignified pastures. *Current Microbiology*, *69*(5), 649–659. https://doi.org/10.1007/s00284-014-0633-5

Ae Kim, S., Hong Park, S., In Lee, S., Owens, C. M., & Ricke, S. C. (2017). Assessment of Chicken Carcass Microbiome Responses During Processing in the Presence of Commercial Antimicrobials Using a Next Generation Sequencing Approach. *Scientific Reports*, *7*, 43354. https://doi.org/10.1038/srep43354

Alexandre, E. M. C., Brandão, T. R. S., & Silva, C. L. M. (2012). Assessment of the impact of hydrogen peroxide solutions on microbial loads and quality factors of red bell peppers, strawberries and watercress. *Food Control*, *27*(2), 362–368. https://doi.org/10.1016/j.foodcont.2012.04.012

Anglen, R. (2018, December 22). *Clues to a deadly medical mystery hide in Arizona's romaine lettuce fields*. Arizona Republic. https://www.azcentral.com/in-depth/news/local/arizona-health/2018/12/19/yuma-california-romaine-lettuce-hiding-answers-deadly-mystery-e-coli/2233659002/

Armstrong, K. B., & Staman, J. A. (2018). *Enforcement of the Food, Drug, and Cosmetic Act: Select Legal Issues*. 23.

Aslam, R., Alam, M. S., & Saeed, P. A. (2020). Sanitization Potential of Ozone and Its Role in Postharvest Quality Management of Fruits and Vegetables. *Food Engineering Reviews*, *12*(1), 48–67. https://doi.org/10.1007/s12393-019-09204-0

Ayala, D. I., Cook, P. W., Franco, J. G., Bugarel, M., Kottapalli, K. R., Loneragan, G. H., Brashears, M. M., & Nightingale, K. K. (2019). A Systematic Approach to Identify and Characterize the Effectiveness and Safety of Novel Probiotic Strains to Control Foodborne Pathogens. *Frontiers in Microbiology*, *10*, 1108. https://doi.org/10.3389/fmicb.2019.01108

Baku, A. (2011, September 12). *European Strategic Action Plan on Antibiotic Resistance*. Regional Commitee for Europe.

Barrett, D. M., Beaulieu, J. C., & Shewfelt, R. (2010). Color, Flavor, Texture, and Nutritional Quality of Fresh-Cut Fruits and Vegetables: Desirable Levels, Instrumental and Sensory

Measurement, and the Effects of Processing. *Critical Reviews in Food Science and Nutrition*, *50*(5), 369–389. https://doi.org/10.1080/10408391003626322

Bartoshuk, L. M., & Klee, H. J. (2013). Better Fruits and Vegetables through Sensory Analysis. *Current Biology*, *23*(9), R374–R378. https://doi.org/10.1016/j.cub.2013.03.038

Bartz, J. A., & Brecht, J. K. (2002). *Postharvest Physiology and Pathology of Vegetables*. CRC Press.

Baumgarten, A., & Spiegel, H. (2004). Phytotoxicity (Plant tolerance). *Ecotix. Environ. Safe*, *30*.

Beecher, C. (2019, March 21). *Yuma romaine growers hopeful harvest will end without E. coli issues*. Food Safety News. https://www.foodsafetynews.com/2019/03/yuma-romaine-growers-hopeful-harvest-will-end-without-e-coli-issues/

Berry, M. E., Wells, M. J., Schmidt, M. J. W., Bosilevac, M. J. M., Fratamico, D. P. M., Hilbert, F., Smulders, F. J. M., Paulsen, P. P., Dewell, P. G., Post, D. L., James, D. W., Bolton, D. D. J., Riley, M. J. M., Foster, T. P., & Buskirk, D. D. D. (2017). *Ensuring safety and quality in the production of beef Volume 1* (P. G. R. Acuff & P. J. S. Dickson, Eds.; 1 edition). Burleigh Dodds Science Publishing.

Bodie, A. R., Dittoe, D. K., Feye, K. M., Knueven, C. J., & Ricke, S. C. (2019). Application of an Alternative Inorganic Acid Antimicrobial for Controlling *Listeria monocytogenes* in Frankfurters. *Frontiers in Sustainable Food Systems*, *3*. https://doi.org/10.3389/fsufs.2019.00034

Braden, K. W., Blanton, J. R., Allen, V. G., Pond, K. R., & Miller, M. F. (2004). Ascophyllum nodosum supplementation: A preharvest intervention for reducing *Escherichia coli* O157:H7 and *Salmonella* spp. in feedlot steers. *Journal of Food Protection*, *67*(9), 1824–1828. https://doi.org/10.4315/0362-028x-67.9.1824

Brake, D. W., & Swanson, K. C. (2018). RUMINANT NUTRITION SYMPOSIUM: Effects of postruminal flows of protein and amino acids on small intestinal starch digestion in beef cattle. *Journal of Animal Science*, *96*(2), 739–750. https://doi.org/10.1093/jas/skx058

Brashears, M., Galyean, M., Loneragan, G., Mann, J., & Killinger-Mann, K. (2003). Prevalence of *Escherichia coli* O157:H7 and performance by beef feedlot cattle given *Lactobacillus* direct-fed microbials. *Journal of Food Protection*, *66*(5), 748–754. https://doi.org/10.4315/0362-028x-66.5.748

Brashears, M. M., & Guillen, L. M. (2014, February 5). Direct-Fed Microbials as Preharvest Interventions in Cattle Production. *Food Safety Magazine*. https://www.foodsafetymagazine.com/magazine-archive1/februarymarch-2014/direct-fed-microbials-as-preharvest-interventions-in-cattle-production/

Burnett, S. L., & Beuchat, L. R. (2001). Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. *Journal of Industrial Microbiology & Biotechnology*, *27*(2), 104–110. https://doi.org/10.1038/sj.jim.7000199

Bush, L., & Perez, M. (2020, February). *Infection by Escherichia coli O157:H7 and Other Enterohemorrhagic E. coli (EHEC)—Infectious Diseases*. Merck Manuals Professional Edition. https://www.merckmanuals.com/professional/infectious-diseases/gram-negative-bacilli/infection-by-escherichia-coli-o157-h7-and-other-enterohemorrhagic-e-coli-ehec

Callaway, T., Carr, M. A., Edrington, T. S., Anderson, R. C., & Nisbet, D. J. (2009). Diet, Escherichia coli O157:H7, and cattle: A review after 10 years. *Current Issues in Molecular Biology*, *11*(2), 67–79.

Callaway, T., Carroll, J. A., Arthington, J. D., Edrington, T. S., Rossman, M. L., Carr, M. A., Krueger, N. A., Ricke, S. C., Crandall, P., & Nisbet, D. J. (2011). *Escherichia coli* O157:H7 populations in ruminants can be reduced by orange peel product feeding. *Journal of Food Protection*, *74*(11), 1917–1921. https://doi.org/10.4315/0362-028X.JFP-11-234

Callaway, T., Carroll, J. A., Arthington, J. D., Pratt, C., Edrington, T. S., Anderson, R. C., Galyean, M. L., Ricke, S. C., Crandall, P., & Nisbet, D. J. (2008). Citrus products decrease growth of *E. coli* O157:H7 and *Salmonella typhimurium* in pure culture and in fermentation with mixed ruminal microorganisms in vitro. *Foodborne Pathogens and Disease*, *5*(5), 621–627. https://doi.org/10.1089/fpd.2008.0088

Callaway, T., Edrington, T. s., Brabban, A. d., Keen, J. e., Anderson, R. c., Rossman, M. l., Engler, M. j., Genovese, K. j., Gwartney, B. l., Reagan, J. o., Poole, T. l., Harvey, R. b., Kutter, E. m., & Nisbet, D. j. (2006). Fecal Prevalence of *Escherichia coli* O157, *Salmonella*, *Listeria*, and Bacteriophage Infecting *E. coli* O157:H7 in Feedlot Cattle in the Southern Plains Region of the United States. *Foodborne Pathogens and Disease*, *3*(3), 234–244. https://doi.org/10.1089/fpd.2006.3.234

Callaway, T., Elder, R. O., Keen, J. E., Anderson, R. C., & Nisbet, D. J. (2003). Forage feeding to reduce preharvest *Escherichia coli* populations in cattle, a review. *Journal of Dairy Science*, *86*(3), 852–860. https://doi.org/10.3168/jds.S0022-0302(03)73668-6

Callaway, T. R., Edrington, T. S., Anderson, R. C., Byrd, J. A., & Nisbet, D. J. (2008). Gastrointestinal microbial ecology and the safety of our food supply as related to Salmonella,. *Journal of Animal Science*, *86*(suppl_14), E163–E172. https://doi.org/10.2527/jas.2007-0457

Camelo, A. F. L., & Nations, F. and A. O. of the U. (2004). *Manual for the Preparation and Sale of Fruits and Vegetables: From Field to Market*. Food & Agriculture Org.

Canavari, M., & Olson, K. D. (Eds.). (2007). *Organic Food: Consumers' Choices and Farmers' Opportunities* (2007 edition). Springer.

Cantwell, M., & Suslow, T. (2002). Postharvest Handling Systems: Fresh-Cut Fruits and Vegetables. In *Postharvest Technology of Horticultural Crops* (3rd ed., pp. 445–464). Library of Congress.

Cantwell, Ma. (2013, June). *Fresh-cut Fruit & Vegetables* [Lecture].

Castro, V. S., Figueiredo, E. E. de S., Stanford, K., McAllister, T., & Conte-Junior, C. A. (2019). Shiga-Toxin Producing *Escherichia Coli* in Brazil: A Systematic Review. *Microorganisms, 7*(5). https://doi.org/10.3390/microorganisms7050137

Center for Veterinary Medicine. (2019a, April 16). *CVM GFI #213 New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food-Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GFI #209*. U.S. Food and Drug Administration. http://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-213-new-animal-drugs-and-new-animal-drug-combination-products-administered-or-medicated-feed

Center for Veterinary Medicine. (2019b). About NARMS. *FDA*. http://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/about-narms

Center for Veterinary Medicine. (2019c). *Veterinary Feed Directive (VFD)*. Food and Drug Administration. http://www.fda.gov/animal-veterinary/development-approval-process/veterinary-feed-directive-vfd

Centers for Disease Control and Prevention. (2017, May). *Defining Moments in MMWR History: 1993 E. coli O157:H7 Hamburger Outbreak*. CDC Stacks. https://stacks.cdc.gov/view/cdc/49221

Centers for Disease Control and Prevention. (2018, June 28). *Multistate Outbreak of E. coli O157:H7 Infections Linked to Romaine Lettuce (Final Update)*. https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html

Centers for Disease Control and Prevention. (2019a). *Antibiotic Resistance Threats in the United States, 2019*. 148.

Centers for Disease Control and Prevention. (2019b, November 22). *List of Selected Multistate Foodborne Outbreak Investigations | Foodborne Outbreaks | Food Safety | CDC*. https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html

Centers for Disease Control and Prevention. (2019c, November 23). *Outbreak of E. coli Infections Linked to Romaine Lettuce | E.coli Infections | November 2019 | E. coli | CDC*. https://www.cdc.gov/ecoli/2019/o157h7-11-19/index.html

Centers for Disease Control and Prevention. (2019d, December 9). *Reports of Selected E. coli Outbreak Investigations*. https://www.cdc.gov/ecoli/outbreaks.html

Centers for Disease Control and Prevention. (2020, February 26). *E. coli (Escherichia coli) | E. coli | CDC*. https://www.cdc.gov/ecoli/index.html

Centers for Disease Control (CDC). (1982). Isolation of *E. coli* O157:H7 from sporadic cases of hemorrhagic colitis—United States. *MMWR. Morbidity and Mortality Weekly Report*, *31*(43), 580, 585.

Chaney, W. E., Maloney, R., Johnson, B. J., Brooks, J. C., Brashears, M. M., & Loneragan, G. H. (2018). Corn-Based Distillers' Grains in Diets for Feedlot Cattle Are Associated with the Burden of *Escherichia coli* O157 in Feces. *Foodborne Pathogens and Disease*, *15*(7), 398–405. https://doi.org/10.1089/fpd.2017.2395

Chapman, P. A., Siddons, C. A., Wright, D. J., Norman, P., Fox, J., & Crick, E. (1993). Cattle as a possible source of verocytotoxin-producing *Escherichia coli* O157 infections in man. *Epidemiology and Infection*, *111*(3), 439–447. https://doi.org/10.1017/s0950268800057162

Charles, D. (2018, August 29). What Sparked An *E. Coli* Outbreak In Lettuce? Scientists Trace A Surprising Source (Morning Edition). In *National Public Radio*. https://www.npr.org/sections/thesalt/2018/08/29/642646707/investigators-track-contaminated-lettuce-outbreak-to-a-cattle-feedlot

Chaucheyras-Durand, F., & Durand, H. (2010, March 1). *Probiotics in animal nutrition and health* [Text]. https://doi.org/info:doi/10.3920/BM2008.1002

Chen, L., & Wang, H. (2016). Advances in the metabolism and regulation of lactic acids in the rumen. *Pratacult. Sci.*, *33*, 972–980.

Chen, Lianmin, Shen, Y., Wang, C., Ding, L., Zhao, F., Wang, M., Fu, J., & Wang, H. (2019). *Megasphaera elsdenii* Lactate Degradation Pattern Shifts in Rumen Acidosis Models. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00162

Chen, Lianmin, Shen, Y., & Wang, H. (2016). Metabolic mechanism of acid production by *Streptococcus bovis* in rumen and its' regulation. *Chinese Journal of Animal Nutrition*, *28*, 665–673.

Cholewińska, P., Czyż, K., Nowakowski, P., & Wyrostek, A. (2020). The microbiome of the digestive system of ruminants – a review. *Animal Health Research Reviews*, 1–12. https://doi.org/10.1017/S1466252319000069

Collins, M. D., & Gibson, G. R. (1999). Probiotics, prebiotics, and synbiotics: Approaches for modulating the microbial ecology of the gut. *The American Journal of Clinical Nutrition*, *69*(5), 1052S-1057S. https://doi.org/10.1093/ajcn/69.5.1052s

Corpet, D. E. (1988). Antibiotic resistance from food. *The New England Journal of Medicine*, *318*(18), 1206–1207.

Cortes-Sanchez, A., & Salgado-Cruz, M. (2017). *Escherichia coli* O157:H7 in the context of foodborne diseases and public health. *Environmental and Experimental Biology*. https://doi.org/10.22364/eeb.15.19

Crittenden, R., & Playne, M. J. (2008). Prebiotics. In *Handbook of Probiotics and Prebiotics* (pp. 533–581). John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470432624.ch7

Davis, E., Brown, D. C., Baker, A., Bos, K., Dirain, M. S., Halbrook, E., Johnson, Z. B., Maxwell, C., & Rehberger, T. (2007). Effect of direct-fed microbial and antibiotic supplementation on gastrointestinal microflora, mucin histochemical characterization, and immune populations of weanling pigs. *Livestock Science - LIVEST SCI*, *108*, 249–253. https://doi.org/10.1016/j.livsci.2007.01.063

DeClerck, J. C., Reeves, N. R., Miller, M. F., Johnson, B. J., Ducharme, G. A., & Rathmann, R. J. (2020). The influence of *Megasphaera elsdenii* on rumen morphometrics of cull cows immediately stepped up to a high-energy finishing diet. *Translational Animal Science*, *4*(1). https://doi.org/10.1093/tas/txz185

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., & Seifert, J. (2017). A Structural and Functional Elucidation of the Rumen Microbiome Influenced by Various Diets and Microenvironments. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01605

Dhakal, J., Aldrich, C. G., & Knueven, C. (2019). Assessing the Efficacy of Sodium Bisulfate and Organic Acid Treatments for Control of *Salmonella Typhimurium* in Rendered Chicken Fat Applied to Pet Foods. *Journal of Food Protection*, *82*(11), 1864–1869. https://doi.org/10.4315/0362-028X.JFP-18-560

Diez-Gonzalez, F., Callaway, T. R., Kizoulis, M. G., & Russell, J. B. (1998). Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science (New York, N.Y.)*, *281*(5383), 1666–1668. https://doi.org/10.1126/science.281.5383.1666

Dittoe, D. K., Atchley, J. A., Feye, K. M., Lee, J. A., Knueven, C. J., & Ricke, S. C. (2019). The Efficacy of Sodium Bisulfate Salt (SBS) Alone and Combined With Peracetic Acid (PAA) as an Antimicrobial on Whole Chicken Drumsticks Artificially Inoculated With *Salmonella Enteritidis*. *Frontiers in Veterinary Science*, *6*. https://doi.org/10.3389/fvets.2019.00006

Du, J., Han, Y., & Linton, R. H. (2003). Efficacy of chlorine dioxide gas in reducing *Escherichia coli* O157:H7 on apple surfaces. *Food Microbiology*, *20*(5), 583–591. https://doi.org/10.1016/S0740-0020(02)00129-6

Economic Research Service. (2013). *2013 Cost Estimates of Foodborne Illnesses*. https://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx

Enany, S. (2018). *Probiotics: Current Knowledge and Future Prospects*. BoD – Books on Demand.

European and Mediterranean Plant Protection Organization. (2014). Phytotoxicity Assessment. *EPPO Bulletin*, *44*(3), 265–273. https://doi.org/10.1111/epp.12134

European Centre for Disease Prevention and Control. (2018, November 15). *Surveillance of antimicrobial resistance in Europe 2017*. European Centre for Disease Prevention and Control. http://ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-resistance-europe-2017

European Centre for Disease Prevention and Control. (2019). *Shiga toxin/verocytotoxin-producing Escherichia coli (STEC/VTEC) infection* (Annual Epidemiological Report for 2017). https://www.ecdc.europa.eu/sites/portal/files/documents/AER_for_2017-STEC-VTEC.pdf

European Commission. (2016, October 17). *General Food Law* [Text]. Food Safety - European Commission. https://ec.europa.eu/food/safety/general_food_law_en

European Commission. (2018). *The Rapid Alert System for Food and Feed 2018 Annual Report*. 53.

Regulation (EC) No 854/2004, Pub. L. No. 32004R0854, 139 OJ L (2004). http://data.europa.eu/eli/reg/2004/854/oj/eng

Fallik, E. (2014). Microbial Quality and Safety of Fresh Produce. In *Postharvest Handling A Systems Approach* (3rd ed.).

Fan, X., Annous, B. A., Sokorai, K. J. B., Burke, A., & Mattheis, J. P. (2006). Combination of Hot-Water Surface Pasteurization of Whole Fruit and Low-Dose Gamma Irradiation of Fresh-Cut Cantaloupe. *Journal of Food Protection*, *69*(4), 912–919. https://doi.org/10.4315/0362-028X-69.4.912

FAO JECFA. (2007). *World Health Organization Compendium of Food Additive Specifications* (Vol. 4).

FDA. (2020). FSMA Final Rule on Produce Safety. *FDA*. http://www.fda.gov/food/food-safety-modernization-act-fsma/fsma-final-rule-produce-safety

Feliziani, E., Lichter, A., Smilanick, J. L., & Ippolito, A. (2016). Disinfecting agents for controlling fruit and vegetable diseases after harvest. *Postharvest Biology and Technology*, *122*, 53–69. https://doi.org/10.1016/j.postharvbio.2016.04.016

Food Marketing Institute. (2004). *Trends in the United States: Consumer Attitudes & the Supermarket*. Research Department, Food Marketing Institute.

Fox, J. T., Depenbusch, B. E., Drouillard, J. S., & Nagaraja, T. G. (2007). Dry-rolled or steam-flaked grain-based diets and fecal shedding of *Escherichia coli* O157 in feedlot cattle. *Journal of Animal Science*, *85*(5), 1207–1212. https://doi.org/10.2527/jas.2006-079

Francis, G. A., Gallone, A., Nychas, G. J., Sofos, J. N., Colelli, G., Amodio, M. L., & Spano, G. (2012). Factors affecting quality and safety of fresh-cut produce. *Critical Reviews in Food Science and Nutrition*, *52*(7), 595–610. https://doi.org/10.1080/10408398.2010.503685

Fuller, R. (1992). History and development of probiotics. In R. Fuller (Ed.), *Probiotics: The scientific basis* (pp. 1–8). Springer Netherlands. https://doi.org/10.1007/978-94-011-2364-8_1

George, A. (2017). Antimicrobial resistance, trade, food safety and security. *One Health*, *5*, 6–8. https://doi.org/10.1016/j.onehlt.2017.11.004

Ghazanfar, S., Khalid, N., & Imran, I. A. and M. (2017). Probiotic Yeast: Mode of Action and Its Effects on Ruminant Nutrition. *Yeast - Industrial Applications*. https://doi.org/10.5772/intechopen.70778

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *The Journal of Nutrition*, *125*(6), 1401–1412. https://doi.org/10.1093/jn/125.6.1401

Gil, M. I., Gómez-López, V. M., Hung, Y.-C., & Allende, A. (2015). Potential of Electrolyzed Water as an Alternative Disinfectant Agent in the Fresh-Cut Industry. *Food and Bioprocess Technology*, *8*(6), 1336–1348. https://doi.org/10.1007/s11947-014-1444-1

Goepfert, J. M., & Hicks, R. (1969). Effect of volatile fatty acids on *Salmonella typhimurium*. *Journal of Bacteriology*, *97*(2), 956–958.

Gragg, S. (2014, October 6). *Enterohemorrhagic Escherichia coli* [Lecture].

Griffin, P. M., & Tauxe, R. V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews*, *13*, 60–98. https://doi.org/10.1093/oxfordjournals.epirev.a036079

Guan, W., Huang, L., & Fan, X. (2010). Acids in Combination with Sodium Dodecyl Sulfate Caused Quality Deterioration of Fresh-Cut Iceberg Lettuce during Storage in Modified Atmosphere Package. *Journal of Food Science*, *75*(8), S435–S440. https://doi.org/10.1111/j.1750-3841.2010.01786.x

Gutierrez, J., Davis, R. E., Lindahl, I. L., & Warwick, E. J. (1959). Bacterial changes in the rumen during the onset of feed-lot bloat of cattle and characteristics of Peptostreptococcus elsdenii n. Sp. *Applied Microbiology*, *7*(1), 16–22.

Hancock, D. D., Besser, T. E., Rice, D. H., Herriott, D. E., & Tarr, P. I. (1997). A longitudinal study of *Escherichia col*i O157 in fourteen cattle herds. *Epidemiology and Infection*, *118*(2), 193–195. https://doi.org/10.1017/s0950268896007212

Haute, S. V., Sampers, I., Holvoet, K., & Uyttendaele, M. (2013). Physicochemical Quality and Chemical Safety of Chlorine as a Reconditioning Agent and Wash Water Disinfectant for Fresh-Cut Lettuce Washing. *Applied and Environmental Microbiology*, *79*(9), 2850–2861. https://doi.org/10.1128/AEM.03283-12

Heiman, K. E., Mody, R. K., Johnson, S. D., Griffin, P. M., & Gould, L. H. (2015). *Escherichia coli* O157 Outbreaks in the United States, 2003–2012. *Emerging Infectious Diseases*, *21*(8), 1293–1301. https://doi.org/10.3201/eid2108.141364

Hernández, J., Benedito, J. L., Abuelo, A., & Castillo, C. (2014). Ruminal acidosis in feedlot: From aetiology to prevention. *TheScientificWorldJournal*, *2014*, 702572. https://doi.org/10.1155/2014/702572

Huntington, G. (1997). Starch utilization by ruminants: From basics to the bunk. *Journal of Animal Science*, *75*, 852–867.

IFOAM. (2016). *The World of Organic Agriculture*. Organic World. https://www.organic-world.net/yearbook/yearbook-2016.html

Interagency Food Safety Analytics Collaboration. (2019). *Foodborne illness source attribution estimates for 2017 for Salmonella, Escherichia coli O157, Listeria monocytogenes, and Campylobacter using multi-year outbreak surveillance data, United States* (p. 14).

Jacob, M. E., Fox, J. T., Drouillard, J. S., Renter, D. G., & Nagaraja, T. G. (2008). Effects of Dried Distillers' Grain on Fecal Prevalence and Growth of *Escherichia coli* O157 in Batch Culture Fermentations from Cattle. *Applied and Environmental Microbiology*, *74*(1), 38–43. https://doi.org/10.1128/AEM.01842-07

Johnson, R. (2016). *The Federal Food Safety System: A Primer*. 22.

Joshi, K., Mahendran, R., Alagusundaram, K., Norton, T., & Tiwari, B. K. (2013). Novel disinfectants for fresh produce. *Trends in Food Science & Technology*, *34*(1), 54–61. https://doi.org/10.1016/j.tifs.2013.08.008

Jouany, J.-P. (2006). Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. *Animal Reproduction Science*, *96*(3–4), 250–264. https://doi.org/10.1016/j.anireprosci.2006.08.005

Jung, Y., Jang, H., Guo, M., Gao, J., & Matthews, K. R. (2017). Sanitizer efficacy in preventing cross-contamination of heads of lettuce during retail crisping. *Food Microbiology*, *64*, 179–185. https://doi.org/10.1016/j.fm.2017.01.005

Kader, A. A. (2002). *Postharvest Technology of Horticultural Crops*. University of California Agriculture and Natural Resources.

Kader, A., & Marita, C. (2010). *Produce Quality Rating Scales and Color Charts* (2nd ed.). UC Davis.

Kaper, J. B., & O'Brien, A. D. (1998). Shiga toxin-producing Escherichia coli: Yesterday, today, and tomorrow. *ASM Press*, 3–5.

Karaca, H., & Velioglu, Y. S. (2007). Ozone Applications in Fruit and Vegetable Processing. *Food Reviews International*, *23*(1), 91–106. https://doi.org/10.1080/87559120600998221

Karp, B. E., Tate, H., Plumblee, J. R., Dessai, U., Whichard, J. M., Thacker, E. L., Hale, K. R., Wilson, W., Friedman, C. R., Griffin, P. M., & McDermott, P. F. (2017). National Antimicrobial Resistance Monitoring System: Two Decades of Advancing Public Health Through Integrated Surveillance of Antimicrobial Resistance. *Foodborne Pathogens and Disease*, *14*(10), 545–557. https://doi.org/10.1089/fpd.2017.2283

Kaur, S., Smith, D. J., & Morgan, M. T. (2015). Chloroxyanion Residue Quantification in Cantaloupes Treated with Chlorine Dioxide Gas. *Journal of Food Protection*, *78*(9), 1708–1718. https://doi.org/10.4315/0362-028X.JFP-14-576

Kebrom, T. H., Woldesenbet, S., Bayabil, H. K., Garcia, M., Gao, M., Ampim, P., Awal, R., & Fares, A. (2019). Evaluation of phytotoxicity of three organic amendments to collard greens using the seed germination bioassay. *Environmental Science and Pollution Research International*, *26*(6), 5454. https://doi.org/10.1007/s11356-018-3928-4

Keen, J. E., Uhlich, G. A., & Elder, R. O. (1999, November 7). *Effects of hay- and grain-based diets on fecal shedding in naturally-acquired enterohemorrhagic E. coli (EHEC) O157 in beef feedlot cattle*. Conference Research Workers in Animal Diseases, Chicago, IL.

Kenney, N. M., Vanzant, E. S., Harmon, D. L., & McLeod, K. R. (2015). Direct-fed microbials containing lactate-producing bacteria influence ruminal fermentation but not lactate utilization in steers fed a high-concentrate diet. *Journal of Animal Science*, *93*(5), 2336–2348. https://doi.org/10.2527/jas.2014-8570

Kim, S. A., Park, S. H., Knueven, C., Basel, R., & Ricke, S. C. (2018). A decontamination approach using a combination of bisulfate of soda and peracetic acid against Listeria innocua inoculated on whole apples. *Food Control*, *84*, 106–110. https://doi.org/10.1016/j.foodcont.2017.07.036

King, D. A., Lucia, L. M., Castillo, A., Acuff, G. R., Harris, K. B., & Savell, J. W. (2005). Evaluation of peroxyacetic acid as a post-chilling intervention for control of *Escherichia coli* O157:H7 and *Salmonella Typhimurium* on beef carcass surfaces. *Meat Science*, *69*(3), 401–407. https://doi.org/10.1016/j.meatsci.2004.08.010

Kintz, E., Byrne, L., Jenkins, C., McCARTHY, N., Vivancos, R., & Hunter, P. (2019). Outbreaks of Shiga Toxin–Producing *Escherichia coli* Linked to Sprouted Seeds, Salad, and Leafy Greens: A Systematic Review. *Journal of Food Protection*, *82*(11), 1950–1958. https://doi.org/10.4315/0362-028X.JFP-19-014

Kitinoja, L., & Gorny, J. (1998). *Postharvest Technology For Fruit & Vegetable Produce Marketers: Economic Opportunities, Quality, & Food Safety*. UC Postharvest Outreach Program. https://ucanr.edu/sites/Postharvest_Technology_Center_/files/231722.pdf

Kitis, M. (2004). Disinfection of wastewater with peracetic acid: A review. *Environment International*, *30*(1), 47–55. https://doi.org/10.1016/S0160-4120(03)00147-8

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., Fridkin, S. K., & Active Bacterial Core surveillance (ABCs) MRSA Investigators. (2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA*, *298*(15), 1763–1771. https://doi.org/10.1001/jama.298.15.1763

Kyriacou, M. C., & Rouphael, Y. (2018). Towards a new definition of quality for fresh fruits and vegetables. *Scientia Horticulturae*, *234*, 463–469. https://doi.org/10.1016/j.scienta.2017.09.046

Laubli, D., & Ottink, N. (2018, April). *In fresh-food retailing, quality matters more than price | McKinsey*. https://www.mckinsey.com/industries/retail/our-insights/in-fresh-food-retailing-quality-matters-more-than-price

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., … Cars, O. (2013). Antibiotic resistance—The need for global solutions. *The Lancet Infectious Diseases*, *13*(12), 1057–1098. https://doi.org/10.1016/S1473-3099(13)70318-9

Lee, S. S., Hsu, J.-T., Mantovani, H. C., & Russell, J. B. (2002). The effect of bovicin HC5, a bacteriocin from *Streptococcus bovi*s HC5, on ruminal methane production in vitro. *FEMS Microbiology Letters*, *217*(1), 51–55. https://doi.org/10.1111/j.1574-6968.2002.tb11455.x

LeJeune, J. T., & Wetzel, A. N. (2007). Preharvest control of *Escherichia col*i O157 in cattle. *Journal of Animal Science*, *85*(13 Suppl), E73-80. https://doi.org/10.2527/jas.2006-612

Lessard, M., Dupuis, M., Gagnon, N., Nadeau, E., Matte, J. J., Goulet, J. J., & Fairbrother, J. M. (2009). Administration of *Pediococcus acidilactic*i or *Saccharomyces cerevisiae* boulardii modulates development of porcine mucosal immunity and reduces intestinal bacterial translocation after *Escherichia coli* challenge. *Journal of Animal Science*, *87*(3), 922–934. https://doi.org/10.2527/jas.2008-0919

Leverstein-van Hall, M. A., Box, A. T. A., Blok, H. E. M., Paauw, A., Fluit, A. C., & Verhoef, J. (2002). Evidence of Extensive Interspecies Transfer of Integron-Mediated Antimicrobial Resistance Genes among Multidrug-Resistant *Enterobacteriaceae* in a Clinical Setting. *The Journal of Infectious Diseases*, *186*(1), 49–56. https://doi.org/10.1086/341078

Li, Y., Slavik, M. F., Walker, J. T., & Xiong, H. (1997). Pre-Chill Spray of Chicken Carcasses to Reduce *Salmonella typhimurium*. *Journal of Food Science*, *62*(3), 605–607. https://doi.org/10.1111/j.1365-2621.1997.tb04441.x

Lim, J. Y., Yoon, J. W., & Hovde, C. J. (2010). A Brief Overview of *Escherichia coli* O157:H7 and Its Plasmid O157. *Journal of Microbiology and Biotechnology*, *20*(1), 5–14.

Linton, A. H., Howe, K., Bennett, P. M., Richmond, M. H., & Whiteside, E. J. (1977). The colonization of the human gut by antibiotic resistant *Escherichia coli* from chickens. *The Journal of Applied Bacteriology*, *43*(3), 465–469. https://doi.org/10.1111/j.1365-2672.1977.tb00773.x

Liu, X.-L., Hao, Y.-Q., Jin, L., Xu, Z.-J., McAllister, T. A., & Wang, Y. (2013). Anti-*Escherichia coli* O157:H7 properties of purple prairie clover and sainfoin condensed tannins. *Molecules (Basel, Switzerland)*, *18*(2), 2183–2199. https://doi.org/10.3390/molecules18022183

Loneragan, G. H., & Brashears, M. M. (2005). Pre-harvest interventions to reduce carriage of *E. coli* O157 by harvest-ready feedlot cattle. *Meat Science*, *71*(1), 72–78. https://doi.org/10.1016/j.meatsci.2005.04.005

Long, M., Feng, W. J., Li, P., Zhang, Y., He, R. X., Yu, L. H., He, J. B., Jing, W. Y., Li, Y. M., Wang, Z., & Liu, G. W. (2014). Effects of the acid-tolerant engineered bacterial strain *Megasphaera elsdenii* H6F32 on ruminal pH and the lactic acid concentration of simulated rumen acidosis in vitro. *Research in Veterinary Science*, *96*(1), 28–29. https://doi.org/10.1016/j.rvsc.2013.11.013

López-Gálvez, F., Gil, M. I., Truchado, P., Selma, M. V., & Allende, A. (2010). Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology*, *27*(2), 199–204. https://doi.org/10.1016/j.fm.2009.09.009

Lowe, R. M. S., Munns, K., Selinger, L. B., Kremenik, L., Baines, D., McAllister, T. A., & Sharma, R. (2010). Factors influencing the persistence of *Escherichia coli* O157:H7 lineages in feces from cattle fed grain versus grass hay diets. *Canadian Journal of Microbiology*, *56*(8), 667–675. https://doi.org/10.1139/w10-051

Luna-Guevara, J. J., Arenas-Hernandez, M. M. P., Martínez de la Peña, C., Silva, J. L., & Luna-Guevara, M. L. (2019). *The Role of Pathogenic E. coli in Fresh Vegetables: Behavior, Contamination Factors, and Preventive Measures* [Review Article]. International Journal of Microbiology. https://doi.org/10.1155/2019/2894328

Mahmoud, B. S. M., Vaidya, N. A., Corvalan, C. M., & Linton, R. H. (2008). Inactivation kinetics of inoculated *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella Poona* on whole cantaloupe by chlorine dioxide gas. *Food Microbiology*, *25*(7), 857–865. https://doi.org/10.1016/j.fm.2008.05.009

Maldonado-Gómez, M. X., Martínez, I., Bottacini, F., O'Callaghan, A., Ventura, M., van Sinderen, D., Hillmann, B., Vangay, P., Knights, D., Hutkins, R. W., & Walter, J. (2016). Stable Engraftment of *Bifidobacterium longum* AH1206 in the Human Gut Depends on Individualized Features of the Resident Microbiome. *Cell Host & Microbe*, *20*(4), 515–526. https://doi.org/10.1016/j.chom.2016.09.001

Matthews, K. R., Kniel, K. E., & Montville, T. J. (2017). *Food Microbiology: An Introduction* (4 edition). ASM Press.

McAllister, T. A., Beauchemin, K. A., Alazzeh, A. Y., Baah, J., Teather, R. M., & Stanford, K. (2011). Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. *Canadian Journal of Animal Science*, *91*(2), 193–211. https://doi.org/10.4141/cjas10047

McDaniel, C. J., Cardwell, D. M., Moeller, R. B., & Gray, G. C. (2014). Humans and cattle: A review of bovine zoonoses. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.)*, *14*(1), 1–19. https://doi.org/10.1089/vbz.2012.1164

McDaniel, C., & Jadeja, R. (2019). *A review of fresh produce outbreaks, current interventions, food safety concerns and potential benefits of novel antimicrobial sodium acid sulfate*.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, *5*(5), 607–625.

Meireles, A., Giaouris, E., & Simões, M. (2016). Alternative disinfection methods to chlorine for use in the fresh-cut industry. *Food Research International*, *82*, 71–85. https://doi.org/10.1016/j.foodres.2016.01.021

Micciche, A. C., Feye, K. M., Rubinelli, P. M., Lee, J. A., Knueven, C. J., & Ricke, S. C. (2019). Comparison of Acid Sanitizers on *Salmonella Typhimurium* Inoculated Commercial Poultry Processing Reuse Water. *Frontiers in Sustainable Food Systems*, *2*. https://doi.org/10.3389/fsufs.2018.00090

Miller, K. A., Van Bibber-Krueger, C. L., & Drouillard, J. S. (2013). Orally dosing steers with Lactipro (*Megasphaera elsdenii*) decreases the quantity of roughages fed during finishing. *Kansas Agricultural Experiment Station Research Reports*, *1*, 55–58. https://doi.org/10.4148/2378-5977.1446

Miryala, S. K., & Ramaiah, S. (2019). Exploring the multi-drug resistance in *Escherichia coli* O157:H7 by gene interaction network: A systems biology approach. *Genomics*, *111*(4), 958–965. https://doi.org/10.1016/j.ygeno.2018.06.002

Montagne, L., Pluske, J. R., & Hampson, D. J. (2003). A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Animal Feed Science and Technology*, *108*(1), 95–117. https://doi.org/10.1016/S0377-8401(03)00163-9

Munns, K. D., Selinger, L. B., Stanford, K., Guan, L., Callaway, T. R., & McAllister, T. A. (2015). Perspectives on super-shedding of *Escherichia coli* O157:H7 by cattle. *Foodborne Pathogens and Disease*, *12*(2), 89–103. https://doi.org/10.1089/fpd.2014.1829

Murano, E. A., Cross, H. R., & Riggs, P. K. (2018). The outbreak that changed meat and poultry inspection systems worldwide. *Animal Frontiers*, *8*(4), 4–8. https://doi.org/10.1093/af/vfy017

Murray, K., Wu, F., Shi, J., Jun Xue, S., & Warriner, K. (2017). Challenges in the microbiological food safety of fresh produce: Limitations of post-harvest washing and the need for alternative interventions. *Food Quality and Safety*, *1*(4), 289–301. https://doi.org/10.1093/fqsafe/fyx027

Musacchi, S., & Serra, S. (2018). Apple fruit quality: Overview on pre-harvest factors. *Scientia Horticulturae*, *234*, 409–430. https://doi.org/10.1016/j.scienta.2017.12.057

Nagaraja, T. G. (2019, Spring). *Manipulation of Ruminal Microbial Population to Improve Fiber Digestion* [DMP 925 Rumen Microbiology].

Nannapaneni, R., Muthaiyan, A., Crandall, P. G., Johnson, M. G., O'Bryan, C. A., Chalova, V. I., Callaway, T. R., Carroll, J. A., Arthington, J. D., Nisbet, D. J., & Ricke, S. C. (2008). Antimicrobial activity of commercial citrus-based natural extracts against *Escherichia coli* O157:H7 isolates and mutant strains. *Foodborne Pathogens and Disease*, *5*(5), 695–699. https://doi.org/10.1089/fpd.2008.0124

National Academies of Sciences, E. (2016). *Nutrient Requirements of Beef Cattle: Eighth Revised Edition*. https://doi.org/10.17226/19014

Naylor, S. W., Low, J. C., Besser, T. E., Mahajan, A., Gunn, G. J., Pearce, M. C., McKendrick, I. J., Smith, D. G. E., & Gally, D. L. (2003). Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infection and Immunity*, *71*(3), 1505–1512. https://doi.org/10.1128/iai.71.3.1505-1512.2003

Neal, J. A., Marquez-Gonzalez, M., Cabrera-Diaz, E., Lucia, L. M., O'Bryan, C. A., Crandall, P. G., Ricke, S. C., & Castillo, A. (2012). Comparison of multiple chemical sanitizers for reducing *Salmonella* and *Escherichia coli* O157:H7 on spinach (Spinacia oleracea) leaves. *Food Research International*, *45*(2), 1123–1128. https://doi.org/10.1016/j.foodres.2011.04.011

Nei, D., Choi, J.-W., Bari, Md. L., Kawasaki, S., Kawamoto, S., & Inatsu, Y. (2009). Efficacy of Chlorine and Acidified Sodium Chlorite on Microbial Population and Quality Changes of Spinach Leaves. *Foodborne Pathogens and Disease*, *6*(5), 541–546. https://doi.org/10.1089/fpd.2008.0237

Nicola, S., & Fontana, E. (2014). Fresh-Cut Produce Quality: Implications for a Systems Approach. In *Postharvest Handling* (3rd ed.).

Nocek, J. E., & Tamminga, S. (1991). Site of Digestion of Starch in the Gastrointestinal Tract of Dairy Cows and Its Effect on Milk Yield and Composition. *Journal of Dairy Science*, *74*(10), 3598–3629. https://doi.org/10.3168/jds.S0022-0302(91)78552-4

Nurmi, E., & Rantala, M. (1973). New aspects of Salmonella infection in broiler production. *Nature*, *241*(5386), 210–211. https://doi.org/10.1038/241210a0

Ohya, T., Marubashi, T., & Ito, H. (2000). Significance of fecal volatile fatty acids in shedding of *Escherichia coli* O157 from calves: Experimental infection and preliminary use of a probiotic product. *The Journal of Veterinary Medical Science*, *62*(11), 1151–1155. https://doi.org/10.1292/jvms.62.1151

Ölmez, H., & Kretzschmar, U. (2009). Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *LWT - Food Science and Technology*, *42*(3), 686–693. https://doi.org/10.1016/j.lwt.2008.08.001

Orsini, F., Maggio, A., Rouphael, Y., & De Pascale, S. (2016). "Physiological quality" of organically grown vegetables. *Scientia Horticulturae*, *208*, 131–139. https://doi.org/10.1016/j.scienta.2016.01.033

Owens, F., & Soderlund, S. (2006). Ruminal and postruminal starch digestions by cattle. *Proc.of Cattle Grain Processing Symp.*

Pahariya, P., Choudhary, R., & Fisher, D. J. (2019). Antimicrobial Effect of Sanitizing Solutions on Fresh Romaine Lettuce. *2019 Boston, Massachusetts July 7- July 10, 2019*. 2019 Boston, Massachusetts July 7- July 10, 2019. https://doi.org/10.13031/aim.201901623

Panos, G. Z., Betsi, G. I., & Falagas, M. E. (2006). Systematic review: Are antibiotics detrimental or beneficial for the treatment of patients with *Escherichia coli* O157:H7 infection? *Alimentary Pharmacology & Therapeutics*, *24*(5), 731–742. https://doi.org/10.1111/j.1365-2036.2006.03036.x

Parish, J., Rivera, D., & Boland, H. (2017). *Understanding the Ruminant Animal Digestive System* [Extension Publication]. Mississippi State University. http://extension.msstate.edu/publications/publications/understanding-the-ruminant-animal-digestive-system

Penn State Extension. (2011, April 24). *Phytotoxicity*. Penn State Extension. https://extension.psu.edu/phytotoxicity

Pliakoni, E., Batziakas, K., & Gude, K. (2019). *Composition and Compositional Changes During Development*. Postharvest Physiology and Technology, Kansas State University.

President's Council of Advisors on Science and Technology. (2015). *National action plan for combating antibiotic-resistant bacteria*. The White House. https://www.cdc.gov/drugresistance/pdf/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf

Produce Safety Alliance. (2017). *Introduction to Produce Safety*.

Produce Safety Alliance (PSA). (2017). *PSA Curriculum | Produce Safety Alliance*. Produce Safety Alliance Grower Training Curriculum. https://producesafetyalliance.cornell.edu/curriculum/

Qadri, O. S., Yousuf, B., & Srivastava, A. K. (2015). Fresh-cut fruits and vegetables: Critical factors influencing microbiology and novel approaches to prevent microbial risks—A review. *Cogent Food & Agriculture*, *1*(1), 1121606. https://doi.org/10.1080/23311932.2015.1121606

Raabis, S., Li, W., & Cersosimo, L. (2019). Effects and immune responses of probiotic treatment in ruminants. *Veterinary Immunology and Immunopathology*, *208*, 58–66. https://doi.org/10.1016/j.vetimm.2018.12.006

Rahal, E. A., Kazzi, N., Nassar, F. J., & Matar, G. M. (2012). *Escherichia coli* O157:H7—Clinical aspects and novel treatment approaches. *Frontiers in Cellular and Infection Microbiology*, *2*. https://doi.org/10.3389/fcimb.2012.00138

Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P., & Silva, C. L. M. (2013). Fresh fruits and vegetables—An overview on applied methodologies to improve its quality and safety. *Innovative Food Science & Emerging Technologies*, *20*, 1–15. https://doi.org/10.1016/j.ifset.2013.07.002

Reinstein, S., Fox, J. T., Shi, X., & Nagaraja, T. G. (2007). Prevalence of *Escherichia coli* O157:H7 in gallbladders of beef cattle. *Applied and Environmental Microbiology*, *73*(3), 1002–1004. https://doi.org/10.1128/AEM.02037-06

Renwick, S. A., Wilson, J. B., Clarke, R. C., Lior, H., Borczyk, A. A., Spika, J., Rahn, K., McFadden, K., Brouwer, A., & Copps, A. (1993). Evidence of direct transmission of *Escherichia coli* O157:H7 infection between calves and a human. *The Journal of Infectious Diseases*, *168*(3), 792–793. https://doi.org/10.1093/infdis/168.3.792

Rico, D., Martín-Diana, A. B., Barat, J. M., & Barry-Ryan, C. (2007). Extending and measuring the quality of fresh-cut fruit and vegetables: A review. *Trends in Food Science & Technology*, *18*(7), 373–386. https://doi.org/10.1016/j.tifs.2007.03.011

Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargrett, N. T., Blake, P. A., & Cohen, M. L. (1983). Hemorrhagic Colitis Associated with a Rare *Escherichia coli* Serotype. *New England Journal of Medicine*, *308*(12), 681–685. https://doi.org/10.1056/NEJM198303243081203

Rossoni, E. M. M., & Gaylarde, C. C. (2000). Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy. *International Journal of Food Microbiology*, *61*(1), 81–85. https://doi.org/10.1016/S0168-1605(00)00369-X

Russell, J. B., & Rychlik, J. L. (2001). Factors That Alter Rumen Microbial Ecology. *Science*, *292*(5519), 1119–1122. https://doi.org/10.1126/science.1058830

Samuelson, K. L., Hubbert, M. E., Galyean, M. L., & Löest, C. A. (2016). Nutritional recommendations of feedlot consulting nutritionists: The 2015 New Mexico State and Texas Tech University survey. *Journal of Animal Science*, *94*(6), 2648–2663. https://doi.org/10.2527/jas.2016-0282

Sanchez, M. (2015). *Food Law and Regulation for Non-Lawyers: A US Perspective*. Springer International Publishing. https://doi.org/10.1007/978-3-319-12472-8

Sapers, G. M. (2014). Chapter 17—Disinfection of Contaminated Produce with Conventional Washing and Sanitizing Technology. In K. R. Matthews, G. M. Sapers, & C. P. Gerba (Eds.), *The Produce Contamination Problem (Second Edition)* (pp. 389–431). Academic Press. https://doi.org/10.1016/B978-0-12-404611-5.00017-8

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.P11101

Schaberg, D. R., & Zervos, M. J. (1986). Intergeneric and interspecies gene exchange in gram-positive cocci. *Antimicrobial Agents and Chemotherapy*, *30*(6), 817–822.

Schamberger, G. P., & Diez-Gonzalez, F. (2004). Characterization of colicinogenic *Escherichia coli* strains inhibitory to enterohemorrhagic *Escherichia col*i. *Journal of Food Protection*, *67*(3), 486–492. https://doi.org/10.4315/0362-028x-67.3.486

Schuenzel, K. M., & Harrison, M. A. (2002). Microbial antagonists of foodborne pathogens on fresh, minimally processed vegetables. *Journal of Food Protection*, *65*(12), 1909–1915. https://doi.org/10.4315/0362-028x-65.12.1909

Selma, M. V., Ibáñez, A. M., Allende, A., Cantwell, M., & Suslow, T. (2008). Effect of gaseous ozone and hot water on microbial and sensory quality of cantaloupe and potential transference of *Escherichia coli* O157:H7 during cutting. *Food Microbiology*, *25*(1), 162–168. https://doi.org/10.1016/j.fm.2007.06.003

Seo, J. K., Kim, S.-W., Kim, M. H., Upadhaya, S. D., Kam, D. K., & Ha, J. K. (2010). Direct-fed Microbials for Ruminant Animals. *Asian-Australasian Journal of Animal Sciences*, *23*(12), 1657–1667. https://doi.org/10.5713/ajas.2010.r.08

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N., & Mizrahi, I. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *The ISME Journal*, *10*(12), 2958–2972. https://doi.org/10.1038/ismej.2016.62

Shah, N. P. (2000). Effects of milk-derived bioactives: An overview. *The British Journal of Nutrition*, *84 Suppl 1*, S3-10. https://doi.org/10.1017/s000711450000218x

Sheu, C. W., & Freese, E. (1973). Lipopolysaccharide layer protection of gram-negative bacteria against inhibition by long-chain fatty acids. *Journal of Bacteriology*, *115*(3), 869–875.

Shewfelt, R. L. (2014). Chapter 14—Measuring Quality and Maturity. In W. J. Florkowski, R. L. Shewfelt, B. Brueckner, & S. E. Prussia (Eds.), *Postharvest Handling (Third Edition)* (pp. 387–410). Academic Press. https://doi.org/10.1016/B978-0-12-408137-6.00014-4

Singh, P., Hung, Y.-C., & Qi, H. (2018). Efficacy of Peracetic Acid in Inactivating Foodborne Pathogens on Fresh Produce Surface. *Journal of Food Science*, *83*(2), 432–439. https://doi.org/10.1111/1750-3841.14028

Smith, J. L., Fratamico, P. M., & Gunther, N. W. (2014). Chapter Three—Shiga Toxin-Producing *Escherichia coli*. In S. Sariaslani & G. M. Gadd (Eds.), *Advances in Applied Microbiology* (Vol. 86, pp. 145–197). Academic Press. https://doi.org/10.1016/B978-0-12-800262-9.00003-2

Smith, K. E., Wilker, P. R., Reiter, P. L., Hedican, E. B., Bender, J. B., & Hedberg, C. W. (2012). Antibiotic treatment of *Escherichia coli* O157 infection and the risk of hemolytic uremic syndrome, Minnesota. *The Pediatric Infectious Disease Journal*, *31*(1), 37–41. https://doi.org/10.1097/INF.0b013e31823096a8

Srinivasan, V., Nguyen, L. T., Headrick, S. I., Murinda, S. E., & Oliver, S. P. (2007). Antimicrobial resistance patterns of Shiga toxin-producing *Escherichia coli* O157:H7 and O157:H7- from different origins. *Microbial Drug Resistance (Larchmont, N.Y.)*, *13*(1), 44–51. https://doi.org/10.1089/mdr.2006.9996

Stanford, K., Croy, D., Bach, S. J., Wallins, G. L., Zahiroddini, H., & McAllister, T. A. (2005). Ecology of Escherichia coli O157:H7 in commercial dairies in southern Alberta. *Journal of Dairy Science*, *88*(12), 4441–4451. https://doi.org/10.3168/jds.S0022-0302(05)73131-3

Stanton, T. B., & Humphrey, S. B. (2011). Persistence of Antibiotic Resistance: Evaluation of a Probiotic Approach Using Antibiotic-Sensitive *Megasphaera elsdenii* Strains To Prevent Colonization of Swine by Antibiotic-Resistant Strains. *Applied and Environmental Microbiology*, *77*(20), 7158–7166. https://doi.org/10.1128/AEM.00647-11

Stephens, T., Loneragan, G., Chichester, L., & Brashears, M. (2007). Prevalence and enumeration of *Escherichia coli* O157 in steers receiving various strains of *Lactobacillus*-based direct-fed microbials. *Journal of Food Protection*, *70*(5), 1252–1255. https://doi.org/10.4315/0362-028x-70.5.1252

Stephens, T., Loneragan, G., Karunasena, E., & Brashears, M. (2007a). Reduction of *Escherichia coli* O157 and *Salmonella* in feces and on hides of feedlot cattle using various doses of a direct-fed microbial. *Journal of Food Protection*, *70*(10), 2386–2391. https://doi.org/10.4315/0362-028x-70.10.2386

Stephens, T., Loneragan, G., Karunasena, E., & Brashears, M. (2007b). Reduction of *Escherichia coli* O157 and *Salmonella* in feces and on hides of feedlot cattle using various doses of a direct-fed microbial. *Journal of Food Protection*, *70*(10), 2386–2391. https://doi.org/10.4315/0362-028x-70.10.2386

Stevens, K., Eastridge, M., Daniels, K., Finney, S., LeShure, S., & Firkins, J. (2017). Effect of oral administration of *Megasphaera elsdenii* on performance of Holstein cows during early lactation. *The Professional Animal Scientist*, *33*(1), 120–126. https://doi.org/10.15232/pas.2016-01540

Subirats, J., Domingues, A., & Topp, E. (2019). Does Dietary Consumption of Antibiotics by Humans Promote Antibiotic Resistance in the Gut Microbiome? *Journal of Food Protection*, *82*(10), 1636–1642. https://doi.org/10.4315/0362-028X.JFP-19-158

Szabó, I., Wieler, L. H., Tedin, K., Scharek-Tedin, L., Taras, D., Hensel, A., Appel, B., & Nöckler, K. (2009). Influence of a Probiotic Strain of *Enterococcus faecium* on *Salmonella enterica* Serovar *Typhimurium* DT104 Infection in a Porcine Animal Infection Model. *Applied and Environmental Microbiology*, *75*(9), 2621–2628. https://doi.org/10.1128/AEM.01515-08

The PEW Charitable Trusts. (2019, May). *Opportunities to Improve Food Safety From Farm to Fork*. https://pew.org/2JYa63a

Theurer, C. B. (1986). Grain processing effects on starch utilization by ruminants. *Journal of Animal Science*, *63*(5), 1649–1662. https://doi.org/10.2527/jas1986.6351649x

Thomas, D. E., & Elliott, E. J. (2013). Interventions for preventing diarrhea-associated hemolytic uremic syndrome: Systematic review. *BMC Public Health*, *13*, 799. https://doi.org/10.1186/1471-2458-13-799

Torrence, M. E. (2018). Introduction to Preharvest Food Safety. In *Preharvest Food Safety* (pp. 1–17). John Wiley & Sons, Ltd. https://doi.org/10.1128/9781555819644.ch1

Tsai, Y.-T., Cheng, P.-C., & Pan, T.-M. (2010). Immunomodulating Activity of *Lactobacillus paracasei* subsp. *Paracasei* NTU 101 in Enterohemorrhagic *Escherichia coli* O157H7-Infected Mice. *Journal of Agricultural and Food Chemistry*, *58*(21), 11265–11272. https://doi.org/10.1021/jf103011z

Unruh, D. (2018). *Addressing and anticipating food safety challenges: Microbiology and policy frameworks for Shiga toxin-producing Escherichia coli (STEC) and Salmonella* [Kansas State University]. https://krex.k-state.edu/dspace/bitstream/handle/2097/38902/DanielUnruh2018.pdf?sequence=3&isAllowed=y

U.S. Food and Drug Administration. (2019). FDA Releases Results of Romaine Lettuce Sampling Assignment in Yuma Growing Region. *FDA*. http://www.fda.gov/food/cfsan-constituent-updates/fda-releases-results-romaine-lettuce-sampling-assignment-yuma-growing-region

USDA. (1997). *Generic HACCP Model for Raw, Ground Meat and Poultry Products* (HACCP-3). http://haccpalliance.org/alliance/haccpmodels/rawgroud.pdf

Vandekinderen, I., Devlieghere, F., Van, J. C., Kerkaert, B., Cucu, T., Ragaert, P., De, J. B., & De, B. M. (2009). Effects of food composition on the inactivation of foodborne microorganisms by chlorine dioxide. *International Journal of Food Microbiology*, *131*(2–3), 138–144. https://doi.org/10.1016/j.ijfoodmicro.2009.02.004

Waldroup, P. W., Oviedo-Rondón, E. O., & C.A.Fritts. (2003). *Comparison of Bio-Mos ® and Antibiotic Feeding Programs in Broiler Diets Containing Copper Sulfate*. https://doi.org/10.3923/ijps.2003.28.31

Walker, B., Barrett, S., Polasky, S., Galaz, V., Folke, C., Engström, G., Ackerman, F., Arrow, K., Carpenter, S., Chopra, K., Daily, G., Ehrlich, P., Hughes, T., Kautsky, N., Levin, S., Mäler, K.-G., Shogren, J., Vincent, J., Xepapadeas, T., & Zeeuw, A. de. (2009). Looming Global-Scale Failures and Missing Institutions. *Science*, *325*(5946), 1345–1346. https://doi.org/10.1126/science.1175325

Wanapat, M. (2007). *Rumen Manipulation to Increase the Efficient Use of Local Feed Resources and Productivity of Ruminants in the Tropics*.

Wang, H., Pan, X., Wang, C., Wang, M., & Yu, L. (2015). Effects of different dietary concentrate to forage ratio and thiamine supplementation on the rumen fermentation and ruminal bacterial community in dairy cows. *Animal Production Science*, *55*(2), 189–193. https://doi.org/10.1071/AN14523

Wang, Y., Xu, Z., Bach, S. J., & McAllister, T. A. (2009). Sensitivity of *Escherichia coli* to Seaweed (*Ascophyllum nodosum*) Phlorotannins and Terrestrial Tannins. *Asian-Australasian Journal of Animal Sciences*, *22*(2), 238–245. https://doi.org/2009.22.2.238

Warburton, R. (2014, June 13). *Peracetic Acid in the Fresh Food Industry*. Food Safety Magazine. https://www.foodsafetymagazine.com/signature-series/peracetic-acid-in-the-fresh-food-industry/

Warriner, K., Ibrahim, F., Dickinson, M., Wright, C., & Waites, W. M. (2003). Interaction of *Escherichia coli* with growing salad spinach plants. *Journal of Food Protection*, *66*(10), 1790–1797. https://doi.org/10.4315/0362-028x-66.10.1790

Watson, R. R., & Preedy, V. R. (2015). *Probiotics, Prebiotics, and Synbiotics: Bioactive Foods in Health Promotion*. Academic Press.

Weimer, P. J., Da Silva Cabral, L., & Cacite, F. (2015). Effects of ruminal dosing of Holstein cows with *Megasphaera elsdenii* on milk fat production, ruminal chemistry, and bacterial strain persistence1. *Journal of Dairy Science*, *98*(11), 8078–8092. https://doi.org/10.3168/jds.2015-9721

Weimer, P. J., & Moen, G. N. (2013). Quantitative analysis of growth and volatile fatty acid production by the anaerobic ruminal bacterium *Megasphaera elsdenii* T81. *Applied Microbiology and Biotechnology*, *97*(9), 4075–4081. https://doi.org/10.1007/s00253-012-4645-4

Wells, J. E., Shackelford, S. D., Berry, E. D., Kalchayanand, N., Guerini, M. N., Varel, V. H., Arthur, T. M., Bosilevac, J. M., Freetly, H. C., Wheeler, T. L., Ferrell, C. L., & Koohmaraie, M. (2009). Prevalence and level of *Escherichia coli* O157:H7 in feces and on hides of feedlot steers fed diets with or without wet distillers grains with solubles. *Journal of Food Protection*, *72*(8), 1624–1633. https://doi.org/10.4315/0362-028x-72.8.1624

Wells, J. G., Davis, B. R., Wachsmuth, I. K., Riley, L. W., Remis, R. S., Sokolow, R., & Morris, G. K. (1983). Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *Journal of Clinical Microbiology*, *18*(3), 512–520.

Whipp, S. C., Rasmussen, M. A., & Cray, W. C. (1994). Animals as a source of *Escherichia coli* pathogenic for human beings. *Journal of the American Veterinary Medical Association*, *204*(8), 1168–1175.

Wick, L. M., Qi, W., Lacher, D. W., & Whittam, T. S. (2005). Evolution of Genomic Content in the Stepwise Emergence of *Escherichia coli* O157:H7. *Journal of Bacteriology*, *187*(5), 1783–1791. https://doi.org/10.1128/JB.187.5.1783-1791.2005

Wilkerson, C., Samadpour, M., Kirk, N. van, & Roberts, M. C. (2004). Antibiotic Resistance and Distribution of Tetracycline Resistance Genes in *Escherichia coli* O157:H7 Isolates from Humans and Bovines. *Antimicrobial Agents and Chemotherapy*, *48*(3), 1066–1067. https://doi.org/10.1128/AAC.48.3.1066-1067.2004

Wisener, L. V., Sargeant, J. M., O'Connor, A. M., Faires, M. C., & Glass-Kaastra, S. K. (2015). The use of direct-fed microbials to reduce shedding of *Escherichia coli* O157 in beef cattle: A systematic review and meta-analysis. *Zoonoses and Public Health*, *62*(2), 75–89. https://doi.org/10.1111/zph.12112

Yang, Y. T., & Swinburne, M. (2016). New Produce Safety Regulations. *Public Health Reports*, *131*(6), 754–757. https://doi.org/10.1177/0033354916669495

Yesil, M., Kasler, D. R., Huang, E., & Yousef, A. E. (2017). Efficacy of Gaseous Ozone Application during Vacuum Cooling against *Escherichia coli* O157:H7 on Spinach Leaves as Influenced by Bacterium Population Size. *Journal of Food Protection*, *80*(7), 1066–1071. https://doi.org/10.4315/0362-028X.JFP-16-284

Younts-Dahl, S. M., Galyean, M. L., Loneragan, G. H., Elam, N. A., & Brashears, M. M. (2004). Dietary supplementation with *Lactobacillus*- and *Propionibacterium*-based direct-fed microbials and prevalence of *Escherichia coli* O157 in beef feedlot cattle and on hides at harvest. *Journal of Food Protection*, *67*(5), 889–893. https://doi.org/10.4315/0362-028x-67.5.889

Younts-Dahl, S., Osborn, G., Galyean, M., Rivera, D., Loneragan, G., & Brashears, M. (2005). Reduction of *Escherichia coli* O157 in finishing beef cattle by various doses of *Lactobacillus acidophilus* in direct-fed microbials. *Journal of Food Protection*, *68*(1), 6–10. https://doi.org/10.4315/0362-028x-68.1.6

Zangari, T., Melton-Celsa, A. R., Panda, A., Smith, M. A., Tatarov, I., De Tolla, L., & O'Brien, A. D. (2014). Enhanced Virulence of the *Escherichia coli* O157:H7 Spinach-Associated Outbreak Strain in Two Animal Models Is Associated with Higher Levels of Stx2 Production after Induction with Ciprofloxacin. *Infection and Immunity*, *82*(12), 4968–4977. https://doi.org/10.1128/IAI.02361-14

Chapter 2 - Impact of the Probiotic Organism *Megasphaera elsdenii* on *Escherichia coli* O157:H7 Prevalence in Finishing Cattle

2.1. Abstract

Feedlot cattle commonly shed the foodborne pathogen *Escherichia coli* O157:H7 in their feces. *Megasphaera elsdenii* (ME), a lactic acid-utilizing bacterium, is commonly administered to cattle to avoid lactate accumulation in the rumen and control ruminal acidosis. The impact of administering ME on foodborne pathogen shedding, specifically *E. coli* O157:H7, has not been explored. The purpose of this study was to quantify *E. coli* O157:H7 prevalence in finishing cattle administered ME. Cattle (n=448) were assigned to 1 of 4 treatments in a randomized complete design with a 2x2 factorial arrangement of treatments containing: ruminally-protected lysine (LYS; Kemin Industries, Des Moines, IA) fed at 0 or 0.45% of diet dry matter; with or without ME. Freeze-dried ME was administered as an oral drench providing 1 x 10^{10} colony forming units (CFU) on day one of the study. On each day thereafter, freeze-dried ME was top-dressed onto basal feed diets to provide 1x10^{7} CFU/steer daily. Rectoanal mucosal swabs (RAMS) were obtained from animals twice before harvest, yielding 896 RAMS. Swabs were incubated in Gram-negative broth at 37^{o}C for 6 hours, subjected to immunomagnetic separation (IMS) with anti-O157 beads, spread-plated onto nt-Chromagar O157, and incubated for 18-24 hours at 37^{o}C. Latex agglutination was used to presumptively identify suspect colonies as *E. coli* O157 and polymerase chain reaction was used for *E. coli* O157:H7 confirmation. The ME x time (sampling period 1 vs 2) interaction was significant (P=0.0323), which indicates that *E. coli* O157:H7 prevalence varied based on ME dose and sampling period. More specifically, a diet containing 20 mL ME + top dressing reduced the odds of *E. coli* O157:H7 prevalence by 50%

during sampling period 1 (P=0.0921) and increased the odds by 23% during sampling period 2 (P=0.6130). Administering ME in cattle diets suggests variable efficacy at reducing *E. coli* O157:H7 in feedlot cattle and additional research is necessary given that this study is the first to investigate the use of ME as a pre-harvest food safety intervention in cattle.

2.2. Introduction

Escherichia coli O157:H7 is estimated to cause over 63,000 foodborne illnesses annually (Mead et al., 1999; Scallan et al., 2011), with cattle representing the main reservoir (Chapman et al., 1993; Hancock et al., 1997; C. J. McDaniel et al., 2014; Renwick et al., 1993; Whipp et al., 1994). *E. coli* O157:H7 is commonly shed by feedlot cattle, with studies showing up to 30% of cattle being asymptomatic carriers (Callaway et al., 2006, 2009; Reinstein et al., 2007; K. Stanford et al., 2005). The United States Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC), and Department of Agriculture (USDA) surveyed foodborne illness data from 1998-2017 and determined that 25.8% of foodborne *E. coli* O157:H7 illnesses were attributable to beef (Interagency Food Safety Analytics Collaboration, 2019). The relationship between cattle and this foodborne pathogen is well documented (Berry et al., 2017; Callaway et al., 2009; Castro et al., 2019; McDaniel et al., 2014; Whipp et al., 1994); however, the influence of animal diet and the host microbiota on shedding of *E. coli* O157:H7 is only beginning to be understood. Pre-harvest interventions aimed at reducing *E. coli* O157:H7 colonization and shedding in cattle, including the manipulation of diet and indigenous microbiota, require additional investigation.

Fasting, diet supplementation, and bacteriophage therapy have been investigated as potential pre-harvest food safety interventions in feedlot cattle (Thomas & Elliott, 2013). Antibiotics, once considered one of our best tools to prevent disease, have become less effective,

with some becoming obsolete (Centers for Disease Control and Prevention, 2019). Historically, producers administered antibiotics to cattle sub-therapeutically as growth promotants; however, in 2013 the U.S. Food and Drug Administration (FDA) released Guidance for Industry #213 containing a timeline for phasing out medically important antimicrobial drugs for use in food-producing animals while bringing these drugs under veterinary oversight through the Veterinary Feed Directive (VFD). This legislation changed antibiotic use in U.S. agriculture (Center for Veterinary Medicine, 2019a, 2019b). Growing concerns over the reliance on antibiotics in agricultural production, coupled with the public health threat of antimicrobial resistance (AMR; Centers for Disease Control and Prevention, 2019) urge investigation into alternative management practices, including the use of probiotics in feedlot operations.

The term probiotic, which is defined as "a live microbial feed supplement, benefiting the animal by improving its gastrointestinal microbial balance or preventing a disease outcome" (Fuller, 1992), describes microbial cultures or culture extracts of bacteria and fungi (Yoon & Stern, 1995). Inclusion of a ruminal probiotic, or a "live culture of microorganisms deliberately introduced into the rumen to improve animal health" (Kmet et al., 1993) has been a common practice since the early 1900s (Metchnikoff, 1908). Probiotics influence gastrointestinal tract microbial function in ruminants (Elam et al., 2003; Khan et al., 2016). Within the United States, the Food and Drug Administration (FDA) simplified the definition in 1989, requiring feed manufactures to use the term direct fed-microbial (DFM), which refers to "a source of live (viable) naturally-occurring microorganisms" (Brashears & Guillen, 2014). The addition of DFMs in cattle diets—a topic extensively studied in the last ten years—has demonstrated efficacy in reducing fecal shedding of *E. coli* O157:H7 (Callaway et al., 2009; Imperial & Ibana, 2016; Stanford et al., 2014; Valeriano et al., 2014; Wisener et al., 2015) with *Lactobacillus*

acidophilus NP51 most notably reducing *E. coli* O157:H7 shedding in cattle by 48-80% (Stephens et al., 2007; Younts-Dahl et al., 2005). In the U.S., DFMs in animal feed represent a significant market, with an estimated value of $4.6 billion in 2019 (Markets and Markets, 2019). Frameworks for characterizing novel probiotic strains (Ayala et al., 2018, 2019; Ren et al., 2019; Reuben et al., 2019) along with exploration of potential probiotic "cocktails" and accurate durations of administration have been the focus of previous research (Brown et al., 2020; Luedtke et al., 2016). Benefits of feeding DFMs are not limited to food safety applications, with enhances in average daily gain (ADG)—along with reductions in fecal *E. coli* counts—in calves also reported (Roodposhti & Dabiri, 2012). Similar data demonstrate a general increase in average daily gain (2.5%) and an estimated 2% improvement in animal efficiency when fed DFMs (Krehbiel et al., 2003). Alternatively, some research illustrates no improvement in animal performance, nutrient digestibility, or reductions of *E. coli* O157:H7 shedding (Encinas et al., 2018; Wilson et al., 2016). Feeding DFMs has shown varying results, which may be due to different strains, concentrations, and experimental designs; however, research indicates these microorganisms have potential to decrease shedding of *E. coli* O157:H7 (Wilson & Krehbiel, 2012), which is why they remain important as pre-harvest food safety interventions in cattle.

Megasphaera elsdenii is a lactic acid-utilizing bacterium present in the rumens of cattle (Chen et al., 2019). This organism, often used as a DFM, reduces lactic acid buildup within the rumen of grain-fed cattle by converting lactic acid to volatile fatty acids (VFAs; acetate, propionate, and butyrate), which are the main source of energy for ruminants (Chen & Wang, 2016; Chen et al., 2016; Weimer & Moen, 2013). Moreover; *M. elsdenii* moderates a common disorder in feedlot cattle known as ruminal acidosis (RA; Long et al., 2014; Weimer et al., 2015), characterized by lactate accumulation within the rumen followed by a drop in the pH of ruminal

fluid (Hernández et al., 2014; Kenney et al., 2015; Wang et al., 2015). As a DFM, *M. elsdenii* demonstrates value as a RA mitigating agent; however, research is needed to determine if this microorganism influences the prevalence of foodborne pathogen shedding—specifically *Escherichia coli* O157:H7—in finishing cattle. Addressing this knowledge gap is, therefore, the principle objective of this study.

2.3. Materials and Methods

2.3.1. Experimental Design

A 2x2 factorial experiment was conducted with crossbred steers (n=448; 352±25 kg initial body weight) blocked by initial body weight and, randomly assigned within block to one of 64 pens. Within each block, pens were randomly assigned to one of the 4 diets, with ¼ of the pens in each block assigned to each diet. Therefore, study pens were grouped in blocks of 4, with every block containing all 4 treatment combinations. Cattle were fed treatment diets consisting of *Megasphaera elsdenii* strain NCIMB 41152 (ME; Lactipro advance, MS Biotec, Wamego, KS) at one of 2 levels (0 or 20 mL ME [+Top Dressing]) in combination with ruminally-protected lysine (LYS; USA Lysine, Kemin Industries, Inc., Des Moines, IA) at one of 2 levels (0 g or 40 g LYS)/steer daily. Each diet was fed to 16 experimental pens consisting of 7 cattle per pen; thus, each dietary treatment was applied to 112 cattle in total.

Steers were sampled on two different occasions, hereafter referred to as sampling period 1 (SP1) and 2 (SP2). For each sampling period, half of the steers were sampled in each of two sampling days (D1 and D2), as is highlighted in Figure 2.1. Prevalence of *E. coli* O157:H7 was measured using rectoanal mucosal swabs (RAMS) obtained from each animal and analyzed using standard laboratory methods (Rice et al., 2003). Steers (n=448) were sampled twice, yielding a total of 896 RAMS samples in this study.

2.3.2. Cattle Diets

Experimental diets are identified in Table 2.1 as follows: -ME/-LYS (0 mL ME + 0 g LYS); -ME/+LYS (0 mL ME + 40 g LYS); +ME/-LYS (20 mL ME + 0 g LYS); and +ME/+LYS (20 mL ME + 40 g LYS). All cattle were started on a diet comprised of (dry matter basis) approximately 50% concentrate/50% roughage, and stepped-up within 10 days to a finishing ration containing 60.4% steam-flaked corn, 30% wet corn gluten feed, 7% forage, and a vitamin-mineral premixed supplement (Rumensin, Elanco Animal Health, Greenfield, IN) that provided monensin at 33 mg/kg diet dry matter. For the final 28 to 42 days before harvest, finishing diets contained 25 mg/kg ractopamine-HCl (Optaflexx, Elanco Animal Health, Greenfield, IN) (Table 2.2). Corn was conditioned by adding moisture (SarTec; Anoka, MN), steamed, and flaked daily utilizing a R & R Machine Works steam-flaker (46 x 91 cm corrugated rolls; Dalhart, TX). Corn was flaked to 360 g/L bulk density, which is consistent with previous research (Horton & Drouillard, 2018). Basal feed diet ingredients (Table 2.2) were analyzed on site daily for dry matter content, retained weekly and composited monthly for nutrient analysis (SDK Labs; Hutchinson, KS). Administration of ME and LYS to basal feed diets occurred during the entire feeding period. Animals were fed once daily *ad libitum*.

M. elsdenii was administered on day 1 as an oral drench, consisting of a 20-mL dose containing 5×10^8 CFU/ml, delivering a targeted amount of 1×10^{10} CFU/steer. On each day thereafter, ME at a concentration of 2×10^8 CFU/g was administered as a top-dressing to the finishing diet, such that each animal would receive a target dose of 1×10^7 CFU/steer daily, Delivered concentrations were based upon manufacturer reported concentrations and not further verified by research personnel. Top dressing was prepared by mixing twelve grams of freeze-dried ME (220 doses) with 16 lb of ground corn, and 0.5 lb of this mixture was delivered to each

ME treatment pen. A lipid-encapsulated, ruminally-protected lysine sulfate (LYS) was mixed within the total rations, and was included for separate analysis of diet effect on performance and carcass characteristics (Veloso et al., 2019). Following 117 (SP1D1), 119 (SP1D2), 124 (SP2D1) and 126 (SP2D2) days on feed, RAMS samples were obtained from each animal and analyzed using standard laboratory methods.

2.3.3. RAMS Sampling

Study personnel obtained a RAMS sample from each animal enrolled in the study during 2 separate sampling periods (SP1 and SP2). Cattle were sampled in cohorts of 224 animals per day, with 2 sampling days per sampling period (SP1D1, SP1D2, SP2D1, and SP2D2). The sampling scheme yielded 2 RAMS samples from each of the 448 enrolled animals, for a total of 896 total RAMS. Collection of RAMS followed protocols previously described (Greenquist et al., 2005, Rice et at., 2003). Briefly, a sterile foam-tipped swab (VWR, Radnor, PA) was inserted 3 to 5 cm into the anus of each animal. The swab vigorously scraped the mucosal surface using a rapid in-and-out motion. Following sampling, study personnel placed the swab into a sterile tube containing 3 mL of Gram-negative broth (GN Broth; Remel, Lenexa, KS) supplemented with 0.05 mg/L of cefixime (VCC Supplement; Millipore Sigma, St. Louis, MO), 10 mg/L of cefsulodin, and 8 mg/L of vancomycin (GNccv). Study personnel stored RAMS+GNccv sample tubes on ice immediately following sampling, followed by transport to the laboratory within 3 hours of concluding sample collection for further microbiological processing.

2.3.4. Microbiological Analysis

Upon arrival at the laboratory, RAMS+GNccv sample tubes were vortexed for 1 minute, 1 mL was transferred into a 9-mL GNccv enrichment tube, vortexed, and incubated at 37^{o}C for 6 hours. Samples were incubated in a programmable incubator that was programmed to hold the

incubated samples at 4°C until subsequent analyses were undertaken the following day. The incubated GNccv enrichment tubes were subjected to immunomagnetic separation (IMS) according to previously published methods (Chaney et al., 2018, Sargeant et al., 2003). Briefly, 1 mL of each enrichment was mixed with 20 µL of Dynabeads ™ anti-*E. coli* O157:H7 (Applied Biosystems; Foster City, CA) and then washed three times in phosphate-buffered saline with PBS Tween™ 20 (Thermo Fisher Scientific, Waltham, MA) using an automatic IMS machine (KingFisher ™ mL Food Protection Purification System; Thermo Fisher Scientific, Waltham, MA) according to manufacturer's specifications. A 50-µL aliquot of PBS-Tween containing the resultant bead-bacteria complexes was spread-plated onto ntChromagar plates (BBL™ CHROMagar™ O157; BD Difco, Franklin Lakes, NJ) supplemented with 5 mg/L novobiocin (Novobiocin supplement; Thermo Fisher Scientific, Waltham, MA) and 2.5 mg/L potassium tellurite (Potassium tellurite hydrate; Millipore Sigma, St. Louis, MO) and incubated at 37°C for 18 to 24 hours. Following incubation, up to 3 colonies per plate suspected as *E. coli* O157:H7 were presumptively confirmed using latex agglutination (Remel ™ RIM *E. coli* O157:H7 Latex Test; Thermo Fisher Scientific, Waltham, MA). From each plate, two presumptively positive colonies were then streaked for isolation on Sorbitol MacConkey agar (CT-SMAC; Remel, Lenexa, KS) supplemented with 0.05 mg/L cefixime and 2.5 mg/L potassium tellurite (CT-Supplement; Milipore Sigma, St. Louis, MO). Plates were incubated at 37°C for 18-24 hours and 1 colony was picked from each plate and transferred into 9 mL tryptic soy broth (TSB; BD Difco, Franklin Lakes, NJ) tubes. TSB tubes were incubated at 37°C for 18 to 24 hours and then 1 mL from each tube was transferred to a microcentrifuge tube containing glycerol, resulting in each *E. coli* O157:H7 presumptively confirmed isolate frozen in duplicate at -80°C in TSB with 10% glycerol. Each frozen isolate was subjected to polymerase chain reaction (PCR) using the

MicroSEQ™ *E. coli* O157:H7 Detection Kit (Applied Biosystems, Foster City, CA) with an ABI 7500 FAST real-time PCR machine (Applied Biosystems, Foster City, CA) to confirm the presence of *E. coli* O157:H7.

2.3.5. Statistical Analysis

Based upon PCR confirmation, the result of each RAMS sample was organized as a binary response (positive or negative for *E. coli* O157:H7). Data were analyzed using the logit linear mixed model with repeated measurements over time. Fixed effects of the model included ME treatment, LYS treatment, time, as well as all 2-way and 3-way interactions. Random effects of the model included block, pen and the model intercept (ear tag; the random intercept term accounts for the correlation of binary responses of individual cattle). Prevalence data are reported based upon this model, taking into account these parameters and referred to as "model-adjusted". Prevalence data which does not consider these parameters represents a crude analysis and is referred to as "not model adjusted". All tests were conducted at the P=0.05 significance level. Comparisons between 2 levels of a given treatment were carried out using 2-sided tests. Distributions of test statistics were approximated using Chi-square distributions. All statistical analysis was performed using the PROC GLIMMIX command of Statistical Analysis Software (SAS version 9.4; Cary, NC).

2.4. Results

The overall, non-adjusted prevalence for *E. coli* O157:H7 was 12.8% (n=28/219) for the -ME/-LYS diet, 22.3% (n=50/224) for the -ME/+LYS diet, 15.9% (n=35/220) for the +ME/-LYS diet, and 14.5% (32/220) for the +ME/+LYS diet. During SP1, the non-adjusted *E. coli* O157:H7 prevalence was 16.4% (n=18/110) for -ME/-LYS, 25.0% (n=28/112) for -ME/+LYS, 13.6% (n=15/110) for +ME/-LYS, and 13.6% (n=15/110) for +ME/+LYS. The non-adjusted *E. coli*

O157:H7 prevalence for each diet during SP2, was as follows: 9.2% (n=10/109) for -ME/-LYS, 19.6% (n=22/112) for -ME/+LYS, 18.2% (n=20/110) for +ME/-LYS, and 15.5% (n=17/110) for +ME/+LYS (Table 2.3).

Inclusion of LYS and ME in cattle diets did not statistically impact *E. coli* O157:H7 prevalence, with model-based *p*-values of 0.2136 and 0.5012, respectively. Additional fixed effects, including time (SP1 vs SP2; *P*=0.5750), and the 2- and 3-way interactions LYS x ME (*P*=0.1832), LYS x time (*P*=0.9081) and LYS x ME x time (*P*=0.3932) did not influence *E. coli* O157:H7 prevalence. The 2-way interaction of ME x time was statistically significant, with a *p*-value of 0.0323; therefore, data will be discussed according to ME at each sampling period.

During SP1, the odds of *E. coli* O157:H7 shedding in cattle fed diets supplemented with ME was observed to be 0.50 as high as that in cattle not fed ME (*P*=0.0921), with a prevalence of 14.7% and 8.0% for 0 mL ME and 20 mL + top dressing ME cattle, respectively. This indicates that a diet containing ME reduced the odds of *E. coli* O157:H7 prevalence by 50% in comparison to a diet not supplemented with ME during SP1. Conversely, during SP2, the odds of *E. coli* O157:H7 in cattle fed ME was observed to be 1.23 as high as cattle not fed ME (*P*=0.6130), which suggests that feeding ME increased the odds of *E. coli* O157:H7 prevalence by 23% in comparison to cattle fed a diet not containing ME. The SP2 prevalence of *E. coli* O157:H7 was 8.9% and 10.8% for 0 mL ME and 20 mL + top dressing ME cattle, respectively.

2.5. Discussion

In the present study, the efficacy of ME administered as a direct fed-microbial in cattle finishing diets to reduce the burden of *E. coli* O157:H7 in cattle was evaluated. Inclusion of ME in cattle finishing diets did not reduce the prevalence of *E. coli* O157:H7 in comparison to cattle fed control diets (*P*=0.5012). The ME x time (SP1 vs SP2) interaction was significant

(*P*=0.0323) indicating *E. coli* O157:H7 prevalence varied based upon ME dose and sampling period (Figure 2.2). More specifically, a diet containing ME reduced the odds of *E. coli* O157:H7 prevalence by 50% during sampling period 1 and increased the odds of *E. coli* O157:H7 prevalence by 23% during sampling period 2. These data suggest that ME may reduce *E. coli* O157:H7 prevalence in cattle inconsistently. This study is the first to investigate ME as a pre-harvest intervention in cattle; therefore, it is difficult to explain why a discrepancy between SP1 and SP2 was observed. Mechanistically, the observed influence of ME on *E. coli* O157:H7 prevalence in cattle is not well understood.

In order to explore the ME x time interaction (*P*=0.0323), along with the difference in odds of *E. coli* O157:H7 prevalence in SP1 vs SP2, it is important to consider the amount of time between when steers were given the oral drench of ME and when RAMs samples were taken. During SP1D1, SP1D2, SP2D1, and SP2D2 samples were collected 117, 119, 124, and 126 days, respectively, following the day 1 oral drench with 5 x 10^8 CFU/mL ME. Although a 7 day interval between SP1 and SP2 sampling days may seem marginal, it must be considered that slight differences in duration of DFM administration may yield different results, which is an area of research requiring further exploration (Brown et al., 2020). It is important to note that properties regarding rumen lactic acid accumulation inform current ME administration guidelines and have not been optimized for potential food safety applications.

It should also be noted that an unavoidable diet change occurred between SP1 and SP2. Due to a malfunctioning steam flaker, all cattle enrolled in the study were fed dry-rolled corn in place of steam-flaked corn for one day. Upon repair of the steam flaker, the cattle were fed a diet consisting of steam flaked and dry-rolled corn in a 1:1 ratio for one day. The following day all enrolled cattle returned to the original diet of steam-flaked corn with SP2D1 and SP2D2

occurring on the second and fourth day of resuming the steam-flaked diet, respectively. While this diet change was brief, it likely altered the amount of starch entering the hindgut (Corona et al., 2005; National Academies of Sciences, 2016). Animals were sampled shortly after resuming their original steam-flaked diets and the impact of this diet change on *E. coli* O157:H7 prevalence is unknown. All animals experienced the same diet change for the same amount of time and treatments were impacted equally.

Inclusion of ruminally-protected lysine to animal diets informed a separate analysis of the effect on animal performance and carcass characteristics (Veloso et al., 2019); however, the inclusion of lysine represents as a preliminary inquiry into potential food safety implications of adding amino acids to feedlot cattle diets. In this study, inclusion of LYS did not significantly impact *E. coli* O157:H7 prevalence, with a model-based *p*-value of 0.2136. Research reported in the literature suggests that the addition of glutamate, lysine, and arginine influences the amino acid-dependent acid resistance systems *of E. coli* O157:H7 (Diez-Gonzalez and Karaibrahimoglu, 2004); thus, future research should investigate the implications of adding lysine to cattle diets on foodborne pathogen prevalence and expression of resistance mechanisms.

Originally isolated and described as *Peptostreptococcus elsdenii* (Gutierrez et al., 1959), ME represents one of the few rumen anaerobic bacteria with a capacity to produce substantial amounts of volatile fatty acids from sugars and lactate (Rogosa, 1971; Weimer & Moen, 2013), aiding moderation of rumen pH (Chen et al., 2019). This bacterium, specifically the NCIMB strain 41125 present in Lactipro Advance, demonstrates the ability to halt rumen acidosis (RA) (DeClerck et al., 2020; Henning et al., 2010;McDaniel, 2009), with significant efficacy observed depending upon strain (Sedighi & Alipour, 2019), amount, and duration of inoculum (Yohe et

al., 2018). Research into this DFM has revealed additional applications; notably, orally dosing steers with ME has been shown to decrease the amount of roughage required during the adaptive step-up period, allowing cattle to be placed onto high-concentrate finishing diets more rapidly (Miller et al., 2013). DeClerck et al. (2020) suggested dosing cattle with ME favorably alters rumen ecology, promoting papillae growth. In the companion study, using ME as a preventative agent for ruminitis and liver abscessation—developed secondarily to RA—did not affect liver abscess incidence ($P<0.07$; Veloso et al., 2019). Following its original isolation in 1959, the past 70 years of research on this organism have shown potential for the promotion of both rumen health and animal productivity.

While the food safety application and microbial interaction of ME in the hindgut have not been documented to date, the function of ME within the rumen is well established, with insights being drawn from that body of work. As previously described, addition of ME to cattle feedlot diets helps alleviate disease states of the rumen. Subacute ruminal acidosis (SARA), a disease state characterized by a reversible rumen pH depression (between pH 5.8 and 5.5) for extended periods, is common in cattle fed high concentrate diets (Mao et al., 2013; Nagaraja & Titgemeyer, 2007; Plaizier et al., 2017) and often remedied by the administration of ME (Chen et al., 2019). Plaizier et al. (2017) reported that cattle fed SARA-inducing challenge diets, comprised of ground alfalfa or ground wheat and barley pellets, experience a change in microbiota diversity and composition of bacteria in both the foregut and hindgut (Plaizier et al., 2017). Notably, the SARA-inducing diet treatments significantly increased 1) the abundance of *E. coli* in cecal digesta and feces, and 2) the ME abundance in the rumen (Plaizier et al., 2017). The current body of knowledge suggests that cattle microbiota populations—in both the rumen and hindgut—are greatly impacted by SARA. While the present study investigated impact of

feeding ME on *E. coli* O157:H7 prevalence, the microbial interaction of ME with other microbes residing throughout the ruminant gastrointestinal tract—including the foodborne pathogen *E. coli* O157:H7—require further investigation.

Research into disease states like SARA and acute RA document lysis of Gram-negative bacteria within the rumen, leading to a release of endotoxins such as lipopolysaccharide (LPS) into the rumen fluid, triggering systemic inflammation (Chen et al., 2019; Chin et al., 2006; Li et al., 2012; Loor et al., 2016; Nagaraja & Titgemeyer, 2007). Additionally, research from Li et al. (2012) demonstrated increases in hindgut *E. coli* populations from cattle fed grain-based SARA-challenge diets, likely increasing the amount of hindgut LPS produced by lysed *E. coli* (Li et al., 2012). LPS alters the hindgut microbiota leading to additional animal disease states, including disruption of intestinal barrier integrity and increased epithelial permeability, resulting in translocation and systemic uptake of LPS (Eckel & Ametaj, 2016; Ghoshal et al., 2009; Guo et al., 2017; Khafipour et al., 2009; Williams et al., 2013). This increase in LPS concentration and *E. coli* population reported in the large intestine of animals exhibiting RA, demonstrates the importance of limiting RA in feedlot cattle. Reducing Gram-negative foodborne pathogens, such as *E. coli* O157:H7, in cattle by feeding ME could be beneficial for both food safety and prevention of RA. The use of DFMs like ME promote positive health outcomes, improve negative disease states, and potentially limit foodborne pathogens in cattle, simultaneously. Application of "systems biology", a concept for the integration of ruminant nutrition and physiology, is necessary in order to discover the complex interactions and properties within components of the cattle system, in this case the nutrition and microbial interactions (Loor et al., 2016).

Specifics regarding the use of DFMs as a pre-harvest food safety intervention in cattle have been described in the literature, with several mechanisms of action being identified. Competitive exclusion, a term describing prevention of establishment or proliferation of certain bacteria via introduction of beneficial bacteria into an ecological niche, represents a mechanism utilized by current DFMs in cattle production (LeJeune & Wetzel, 2007; McAllister et al., 2011). In cattle, *L. acidophilus* strain NP51 has been extensively studied, with the competitive exclusion qualities of this DFM demonstrating a reduction of *E. coli* O157:H7 shedding in cattle by 48 to 80% when fed at 1×10^9 CFU (Brashears et al., 2003; Stephens et al., 2007; Stephens et al., 2007; Younts-Dahl et al., 2005; Younts-Dahl et al., 2004). Another strategy includes the production of bacteriocins, organic acids, or other toxic compounds (Callaway et al., 2004). A probiotic culture containing *L. acidophilus* and *Streptococcus bovis* reduced *E. coli* O157:H7 shedding via increased production of Gram negative-toxic volatile fatty acids (VFAs) in infected calves (Ohya et al., 2000). Research on DFMs commonly suggests VFA production as the mechanism of action for reduced *E. coli* O157:H7 shedding in cattle (Brashears et al., 2003; Callaway et al., 2009; Tabe et al., 2008). Bacteriocins, have been isolated from non-pathogenic strains of *E. coli* (Schamberger & Diez-Gonzalez, 2004) containing potential anti-*E. coli* O157:H7 mechanisms. Additionally, bacteriocins from *L. paracasei, S. bovis,* and *L. rhamnosus* exhibiting a similar mechanism, have been isolated, yet are not fully understood (Lee et al., 2002; McAllister et al., 2011; Tsai et al., 2010). Heightened immune responses caused by DFMs—documented in the literature—help to eliminate pathogens from the ruminant hindgut (Davis et al., 2007; Lessard et al., 2009; McAllister et al., 2011; Raabis et al., 2019; Szabó et al., 2009). The data described herein suggest that ME may reduce *E. coli* O157:H7 shedding in cattle. Given that ME is a lactate utilizer producing VFAs from lactate in the rumen (Weimer &

Moen, 2013), a reasonable hypothesis would be that cattle fed diets supplemented with ME may experience an increase in VFA production in the hindgut, consequently decreasing *E. coli* O157:H7 shedding in cattle. Future research should focus on investigating this hypothesis, probing at ME mode of action and efficacy as a pre-harvest food safety intervention.

2.6. Conclusion

The inclusion of direct fed-microbials in animal diets has been widely used to enhance ruminant production and provides a practical means for the control of foodborne pathogens in cattle. To our knowledge, this was the first study to investigate the impact of feeding *Megasphaera elsdenii* on *Escherichia coli* O157:H7 shedding in cattle. The data described herein suggest that *M. elsdenii* may reduce *E. coli* O157:H7. This study indicates a preliminary investigation with additional research required to gain insight into the ability of *M. elsdenii* to reduce *E. coli* O157:H7 populations in cattle.

2.7. References

Alvarez Encinas, C. M., Villalobos, G., Dominguez Viveros, J., Corral Flores, G., Alvarez Almora, E., & Castillo Rangel, F. (2018). Animal performance and nutrient digestibility of feedlot steers fed a diet supplemented with a mixture of direct-fed microbials and digestive enzymes. *Revista Brasileira De Zootecnia-Brazilian Journal of Animal Science*, *47*, e20170121. https://doi.org/10.1590/rbz4720170121

Ayala, D. I., Chen, J. C., Bugarel, M., Loneragan, G. H., den Bakker, H. C., Kottapalli, K. R., Brashears, M. M., & Nightingale, K. K. (2018). Molecular detection and quantification of viable probiotic strains in animal feedstuffs using the commercial direct fed microbial Lactobacillus animalis NP51 as a model. *Journal of Microbiological Methods*, *149*, 36–43. https://doi.org/10.1016/j.mimet.2018.04.012

Ayala, Diana I., Cook, P. W., Franco, J. G., Bugarel, M., Kottapalli, K. R., Loneragan, G. H., Brashears, M. M., & Nightingale, K. K. (2019). A Systematic Approach to Identify and Characterize the Effectiveness and Safety of Novel Probiotic Strains to Control Foodborne Pathogens. *Frontiers in Microbiology*, *10*, 1108. https://doi.org/10.3389/fmicb.2019.01108

Berry, M. E., Wells, M. J., Schmidt, M. J. W., Bosilevac, M. J. M., Fratamico, D. P. M., Hilbert, F., Smulders, F. J. M., Paulsen, P. P., Dewell, P. G., Post, D. L., James, D. W., Bolton, D. D. J., Riley, M. J. M., Foster, T. P., & Buskirk, D. D. D. (2017). *Ensuring safety and quality in the production of beef Volume 1* (P. G. R. Acuff & P. J. S. Dickson, Eds.; 1 edition). Burleigh Dodds Science Publishing.

Brashears, M., Jaroni, D., & Trimble, J. (2003). Isolation, Selection, and Characterization of Lactic Acid Bacteria for a Competitive Exclusion Product To Reduce Shedding of *Escherichia coli* O157:H7 in Cattle. *Journal of Food Protection*, *66*(3), 355–363. https://doi.org/10.4315/0362-028X-66.3.355

Brashears, M. M., Galyean, M. L., Loneragan, G. H., Mann, J. E., & Killinger-Mann, K. (2003). Prevalence of *Escherichia coli* O157:H7 and performance by beef feedlot cattle given Lactobacillus direct-fed microbials. *Journal of Food Protection*, *66*(5), 748–754. https://doi.org/10.4315/0362-028x-66.5.748

Brashears, Mindy M., & Guillen, L. M. (2014, February 5). Direct-Fed Microbials as Preharvest Interventions in Cattle Production. *Food Safety Magazine*. https://www.foodsafetymagazine.com/magazine-archive1/februarymarch-2014/direct-fed-microbials-as-preharvest-interventions-in-cattle-production/

Brown, T. R., Edrington, T. S., Genovese, K. J., He, H. L., Anderson, R. C., & Nisbet, D. J. (2020). Evaluation of the Efficacy of Three Direct Fed Microbial Cocktails To Reduce Fecal Shedding of *Escherichia coli* O157:H7 in Naturally Colonized Cattle and Fecal Shedding and Peripheral Lymph Node Carriage of Salmonella in Experimentally Infected Cattle. *Journal of Food Protection*, *83*(1), 28–36. https://doi.org/10.4315/0362-028X.JFP-19-208

Callaway, T., Anderson, R. C., Edrington, T. S., Genovese, K. J., Harvey, R. B., Poole, T. L., & Nisbet, D. J. (2004). Recent pre-harvest supplementation strategies to reduce carriage and shedding of zoonotic enteric bacterial pathogens in food animals. *Animal Health Research Reviews*, *5*(1), 35–47. https://doi.org/10.1079/ahr200462

Callaway, T., Carr, M. A., Edrington, T. S., Anderson, R. C., & Nisbet, D. J. (2009). Diet, *Escherichia coli* O157:H7, and cattle: A review after 10 years. *Current Issues in Molecular Biology*, *11*(2), 67–79.

Callaway, T., Edrington, T. s., Brabban, A. d., Keen, J. e., Anderson, R. c., Rossman, M. l., Engler, M. j., Genovese, K. j., Gwartney, B. l., Reagan, J. o., Poole, T. l., Harvey, R. b., Kutter, E. m., & Nisbet, D. j. (2006). Fecal Prevalence of *Escherichia coli* O157, Salmonella, Listeria, and Bacteriophage Infecting *E. coli* O157:H7 in Feedlot Cattle in the Southern Plains Region of the United States. *Foodborne Pathogens and Disease*, *3*(3), 234–244. https://doi.org/10.1089/fpd.2006.3.234

Castro, V. S., Figueiredo, E. E. de S., Stanford, K., McAllister, T., & Conte-Junior, C. A. (2019). Shiga-Toxin Producing *Escherichia Coli* in Brazil: A Systematic Review. *Microorganisms*, *7*(5). https://doi.org/10.3390/microorganisms7050137

Center for Veterinary Medicine. (2019a, April 16). *CVM GFI #213 New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food-Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GFI #209*. U.S. Food and Drug Administration. http://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-213-new-animal-drugs-and-new-animal-drug-combination-products-administered-or-medicated-feed

Center for Veterinary Medicine. (2019b). *Veterinary Feed Directive (VFD)*. Food and Drug Administration. http://www.fda.gov/animal-veterinary/development-approval-process/veterinary-feed-directive-vfd

Centers for Disease Control and Prevention. (2019). *Antibiotic Resistance Threats in the United States, 2019*. 148.

Chaney, W. E., Maloney, R., Johnson, B. J., Brooks, J. C., Brashears, M. M., & Loneragan, G. H. (2018). Corn-Based Distillers' Grains in Diets for Feedlot Cattle Are Associated with the Burden of *Escherichia coli* O157 in Feces. *Foodborne Pathogens and Disease*, *15*(7), 398–405. https://doi.org/10.1089/fpd.2017.2395

Chapman, P. A., Siddons, C. A., Wright, D. J., Norman, P., Fox, J., & Crick, E. (1993). Cattle as a possible source of verocytotoxin-producing *Escherichia coli* O157 infections in man. *Epidemiology and Infection*, *111*(3), 439–447. https://doi.org/10.1017/s0950268800057162

Chen, L., & Wang, H. (2016). Advances in the metabolism and regulation of lactic acids in the rumen. *Pratacult. Sci.*, *33*, 972–980.

Chen, Lianmin, Shen, Y., Wang, C., Ding, L., Zhao, F., Wang, M., Fu, J., & Wang, H. (2019). *Megasphaera elsdenii* Lactate Degradation Pattern Shifts in Rumen Acidosis Models. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00162

Chen, Lianmin, Shen, Y., & Wang, H. (2016). Metabolic mechanism of acid production by *Streptococcus bovis* in rumen and it's regulation. *Chinese Journal of Animal Nutrition*, *28*, 665–673.

Chin, A. C., Flynn, A. N., Fedwick, J. P., & Buret, A. G. (2006). The role of caspase-3 in lipopolysaccharide-mediated disruption of intestinal epithelial tight junctions. *Canadian Journal of Physiology and Pharmacology*, *84*(10), 1043–1050. https://doi.org/10.1139/y06-056

Corona, L., Rodriguez, S., Ware, R. A., & Zinn, R. A. (2005). Comparative Effects of Whole, Ground, Dry-Rolled, and Steam-Flaked Corn on Digestion and Growth Performance in Feedlot Cattle. *The Professional Animal Scientist*, *21*(3), 200–206. https://doi.org/10.15232/S1080-7446(15)31203-1

Davis, E., Brown, D. C., Baker, A., Bos, K., Dirain, M. S., Halbrook, E., Johnson, Z. B., Maxwell, C., & Rehberger, T. (2007). Effect of direct-fed microbial and antibiotic supplementation on gastrointestinal microflora, mucin histochemical characterization, and immune populations of weanling pigs. *Livestock Science - LIVEST SCI*, *108*, 249–253. https://doi.org/10.1016/j.livsci.2007.01.063

DeClerck, J. C., Reeves, N. R., Miller, M. F., Johnson, B. J., Ducharme, G. A., & Rathmann, R. J. (2020). The influence of *Megasphaera elsdenii* on rumen morphometrics of cull cows immediately stepped up to a high-energy finishing diet. *Translational Animal Science*, *4*(1). https://doi.org/10.1093/tas/txz185

Diez-Gonzalez, F., & Karaibrahimoglu, Y. (2004). Comparison of the glutamate-, arginine- and lysine-dependent acid resistance systems in *Escherichia coli* O157:H7. *Journal of Applied Microbiology*, *96*(6), 1237–1244. https://doi.org/10.1111/j.1365-2672.2004.02251.x

Eckel, E. F., & Ametaj, B. N. (2016). Invited review: Role of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. *Journal of Dairy Science*, *99*(8), 5967–5990. https://doi.org/10.3168/jds.2015-10727

Elam, N. A., Gleghorn, J. F., Rivera, J. D., Galyean, M. L., Defoor, P. J., Brashears, M. M., & Younts-Dahl, S. M. (2003). Effects of live cultures of *Lactobacillus acidophilus* (strains NP45 and NP51) and *Propionibacterium freudenreichii* on performance, carcass, and intestinal characteristics, and *Escherichia coli* strain O157 shedding of finishing beef steers. *Journal of Animal Science*, *81*(11), 2686–2698. https://doi.org/10.2527/2003.81112686x

Fuller, R. (1992). History and development of probiotics. In R. Fuller (Ed.), *Probiotics: The scientific basis* (pp. 1–8). Springer Netherlands. https://doi.org/10.1007/978-94-011-2364-8_1

Ghoshal, S., Witta, J., Zhong, J., Villiers, W. de, & Eckhardt, E. (2009). Chylomicrons promote intestinal absorption of lipopolysaccharides. *Journal of Lipid Research*, *50*(1), 90–97. https://doi.org/10.1194/jlr.M800156-JLR200

Greenquist, M. A., Drouillard, J. S., Sargeant, J. M., Depenbusch, B. E., Shi, X., Lechtenberg, K. F., & Nagaraja, T. G. (2005). Comparison of Rectoanal Mucosal Swab Cultures and Fecal Cultures for Determining Prevalence of Escherichia coli O157:H7 in Feedlot Cattle. *Applied and Environmental Microbiology*, *71*(10), 6431–6433. https://doi.org/10.1128/AEM.71.10.6431-6433.2005

Guo, J., Chang, G., Zhang, K., Xu, L., Jin, D., Bilal, M. S., & Shen, X. (2017). Rumen-derived lipopolysaccharide provoked inflammatory injury in the liver of dairy cows fed a high-concentrate diet. *Oncotarget*, *8*(29), 46769–46780. https://doi.org/10.18632/oncotarget.18151

Gutierrez, J., Davis, R. E., Lindahl, I. L., & Warwick, E. J. (1959). Bacterial changes in the rumen during the onset of feed-lot bloat of cattle and characteristics of Peptostreptococcus elsdenii n. Sp. *Applied Microbiology*, *7*(1), 16–22.

Hancock, D. D., Besser, T. E., Rice, D. H., Herriott, D. E., & Tarr, P. I. (1997). A longitudinal study of *Escherichia coli* O157 in fourteen cattle herds. *Epidemiology and Infection*, *118*(2), 193–195. https://doi.org/10.1017/s0950268896007212

Henning, P. H., Horn, C. H., Steyn, D. G., Meissner, H. H., & Hagg, F. M. (2010). The potential of *Megasphaera elsdenii* isolates to control ruminal acidosis. *Animal Feed Science and Technology*, *157*(1), 13–19. https://doi.org/10.1016/j.anifeedsci.2009.12.011

Hernández, J., Benedito, J. L., Abuelo, A., & Castillo, C. (2014). Ruminal acidosis in feedlot: From aetiology to prevention. *TheScientificWorldJournal*, *2014*, 702572. https://doi.org/10.1155/2014/702572

Horton, L., & Drouillard, J. (2018). 57 Effects of particle size and heat treatment on in situ and in vitro digestion of dry-rolled Enogen® Feed Corn. *Journal of Animal Science*, *96*, 391–391. https://doi.org/10.1093/jas/sky404.857

Imperial, I. C. V. J., & Ibana, J. A. (2016). Addressing the Antibiotic Resistance Problem with Probiotics: Reducing the Risk of Its Double-Edged Sword Effect. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01983

Interagency Food Safety Analytics Collaboration. (2019). Foodborne illness source attribution estimates for 2017 for *Salmonella, Escherichia coli O157, Listeria monocytogenes,* and *Campylobacter* using multi-year outbreak surveillance data, United States (p. 14).

Kenney, N. M., Vanzant, E. S., Harmon, D. L., & McLeod, K. R. (2015). Direct-fed microbials containing lactate-producing bacteria influence ruminal fermentation but not lactate utilization in steers fed a high-concentrate diet. *Journal of Animal Science*, *93*(5), 2336–2348. https://doi.org/10.2527/jas.2014-8570

Khafipour, E., Krause, D. O., & Plaizier, J. C. (2009). A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *Journal of Dairy Science*, *92*(3), 1060–1070. https://doi.org/10.3168/jds.2008-1389

Khan, R. U., Naz, S., Dhama, K., Karthik, K., Tiwari, R., Abdelrahma, M. M., Alhidary, I., & Zahoor, A. (2016). Direct-Fed Microbial: Beneficial Applications, Modes of Action and Prospects as a Safe Tool for Enhancing Ruminant Production and Safeguarding Health. https://doi.org/10.3923/ijp.2016.220.231

Kmet, V., Flint, H. J., & Wallace, R. J. (1993). Probiotics and manipulation of rumen development and function. *Archiv Fur Tierernahrung*, *44*(1), 1–10. https://doi.org/10.1080/17450399309386053

Krehbiel, C. R., Rust, S. R., Zhang, G., & Gilliland, S. E. (2003). Bacterial direct-fed microbials in ruminant diets: Performance response and mode of action. *Journal of Animal Science*, *81*(14_suppl_2), E120–E132. https://doi.org/10.2527/2003.8114_suppl_2E120x

Lee, S. S., Hsu, J.-T., Mantovani, H. C., & Russell, J. B. (2002). The effect of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, on ruminal methane production in vitro. *FEMS Microbiology Letters*, *217*(1), 51–55. https://doi.org/10.1111/j.1574-6968.2002.tb11455.x

LeJeune, J. T., & Wetzel, A. N. (2007). Preharvest control of *Escherichia coli* O157 in cattle. *Journal of Animal Science*, *85*(13 Suppl), E73-80. https://doi.org/10.2527/jas.2006-612

Lessard, M., Dupuis, M., Gagnon, N., Nadeau, E., Matte, J. J., Goulet, J. J., & Fairbrother, J. M. (2009). Administration of *Pediococcus acidilactici* or *Saccharomyces cerevisiae boulardii* modulates development of porcine mucosal immunity and reduces intestinal bacterial translocation after *Escherichia coli* challenge. *Journal of Animal Science*, *87*(3), 922–934. https://doi.org/10.2527/jas.2008-0919

Li, S., Khafipour, E., Krause, D. O., Kroeker, A., Rodriguez-Lecompte, J. C., Gozho, G. N., & Plaizier, J. C. (2012). Effects of subacute ruminal acidosis challenges on fermentation and endotoxins in the rumen and hindgut of dairy cows. *Journal of Dairy Science*, *95*(1), 294–303. https://doi.org/10.3168/jds.2011-4447

Long, M., Feng, W. J., Li, P., Zhang, Y., He, R. X., Yu, L. H., He, J. B., Jing, W. Y., Li, Y. M., Wang, Z., & Liu, G. W. (2014). Effects of the acid-tolerant engineered bacterial strain *Megasphaera elsdenii* H6F32 on ruminal pH and the lactic acid concentration of simulated rumen acidosis in vitro. *Research in Veterinary Science*, *96*(1), 28–29. https://doi.org/10.1016/j.rvsc.2013.11.013

Loor, J. J., Elolimy, A. A., & McCann, J. C. (2016). Dietary impacts on rumen microbiota in beef and dairy production. *Animal Frontiers*, *6*(3), 22–29. https://doi.org/10.2527/af.2016-0030

Luedtke, B. E., Bosilevac, J. M., Harhay, D. M., & Arthur, T. M. (2016). Effect of Direct-Fed Microbial Dosage on the Fecal Concentrations of Enterohemorrhagic *Escherichia coli* in Feedlot Cattle. *Foodborne Pathogens and Disease*, *13*(4), 190–195. https://doi.org/10.1089/fpd.2015.2063

Mao, S. Y., Zhang, R. Y., Wang, D. S., & Zhu, W. Y. (2013). Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. *Anaerobe*, *24*, 12–19. https://doi.org/10.1016/j.anaerobe.2013.08.003

Markets and Markets. (2019, April). Probiotics in Animal Feed Market by Livestock, Source, Form, and Region—Global Forecast 2025. https://www.marketsandmarkets.com/Market-Reports/probiotics-animal-feed-market-85832335.html?gclid=CjwKCAiAvonyBRB7EiwAadauqQeDMGT55u-SoJSS1AptQDXv4_LkxqUnwDfao_-N8QsVqA_0Ao3R4xoChKwQAvD_BwE

McAllister, T. A., Beauchemin, K. A., Alazzeh, A. Y., Baah, J., Teather, R. M., & Stanford, K. (2011). Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. *Canadian Journal of Animal Science*, *91*(2), 193–211. https://doi.org/10.4141/cjas10047

McDaniel, C. J., Cardwell, D. M., Moeller, R. B., & Gray, G. C. (2014). Humans and cattle: A review of bovine zoonoses. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.)*, *14*(1), 1–19. https://doi.org/10.1089/vbz.2012.1164

McDaniel, M. R. (2009). The effects of dosing feedlot cattle with *Megasphaera elsdenii* strain NCIMB 41125 prior to the introduction of a grain-rich diet [Thesis, Kansas State University]. https://krex.k-state.edu/dspace/handle/2097/1666

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, *5*(5), 607–625.

Metchnikoff, I. I. (1908). *The Prolongation of Life: Optimistic Studies*. Springer Publishing Company.

Miller, K. A., Van Bibber-Krueger, C. L., & Drouillard, J. S. (2013). Orally dosing steers with Lactipro (*Megasphaera elsdenii*) decreases the quantity of roughages fed during finishing. *Kansas Agricultural Experiment Station Research Reports*, *1*, 55–58. https://doi.org/10.4148/2378-5977.1446

Nagaraja, T. G., & Titgemeyer, E. C. (2007). Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. *Journal of Dairy Science*, *90 Suppl 1*, E17-38. https://doi.org/10.3168/jds.2006-478

National Academies of Sciences, E. (2016). *Nutrient Requirements of Beef Cattle: Eighth Revised Edition*. https://doi.org/10.17226/19014

Ohya, T., Marubashi, T., & Ito, H. (2000). Significance of fecal volatile fatty acids in shedding of *Escherichia coli* O157 from calves: Experimental infection and preliminary use of a probiotic product. *The Journal of Veterinary Medical Science*, *62*(11), 1151–1155. https://doi.org/10.1292/jvms.62.1151

Plaizier, J. C., Li, S., Tun, H. M., & Khafipour, E. (2017). Nutritional Models of Experimentally-Induced Subacute Ruminal Acidosis (SARA) Differ in Their Impact on Rumen and Hindgut Bacterial Communities in Dairy Cows. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.02128

Raabis, S., Li, W., & Cersosimo, L. (2019). Effects and immune responses of probiotic treatment in ruminants. *Veterinary Immunology and Immunopathology*, *208*, 58–66. https://doi.org/10.1016/j.vetimm.2018.12.006

Reinstein, S., Fox, J. T., Shi, X., & Nagaraja, T. G. (2007). Prevalence of *Escherichia coli* O157:H7 in gallbladders of beef cattle. *Applied and Environmental Microbiology*, *73*(3), 1002–1004. https://doi.org/10.1128/AEM.02037-06

Ren, H., Saliu, E.-M., Zentek, J., Goodarzi Boroojeni, F., & Vahjen, W. (2019). Screening of Host Specific Lactic Acid Bacteria Active Against *Escherichia coli* From Massive Sample Pools With a Combination of in vitro and ex vivo Methods. *Frontiers in Microbiology*, *10*, 2705. https://doi.org/10.3389/fmicb.2019.02705

Renwick, S. A., Wilson, J. B., Clarke, R. C., Lior, H., Borczyk, A. A., Spika, J., Rahn, K., McFadden, K., Brouwer, A., & Copps, A. (1993). Evidence of direct transmission of *Escherichia coli* O157:H7 infection between calves and a human. *The Journal of Infectious Diseases*, *168*(3), 792–793. https://doi.org/10.1093/infdis/168.3.792

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, R.-U., & Jahid, I. K. (2019). Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. *BMC Microbiology*, *19*(1), 253. https://doi.org/10.1186/s12866-019-1626-0

Rice, D. H., Sheng, H. Q., Wynia, S. A., & Hovde, C. J. (2003). Rectoanal Mucosal Swab Culture Is More Sensitive Than Fecal Culture and Distinguishes *Escherichia coli* O157:H7-Colonized Cattle and Those Transiently Shedding the Same Organism. *Journal of Clinical Microbiology*, *41*(11), 4924–4929. https://doi.org/10.1128/JCM.41.11.4924-4929.2003

Rogosa, M. (1971). Transfer of *Peptostreptococcus elsdenii* Gutierrez et al. To a New Genus, *Megasphaera* [*M. elsdenii* (Gutierrez et al.) comb. Nov.]. *International Journal of Systematic and Evolutionary Microbiology*, *21*(2), 187–189. https://doi.org/10.1099/00207713-21-2-187

Roodposhti, P. M., & Dabiri, N. (2012). Effects of Probiotic and Prebiotic on Average Daily Gain, Fecal Shedding of *Escherichia coli*, and Immune System Status in Newborn Female Calves. *Asian-Australasian Journal of Animal Sciences*, *25*(9), 1255–1261. https://doi.org/10.5713/ajas.2011.11312

Sargeant, J. M., Sanderson, M. W., Smith, R. A., & Griffin, D. D. (2003). *Escherichia coli* O157 in feedlot cattle feces and water in four major feeder-cattle states in the USA. *Preventive Veterinary Medicine*, *61*(2), 127–135. https://doi.org/10.1016/s0167-5877(03)00166-1

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.P11101

Schamberger, G. P., & Diez-Gonzalez, F. (2004). Characterization of colicinogenic Escherichia coli strains inhibitory to enterohemorrhagic *Escherichia coli*. *Journal of Food Protection*, *67*(3), 486–492. https://doi.org/10.4315/0362-028x-67.3.486

Sedighi, R., & Alipour, D. (2019). Assessment of probiotic effects of isolated *Megasphaera elsdenii* strains in Mehraban sheep and Holstein lactating cows. *Animal Feed Science and Technology*, *248*, 126–131. https://doi.org/10.1016/j.anifeedsci.2019.01.007

Stanford, K., Croy, D., Bach, S. J., Wallins, G. L., Zahiroddini, H., & McAllister, T. A. (2005). Ecology of *Escherichia coli* O157:H7 in commercial dairies in southern Alberta. *Journal of Dairy Science*, *88*(12), 4441–4451. https://doi.org/10.3168/jds.S0022-0302(05)73131-3

Stanford, Kim, Bach, S., Baah, J., & McAllister, T. I. M. (2014). A Mixture of *Lactobacillus casei*, *Lactobacillus lactis*, and *Paenibacillus polymyxa* Reduces *Escherichia coli* O157:H7 in Finishing Feedlot Cattle. *Journal of Food Protection*, *77*(5), 738–744. https://doi.org/10.4315/0362-028X.JFP-13-433

Stephens, T., Loneragan, G., Chichester, L., & Brashears, M. (2007). Prevalence and enumeration of *Escherichia coli* O157 in steers receiving various strains of *Lactobacillus*-based direct-fed microbials. *Journal of Food Protection*, *70*(5), 1252–1255. https://doi.org/10.4315/0362-028x-70.5.1252

Stephens, T., Loneragan, G., Karunasena, E., & Brashears, M. (2007). Reduction of *Escherichia coli* O157 and *Salmonella* in feces and on hides of feedlot cattle using various doses of a direct-fed microbial. *Journal of Food Protection*, *70*(10), 2386–2391. https://doi.org/10.4315/0362-028x-70.10.2386

Szabó, I., Wieler, L. H., Tedin, K., Scharek-Tedin, L., Taras, D., Hensel, A., Appel, B., & Nöckler, K. (2009). Influence of a Probiotic Strain of *Enterococcus faecium* on *Salmonella enterica* Serovar *Typhimurium* DT104 Infection in a Porcine Animal Infection Model. *Applied and Environmental Microbiology*, *75*(9), 2621–2628. https://doi.org/10.1128/AEM.01515-08

Tabe, E., Oloya, J., Doetkott, D., Bauer, M., Gibbs, P., & Khaitsa, M. (2008). Comparative Effect of Direct-Fed Microbials on Fecal Shedding of *Escherichia coli* O157:H7 and *Salmonella* in Naturally Infected Feedlot Cattle | Journal of Food Protection | Allen Press. *Journal of Food Protection*, *71*(3). https://meridian.allenpress.com/jfp/article/71/3/539/172825/Comparative-Effect-of-DirectFed-Microbials-on

Thomas, D. E., & Elliott, E. J. (2013). Interventions for preventing diarrhea-associated hemolytic uremic syndrome: Systematic review. *BMC Public Health*, *13*, 799. https://doi.org/10.1186/1471-2458-13-799

Tsai, Y.-T., Cheng, P.-C., & Pan, T.-M. (2010). Immunomodulating Activity of *Lactobacillus paracasei* subsp. *Paracasei* NTU 101 in Enterohemorrhagic *Escherichia coli* O157H7-Infected Mice. *Journal of Agricultural and Food Chemistry*, *58*(21), 11265–11272. https://doi.org/10.1021/jf103011z

Valeriano, V. D., Parungao-Balolong, M. M., & Kang, D.-K. (2014). In vitro evaluation of the mucin-adhesion ability and probiotic potential of *Lactobacillus mucosae* LM1. *Journal of Applied Microbiology*, *117*(2), 485–497. https://doi.org/10.1111/jam.12539

Veloso, V. de A., Horton, L., Baker, A., Aperce, C., & Drouillard, J. (2019). 333 Effects of ruminally-protected lysine and *Megasphaera elsdenii* on performance and carcass characteristics of finishing cattle. *Journal of Animal Science*, *97*(Supplement_2), 134–134. https://doi.org/10.1093/jas/skz122.238

Wang, H., Pan, X., Wang, C., Wang, M., & Yu, L. (2015). Effects of different dietary concentrate to forage ratio and thiamine supplementation on the rumen fermentation and ruminal bacterial community in dairy cows. *Animal Production Science*, *55*(2), 189–193. https://doi.org/10.1071/AN14523

Weimer, P. J., Da Silva Cabral, L., & Cacite, F. (2015). Effects of ruminal dosing of Holstein cows with *Megasphaera elsdenii* on milk fat production, ruminal chemistry, and bacterial strain persistence1. *Journal of Dairy Science*, *98*(11), 8078–8092. https://doi.org/10.3168/jds.2015-9721

Weimer, P. J., & Moen, G. N. (2013). Quantitative analysis of growth and volatile fatty acid production by the anaerobic ruminal bacterium *Megasphaera elsdenii* T81. *Applied Microbiology and Biotechnology*, *97*(9), 4075–4081. https://doi.org/10.1007/s00253-012-4645-4

Whipp, S. C., Rasmussen, M. A., & Cray, W. C. (1994). Animals as a source of *Escherichia coli* pathogenic for human beings. *Journal of the American Veterinary Medical Association*, *204*(8), 1168–1175.

Williams, J. M., Duckworth, C. A., Watson, A. J. M., Frey, M. R., Miguel, J. C., Burkitt, M. D., Sutton, R., Hughes, K. R., Hall, L. J., Caamaño, J. H., Campbell, B. J., & Pritchard, D. M. (2013). A mouse model of pathological small intestinal epithelial cell apoptosis and

shedding induced by systemic administration of lipopolysaccharide. *Disease Models & Mechanisms*, *6*(6), 1388–1399. https://doi.org/10.1242/dmm.013284

Wilson, B. K., Holland, B. P., Step, D. L., Jacob, M. E., VanOverbeke, D. L., Richards, C. J., Nagaraja, T. G., & Krehbiel, C. R. (2016). Feeding wet distillers grains plus solubles with and without a direct-fed microbial to determine performance, carcass characteristics, and fecal shedding of O157:H7 in feedlot heifers. *Journal of Animal Science*, *94*(1), 297–305. https://doi.org/10.2527/jas.2015-9601

Wilson, B., & Krehbiel, C. (2012). *Current and Future Status of Practical Applications: Beef Cattle* (pp. 137–152). https://doi.org/10.1007/978-1-4614-1311-0_9

Wisener, L. V., Sargeant, J. M., O'Connor, A. M., Faires, M. C., & Glass-Kaastra, S. K. (2015). The use of direct-fed microbials to reduce shedding of *Escherichia coli* O157 in beef cattle: A systematic review and meta-analysis. *Zoonoses and Public Health*, *62*(2), 75–89. https://doi.org/10.1111/zph.12112

Yohe, T. T., Enger, B. D., Wang, L., Tucker, H. L. M., Ceh, C. A., Parsons, C. L. M., Yu, Z., & Daniels, K. M. (2018). Short communication: Does early-life administration of a *Megasphaera elsdenii* probiotic affect long-term establishment of the organism in the rumen and alter rumen metabolism in the dairy calf? *Journal of Dairy Science*, *101*(2), 1747–1751. https://doi.org/10.3168/jds.2017-12551

Yoon, I. K., & Stern, M. D. (1995). Influence of direct-fed microbials on ruminal microbial fermentation and performance of ruminants—A Review -. *Asian-Australasian Journal of Animal Sciences*, *8*(6), 533–555. https://doi.org/1995.8.6.533

Younts-Dahl, S. M., Galyean, M. L., Loneragan, G. H., Elam, N. A., & Brashears, M. M. (2004). Dietary supplementation with Lactobacillus- and Propionibacterium-based direct-fed microbials and prevalence of *Escherichia coli* O157 in beef feedlot cattle and on hides at harvest. *Journal of Food Protection*, *67*(5), 889–893. https://doi.org/10.4315/0362-028x-67.5.889

Younts-Dahl, S., Osborn, G., Galyean, M., Rivera, D., Loneragan, G., & Brashears, M. (2005). Reduction of *Escherichia coli* O157 in finishing beef cattle by various doses of *Lactobacillus acidophilus* in direct-fed microbials. *Journal of Food Protection*, *68*(1), 6–10. https://doi.org/10.4315/0362-028x-68.1.6

2.8. Tables & Figures

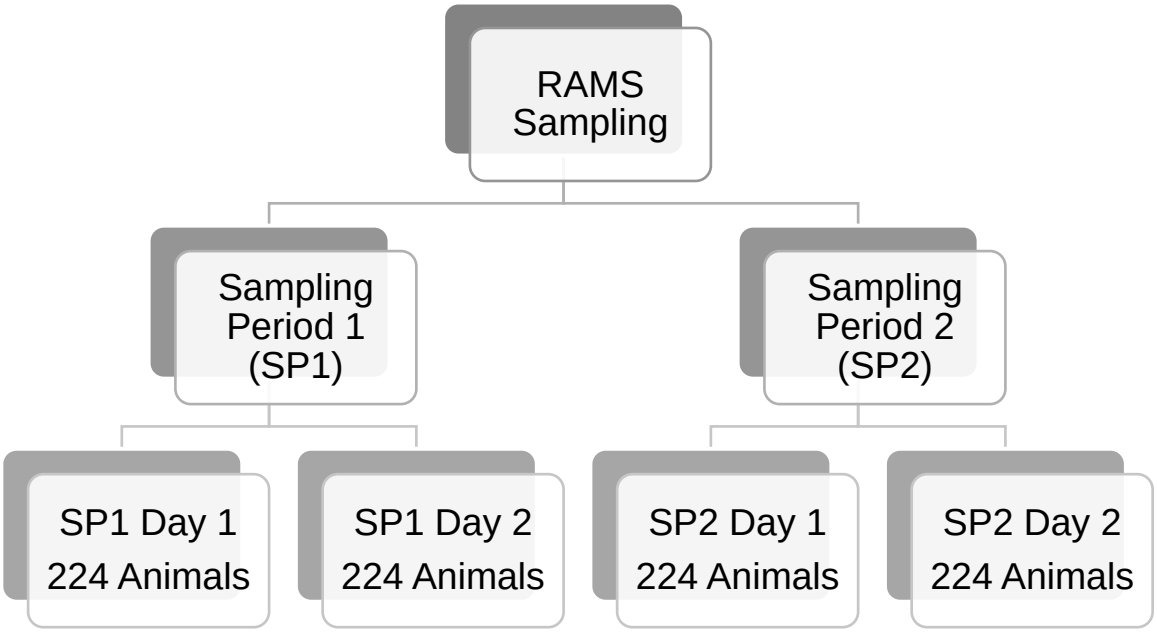

Figure 2.1. RAMS sampling scheme for sampling periods 1 and 2.

SP1D1 (May 29, 2018) and SP1D2 (May 31, 2018) samples were collected from cattle after 117 and 119 days on feed, respectively. SP2D1 (June 5, 2018) and SP2D2 (June 7, 2019) samples were collected after 124 and 126 days on feed, respectively.

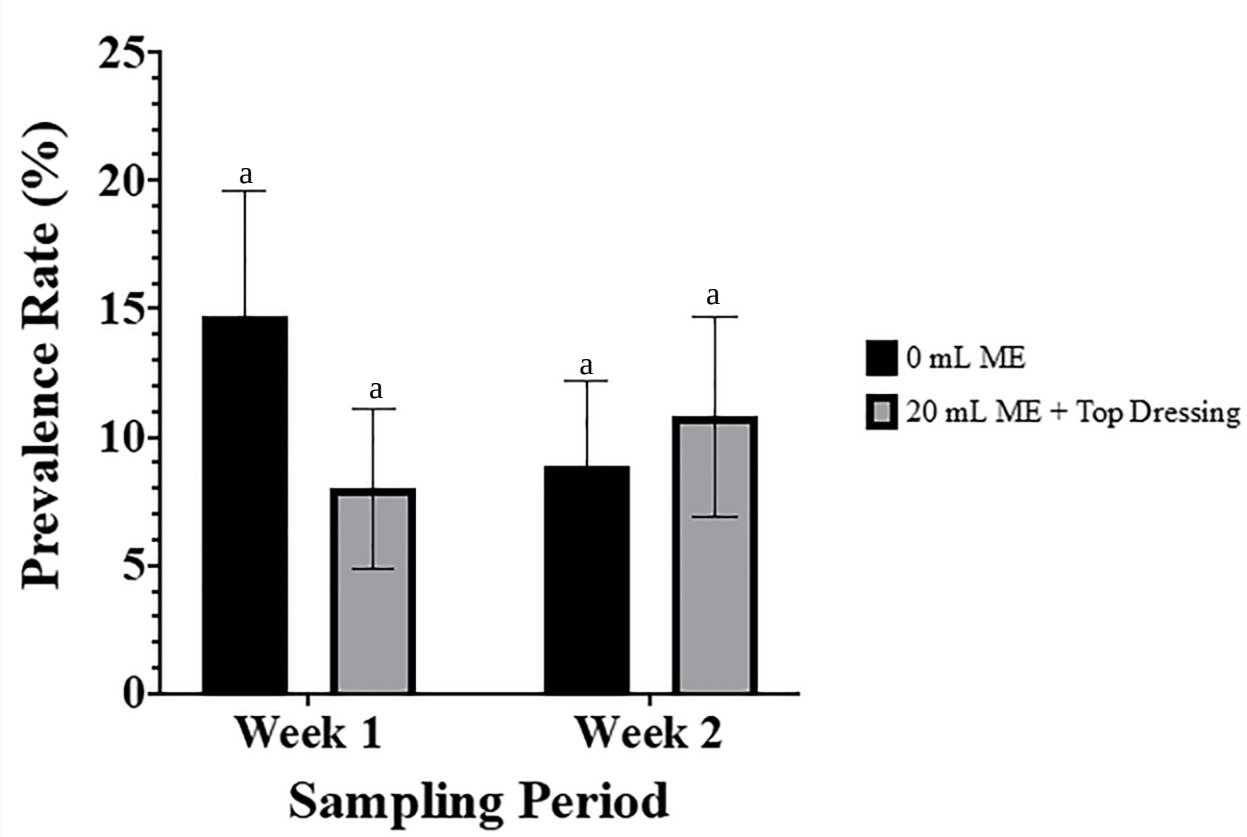

Figure 2.2. Model-adjusted *E. coli* O157:H7 prevalence rates from cattle RAMS samples collected during each sampling period.

The ME x time interaction was significant (P=0.0323). P-values for testing if the odds ratio is 1 were P=0.0921 and P=0.6130 for week1 and week 2, respectively. Error bars represent standard error of the mean.

[a] Values with the same superscript do not differ statistically at the $P \leq 0.05$ threshold.

Table 2.1. Cattle diet treatment design using a 2x2 factorial arrangement.

Each treatment was assigned to 16 pens (7 head per pen) for a total of 112 cattle per treatment and fed throughout the feeding period.

		Lysine	
		0 g	40 g
Megasphaera elsdenii	0 mL	-ME/-LYS	-ME/+LYS
	20 mL + Top Dressing	+ME/-LYS	+ME/+LYS

Table 2.2. Composition of finishing diet fed to steers for 117 (SP1D1), 119 (SP1D2), 124 (SP2D1) and 126 (SP2D2) days.

Item	% of dry matter
Ingredient[1]	
Steam-flaked corn	60
Sweet Bran	30
Wheat straw	7
Supplement[2,3]	3
Nutrient Composition[†,*]	
Crude protein	13.71
Acid detergent fiber	9.05
Neutral detergent fiber	21.41
Fat	2.99
Calcium	0.76
Phosphorus	0.44
Potassium	1.24

[1]Diets were top dressed with 400mg/animal daily of ractopamine (Optaflexx; Elanco Animal Health, Greenfield, IN) within a ground corn carrier and 1% soybean oil for the final 5 weeks on feed.

[2] Consisted of urea, salt, limestone, trace mineral premix, vitamin premix, and potassium chloride to provide (on total diet DM basis), 36.4 mg/kg monensin (Rumensin;Elanco Animal Health), 0.25% salt, 0.15 mg/kg cobalt, 10 mg/kg copper, 0.5 mg/kg iodine, 20 mg/kg manganese, 0.10 mg/kg selenium, 30 mg/kg zinc, 1000 IU vitamin A, and 7 IU vitamin E.

[3]Ruminally protected lysine (LYS; USA Lysine, Kemin Industries, Inc., Des Moines, IA) was premixed in supplement of LYS treated group.

[†]Analyzed by SDK Laboratories, Hutchinson, KS. Ingredients were analyzed by month as composites from weekly samples (5 composites/ingredient).

[*]Original formulation based on the previously established guidelines for feedlot cattle in Nutrient Requirements of Beef Cattle, 8th Revised Edition (National Academies of Sciences, 2016).

Table 2.3. Summary of *E. coli* O157:H7 binary responses from RAMS samples collected from cattle fed each diet during sampling periods 1 and 2.

Prevalence rates are not model-adjusted.

			Sampling Day 1			Sampling Day 2		
Lysine	***M. elsdenii***	**Diet**	**N**	**EC* Pos.**	**Prevalence**	**N**	**EC* Pos.**	**Prevalence**
0 g	0 mL	-ME/-LYS	110	18	16.4%	109	10	9.2%
	20 mL	+ME/-LYS	110	15	13.6%	110	20	18.2%
40 g	0 mL	-ME/+LYS	112	28	25.0%	112	22	19.6%
	20 mL	+ME/+LYS	110	15	13.6%	110	17	15.5%

*EC Pos. represents *E. coli* O157:H7 positive samples.

Chapter 3 - Reduction of *Escherichia coli* O157:H7 in Finishing Cattle Fed Enogen® Feed Corn

3.1. Abstract

Cattle are recognized as the principal reservoir for *Escherichia coli* O157:H7 and pre-harvest food safety efforts often focus on decreasing shedding of this pathogen in cattle feces. Enogen® Feed corn (EFC; Syngenta Seeds, LLC) is genetically modified for amplified expression of α-amylase. Research demonstrates improved feed efficiency in cattle fed EFC; however, the potential foodborne pathogen shedding impact has not been investigated. This study explored the effect of finishing diets containing EFC on *E. coli* O157:H7 prevalence in cattle. A 2x2 factorial experiment was conducted with steers (n=960) fed diets consisting of 2 types of silage (EFC or Control) and grain (EFC or Control), fed daily *ad libitum*. Rectoanal mucosal swabs (RAMS) were collected pre-harvest, incubated in Gram-negative broth at 37°C for 6 hours, subjected to immunomagnetic separation (IMS) using anti-O157 beads, spread-plated onto CHROMagar O157 supplemented with 5 mg/L novobiocin and 2.5 mg/L potassium tellurite, and incubated at 37°C for 18 to 24 hours. Colonies were identified as *E. coli* O157 via latex agglutination and confirmed as *E. coli* O157:H7 using polymerase chain reaction (PCR). *E. coli* O157:H7 prevalence rates ranged from 0.0% (n=0/75) to 10% (n=8/80) depending on sampling day. Tests for the silage x corn interaction and the main effect of silage had *p*-values of *P*=0.5308 and *P*=1.0000, respectively. Test for the main effect of grain had a *p*-value of 0.0706, with EFC reducing the odds of *E. coli* O157:H7 prevalence by 43.3% compared to a control corn diet. Cattle diets containing Enogen® Feed corn reduced the odds of *E. coli* O157:H7 prevalence in feedlot cattle.

3.2. Introduction

Estimated to cause over 63,000 foodborne illnesses annually, *Escherichia coli* O157:H7 represents a significant public health threat (Mead et al., 1999; Scallan et al., 2011). Cattle are recognized as the principal reservoir for *E. coli* O157:H7 (Chapman et al., 1993; Hancock et al., 1997; McDaniel et al., 2014; Renwick et al., 1993; Whipp et al., 1994), with studies showing up to 30% of cattle serving as asymptomatic carriers (Callaway et al., 2006; Callaway et al., 2009; Reinstein et al., 2007; Stanford et al., 2005). The United States (U.S.) Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC), and Department of Agriculture (USDA) surveyed foodborne illness data from 1998 to 2017 and determined that 25.8% of foodborne *E. coli* O157:H7 illnesses were attributable to beef (Interagency Food Safety Analytics Collaboration, 2019). Methods of reducing *E. coli* O157:H7 in cattle, prior to their entrance into the food chain, are important for decreasing the risk of foodborne illness in humans.

Within the feedlot segment of U.S. cattle production, animals are routinely fed diets containing high concentrations of grain to optimize feed efficiency (Callaway et al., 2003; National Academies of Sciences, 2016). Ruminants have evolved to eat cellulose-containing plant material, but the rumen microbiota degrade starch (Huntington, 1997; Huntington et al., 2006; Owens et al., 1986; Westreicher-Kristen et al., 2018). Corn, the most widely utilized source of grain in U.S. cattle finishing diets (Samuelson et al., 2016), is primarily comprised of starch. Steam-flaked corn is readily digested (Corona et al., 2005) and enhances average daily gain and efficiency of finishing cattle (Gonzolaz-Vizcarra et al., 2017), yet has been shown to increase *E. coli* O157:H7 shedding in cattle compared to dry-rolled corn (Fox et al., 2007). Steam-flaked corn digestion occurs primarily in the rumen while dry-rolled corn allows more

starch to escape digestion in the rumen (National Academies of Sciences, 2016; F. Owens & Soderlund, 2006). Starch that escapes ruminal degradation often leads to a secondary fermentation in the hindgut (Brake & Swanson, 2018; Huntington, 1997; Nocek & Tamminga, 1991; Owens & Soderlund, 2006; Theurer, 1986) encouraging production of volatile fatty acids (VFA) that are inhibitory to *E. coli* (Huntington, 1997; Van Kessel et al., 2002) and in some cases, *E. coli* O157:H7 (Ohya et al., 2000). Cattle diets consisting of grain have a substantial effect on metabolism, animal health, and the ruminal microbial ecosystem (Deusch et al., 2017; Russell & Rychlik, 2001; Shabat et al., 2016), with research showing cattle fed high concentrate (grain) diets shed variable concentrations of *E. coli* O157:H7 (Tkalcic et al., 2000). Cattle fed finishing diets with high concentrations of distiller's grains have demonstrated increased levels of *E. coli* O157:H7 shedding (Chaney et al., 2018). Alternatively, cattle fed direct fed-microbials (DFM)—a term describing probiotics used within commercial agriculture production—(Brashears & Guillen, 2014; Yoon & Stern, 1995) or the inclusion of dry-rolled grains (Fox et al., 2007) both demonstrate reductions in *E. coli* O157:H7, suggesting feed modification may be a practical mitigation strategy. Diet manipulations that increase post-ruminal fermentation and favor production of VFAs serve as a potential pre-harvest food safety intervention by decreasing foodborne pathogen shedding in cattle (Lowe et al., 2010; Munns et al., 2015; Wolin, 1969).

Increased starch digestion maximizes cattle feeding efficiency for feedlot producers. Enogen® feed corn (EFC; Syngenta Seeds, LLC), launched in 2011, contains a thermotolerant α-amylase enzyme trait that improves cattle digestion by maximizing starch availability (Erickson et al., 2018; Jolly-Breithaupt et al., 2019). Research suggests that EFC increases feed efficiency and energy availability (Johnson et al., 2018, 2019; Jolly-Breithaupt et al., 2019) potentially altering post-ruminal fermentation and impacting foodborne pathogen populations in the hindgut.

The potential food safety application of EFC has not been explored and addressing this knowledge gap by comparing *E. coli* O157:H7 prevalence in cattle fed finishing diets containing EFC fed as steam-flaked corn and corn silage is the objective of this study.

3.3. Materials and Methods

3.3.1. Experimental Design

A 2x2 factorial experiment was conducted with steers (n=960; 388±7.4 kg initial body weight) blocked by incoming body weight and assigned at random within block to one of 48 pens. Within each block, pens were assigned to one of 4 diets at random. Cattle were fed diets consisting of 2 types of silage (EFC or Control) and 2 types of corn (EFC or Control) in a 2x2 factorial arrangement of treatments. Each diet was applied to 12 pens consisting of 15 or 25 cattle per pen; therefore, each diet treatment was supplied to 240 cattle in total. Prevalence of *E. coli* O157:H7 was measured using rectoanal mucosal swabs (RAMS), obtained from each animal and analyzed using standard laboratory methods. Cattle were sampled in cohorts of 300-340 cattle per day, for a total of 3 sampling days, yielding 960 RAMS samples.

3.3.2. Cattle Diets

All cattle were fed finishing diets for 62 (sampling day 1), 78 (sampling day 2), and 93 (sampling day 3) days, in accordance with the blocks to which they were assigned. The treatments were as follows: control silage with control corn (CSCC), Enogen® silage with control corn (ESCC), control silage with Enogen® corn (CSEC), and Enogen® silage with Enogen® corn (ESEC).

Steam-flaked Enogen® feed corn (EFC; Syngenta Seeds, LLC) was sourced from the study sponsor while mill-run control corn (CON) was sourced locally. Corn was conditioned by adding moisture (SarTec; Anoka, MN), steamed, and flaked daily utilizing a R & R Machine

Works steam-flaker (46 x 91 cm corrugated rolls; Dalhart, TX). The CON corn was flaked to 360 g/L bulk density while EFC was flaked to 386 g/L; thus, yielding similar starch availabilities for both grains (Horton & Drouillard, 2018). Analysis of starch availability was performed daily using previously described methods (Horton, 2018), to monitor steam-flaking procedures and to achieve similar starch availability of flakes between both EFC and CON corn. Corn silages were planted at Kansas State University and harvested using a commercial harvester (John Deere, Model 7980; Moline, IL). Whole chopped corn plant was transported to the Beef Cattle Research Center (BCRC) and packed into agricultural bags (Hitec Bag; Plastika Kritis; Iraklion, Greece) for ensiling and storage.

Diet dry matter composition included: 8% corn silage (EFC or CON), 2% ground alfalfa hay, 74.5% flaked corn (EFC or CON), 12% Sweet Bran (Cargill Corp.; Wayzata, MN), and 3.5% supplement (Table 3.1). The supplement consisted of urea, salt, limestone, trace mineral premix, potassium chloride, and monensin (Rumensin; Elanco Animal Health, Greenfield, IN). Diets were top dressed 35 days prior to slaughter with ractopamine (Optaflexx; Elanco Animal Health, Greenfield, IN) at the rate of 400mg/animal using a ground corn carrier. Animal diets (Table 3.1), including silage (Table 3.2) and grain (Table 3.3), were analyzed for nutrient composition in a monthly composite for each ingredient (SDK Labs; Hutchinson, KS). Animals were fed once daily *ad libitum*. Feed efficiency and animal performance metrics were evaluated during this study, as outlined by the companion study (Baker et al., 2019).

3.3.3. RAMS Sampling

A RAMS sample was obtained from each animal enrolled in the study. Cattle were sampled in cohorts of 300-340 animals per day, with a total of 3 sampling days. Collection of RAMS samples followed protocols previously described (Greenquist et al., 2005, Rice et al.,

2003). Briefly, a sterile foam-tipped swab (VWR, Radnor, PA) was inserted 3 to 5 cm into the anus of each animal. The swab vigorously scraped the mucosal surface using a rapid in-and-out motion. Following sampling, the swab was placed into a sterile tube containing 3 mL of Gram-negative broth (GN Broth; Remel, Lenexa, KS) supplemented with 0.05 mg/L of cefixime (VCC Supplement; Millipore Sigma, St. Louis, MO), 10 mg/L of cefsulodin, and 8 mg/L of vancomycin (GNccv). The RAMS were stored on ice immediately following sampling and transported to the laboratory within 3 hours of completing all sample collection for further processing.

3.3.4. Microbiological Analysis

Upon RAMS+GNccv sample arrival at the laboratory, sample tubes were vortexed for 1 minute, 1 mL was transferred into a 9 mL GNccv enrichment tube, vortexed, and incubated at 37^{o}C for 6 hours. Samples were incubated in a programmable incubator which held the incubated samples at 4^{o}C until subsequent analyses the following day. The incubated GNccv enrichment tubes underwent immunomagnetic separation (IMS) following previously published methods (Chaney et al., 2018, Sargeant et al., 2003). In brief, 1 mL of each enrichment was mixed with 20 µL of anti-*E. coli* O157:H7 Dynabeads ™ (Applied Biosystems; Foster City, CA) and then washed three times in phosphate-buffered saline with PBS Tween 20 (Thermo Fisher Scientific, Waltham, MA) using an automatic IMS machine (KingFisher ™ mL Food Protection Purification System; Thermo Fisher Scientific, Waltham, MA) according to manufacturer's guidelines. A 50-µL aliquot of PBS-Tween containing the resultant bead-bacteria complexes was then spread-plate on ntChromagar plates (BBL™ CHROMagar™ O157; BD Difco, Franklin Lakes, NJ) supplemented with 5 mg/L of novobiocin (Novobiocin supplement; Thermo Fisher Scientific, Waltham, MA) and 2.5 mg/L of potassium tellurite (Potassium tellurite hydrate;

Millipore Sigma, St. Louis, MO) and incubated at 37ºC for 18 to 24 hours. After incubation, study personnel carefully chose up to 3 colonies suspected as *E. coli* O157:H7 for presumptive confirmation via latex agglutination testing (Remel ™ RIM *E. coli* O157:H7 Latex Test; Thermo Fisher Scientific, Waltham, MA). From each ntChromagar plate, two presumptively positive colonies were streaked for isolation on Sorbitol MacConkey agar (CT-SMAC; Remel, Lenexa, KS) supplemented with 0.05 mg/L of cefixime and 2.5 mg/L of potassium tellurite (CT-Supplement; Milipore Sigma, St. Louis, MO). CT-SMAC plates were incubated for 18-24 hours at 37ºC. A single colony was picked from each plate and transferred into 9 mL tryptic soy broth (TSB; BD Difco, Franklin Lakes, NJ) tubes. All TSB tubes were incubated for 18 to 24 hours at 37ºC and 1 mL from each tube was transferred to a microcentrifuge tube containing glycerol, such that the resultant concentration of glycerol was 10%. Each presumptively confirmed *E. coli* O157:H7 isolate was frozen in duplicate at -80ºC. Polymerase chain reaction (PCR) was performed on isolates using the MicroSEQ™ *E. coli* O157:H7 Detection Kit (Applied Biosystems, Foster City, CA) with an ABI 7500 FAST real-time PCR machine (Applied Biosystems, Foster City, CA) to confirm the presence of *E. coli* O157:H7.

3.3.5. Statistical Analysis

Following PCR confirmation, the result for each animal was organized as a binary response (positive or negative for *E. coli* O157:H7). Due to the low prevalence rate, the exact conditional logistic regression approach (logit linear model) was used to evaluate the main effects of silage and corn as well as their interaction. Sampling date served as the stratifying variable for the conditional inference. The effect of pen was ignored in the statistical analysis due to the low prevalence rate. Prevalence data are reported based upon this model, considering these parameters and referred to as “model-adjusted”. Prevalence data which does not reflect these

parameters represents a crude analysis and is referred to as “not model adjusted”. All tests were conducted at the *P*=0.05 significance level. Diet comparison was carried out using 2-sided tests. When the interaction between silage and corn was evaluated, fixed effects of the logit model included silage, corn, and silage-by-corn interaction. The exact *p*-value was used to test for interaction. In the case that the interaction was not significant, fixed effects of the logit model included the main effects of silage and corn only. Exact *p*-values were used to test for main effects. Statistical analyses were performed using Statistical Analysis Software (SAS version 9.4; Cary, NC) PROC LOGISTIC with sampling date in the STRATA statement.

3.4. Results

The overall, non-adjusted *E. coli* O157:H7 prevalence rates (Table 3.4) for each diet combination were low, ranging from 0.0% (n=0/75) to 10% (n=8/80) depending on sampling day. Summarized in Table 3.4, diet ESEC yielded 5.0% (n=4/80), 3.6% (n=3/84), and 0.0% (n=0/75) *E. coli* O157:H7 prevalence for sampling days 1, 2, and 3, respectively. Diet CSCC yielded 10.0% (n=8/80), 3.6% (n=3/84), 2.7% (n=2/75) *E. coli* O157:H7 prevalence for sampling days 1, 2, and 3, respectively. Diet ESCC yielded 8.8% (n=7/80), 8.3% (n=7/84), 2.7% (n=2/73) *E. coli* O157:H7 prevalence for sampling days 1, 2, and 3, respectively. Diet CSEC yielded 3.8% (n=3/80), 5.9% (n=5/85), 2.7% (n=2/75) *E. coli* O157:H7 prevalence for sampling days 1, 2, and 3, respectively. Diet CSCC yielded 10.0% (n=8/80), 3.6% (n=3/84), 2.7% (n=2/75) *E. coli* O157:H7 prevalence for sampling days 1, 2, and 3, respectively.

The statistical model followed the conditional logistic regression approach with sampling date as the stratifying variable. The model assumes no interaction between corn x sampling date, silage x sampling date, and silage x corn x sampling date because the low prevalence rate observed in this study prevents testing 2-way and 3-way interactions of the sampling date

stratifying variable. Tests for the silage x corn interaction were not significant (P=0.5308). Tests for the main effect of silage (EFC and CON) were not significant (P=1.0000); thus, no silage effect on *E. coli* O157:H7 prevalence in finishing cattle was observed. The *p*-value for the main effect of corn (with levels EFC and CON) was 0.0706, which was not statistically significant at the $P \leq 0.05$ level.

The exact conditional logistic regression approach (logit linear model) yields odds ratio data, similar to the ratio of prevalence rates, both of which are commonly used measures of association in research studies (Tamhane et al., 2016). Analysis using odds ratio provided further comparison between the 2 levels of each main effect (grain and silage). The odds of *E. coli* O157:H7 positive in cattle fed with EFC silage was observed to be 1.004 as high as that in cattle fed CON silage. The odds of *E. coli* O157:H7 in cattle fed EFC corn is observed to be 0.567 as high as cattle fed with CON corn; in other words, a diet containing EFC reduced the odds of *E. coli* O157:H7 prevalence by 43.3% in comparison to a CON corn diet.

3.5. Discussion

In the present study, the impact of feeding EFC as steam-flaked corn and silage in cattle finishing diets to reduce the burden of *E. coli* O157:H7 in feedlot cattle was analyzed. A 43.3% reduction in the odds of *E. coli* O157:H7 prevalence in cattle fed diets containing EFC grain versus a CON grain diet was observed. A possible explanation for this observed reduction of *E. coli* O157:H7 could be due to increased production of VFAs, a known Gram negative bacteria toxicant (Hentges, 1992), due to changes in post-ruminal fermentation within the hindgut; however, VFA data were not collected as a part of this experiment, representing only a hypothesis. The data described herein are critical for the beef and feed grain industries—in

addition to the public health community—as pre-harvest reductions of *E. coli* O157:H7 in cattle are of great interest in order to reduce foodborne illness outbreaks.

Shifts in *E. coli* O157:H7 prevalence based on feed composition could be explained by changes in post-ruminal starch digestibility. Published data demonstrates increased hindgut starch digestion leads to increased hindgut VFA concentration, making it a less suitable site for *E. coli* O157:H7 proliferation (Buchko et al., 2000; Callaway et al., 2004, 2009), a mechanism first presented in 1969 by Dr. Meyer Wolin (Wolin, 1969). Increases in post-ruminal starch digestion can lead to increased VFAs and decreased pH within the hindgut (Van Kessel et al., 2002); however, this starch digestion and absorption is limited by the animal's ability to secrete sufficient amounts of α-amylase from the pancreas (National Academies of Sciences, 2016). Volatile fatty acids have been reported to have an inhibitory effect on *E. coli* O157:H7 shedding in cattle (Kudva et al., 2014; Lowe et al., 2010; Munns et al., 2015; Ohya et al., 2000; Rasmussen et al., 1993; Shin et al., 2002). Previous data on EFC used in finishing diets show an increase in post-ruminal starch digestion (Harris et al., 2016; Jolly-Breithaupt et al., 2016, 2018) and improved feed efficiency (Johnson et al., 2018, 2019); however, data on the influence of EFC on total tract starch digestibility is limited. If EFC corn increases hindgut starch fermentation and subsequent VFA production this may be a potential explanation for the reduced odds of *E. coli* O157:H7 prevalence observed in cattle fed the EFC vs CON corn, yet this requires further investigation.

Sampling of VFA concentrations in the hindgut throughout animal feeding was beyond the scope of this study and is a limitation, but also represents an opportunity for future research. More specifically, if VFA quantities and composition are known (% acetic, propionic, butyric, etc.) in conjunction with *E. coli* O157:H7 shedding, the resultant data can be used to inform anti-

E. coli O157:H7 mitigation strategies, as well as future research. Mao et al. (2012) demonstrated that alteration of VFA concentrations in the hindgut significantly impacts the fecal microbiome and, potentially, animal health and productivity (Mao et al., 2012); however, this concept is not well understood. Additionally, an overabundance of fermentable carbohydrate in the hindgut can lead to hindgut acidosis, which is characterized by damage to the gut epithelium, increased production of lactic acid and VFAs, and decreased fecal pH (Gressley et al., 2011). The difficult challenge of maintaining hindgut fermentation while also balancing ruminant nutrition, health, productivity, and food safety must be addressed in subsequent research studies.

Modification of feed to influence cattle microbiota was first demonstrated in 1998 by shifting animal diets containing high grain to a period of hay-based rations, thereby significantly reducing acid resistant *E. coli* by 10^6 fold after 5 days (Diez-Gonzalez et al., 1998). Further research provided additional evidence for this influence on *E. coli* prevalence, but reported that switching cattle from grain to hay decreased body weight gain (approximately 1.25lb/ head per day) (Keen et al., 1999). This research posed many questions on the effect animal diets have on foodborne pathogen prevalence—some of which have yet to be answered—and illustrates the difficulty in balancing animal efficiency and food safety. The research described herein suggests that EFC decreases the odds of *E. coli* O157:H7 prevalence in cattle without compromising efficiency. In fact, research has shown that EFC increases feed efficiency and average daily gain by up to 5.50% and 6.00% respectively, when fed as grain or silage (Johnson et al., 2018, 2019; Jolly-Breithaupt et al., 2019).

Recently, research on the food safety implications of modifying cattle diets has increased. Inclusion of distiller's grains (DGs) in cattle feed has shown to be a cost effective, nutrient dense feed supplement; however, reports indicate an association with increased *E. coli* O157:H7

prevalence depending upon the percentage of DGs included (Chaney et al., 2018; Jacob et al., 2008; Wells et al., 2009). Purple prairie clover and sainfoin—two legume forages—have demonstrated potential anti-*E. coli* O157:H7 action (Liu et al., 2013). Additionally, supplements containing seaweed, specifically *Ascophyllum nodosum*, decreased the prevalence of *E. coli* O157:H7 due to its bacteriostatic and bactericidal characteristics (Braden et al., 2004; Wang et al., 2009). Orange peel and pulp—a common citrus byproduct—reduced *E. coli* O157:H7 in the gastrointestinal tract of ruminants (Callaway et al., 2011), with at least 2 log reductions of *E. coli* O157:H7 and *Salmonella* observed in inoculated ruminal fluid (Callaway et al., 2008; Nannapaneni et al., 2008). Impacts of the aforementioned feed supplements are only beginning to be understood, thus requiring additional research on animal efficiency and anti-*E. coli* O157:H7 activity *in vivo*. Regardless, feed modification continues to be a growing area of research, with substantial pre-harvest food safety potential.

3.6 Conclusion

The United States food supply is not immune to the persistence of food safety threats. Improvements in postharvest sanitation, slaughter, and processing methods reduce carcass contamination; however, pre-harvest interventions aimed at reducing *E. coli* O157:H7 populations in the animal—prior to arrival at the abattoir—will enhance the effectiveness of in-plant interventions (Callaway et al., 2009). Additionally, this pre-harvest control technology provides a practical means to control foodborne pathogens, intervening at the farm level (LeJeune & Wetzel, 2007). The present study was designed to address the need for novel pre-harvest interventions, and the data described herein suggest that feeding Enogen® feed corn may reduce *E. coli* O157:H7 carriage in cattle. To our knowledge, this was the first research study undertaken to determine if a food safety benefit exists by feeding Enogen® feed corn. Therefore,

these data are limited in scope and further research is necessary. Future research should also include additional analyses, such as quantifying VFA production in the hindgut, in order to probe at a possible mechanism of action.

3.7. References

Baker, A., Veloso, V. de A., Baker, A., & Barros, L. (2019). 343 Feedlot performance and carcass characteristics of steers fed diets containing steam-flaked grain and corn silage from Enogen® Feed Corn. *Journal of Animal Science*, *97*(Supplement_2), 137–137. https://doi.org/10.1093/jas/skz122.243

Braden, K. W., Blanton, J. R., Allen, V. G., Pond, K. R., & Miller, M. F. (2004). Ascophyllum nodosum supplementation: A preharvest intervention for reducing *Escherichia coli* O157:H7 and Salmonella spp. in feedlot steers. *Journal of Food Protection*, *67*(9), 1824–1828. https://doi.org/10.4315/0362-028x-67.9.1824

Brake, D. W., & Swanson, K. C. (2018). RUMINANT NUTRITION SYMPOSIUM: Effects of postruminal flows of protein and amino acids on small intestinal starch digestion in beef cattle. *Journal of Animal Science*, *96*(2), 739–750. https://doi.org/10.1093/jas/skx058

Brashears, M. M., & Guillen, L. M. (2014, February 5). Direct-Fed Microbials as Preharvest Interventions in Cattle Production. *Food Safety Magazine*. https://www.foodsafetymagazine.com/magazine-archive1/februarymarch-2014/direct-fed-microbials-as-preharvest-interventions-in-cattle-production/

Buchko, S. J., Holley, R. A., Olson, W. O., Gannon, V. P., & Veira, D. M. (2000). The effect of different grain diets on fecal shedding of *Escherichia coli* O157:H7 by steers. *Journal of Food Protection*, *63*(11), 1467–1474. https://doi.org/10.4315/0362-028x-63.11.1467

Callaway, T., Anderson, R. C., Edrington, T. S., Genovese, K. J., Harvey, R. B., Poole, T. L., & Nisbet, D. J. (2004). Recent pre-harvest supplementation strategies to reduce carriage and shedding of zoonotic enteric bacterial pathogens in food animals. *Animal Health Research Reviews*, *5*(1), 35–47. https://doi.org/10.1079/ahr200462

Callaway, T., Carr, M. A., Edrington, T. S., Anderson, R. C., & Nisbet, D. J. (2009). Diet, *Escherichia coli* O157:H7, and cattle: A review after 10 years. *Current Issues in Molecular Biology*, *11*(2), 67–79.

Callaway, T., Carroll, J. A., Arthington, J. D., Edrington, T. S., Rossman, M. L., Carr, M. A., Krueger, N. A., Ricke, S. C., Crandall, P., & Nisbet, D. J. (2011). *Escherichia coli* O157:H7 populations in ruminants can be reduced by orange peel product feeding. *Journal of Food Protection*, *74*(11), 1917–1921. https://doi.org/10.4315/0362-028X.JFP-11-234

Callaway, T., Carroll, J. A., Arthington, J. D., Pratt, C., Edrington, T. S., Anderson, R. C., Galyean, M. L., Ricke, S. C., Crandall, P., & Nisbet, D. J. (2008). Citrus products decrease growth of *E. coli* O157:H7 and *Salmonella typhimurium* in pure culture and in fermentation with mixed ruminal microorganisms in vitro. *Foodborne Pathogens and Disease*, *5*(5), 621–627. https://doi.org/10.1089/fpd.2008.0088

Callaway, T., Edrington, T. s., Brabban, A. d., Keen, J. e., Anderson, R. c., Rossman, M. l., Engler, M. j., Genovese, K. j., Gwartney, B. l., Reagan, J. o., Poole, T. l., Harvey, R. b., Kutter, E. m., & Nisbet, D. j. (2006). Fecal Prevalence of *Escherichia coli* O157, *Salmonella*, *Listeria*, and Bacteriophage Infecting E. coli O157:H7 in Feedlot Cattle in the Southern Plains Region of the United States. *Foodborne Pathogens and Disease*, *3*(3), 234–244. https://doi.org/10.1089/fpd.2006.3.234

Callaway, T., Elder, R. O., Keen, J. E., Anderson, R. C., & Nisbet, D. J. (2003). Forage feeding to reduce preharvest *Escherichia coli* populations in cattle, a review. *Journal of Dairy Science*, *86*(3), 852–860. https://doi.org/10.3168/jds.S0022-0302(03)73668-6

Chaney, W. E., Maloney, R., Johnson, B. J., Brooks, J. C., Brashears, M. M., & Loneragan, G. H. (2018). Corn-Based Distillers' Grains in Diets for Feedlot Cattle Are Associated with the Burden of *Escherichia coli* O157 in Feces. *Foodborne Pathogens and Disease*, *15*(7), 398–405. https://doi.org/10.1089/fpd.2017.2395

Chapman, P. A., Siddons, C. A., Wright, D. J., Norman, P., Fox, J., & Crick, E. (1993). Cattle as a possible source of verocytotoxin-producing *Escherichia col*i O157 infections in man. *Epidemiology and Infection*, *111*(3), 439–447. https://doi.org/10.1017/s0950268800057162

Corona, L., Rodriguez, S., Ware, R. A., & Zinn, R. A. (2005). Comparative Effects of Whole, Ground, Dry-Rolled, and Steam-Flaked Corn on Digestion and Growth Performance in Feedlot Cattle. *The Professional Animal Scientist*, *21*(3), 200–206. https://doi.org/10.15232/S1080-7446(15)31203-1

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., & Seifert, J. (2017). A Structural and Functional Elucidation of the Rumen Microbiome Influenced by Various Diets and Microenvironments. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01605

Diez-Gonzalez, F., Callaway, T. R., Kizoulis, M. G., & Russell, J. B. (1998). Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science (New York, N.Y.)*, *281*(5383), 1666–1668. https://doi.org/10.1126/science.281.5383.1666

Erickson, G., Martin, D., & Watson, E. (2018, October 2). *Improving Profic Potential Through Rations With More Available Energy*. https://www.drovers.com/webinars/improving-profit-potential-through-rations-more-available-energy

Fox, J. T., Depenbusch, B. E., Drouillard, J. S., & Nagaraja, T. G. (2007). Dry-rolled or steam-flaked grain-based diets and fecal shedding of *Escherichia coli* O157 in feedlot cattle. *Journal of Animal Science*, *85*(5), 1207–1212. https://doi.org/10.2527/jas.2006-079

Goepfert, J. M., & Hicks, R. (1969). Effect of volatile fatty acids on *Salmonella typhimurium*. *Journal of Bacteriology*, *97*(2), 956–958.

Gonzolaz-Vizcarra, V. M., Plascencia, A., Ramos-Avina, D., & Zinn, R. A. (2017). Influence of substituting steam-flaked corn for dry rolled corn on feedlot cattle growth performance when cattle are allowed either ad libitum or restricted access to the finishing diet. *Asian-Australasian Journal of Animal Sciences*, *30*(11), 1563–1567.

Greenquist, M. A., Drouillard, J. S., Sargeant, J. M., Depenbusch, B. E., Shi, X., Lechtenberg, K. F., & Nagaraja, T. G. (2005). Comparison of Rectoanal Mucosal Swab Cultures and Fecal Cultures for Determining Prevalence of Escherichia coli O157:H7 in Feedlot Cattle. *Applied and Environmental Microbiology*, *71*(10), 6431–6433. https://doi.org/10.1128/AEM.71.10.6431-6433.2005

Gressley, T. F., Hall, M. B., & Armentano, L. E. (2011). Ruminant Nutrition Symposium: Productivity, digestion, and health responses to hindgut acidosis in ruminants. *Journal of Animal Science*, *89*(4), 1120–1130. https://doi.org/10.2527/jas.2010-3460

Hancock, D. D., Besser, T. E., Rice, D. H., Herriott, D. E., & Tarr, P. I. (1997). A longitudinal study of *Escherichia coli* O157 in fourteen cattle herds. *Epidemiology and Infection*, *118*(2), 193–195. https://doi.org/10.1017/s0950268896007212

Harris, M., Jolly-Breithaupt, M., Nuttelman, B., Burken, D., Erickson, G., Rush, I., & Luebbe, M. (2016). *Evaluating Syngenta Enhanced Feed Corn on Finishing Cattle Performance and Carcass Characteristics*. *2016 Nebraska Beef Cattle Report*.

Hentges, D. J. (1992). Gut flora and disease resistance. In R. Fuller (Ed.), *Probiotics: The scientific basis* (pp. 87–110). Springer Netherlands. https://doi.org/10.1007/978-94-011-2364-8_5

Horton, L., & Drouillard, J. (2018). 57 Effects of particle size and heat treatment on *in situ* and *in vitro* digestion of dry-rolled Enogen® Feed Corn. *Journal of Animal Science*, *96*, 391–391. https://doi.org/10.1093/jas/sky404.857

Horton, L. M. (2018). Processing methods for high-amylase corn: Impact on ruminal digestion and feedlot cattle performance. Kansas State University.

Huntington, G. (1997). Starch utilization by ruminants: From basics to the bunk. *Journal of Animal Science*, *75*, 852–867.

Huntington, G, Harmon, D. L., & Richards, C. J. (2006). Sites, rates, and limits of starch digestion and glucose metabolism in growing cattle. *Journal of Animal Science*, *84 Suppl*, E14-24. https://doi.org/10.2527/2006.8413_supple14x

Interagency Food Safety Analytics Collaboration. (2019). Foodborne illness source attribution estimates for 2017 for *Salmonella*, *Escherichia coli* O157, *Listeria monocytogenes*, and *Campylobacter* using multi-year outbreak surveillance data, United States (p. 14).

Jacob, M. E., Fox, J. T., Drouillard, J. S., Renter, D. G., & Nagaraja, T. G. (2008). Effects of Dried Distillers' Grain on Fecal Prevalence and Growth of *Escherichia coli* O157 in

Batch Culture Fermentations from Cattle. *Applied and Environmental Microbiology*, *74*(1), 38–43. https://doi.org/10.1128/AEM.01842-07

Johnson, M., Spore, T., Montgomery, S., Hollenbeck, W., Wahl, R., Watson, E., & Blasi, D. (2019). Syngenta Enogen Feed Corn Silage Containing an Alpha Amylase Expression Trait Improves Feed Efficiency in Growing Calf Diets. *Kansas Agricultural Experiment Station Research Reports*, *5*(1). https://doi.org/10.4148/2378-5977.7719

Johnson, M., Spore, T., Montgomery, S., Weibert, C., Garzon, J., Hollenbeck, W., Wahl, R., Watson, E., & Blasi, D. (2018). Syngenta Enhanced Feed Corn (Enogen) Containing an Alpha Amylase Expression Trait Improves Feed Efficiency in Growing Calf Diets. *Kansas Agricultural Experiment Station Research Reports*, *4*(1). https://doi.org/10.4148/2378-5977.7543

Jolly-Breithaupt, M., Bittner, C., Burken, D., Erickson, G., Macdonald, J., & Luebbe, M. (2016). Evaluating Syngenta Enhanced Feed Corn Processed as Dry- Rolled or High- Moisture Corn on Cattle Performance and Carcass Characteristics. *2016 Nebraska Beef Cattle Report*.

Jolly-Breithaupt, M. L., Bittner, C. J., Hilscher, F. H., Erickson, G. E., MacDonald, J. C., & Luebbe, M. K. (2018). Impact of Syngenta Enogen Feed Corn on Finishing Cattle Performance and Carcass Characteristics. *2018 Nebraska Beef Cattle Report*, 3.

Keen, J. E., Uhlich, G. A., & Elder, R. O. (1999, November 7). Effects of hay- and grain-based diets on fecal shedding in naturally-acquired enterohemorrhagic *E. coli (*EHEC*)* O157 in beef feedlot cattle. Conference Research Workers in Animal Diseases, Chicago, IL.

Kudva, I., Stanton, T., & Lippolis, J. (2014). The *Escherichia coli* O157:H7 bovine rumen fluid proteome reflects adaptive bacterial responses | BMC Microbiology | Full Text. *BMC Microbiology*, *14*(28). https://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-2180-14-48

LeJeune, J. T., & Wetzel, A. N. (2007). Preharvest control of *Escherichia coli* O157 in cattle. *Journal of Animal Science*, *85*(13 Suppl), E73-80. https://doi.org/10.2527/jas.2006-612

Liu, X.-L., Hao, Y.-Q., Jin, L., Xu, Z.-J., McAllister, T. A., & Wang, Y. (2013). Anti-*Escherichia coli* O157:H7 properties of purple prairie clover and sainfoin condensed tannins. *Molecules (Basel, Switzerland)*, *18*(2), 2183–2199. https://doi.org/10.3390/molecules18022183

Lowe, R. M. S., Munns, K., Selinger, L. B., Kremenik, L., Baines, D., McAllister, T. A., & Sharma, R. (2010). Factors influencing the persistence of *Escherichia coli* O157:H7 lineages in feces from cattle fed grain versus grass hay diets. *Canadian Journal of Microbiology*, *56*(8), 667–675. https://doi.org/10.1139/w10-051

Mao, S., Zhang, R., Wang, D., & Zhu, W. (2012). The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during

subacute rumen acidosis in dairy cows. *BMC Veterinary Research*, *8*, 237. https://doi.org/10.1186/1746-6148-8-237

McDaniel, C. J., Cardwell, D. M., Moeller, R. B., & Gray, G. C. (2014). Humans and cattle: A review of bovine zoonoses. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.)*, *14*(1), 1–19. https://doi.org/10.1089/vbz.2012.1164

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, *5*(5), 607–625.

Munns, K. D., Selinger, L. B., Stanford, K., Guan, L., Callaway, T. R., & McAllister, T. A. (2015). Perspectives on super-shedding of *Escherichia coli* O157:H7 by cattle. *Foodborne Pathogens and Disease*, *12*(2), 89–103. https://doi.org/10.1089/fpd.2014.1829

Nannapaneni, R., Muthaiyan, A., Crandall, P. G., Johnson, M. G., O'Bryan, C. A., Chalova, V. I., Callaway, T. R., Carroll, J. A., Arthington, J. D., Nisbet, D. J., & Ricke, S. C. (2008). Antimicrobial activity of commercial citrus-based natural extracts against *Escherichia coli* O157:H7 isolates and mutant strains. *Foodborne Pathogens and Disease*, *5*(5), 695–699. https://doi.org/10.1089/fpd.2008.0124

National Academies of Sciences, E. (2016). *Nutrient Requirements of Beef Cattle: Eighth Revised Edition*. https://doi.org/10.17226/19014

Nocek, J. E., & Tamminga, S. (1991). Site of Digestion of Starch in the Gastrointestinal Tract of Dairy Cows and Its Effect on Milk Yield and Composition. *Journal of Dairy Science*, *74*(10), 3598–3629. https://doi.org/10.3168/jds.S0022-0302(91)78552-4

Ohya, T., Marubashi, T., & Ito, H. (2000). Significance of fecal volatile fatty acids in shedding of *Escherichia coli* O157 from calves: Experimental infection and preliminary use of a probiotic product. *The Journal of Veterinary Medical Science*, *62*(11), 1151–1155. https://doi.org/10.1292/jvms.62.1151

Owens, F. N., Zinn, R. A., & Kim, Y. K. (1986). Limits to starch digestion in the ruminant small intestine. *Journal of Animal Science*, *63*(5), 1634–1648. https://doi.org/10.2527/jas1986.6351634x

Owens, F., & Soderlund, S. (2006). Ruminal and postruminal starch digestions by cattle. *Proc.of Cattle Grain Processing Symp.*

Rasmussen, M., Cray, W., Casey, T., & Whipp, S. (1993). Rumen contents as a reservoir of enterohemorrhagic *Escherichia coli*. *FEMS Microbiology Letters*, *114*(1), 79–84.

Reinstein, S., Fox, J. T., Shi, X., & Nagaraja, T. G. (2007). Prevalence of *Escherichia coli* O157:H7 in gallbladders of beef cattle. *Applied and Environmental Microbiology*, *73*(3), 1002–1004. https://doi.org/10.1128/AEM.02037-06

Renwick, S. A., Wilson, J. B., Clarke, R. C., Lior, H., Borczyk, A. A., Spika, J., Rahn, K., McFadden, K., Brouwer, A., & Copps, A. (1993). Evidence of direct transmission of *Escherichia coli* O157:H7 infection between calves and a human. *The Journal of Infectious Diseases*, *168*(3), 792–793. https://doi.org/10.1093/infdis/168.3.792

Rice, D. H., Sheng, H. Q., Wynia, S. A., & Hovde, C. J. (2003). Rectoanal Mucosal Swab Culture Is More Sensitive Than Fecal Culture and Distinguishes Escherichia coli O157:H7-Colonized Cattle and Those Transiently Shedding the Same Organism. *Journal of Clinical Microbiology*, *41*(11), 4924–4929. https://doi.org/10.1128/JCM.41.11.4924-4929.2003

Russell, J. B., & Rychlik, J. L. (2001). Factors That Alter Rumen Microbial Ecology. *Science, 292*(5519), 1119–1122. https://doi.org/10.1126/science.1058830

Samuelson, K. L., Hubbert, M. E., Galyean, M. L., & Löest, C. A. (2016). Nutritional recommendations of feedlot consulting nutritionists: The 2015 New Mexico State and Texas Tech University survey. *Journal of Animal Science*, *94*(6), 2648–2663. https://doi.org/10.2527/jas.2016-0282

Sargeant, J. M., Sanderson, M. W., Smith, R. A., & Griffin, D. D. (2003). *Escherichia coli* O157 in feedlot cattle feces and water in four major feeder-cattle states in the USA. *Preventive Veterinary Medicine*, *61*(2), 127–135. https://doi.org/10.1016/s0167-5877(03)00166-1

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.P11101

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N., & Mizrahi, I. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *The ISME Journal*, *10*(12), 2958–2972. https://doi.org/10.1038/ismej.2016.62

Shin, R., Suzuki, M., & Morishita, Y. (2002). Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic *Escherichia coli* O157:H7. *Journal of Medical Microbiology*, *51*(3), 201–206. https://doi.org/10.1099/0022-1317-51-3-201

Stanford, K., Croy, D., Bach, S. J., Wallins, G. L., Zahiroddini, H., & McAllister, T. A. (2005). Ecology of *Escherichia coli* O157:H7 in commercial dairies in southern Alberta. *Journal of Dairy Science*, *88*(12), 4441–4451. https://doi.org/10.3168/jds.S0022-0302(05)73131-3

Tamhane, A., Westfall, A., Burkholder, G., & Cutter, G. (2016). Prevalence Odds Ratio versus Prevalence Ratio: Choice Comes with Consequences. *Statistics in Medicine*, *35*(30). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5135596/

Theurer, C. B. (1986). Grain processing effects on starch utilization by ruminants. *Journal of Animal Science*, *63*(5), 1649–1662. https://doi.org/10.2527/jas1986.6351649x

Tkalcic, S., Brown, C. A., Harmon, B. G., Jain, A. V., Mueller, E. P. O., Parks, A., Jacobsen, K. L., Martin, S. A., Zhao, T., & Doyle, M. P. (2000). Effects of Diet on Rumen Proliferation and Fecal Shedding of *Escherichia coli* O157:H7 in Calves. *Journal of Food Protection*, *63*(12), 1630–1636. https://doi.org/10.4315/0362-028X-63.12.1630

Van Kessel, J. S., Nedoluha, P. C., Williams-Campbell, A., Baldwin, R. L., & McLeod, K. R. (2002). Effects of ruminal and postruminal infusion of starch hydrolysate or glucose on the microbial ecology of the gastrointestinal tract in growing steers,. *Journal of Animal Science*, *80*(11), 3027–3034. https://doi.org/10.2527/2002.80113027x

Wang, Y., Xu, Z., Bach, S. J., & McAllister, T. A. (2009). Sensitivity of *Escherichia coli* to Seaweed (*Ascophyllum nodosum*) Phlorotannins and Terrestrial Tannins. *Asian-Australasian Journal of Animal Sciences*, *22*(2), 238–245. https://doi.org/2009.22.2.238

Wells, J. E., Shackelford, S. D., Berry, E. D., Kalchayanand, N., Guerini, M. N., Varel, V. H., Arthur, T. M., Bosilevac, J. M., Freetly, H. C., Wheeler, T. L., Ferrell, C. L., & Koohmaraie, M. (2009). Prevalence and level of *Escherichia coli* O157:H7 in feces and on hides of feedlot steers fed diets with or without wet distillers grains with solubles. *Journal of Food Protection*, *72*(8), 1624–1633. https://doi.org/10.4315/0362-028x-72.8.1624

Westreicher-Kristen, E., Robbers, K., Blank, R., Tröscher, A., Dickhoefer, U., Wolffram, S., & Susenbeth, A. (2018). Postruminal digestion of starch infused into the abomasum of heifers with or without exogenous amylase administration. *Journal of Animal Science*, *96*(5), 1939–1951. https://doi.org/10.1093/jas/sky082

Whipp, S. C., Rasmussen, M. A., & Cray, W. C. (1994). Animals as a source of *Escherichia coli* pathogenic for human beings. *Journal of the American Veterinary Medical Association*, *204*(8), 1168–1175.

Wolin, M. J. (1969). Volatile Fatty Acids and the Inhibition of *Escherichia coli* Growth by Rumen Fluid. *Applied Microbiology*, *17*(1), 83–87.

Yoon, I. K., & Stern, M. D. (1995). Influence of direct-fed microbials on ruminal microbial fermentation and performance of ruminants—A Review -. *Asian-Australasian Journal of Animal Sciences*, *8*(6), 533–555. https://doi.org/1995.8.6.533

3.8. Tables & Figures

Table 3.1. Finishing diet composition fed to steers for 62 (sampling day 1), 78 (sampling day 2), and 93 (sampling 3) days during the finishing period.

Composition of finishing diets fed to steers[a].				
	CON silage		**EFC silage**	
Item	**CON grain**	**EFC grain**	**CON grain**	**EFC grain**
Ingredient, % of DM				
Control steam-flaked corn	74.5	0.0	74.5	0.0
EFC steam-flaked corn	0.0	74.5	0.0	74.5
Control corn silage	8.0	8.0	0.0	0.0
Enogen® corn silage	0.0	0.0	8.0	8.0
Ground alfalfa	2.0	2.0	2.0	2.0
Sweet Bran®	12.0	12.0	12.0	12.0
Supplement[b]	3.5	3.5	3.5	3.5
Analyzed nutrients[c], % of DM				
Crude protein	14.04	14.26	13.89	14.11
Acid detergent fiber	7.43	8.31	6.79	7.58
Ether extract	2.96	4.34	3.00	4.39
Calcium	0.71	0.72	0.71	0.72
Phosphorous	0.29	0.37	0.28	0.37
Potassium	0.75	0.87	0.68	0.80

[a] Diets were top dressed with 400 mg/animal daily of ractopamine (Optaflexx, Elanco Animal Health, Greenfield, IN) in a ground corn carrier for 35 d prior to harvest.
[b] Consisted of urea, salt, limestone, trace mineral premix, and potassium chloride, and provided 36.4 mg/kg monensin (Rumensin; Elanco Animal Health) in the total diet dry matter.
[c] Analyzed nutrient composition of monthly composite of each ingredient in total diet by SDK Labs (Hutchinson, KS)
[d] Animals were sampled in cohorts of 300-340 animals on 3 sampling days (6/12/18, 6/28/2018, and 7/13/2018)

Table 3.2. Silage analyzed nutrients and composition fed to steers for 62 (sampling day 1), 78 (sampling day 2), and 93 (sampling day 3) days during the finishing period.

Composition of silages fed to steers.		
Item	**CON silage**	**EFC silage**
Dry matter	26.96	35.67
Acid detergent fiber	22.15	32.78
Neutral detergent fiber	57.47	38.21
Starch	2.79	30.73
Ash	0.0	0.0
pH	3.93	3.78
Analyzed nutrients composition, % of DM		
Crude protein	9.15	7.26
Acid detergent fiber	36.15	27.07
Ether extract	2.04	2.63
Calcium	0.39	0.30
Phosphorous	0.24	0.19
Potassium	2.28	1.29

Table 3.3. Nutrient composition of Enogen® Feed Corn (EFC) and control (CON) corn fed to steers for 62 (sampling day 1), 78 (sampling day 2), and 93 (sampling day 3) days during the finishing period.

Composition of grain fed to steers.		
Analyzed nutrient composition, % DM	**CON corn**	**EFC corn**
Crude protein	8.81	9.11
Acid detergent fiber	2.84	4.02
Ether extract	3.20	5.05
Calcium	0.002	0.011
Phosphorous	0.178	0.293
Potassium	0.307	0.468

Table 3.4. Summary of *E. coli* O157:H7 (EC) binary responses from RAMS samples collected from cattle fed each diet sampling days 1, 2, and 3. Prevalence rates are not model adjusted.

		Sampling Day 1			Sampling Day 2			Sampling Day 3		
Silage	**Corn**	**N**	**EC* Pos.**	**Prevalence**	**N**	**EC* Pos.**	**Prevalence**	**N**	**EC* Pos.**	**Prevalence**
Enogen	Enogen	80	4	5.0%	84	3	3.6%	75	0	0.0%
	Control	80	7	8.8%	84	7	8.3%	73	2	2.7%
Control	Enogen	80	3	3.8%	85	5	5.9%	75	2	2.7%
	Control	80	8	10.0%	84	3	3.6%	75	2	2.7%

*EC Pos. represents *E. coli* O157:H7 positive samples.

Chapter 4 - Sodium Bisulfate and Peroxyacetic Acid Reduce *Escherichia coli* O157:H7 Populations on Fresh-Cut Romaine Lettuce When Applied as a Postharvest Wash Intervention

4.1. Abstract

Romaine lettuce has recently been implicated in multiple outbreaks of *Escherichia coli* O157:H7. Sodium hypochlorite (chlorine), commonly used to control foodborne pathogens in fresh-cut romaine lettuce wash water, is only moderately effective at decontaminating the product surface and research exploring novel interventions is necessary to improve fresh-cut romaine lettuce safety. In order to address this need, the purpose of this study was twofold: 1) evaluate sodium bisulfate (SBS) and peroxyacetic acid (PAA) effect on product quality in comparison to chlorine, water, and unwashed control treatments, and 2) quantify the reduction of *E. coli* O157:H7 on lettuce using a blend of SBS and PAA as a postharvest intervention. Lettuce exposed to one of six triple-wash treatments (30 seconds/wash and 90 seconds total exposure): municipal tap water, 80 ppm PAA, 0.75% SBS, 80 ppm PAA+0.75% SBS, chlorine (free chlorine; 10 ppm wash 1, 20-30 ppm wash 2 & 3), and an unwashed control. Washed lettuce was centrifuged to <2% additional moisture by weight, packaged in retail display packages, and stored at 7°C. The impact of each treatment on product quality and artificially inoculated *E. coli* O157:H7 populations was determined throughout shelf-life in two separate experiments. Quality was evaluated using visual, phytotoxic, browning, and color measurements. *E. coli* O157:H7 was enumerated on days 0, 1, 3, 5, 7, 10, 12, and 14, plating on Sorbitol MacConkey supplemented with cefixime and tellurite. Colorless *E. coli* O157:H7 colonies were counted following 37°C incubation for 18-24 hours. Treatment had no significant effect on phytotoxicity (P=0.1840) or

brownness & discoloration (P=0.0580). A significant treatment effect on overall visual quality was observed (P<0.0001); however, no mean quality scores were below the salable threshold of ≤4. Quality data indicate that SBS and SBS+PAA postharvest washes did not negatively influence fresh-cut romaine lettuce quality. Microbiological analysis revealed treatment was a statistically significant variable (P<0.0001). All treatments achieved significant reductions compared to the unwashed control (P<0.0001), with SBS, PAA, and SBS+PAA significantly more effective than chlorine at reducing *E. coli* O157:H7 (P<0.05). While SBS yielded the largest reduction in comparison to the unwashed control (2.2 log CFU/g), SBS, PAA, and SBS+PAA were not statistically different (P>0.05). PAA was not more effective than washing with water (P=0.2783). Application of SBS, PAA, and SBS+PAA represent effective chlorine alternatives for reducing *E. coli* O157:H7 on fresh-cut romaine lettuce.

4.2. Introduction

Escherichia coli O157:H7 is estimated to cause over 63,000 foodborne illnesses annually (Scallan et al., 2011) with 255 outbreaks (65% total outbreaks from 2003-2012) where food was identified as the transmission route (Heiman et al., 2015). Romaine lettuce has been implicated in numerous outbreaks of *E. coli* O157:H7, including the 2018 multistate outbreak originating from the Yuma, AZ region of the United States that infected 210 people, hospitalized 96 people (27 of those developed Hemolytic Uremic Syndrome (HUS)), and resulted in 5 deaths (Centers for Disease Control and Prevention, 2018). A recent 2019 outbreak of *E. coli* O157:H7 in fresh-cut romaine lettuce, originating from Salinas Valley CA, resulted in 167 cases, 85 hospitalizations, and 15 cases of HUS (Centers for Disease Control and Prevention, 2019c). Outbreaks of foodborne illness associated with romaine lettuce consumption are becoming increasingly common and present a significant public health threat.

Romaine lettuce, particularly fresh-cut, lacks a thermal processing step, leading to reliance on postharvest interventions to reduce the risk of contamination prior to consumption (Gil et al., 2015; Schuenzel & Harrison, 2002). Additionally, diverse surface morphologies, commodity epidermis abrasions, increases in consumer preferences for fresh produce, changes in production methods, and emergence of pathogens not previously associated with fresh produce have all contributed to increases in produce-related outbreaks (Burnett & Beuchat, 2001; C. McDaniel & Jadeja, 2019; Meireles et al., 2016). Fresh-cut produce handling, processing, and packaging influence a greater potential for microbial contamination compared to their unaltered counterparts (Qadri et al., 2015). Sodium hypochlorite (chlorine) is frequently used in fresh-cut romaine lettuce wash water to reduce the risk of contamination of foodborne pathogens (Feliziani et al., 2016); however, increases in produce outbreaks challenge its efficacy (Centers for Disease Control and Prevention, 2019b; Ölmez & Kretzschmar, 2009). Production of toxic disinfection byproducts following the reaction of chlorine with organic matter is concerning (Joshi et al., 2013) and even led to the ban of chlorine-based sanitizers in many countries of the European Union (Haute et al., 2013; Meireles et al., 2016).

A common chlorine alternative, peroxyacetic acid (PAA), is an organic acid consisting of acetic acid and hydrogen peroxide (Kitis, 2004). This antimicrobial compound demonstrates a wide spectrum of effectiveness, creating reactive oxygen species that damage bacterial cell membranes and denature proteins and enzymes (Kitis, 2004; Ölmez & Kretzschmar, 2009; Vandekinderen et al., 2009; Warburton, 2014). Recent research has described reductions of generic *E. coli* on romaine lettuce of 4.07 log CFU/g, following a 5 minute exposure to 100 ppm of PAA (Pahariya et al., 2019). In a similar study, efficacy of PAA against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* was evaluated against chlorine-based

sanitizers, with 100mg/L producing a reduction of 2.2 log CFU/g of *E. coli* O157:H7 and near-neutral electrolyzed water producing 2.3 log CFU/g reductions (Singh et al., 2018). Current literature has explored the application of PAA to fresh produce; however, PAA efficacy—alone or in combination with sodium bisulfate—against *E. coli* O157:H7 on fresh-cut romaine lettuce, the principle research question posed by this study, has yet to be evaluated.

Sodium bisulfate (SBS), once dissolved in water, lowers pH (Kim et al., 2018) creating an osmotic effect, which stresses bacterial cells, and often results in cell death (Y. Li et al., 1997). Declines in cytoplasmic pH requires enteric bacteria to expend energy to maintain homeostasis, stressing the bacteria and leading to cell death (Laury, et al., 2009). SBS is a natural food acid with GRAS status (Generally Recognized as Safe; 21 CFR 582.1095) that has been used in combination with PAA as an effective postharvest treatment against *Listeria innocua* on inoculated whole apples (Kim et al., 2018) and *Salmonella* spp. on whole chicken drumsticks (Dittoe et al., 2019). Additionally, both the Food and Agriculture Organization of the United Nations and the World Health Organization have recommended the use of SBS with no restrictions (FAO JECFA, 2007); thus, the use of SBS as a potential fresh-cut romaine lettuce intervention warrants investigation.

Research on postharvest interventions primarily focuses on efficacy of reducing foodborne pathogens; however, a need exists to investigate both produce quality and antimicrobial efficacy simultaneously (Ramos et al., 2013). Within the literature, several antimicrobial produce interventions, depending on concentration and duration of application, have shown deleterious effects on produce quality attributes including color, firmness, texture, along with visual and sensory quality (Alexandre et al., 2012; Guan et al., 2010; Karaca & Velioglu, 2007; Ramos et al., 2013). Conversely, treatments such as chlorine dioxide, acidified

sodium chlorite, hot-water surface pasteurization, and irradiation have shown little to no observable changes in produce quality (Fan et al., 2006; López-Gálvez et al., 2010; Nei et al., 2009; Sapers, 2014); thus, including both quality and microbial analyses is necessary when evaluating novel interventions.

The objective of this study was twofold: 1) evaluate the impact of SBS and PAA, alone or in combination, on visual, phytotoxic, browning, and color quality parameters of fresh-cut romaine lettuce compared to chlorine, water, and unwashed control treatments, and 2) quantify the reduction of *E. coli* O157:H7 on fresh-cut romaine lettuce using a novel blend of SBS and PAA as a postharvest intervention.

4.3. Materials and Methods

4.3.1 Quality Analysis

Fresh-cut romaine lettuce quality analysis was performed first utilizing non-inoculated product, and the quality data obtained informed the intervention concentrations used in the subsequent inoculation study.

4.3.1.1. Romaine Lettuce Preparation and Treatments

Fresh, field packed, unwashed romaine lettuce (*Lactuca sativa*) was purchased from a local wholesale supplier, cut into 2-inch sections using sterile tools, and held at 4^{o}C for approximately 2 hours until treatment application. Fresh-cut romaine lettuce was washed with one of ten treatments: 1) municipal tap water, 2) 80 ppm PAA, 3) 150 ppm total chlorine, 4) 0.25% SBS, 5) 0.5% SBS, 6) 0.75% SBS, 7) 0.25% SBS+ 80 ppm PAA, 8) 0.5% SBS + 80 ppm PAA, 9) 0.75% SBS + 80 ppm PAA, and 10) an unwashed control.

4.3.1.2. Treatment Preparation and Washing Procedure

Fresh-cut romaine lettuce was separated into 250g portions, and each portion was randomly assigned to one of the study treatments, and treated using the following parameters: control water wash applied for 2 minutes; chlorine wash applied for 2 minutes followed by a 10 second water wash; and SBS, PAA, and SBS+PAA applied for 2 minutes. To prepare all treatments, 1.5 gallons of municipal tap water was added to each 2.5-gal plastic tub (Thermo Scientific, Rochester, NY) and mixed with the appropriate chemical, yielding ten treatments. Treatment 1 consisted of municipal tap water; treatment 2 was prepared by adding 4.5 mL of a commercial produce wash (Tsunami 100^{TM}, Ecolab, St. Paul, MN) containing 15% PAA into the plastic tub and measured using PAA kit 311 (Ecolab, St. Paul, MN) according to manufacturer's guidelines in order to achieve a target concentration of 80±5 ppm of PAA (21 CFR § 173.315). Treatments 4, 5, and 6 were prepared by mixing 28.39, 56.78, and 85.17g of SBS (pHase^{TM}, Jones Hamilton Co., Walbridge, OH) for 3 minutes to achieve a target concentration of 0.25, 0.5, and 0.75% SBS weight by volume, respectively. Combining steps outlined above for SBS treatments 4, 5, and 6 along with PAA treatment 2, yielded treatments 7, 8, and 9. Treatment 3 was prepared with the addition of germicidal bleach (Clorox Professional Products Company, Oakland, CA) to achieve a target concentration of 150 ppm total chlorine. Total chlorine was measured using an ultra-high range chlorine meter (Hanna Instruments, Woonsocket, RI). The pH of the chlorine solution was determined using a pH meter (Accumet^{TM} AE150 Benchtop pH Meter, Fisher Scientific, Waltham, MA) and adjusted using 1 N HCl or 1 N NaOH (ThermoFisher Scientific Inc., Asheville, NC) to achieve pH 6.5-7.0.

A total of 250g fresh-cut, non-inoculated lettuce product was placed into slotted containers (8.6 cm x 24.1 cm x 19 cm; InterDesign, Solon, OH) and submerged into 1 of 10 treatments, following the previously mentioned parameters. Following washing, lettuce was

dried via centrifugation using a salad spinner (26 cm diameter; Prepworks®, Kent, WA) that was pulled 50 times to remove excess liquid, thereby achieving <2% additional moisture by weight. Product was packed into retail display packages (American Packaging Corporation, Columbus, WI; structure: 48ga. OPP/1.5 mil linear low-density polyethylene (LLDPE), roll width: 19.5", OTR: 120cc/100in2/day) designed for fresh-cut romaine lettuce (21.66 x 14.78 cm; 50 g lettuce) and stored at 7°C in a low temperature incubator (ThermoFisher Scientific Inc., Asheville, NC) to mimic retail display conditions (O'Beirne et al., 2015). The ratio of packaging size to lettuce weight was chosen to simulate the ratio in larger packages of commercially sold fresh-cut romaine lettuce.

4.3.1.3. Visual Quality Evaluation

Quality was evaluated by trained personnel using the following parameters and rating scales:

(1) Overall visual quality (OVQ) was evaluated by rating 10 randomly selected leaves using a previously developed scale (Kader and Cantwell, 2010) with ratings of 1 to 9 with 9 being excellent; scores ≤4 indicating an unsalable product (Table 4.1).

(2) Browning and Discoloration was evaluated by rating the 50 g of fresh-cut romaine lettuce in each package using a previously developed scale (Cantwell, 2013) with ratings of 1 to 5 with 1= less than 5% of leaves showing visible defects and 5= ≥30% (Figure 4.1)

(3) Phytotoxic effects—caused by high concentrations of chemical compounds, i.e., pesticides, produce washes, or phytotoxins—were evaluated by rating 10 randomly selected leaves using a newly developed phytotoxicity scale (Figure 4.2). Special attention was drawn to deformation, necrosis, or yellowing of leaves and leaf edges.

A score of 1= no observable phytotoxic effects and 5=>75% total leaf area with observable phytotoxic effects.

For OVQ and phytotoxicity measurements, 10 pieces of leaves were selected randomly and evaluated against the scale. For browning and discoloration measurements, 50g of fresh-cut product was evaluated and scored against the scale. Quality evaluations were conducted immediately after removal from incubation at 7^{o}C with 85% relative humidity (Forma Environmental Chambers; ThermoFisher Scientific Inc., Asheville, NC), on days 0, 1, 3, 5, 7, and 10.

4.3.1.4. Color Measurements

In addition to visual evaluations, color was determined using an A5 Chroma-Meter Minolta CR-400 (Minolta Co. Ltd., Osaka, Japan) and expressed as CIELAB color system, and L* (lightness), a* (-greenness to +redness), and b*(-blueness to +yellowness) readings were collected. Additionally, the intensity of color saturation, Chroma [$C^* = (a^{*2} + b^{*2})^{0.5}$], and hue angle [$h^o = 180^o + \tan^{-1}(b^*/a^*)$] were calculated from the a* and b* values as previously outlined (Lancaster et al., 1997; McGuire, 1992). A single color measurement was taken on 10 separate pieces of lettuce from each treatment, on each measurement day. Chroma-Meter was calibrated using manufacturer's standard white tile.

4.3.2 Microbiological Analysis

4.3.2.1. Culture Preparation

Frozen stock cultures of *Escherichia coli* O157:H7, stored in tryptic soy broth (TSB; BD Difco, Franklin Lakes, NJ) with 15% glycerol at -80^{o}C, were used to create the inoculum cocktail. Cultures were initially isolated from fellow researchers at Kansas State University and were originally isolated from cattle. The cocktail consisted of four *E. coli* O157:H7 strains, and

included 2 pansusceptible and 2 resistant to one or more of the following veterinary antimicrobials: cefoxitin, azithromycin, chloramphenicol, tetracycline, ceftriaxone, amoxicillin/clavulanic acid, ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, sulfisoxazole, trimethoprim/sulfamethoxazole, kanamycin, ampicillin or streptomycin. In addition to susceptibility testing, all strains were previously confirmed and serotyped. Each strain was activated from frozen storage by streaking onto tryptic soy agar (TSA; BD Difco, Franklin Lakes, NJ) and incubating at 37ºC for 24 h. One colony from each plate was transferred to a 9 mL TSB tube, with a single TSB tube for each strain (4 TSB tubes total), and incubated at 37ºC for 18 h. For each strain tube, 5.2 mL was combined and added to an empty centrifuge tube and centrifuged at 5,500 x *g* for 15 min at 4ºC (Allegra X-30R, Beckman Coulter, Brea, CA). The supernatant was discarded, and the resultant pellet was resuspended in 20.9 mL of buffered peptone water (BPW; Difco, Franklin Lakes, NJ) and diluted in 20.88 L of BPW, to achieve a target inoculum titer of 6.0 log CFU/mL.

4.3.2.2. Romaine Lettuce Preparation and Inoculation

Fresh-cut romaine lettuce (2400 g total), procured and prepared as described in section 4.3.1.1, was inoculated via submersion with an *E. coli* O157:H7 cocktail as previously described (Gragg & Brashears, 2010). Lettuce was submerged in inoculum for 20 minutes then dried on four stainless steel trays with grates under a biosafety cabinet for 60 minutes. To facilitate even drying, the lettuce was rotated using sterile tongs after 30 minutes of drying.

4.3.2.3. Wash Preparation and Application

Following inoculation and drying, the inoculated lettuce was subjected to postharvest wash treatments. Each treatment was applied to the lettuce in a sequence of three washes, simulating a triple-wash industry application. The treatments included: 1) municipal tap water, 2)

80 ppm PAA, 3) chlorine (free chlorine; 10 ppm wash 1, 20-30 ppm wash 2 & 3), 4) 0.75% SBS, 5) 0.75% SBS + 80 ppm PAA, and 6) an unwashed control. Treatments were prepared according to the washing protocols outlined in the quality analysis section, with minor variations. Each wash consisted of gentle, manual agitation for 30 seconds, with a 10 second drain period separating each wash, yielding 90 seconds of total exposure. Control of wash water temperatures for all treatments were maintained at <60^{o}F for washes 1 and 2, and 36±3^{o}F for wash 3.

Inoculated lettuce was washed and centrifuged as described above. To protect against aerosol generation, the salad spinner was placed in a biohazard bag and centrifugation occurred under a biosafety cabinet (BSC), with the biohazard bag opening aimed inside the BSC and closed tightly by hand during operation. Following centrifugation, the salad spinner was removed from the biohazard bag and opened under the BSC for sampling and packaging. The product was packaged as described in section 4.3.1. in retail display packages (American Packaging Corporation, Columbus, WI; structure: 48ga. OPP/1.5 mil LLDPE, roll width: 19.5", OTR: 120cc/100in2/day) designed for fresh-cut romaine lettuce (21.66 x 14.78 cm; 50 g lettuce). Utilizing a passive atmosphere approach, each package was flushed with nitrogen (Matheson Gas, St Joseph, MO) prior to sealing, achieving 3-5% oxygen, and packaging atmosphere was confirmed using a portable gas analyzer (model 900141; Bridge Analyzers, Alameda, CA). Sealed packages were stored at 7^{o}C in a low temperature incubator (ThermoFisher Scientific Inc., Asheville, NC) to mimic retail display conditions (O'Beirne et al., 2015).

4.3.2.4. *E. coli* O157:H7 Enumeration

E. coli O157:H7 populations were enumerated on days 0, 1 , 3 , 5 , 7 , 10, 12, and 14 by homogenizing (Stomacher® 400 Circulator, Seward, Bohemia, NY) 25g of product with 225mL of Dey-Engley neutralizing broth (DNB; BD BBL ^{TM}, New Jersey) for 1 minute at 230 rpm.

Homogenized samples were diluted with 0.1% peptone water (PW; BD Difco, Franklin Lakes, NJ), spread plated onto Sorbitol MacConkey agar (CT-SMAC; Remel, Lenexa, KS) supplemented with cefixime and tellurite (CT-Supplement, Milipore Sigma, St. Louis, MO), and incubated at 37°C for 18-24 hours.

4.4. Statistical Analysis

Three replications of both quality and microbiological studies were completed. For each study, data were statistically analyzed using the MIXED procedure with Tukey's adjustment of Statistical Analysis Software (SAS 9.4; Cary, NC). Statistical significance of the main effects of day and treatment, as well as the day x treatment interaction, was evaluated at the $P \leq 0.05$ threshold.

During the microbiology portion of the study, increased background flora growth was observed on the CT-SMAC plates after sampling day 10. This resulted in difficulty obtaining accurate plate counts and questionable plate count data from days 12 and 14. Therefore, these data were eliminated from the data set, and statistical analyses were performed on data from days 0 through 10 only.

4.5. Results

4.5.1 Quality Analysis

4.5.1.1. Overall Visual Quality

The main effects of sampling day ($P<0.0001$, Figure 4.3) and treatment ($P<0.0001$; Figure 4.4) were significant for OVQ score. OVQ declined throughout the shelf-life, as is shown in Figure 4.4. Although treatment significantly impacted OVQ, no treatment was different than the unwashed control or below the salable threshold of ≤4. The treatment x day interaction ($P=0.4361$) was not significant; therefore, data are only presented for main effects.

4.5.1.2. Browning

Browning score varied according to sampling day ($P<0.0001$), as is depicted in Figure 4.5. Browning increased during the shelf-life, with a notable and significant increase occurring after sampling day 3. Treatment ($P=0.0580$) did not impact browning and the treatment x day interaction ($P=0.3151$) was also not significant.

4.5.1.3. Phytotoxicity

The main effect of treatment did not impact phytotoxicity score ($P=0.1840$) and the treatment x day interaction was also not significant ($P=0.2664$). As Figure 4.6 illustrates, sampling day was a significant main effect ($P=0.0298$), with phytotoxicity increasing throughout the shelf-life. However, the adjusted *p*-values for multiple comparisons, calculated using Tukey's adjustment, did not result in significant differences between sampling days, indicating no statistical difference in phytotoxicity.

4.5.1.4. Color

Treatment had no statistically significant effect on L* ($P=0.9880$), a* ($P=0.1650$), b*($P=0.8518$), or C* ($P=0.7146$), which indicates that lettuce color was not affected by treatment. The main effect of day was significant for L* ($P=0.0163$), a* ($P=0.0030$), b*($P<0.0001$), and C* ($P<0.0001$), indicating that color varied throughout shelf-life. The treatment x day interaction was not significant for L* ($P=0.8871$), a* ($P=0.5347$), b*($P=0.8653$), or C* ($P=0.8282$).

4.5.1.5. Treatment Selection for Inoculated Study

The compilation of diverse quality data collected suggests that that all treatments are acceptable as a postharvest wash for fresh-cut romaine lettuce. Therefore, the highest concentration of SBS (0.75% w/v) was selected for use in the subsequent inoculation study, as it

was hypothesized that the strongest concentration would be the most effective at reducing *E. coli* O157:H7 on fresh-cut romaine lettuce.

4.5.2. Microbiological Analysis

The main effect of sampling day was significant ($P<0.0001$; Figure 4.7), with *E. coli* O157:H7 populations declining throughout the storage period. The effect of treatment was a significant main effect ($P<0.0001$; Figure 4.8), indicating differences in efficacy exist between treatments. All treatments significantly reduced *E. coli* O157:H7 populations in comparison to the unwashed control, with SBS+PAA, PAA, and SBS yielding reductions of 2.3 log CFU/g, 1.9 log CFU/g, and 2.2 log CFU/g, respectively. SBS ($P=0.0032$) and SBS+PAA ($P=0.0002$) were both more effective at reducing *E. coli* O157:H7 populations compared to the water wash treatment; however, PAA ($P=0.2783$) and chlorine ($P=0.3284$) were as effective as washing with water. Reductions achieved by SBS, PAA, and SBS+PAA were statistically the same as one another ($P>0.05$) and all were more effective than the industry standard chlorine wash ($P<0.05$). Because the treatment x sampling day interaction was not significant ($P=0.9421$), data are only presented according to the significant main effects.

4.6. Discussion

The present study investigated the effect of washing fresh-cut romaine lettuce with concentrations of SBS, PAA, SBS+PAA, and chlorine on lettuce quality and inoculated *E. coli* O157:H7 populations. Quality data indicated that all treatments were acceptable for lettuce, and these data informed treatment concentration selection for use in the subsequent inoculation study. When applied to inoculated product, all treatments achieved significant reductions of *E. coli* O157:H7 in comparison to the unwashed control. SBS+PAA produced the greatest reduction (2.3 CFU/g), followed by SBS (2.2 CFU/g), and PAA (1.9 CFU/g); however, these reductions

were statistically similar to one another (P>0.05). To our knowledge, this represents the first study analyzing the efficacy of SBS and PAA, alone or in combination, against *E. coli* O157:H7 for application as a postharvest wash intervention for fresh-cut romaine lettuce.

Research in the literature has explored SBS and PAA applications separately in whole apples, poultry processing, frankfurters, chicken drumsticks, and pet food (Bodie et al., 2019; Dhakal et al., 2019; Dittoe et al., 2019; Kim et al., 2018; Micciche et al., 2019). Kim et. al, demonstrated a 4.30 log CFU/g reduction of *Listeria innocua* on whole apples using 1% SBS+60 ppm PAA (Kim et al., 2018) while Dittoe et. al, achieved a 5.42 log CFU/g reduction on whole chicken drumsticks using 1% SBS+200 ppm PAA (Dittoe et al., 2019). In poultry, SBS alone or in combination with PAA yielded pathogen reductions that were not statistically different (Dittoe et al., 2019). Comparison of the present study to SBS and PAA efficacy reported in the literature presents a difficult challenge, as reductions were observed using different food matrices, concentrations, and foodborne pathogens. Presently, efficacy of PAA, SBS, and SBS+PAA have not been explored for fresh-cut romaine lettuce; in this regard, the research presented herein provides foundational understanding for how these interventions may be used to effectively control *E. coli* O157:H7 as a postharvest intervention for fresh-cut romaine lettuce.

Recently, analysis of treatment efficacy on lettuce, spinach, and other leafy greens has compared both alternative and traditional methodologies. Jung et al (2017), reported that soaking romaine heads in electrolyzed water yielded significant reductions of *Salmonella enterica* (2.8 ± 1.5 log CFU/g), *Listeria monocytogenes* (2.6 ± 0.7 log CFU/g), and *E. coli* O157:H7 (3.4 ± 1.1 log CFU/g) compared to municipal tap water, lactic and phosphoric acid-based sanitizers, and a citric acid-based sanitizer (Jung et al., 2017). The 3.4 log CFU/g reduction in *E. coli* O157:H7 reported by Jung et al. (2017) exceeds the <2.5 log CFU/g reductions achieved in the present

study. Pahariya et al. (2019) compared acidified sodium chlorite and sodium hypochlorite to PAA, with PAA producing significant reductions of generic *E. coli* (4.07 log CFU/g) after soaking fresh-cut romaine lettuce for 5 minutes (Pahariya et al., 2019). When comparing PAA and chlorine efficacy against *E. coli* O157:H7 in romaine lettuce, Singh et al, observed a 2.2 and 2.1 log CFU/g reduction, respectively (Singh et al., 2018), which is similar to the reductions observed by PAA and chlorine treatments in the present study. Differences in methodology are often attributed to variations in foodborne pathogen reductions. In the present experiment, fresh-cut romaine lettuce was inoculated via submersion (Gragg & Brashears, 2010) in an *E. coli* O157:H7 cocktail as a "worst-case scenario", which also mimics a contamination scenario wherein the romaine becomes contaminated while submerged during washing and may also lead to internalization. Similar studies utilized pipette spot inoculation on romaine lettuce leaves or leaf punches (J. L. Banach et al., 2020; Jung et al., 2017; Pahariya et al., 2019; Qi & Hung, 2019), were inoculated using a pressurized sprayer (Luo et al., 2012), or spot inoculated then surface spread using an un-inoculated leaf as an applicator (Singh et al., 2018). Variation in inoculation methodology likely contributes to reported differences in treatment efficacy and repeating this study utilizing different approaches to inoculation presents an opportunity for future research.

Leafy green quality, particularly fresh-cut, is heavily influenced by environmental changes, harsh handling and processing, and water loss (Qadri et al., 2015). Investigation into any produce safety intervention for use on leafy greens requires both microbial and quality analyses. In the present study, treatment did not significantly impact color, browning, or phytotoxicity of fresh-cut romaine lettuce. Treatment did impact the OVQ, with differences observed among treatments; however, all treatments were not significantly different than the

control and all treated fresh-cut romaine lettuce was rated above the salable threshold of ≤4. Fan et. al, (2009) observed that SBS, when used as a postharvest wash treatment, demonstrated an anti-browning effect in apples, and that effect increased as concentration increased (Fan et al., 2009). Salgado et. al, (2014) reported significantly higher OVQ of romaine lettuce treated with chlorine in comparison to PAA and other washes (Salgado et al., 2014); however, this was not observed in the present study. Recently, activated persulfate demonstrated a 3.5 log CFU/g reduction of *E. coli* O157:H7 and *Listeria monocytogenes* populations without causing significant changes in romaine lettuce color quality (Qi & Hung, 2019). In a review by Ramos et al. (2013), postharvest interventions consisting of hydrogen peroxide, ozone, organic acids, and electrolyzed water reportedly caused significant negative impacts on the quality of fruits and vegetables, regardless of their potential to reduce foodborne pathogens (Ramos et al., 2013). The body of literature suggests that postharvest washes may be effective at reducing populations of *E. coli* O157:H7 and other pathogens; however, the impact on product quality must always be considered.

The antimicrobial efficacy of chlorine, the most widely used treatment in the fresh-cut industry, has been challenged due to increasing numbers of produce related outbreaks (Centers for Disease Control and Prevention, 2019b; Kintz et al., 2019; C. McDaniel & Jadeja, 2019; Ölmez & Kretzschmar, 2009). Moreover, chlorine has been shown to react with organic matter, producing carcinogenic halogenated disinfection byproducts (Batterman et al., 2000; Hua & Reckhow, 2007; Singer Philip C., 1994). Environmental concerns, particularly the association of chlorine with high amounts of wastewater production and very high levels of biological oxygen demand, have led to a chlorine ban in the European Union for use in organic production (Ölmez & Kretzschmar, 2009). Food safety, health, and environmental risks associated with the use of

chlorine in postharvest wash water necessitate research for validated, effective alternatives for the produce industry. In the present study, SBS, PAA, and SBS+PAA washes were more effective than the industry standard chlorine at reducing *E. coli* O157:H7 on fresh-cut romaine lettuce. This combined with the fact that SBS, PAA, and SBS+PAA did not negatively influence product quality suggests that these treatments may be effective alternatives for postharvest washing of fresh-cut romaine lettuce.

4.7. Conclusion

This research demonstrates that including SBS, PAA, and SBS+PAA in postharvest wash water is effective at reducing *E. coli* O157:H7 on fresh-cut romaine lettuce and did not negatively influence product quality. There is opportunity for future research to investigate efficacy of these treatments as postharvest food safety interventions in other produce or meat products. Ground beef, sprouts, pork products, and pre-cut melon applications should all be considered given their implication in recent outbreaks (Centers for Disease Control and Prevention, 2019b; Reiley, 2019). Additionally, future research efforts focused on determining antimicrobial efficacy of SBS, PAA, and SBS+PAA at reducing foodborne pathogens as organic load increases in produce wash water would provide practical information that may improve adoption of these treatments as postharvest interventions. As increases in produce-associated foodborne illness outbreaks are reported, investigation into new and effective interventions is warranted in order to protect public health.

4.8. References

Alexandre, E. M. C., Brandão, T. R. S., & Silva, C. L. M. (2012). Assessment of the impact of hydrogen peroxide solutions on microbial loads and quality factors of red bell peppers, strawberries and watercress. *Food Control*, *27*(2), 362–368. https://doi.org/10.1016/j.foodcont.2012.04.012

Banach, J. L., van Bokhorst-van de Veen, H., van Overbeek, L. S., van der Zouwen, P. S., Zwietering, M. H., & van der Fels-Klerx, H. J. (2020). Effectiveness of a peracetic acid solution on *Escherichia coli* reduction during fresh-cut lettuce processing at the laboratory and industrial scales. *International Journal of Food Microbiology*, 108537. https://doi.org/10.1016/j.ijfoodmicro.2020.108537

Batterman, S., Zhang, L., & Wang, S. (2000). Quenching of chlorination disinfection by-product formation in drinking water by hydrogen peroxide. *Water Research*, *34*(5), 1652–1658. https://doi.org/10.1016/S0043-1354(99)00294-8

Bodie, A. R., Dittoe, D. K., Feye, K. M., Knueven, C. J., & Ricke, S. C. (2019). Application of an Alternative Inorganic Acid Antimicrobial for Controlling *Listeria monocytogenes* in Frankfurters. *Frontiers in Sustainable Food Systems*, *3*. https://doi.org/10.3389/fsufs.2019.00034

Burnett, S. L., & Beuchat, L. R. (2001). Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. *Journal of Industrial Microbiology & Biotechnology*, *27*(2), 104–110. https://doi.org/10.1038/sj.jim.7000199

Cantwell, M., & Suslow, T. (2002). Postharvest Handling Systems: Fresh-Cut Fruits and Vegetables. In *Postharvest Technology of Horticultural Crops* (3rd ed., pp. 445–464). Library of Congress.

Cantwell, Ma. (2013, June). *Fresh-cut Fruit & Vegetables* [Lecture].

Centers for Disease Control and Prevention. (2018, June 28). *Multistate Outbreak of E. coli O157:H7 Infections Linked to Romaine Lettuce (Final Update)*. https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html

Centers for Disease Control and Prevention. (2019a, November 22). *List of Selected Multistate Foodborne Outbreak Investigations | Foodborne Outbreaks | Food Safety | CDC*. https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html

Centers for Disease Control and Prevention. (2019b, November 23). *Outbreak of E. coli Infections Linked to Romaine Lettuce | E.coli Infections | November 2019 | E. coli | CDC*. https://www.cdc.gov/ecoli/2019/o157h7-11-19/index.html

Dhakal, J., Aldrich, C. G., & Knueven, C. (2019). Assessing the Efficacy of Sodium Bisulfate and Organic Acid Treatments for Control of *Salmonella Typhimurium* in Rendered

Chicken Fat Applied to Pet Foods. *Journal of Food Protection*, *82*(11), 1864–1869. https://doi.org/10.4315/0362-028X.JFP-18-560

Dittoe, D. K., Atchley, J. A., Feye, K. M., Lee, J. A., Knueven, C. J., & Ricke, S. C. (2019). The Efficacy of Sodium Bisulfate Salt (SBS) Alone and Combined With Peracetic Acid (PAA) as an Antimicrobial on Whole Chicken Drumsticks Artificially Inoculated With *Salmonella Enteritidis*. *Frontiers in Veterinary Science*, *6*. https://doi.org/10.3389/fvets.2019.00006

Fan, X., Annous, B. A., Sokorai, K. J. B., Burke, A., & Mattheis, J. P. (2006). Combination of Hot-Water Surface Pasteurization of Whole Fruit and Low-Dose Gamma Irradiation of Fresh-Cut Cantaloupe. *Journal of Food Protection*, *69*(4), 912–919. https://doi.org/10.4315/0362-028X-69.4.912

Fan, X., Sokorai, K. J. B., Liao, C.-H., Cooke, P., & Zhang, H. Q. (2009). Antibrowning and Antimicrobial Properties of Sodium Acid Sulfate in Apple Slices. *Journal of Food Science*, *74*(9), M485–M492. https://doi.org/10.1111/j.1750-3841.2009.01362.x

FAO JECFA. (2007). *World Health Organization Compendium of Food Additive Specifications* (Vol. 4).

Feliziani, E., Lichter, A., Smilanick, J. L., & Ippolito, A. (2016). Disinfecting agents for controlling fruit and vegetable diseases after harvest. *Postharvest Biology and Technology*, *122*, 53–69. https://doi.org/10.1016/j.postharvbio.2016.04.016

Gil, M. I., Gómez-López, V. M., Hung, Y.-C., & Allende, A. (2015). Potential of Electrolyzed Water as an Alternative Disinfectant Agent in the Fresh-Cut Industry. *Food and Bioprocess Technology*, *8*(6), 1336–1348. https://doi.org/10.1007/s11947-014-1444-1

Gragg, S. E., & Brashears, M. M. (2010). Reduction of *Escherichia coli* O157:H7 in Fresh Spinach, Using Lactic Acid Bacteria and Chlorine as a Multihurdle Intervention. *Journal of Food Protection*, *73*(2), 358–361. https://doi.org/10.4315/0362-028X-73.2.358

Guan, W., Huang, L., & Fan, X. (2010). Acids in Combination with Sodium Dodecyl Sulfate Caused Quality Deterioration of Fresh-Cut Iceberg Lettuce during Storage in Modified Atmosphere Package. *Journal of Food Science*, *75*(8), S435–S440. https://doi.org/10.1111/j.1750-3841.2010.01786.x

Haute, S. V., Sampers, I., Holvoet, K., & Uyttendaele, M. (2013). Physicochemical Quality and Chemical Safety of Chlorine as a Reconditioning Agent and Wash Water Disinfectant for Fresh-Cut Lettuce Washing. *Applied and Environmental Microbiology*, *79*(9), 2850–2861. https://doi.org/10.1128/AEM.03283-12

Heiman, K. E., Mody, R. K., Johnson, S. D., Griffin, P. M., & Gould, L. H. (2015). *Escherichia coli* O157 Outbreaks in the United States, 2003–2012. *Emerging Infectious Diseases*, *21*(8), 1293–1301. https://doi.org/10.3201/eid2108.141364

Hua, G., & Reckhow, D. A. (2007). Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water Research*, *41*(8), 1667–1678. https://doi.org/10.1016/j.watres.2007.01.032

Joshi, K., Mahendran, R., Alagusundaram, K., Norton, T., & Tiwari, B. K. (2013). Novel disinfectants for fresh produce. *Trends in Food Science & Technology*, *34*(1), 54–61. https://doi.org/10.1016/j.tifs.2013.08.008

Jung, Y., Jang, H., Guo, M., Gao, J., & Matthews, K. R. (2017). Sanitizer efficacy in preventing cross-contamination of heads of lettuce during retail crisping. *Food Microbiology*, *64*, 179–185. https://doi.org/10.1016/j.fm.2017.01.005

Kader, A., & Marita, C. (2010). *Produce Quality Rating Scales and Color Charts* (2nd ed.). UC Davis.

Karaca, H., & Velioglu, Y. S. (2007). Ozone Applications in Fruit and Vegetable Processing. *Food Reviews International*, *23*(1), 91–106. https://doi.org/10.1080/87559120600998221

Kim, S. A., Park, S. H., Knueven, C., Basel, R., & Ricke, S. C. (2018). A decontamination approach using a combination of bisulfate of soda and peracetic acid against *Listeria innocua* inoculated on whole apples. *Food Control*, *84*, 106–110. https://doi.org/10.1016/j.foodcont.2017.07.036

Kintz, E., Byrne, L., Jenkins, C., McCARTHY, N., Vivancos, R., & Hunter, P. (2019). Outbreaks of Shiga Toxin–Producing *Escherichia coli* Linked to Sprouted Seeds, Salad, and Leafy Greens: A Systematic Review. *Journal of Food Protection*, *82*(11), 1950–1958. https://doi.org/10.4315/0362-028X.JFP-19-014

Kitis, M. (2004). Disinfection of wastewater with peracetic acid: A review. *Environment International*, *30*(1), 47–55. https://doi.org/10.1016/S0160-4120(03)00147-8

Lancaster, J. E., Lister, C. E., Reay, P. F., & Triggs, C. M. (1997). Influence of Pigment Composition on Skin Color in a Wide Range of Fruit and Vegetables. *Journal of the American Society for Horticultural Science*, *122*(4), 594–598. https://doi.org/10.21273/JASHS.122.4.594

Laury, A. M., Alvarado, M. V., Nace, G., Alvarado, C. Z., Brooks, J. C., Echeverry, A., & Brashears, M. M. (2009). Validation of a lactic acid- and citric acid-based antimicrobial product for the reduction of *Escherichia coli* O157: H7 and *Salmonella* on beef tips and whole chicken carcasses. *Journal of Food Protection*, *72*(10), 2208-2211.

Lj, Y., Slavik, M. F., Walker, J. T., & Xiong, H. (1997). Pre-Chill Spray of Chicken Carcasses to Reduce *Salmonella typhimurium*. *Journal of Food Science*, *62*(3), 605–607. https://doi.org/10.1111/j.1365-2621.1997.tb04441.x

López-Gálvez, F., Gil, M. I., Truchado, P., Selma, M. V., & Allende, A. (2010). Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot

be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology*, *27*(2), 199–204. https://doi.org/10.1016/j.fm.2009.09.009

Luo, Y., Nou, X., Millner, P., Zhou, B., Shen, C., Yang, Y., Wu, Y., Wang, Q., Feng, H., & Shelton, D. (2012). A pilot plant scale evaluation of a new process aid for enhancing chlorine efficacy against pathogen survival and cross-contamination during produce wash. *International Journal of Food Microbiology*, *158*(2), 133–139. https://doi.org/10.1016/j.ijfoodmicro.2012.07.008

McDaniel, C., & Jadeja, R. (2019). *A review of fresh produce outbreaks, current interventions, food safety concerns and potential benefits of novel antimicrobial sodium acid sulfate*.

McGuire, R. G. (1992). Reporting of Objective Color Measurements. *HortScience*, *27*(12), 1254–1255. https://doi.org/10.21273/HORTSCI.27.12.1254

Meireles, A., Giaouris, E., & Simões, M. (2016). Alternative disinfection methods to chlorine for use in the fresh-cut industry. *Food Research International*, *82*, 71–85. https://doi.org/10.1016/j.foodres.2016.01.021

Micciche, A. C., Feye, K. M., Rubinelli, P. M., Lee, J. A., Knueven, C. J., & Ricke, S. C. (2019). Comparison of Acid Sanitizers on *Salmonella Typhimurium* Inoculated Commercial Poultry Processing Reuse Water. *Frontiers in Sustainable Food Systems*, *2*. https://doi.org/10.3389/fsufs.2018.00090

Nei, D., Choi, J.-W., Bari, Md. L., Kawasaki, S., Kawamoto, S., & Inatsu, Y. (2009). Efficacy of Chlorine and Acidified Sodium Chlorite on Microbial Population and Quality Changes of Spinach Leaves. *Foodborne Pathogens and Disease*, *6*(5), 541–546. https://doi.org/10.1089/fpd.2008.0237

O'Beirne, D., Gomez-Lopez, V., Tudela, J. A., Allende, A., & Gil, M. I. (2015). Effects of oxygen-depleted atmospheres on survival and growth of *Listeria monocytogenes* on fresh-cut Iceberg lettuce stored at mild abuse commercial temperatures. *Food Microbiology*, *48*, 17–21. https://doi.org/10.1016/j.fm.2014.11.012

Ölmez, H., & Kretzschmar, U. (2009). Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. *LWT - Food Science and Technology*, *42*(3), 686–693. https://doi.org/10.1016/j.lwt.2008.08.001

Pahariya, P., Choudhary, R., & Fisher, D. J. (2019). Antimicrobial Effect of Sanitizing Solutions on Fresh Romaine Lettuce. *2019 Boston, Massachusetts July 7- July 10, 2019*. 2019 Boston, Massachusetts July 7- July 10, 2019. https://doi.org/10.13031/aim.201901623

Qadri, O. S., Yousuf, B., & Srivastava, A. K. (2015). Fresh-cut fruits and vegetables: Critical factors influencing microbiology and novel approaches to prevent microbial risks—A review. *Cogent Food & Agriculture*, *1*(1), 1121606. https://doi.org/10.1080/23311932.2015.1121606

Qi, H., & Hung, Y.-C. (2019). Effectiveness of activated persulfate in removal of foodborne pathogens from romaine lettuce. *Food Control*, *106*, 106708. https://doi.org/10.1016/j.foodcont.2019.106708

Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P., & Silva, C. L. M. (2013). Fresh fruits and vegetables—An overview on applied methodologies to improve its quality and safety. *Innovative Food Science & Emerging Technologies*, *20*, 1–15. https://doi.org/10.1016/j.ifset.2013.07.002

Reiley, L. (2019, April 25). 2018 saw the most multistate outbreaks of foodborne illness in more than a decade, CDC says. *Washington Post*. https://www.washingtonpost.com/business/2019/04/25/cdc-releases-its-annual-report-card-foodborne-illness-did-not-have-passing-grade/

Salgado, S. P., Pearlstein, A. J., Luo, Y., & Feng, H. (2014). Quality of Iceberg (Lactuca sativa L.) and Romaine (*L. sativa L. var. Longifolial*) lettuce treated by combinations of sanitizer, surfactant, and ultrasound. *LWT - Food Science and Technology*, *56*(2), 261–268. https://doi.org/10.1016/j.lwt.2013.11.038

Sapers, G. M. (2014). Chapter 17—Disinfection of Contaminated Produce with Conventional Washing and Sanitizing Technology. In K. R. Matthews, G. M. Sapers, & C. P. Gerba (Eds.), *The Produce Contamination Problem (Second Edition)* (pp. 389–431). Academic Press. https://doi.org/10.1016/B978-0-12-404611-5.00017-8

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.P11101

Schuenzel, K. M., & Harrison, M. A. (2002). Microbial antagonists of foodborne pathogens on fresh, minimally processed vegetables. *Journal of Food Protection*, *65*(12), 1909–1915. https://doi.org/10.4315/0362-028x-65.12.1909

Singer Philip C. (1994). Control of Disinfection By-Products in Drinking Water. *Journal of Environmental Engineering*, *120*(4), 727–744. https://doi.org/10.1061/(ASCE)0733-9372(1994)120:4(727)

Singh, P., Hung, Y.-C., & Qi, H. (2018). Efficacy of Peracetic Acid in Inactivating Foodborne Pathogens on Fresh Produce Surface. *Journal of Food Science*, *83*(2), 432–439. https://doi.org/10.1111/1750-3841.14028

Vandekinderen, I., Devlieghere, F., Van, J. C., Kerkaert, B., Cucu, T., Ragaert, P., De, J. B., & De, B. M. (2009). Effects of food composition on the inactivation of foodborne microorganisms by chlorine dioxide. *International Journal of Food Microbiology*, *131*(2–3), 138–144. https://doi.org/10.1016/j.ijfoodmicro.2009.02.004

Warburton, R. (2014, June 13). *Peracetic Acid in the Fresh Food Industry*. Food Safety Magazine. https://www.foodsafetymagazine.com/signature-series/peracetic-acid-in-the-fresh-food-industry/

4.9. Tables & Figures

Table 4.1. Overall visual quality rating scale for romaine lettuce.

Scale considers water loss, color, discoloration, flavor, and other visual quality parameters. A value of $\leq$4 represents an unsalable product. Adapted from Kader and Cantwell (2010) and created by Jeff Brecht.

Romaine Lettuce Quality Rating Scale	
Rating	**Definition**
9	Fresh appearance with bright green color; crisp texture; no discoloration or decay.
7	Slight loss of green color; no russeting; slight water loss not objectionable; slight butt-end discoloration
5	Noticeable loss of green color; noticeable water loss on outer leaves; moderate discoloration of butt-end
3	More yellow than green; outer leaves limp and need trimming; severe discoloration; decay may be noticeable. May have bitter flavor.
1	Yellow. Severe wilting and extreme discoloration; soft rot or fungal mycelium on outer leaves.

Figure 4.1. Discoloration and browning rating scale for fresh-cut romaine lettuce.

The scale ranks from 1-5, where 1= less than 5% of leaves show visible defects, 2= 5-10% of leaves show visible defects, 3= 10-20% of leaves show visible defects, 4= 20-30% of leaves show visible defects, 5= ≥30% of leaves show visible defects. Scale was adapted from Cantwell (2013) as derived from Cantwell and Suslow (2002).

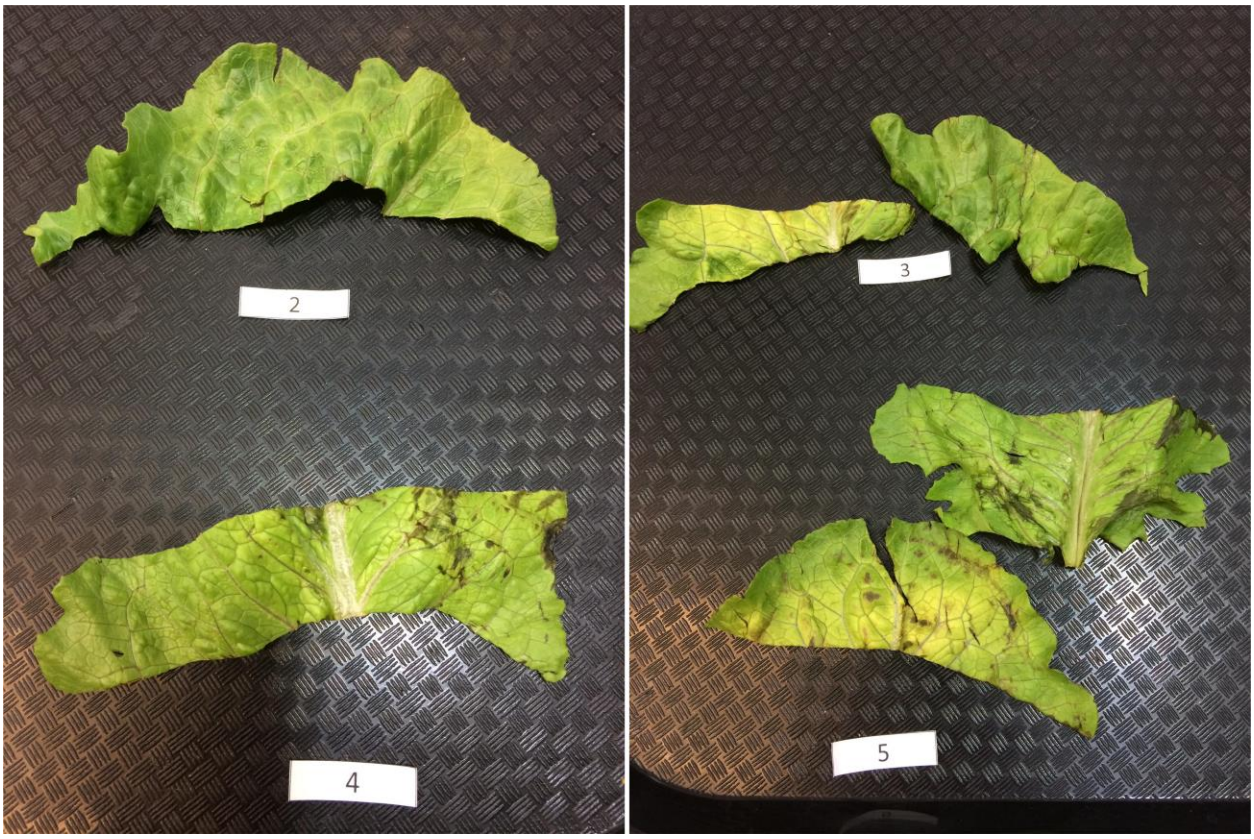

Figure 4.2. Phytotoxicity scale for fresh-cut romaine lettuce evaluating discoloration, deformation, necrosis, or yellowing of leaves.

The scale ranks product from 1-5, where 1 = no observable phytotoxic defects (not pictured), 2= 25% total leaf area with observable phytotoxic defects (discoloration, deformation, necrosis, yellowing of leaves or leaf edges), 3= 50% total leaf area with observable phytotoxic defects, 4= 75% of total leaf area with observable phytotoxic defects, 5= >75% total leaf area with observable phytotoxic defects.

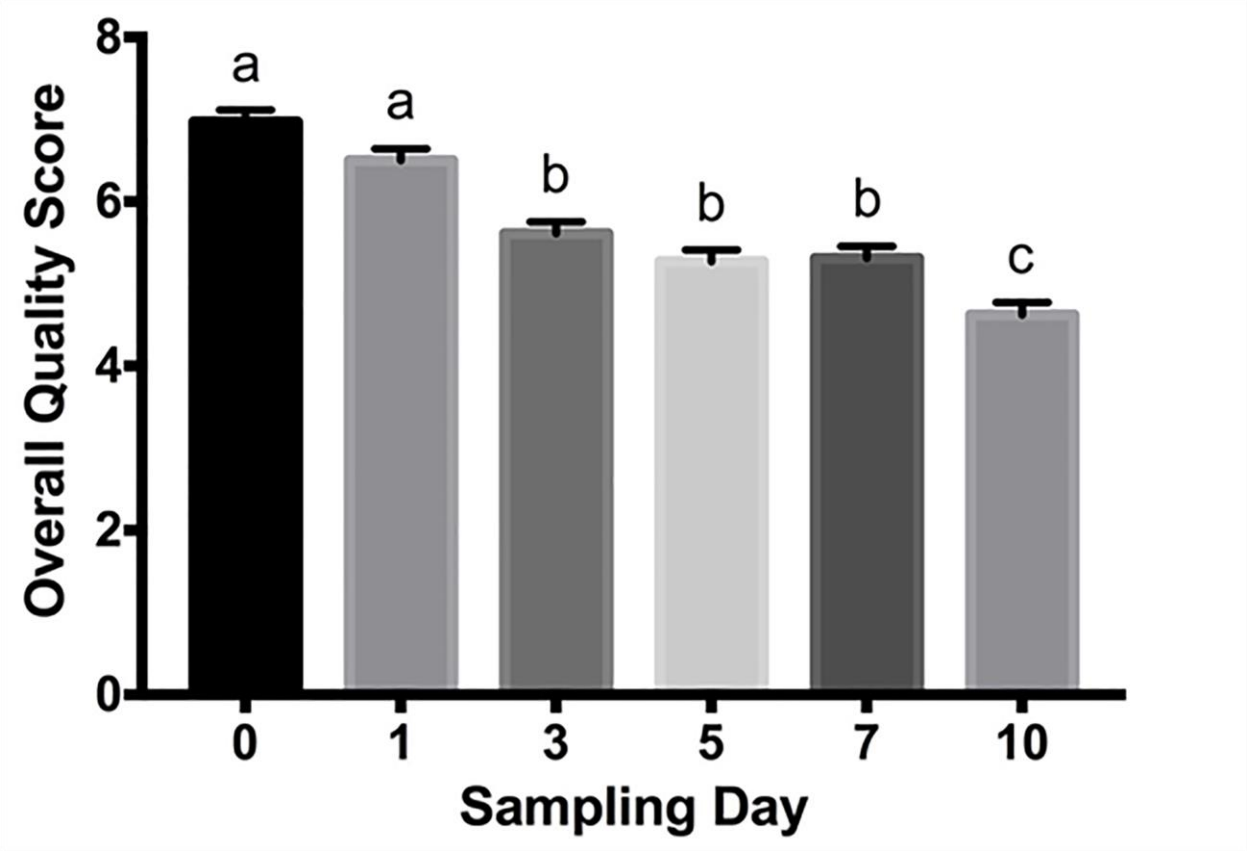

Figure 4.3. Overall visual quality of fresh-cut romaine lettuce following treatment with postharvest wash interventions, objectively measured utilizing the overall visual quality scale (Table 4.1) throughout a 10-day storage period (7°C).

The main effect of sampling day was significant ($P<0.0001$). A value of ≤4 represents an unsalable product.

Error bars represent the standard error of the mean.

[a,b,c] Observations with a common superscript do not differ statistically at the $P\leq0.05$ threshold.

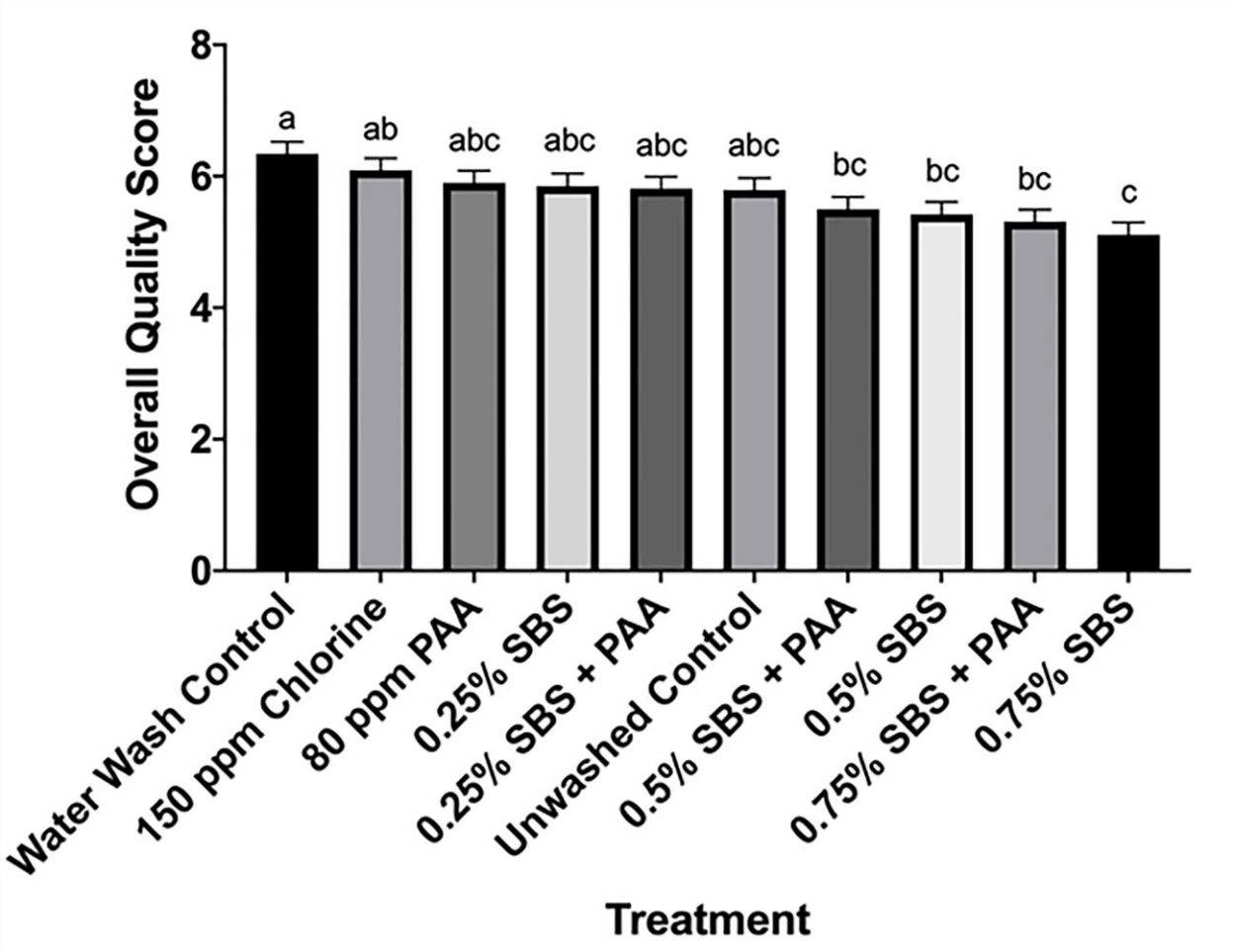

Figure 4.4. Overall visual quality of fresh-cut romaine lettuce following treatment with postharvest wash interventions, objectively measured utilizing the overall visual quality scale (Table 4.1) throughout a 10-day storage period (7°C).

The main effect of treatment was significant ($P<0.0001$). A value of ≤4 represents an unsalable product. Although treatment significantly impacted overall visual quality, no treatment was different than the unwashed control or below the salable threshold of ≤4.

Error bars represent the standard error of the mean.

[a,b,c] Observations with a common superscript do not differ statistically at the $P\leq0.05$ threshold.

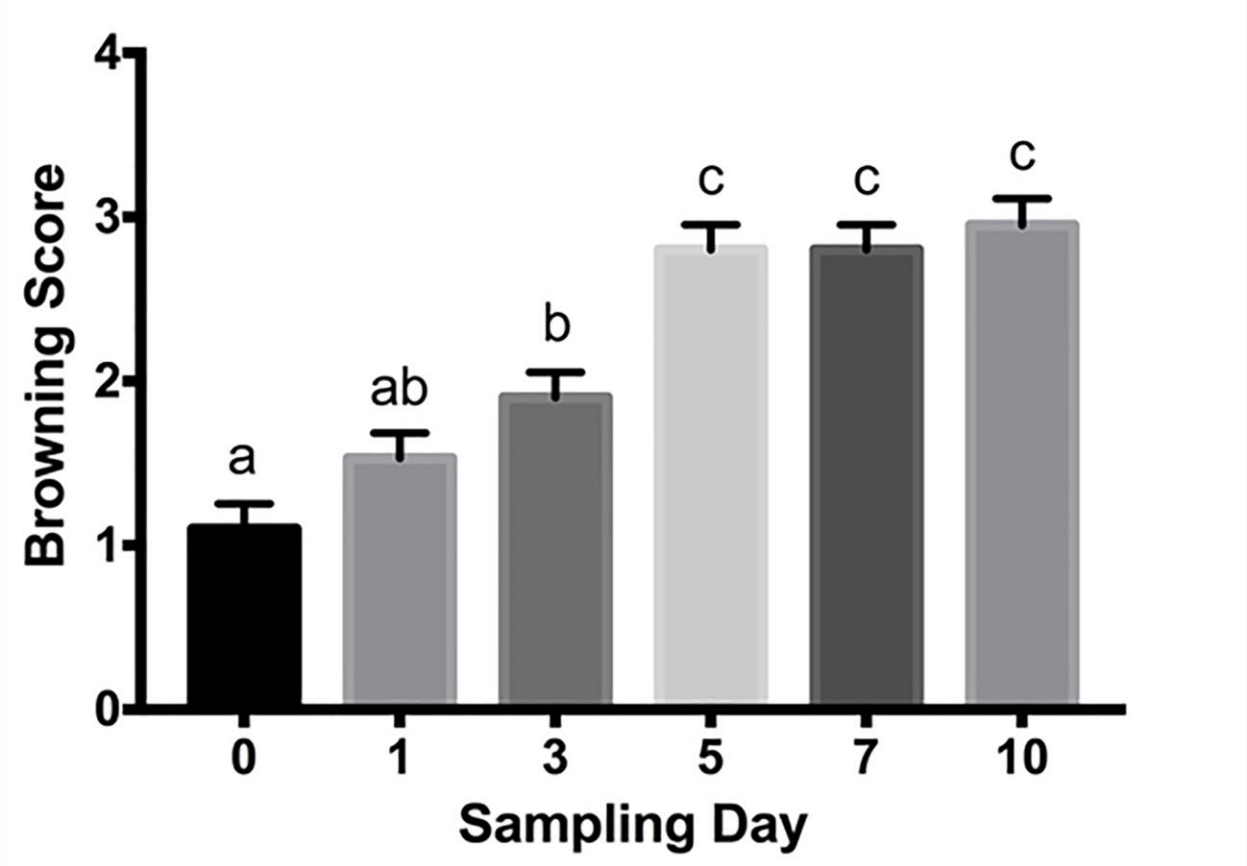

Figure 4.5. Discoloration and browning rating of fresh-cut romaine lettuce following treatment with postharvest wash interventions, objectively measured utilizing the discoloration and browning scale (Figure 4.1) throughout a 10-day storage period (7°C).

The main effect of sampling day was significant ($P<0.0001$).

Error bars represent the standard error of the mean.

[a,b,c] Observations with a common superscript do not differ statistically at the $P \leq 0.05$ threshold.

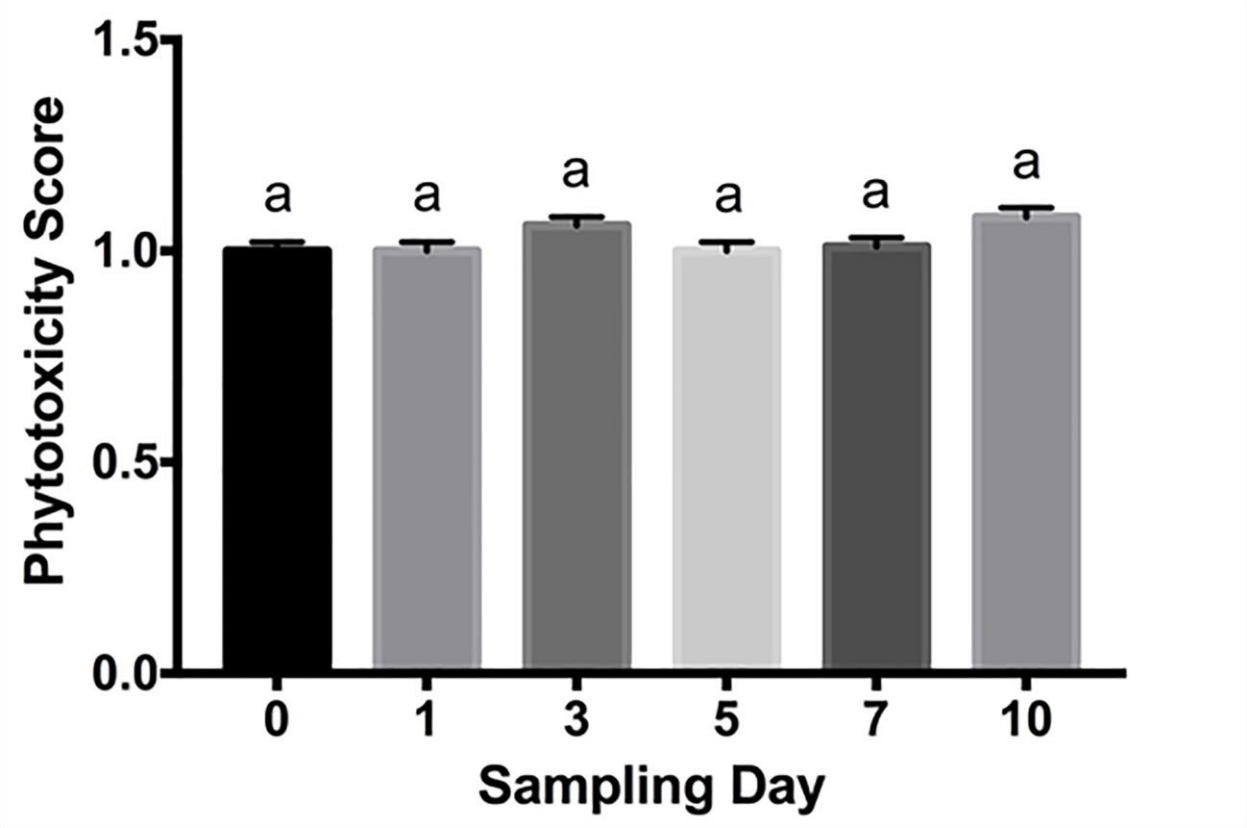

Figure 4.6. Phytotoxicity rating of fresh-cut romaine lettuce following treatment with postharvest wash interventions, objectively measured utilizing the phytotoxicity scale (Figure 4.2) throughout a 10-day storage period (7°C).

The main effect of sampling day was significant (P=0.0298). The adjusted p-values for multiple comparisons, calculated using Tukey's adjustment, did not result in significant differences between sampling days, indicating no statistical difference in phytotoxicity.

Error bars represent the standard error of the mean.

[a] Observations with a common superscript do not differ statistically different at the $P \leq 0.05$ threshold.

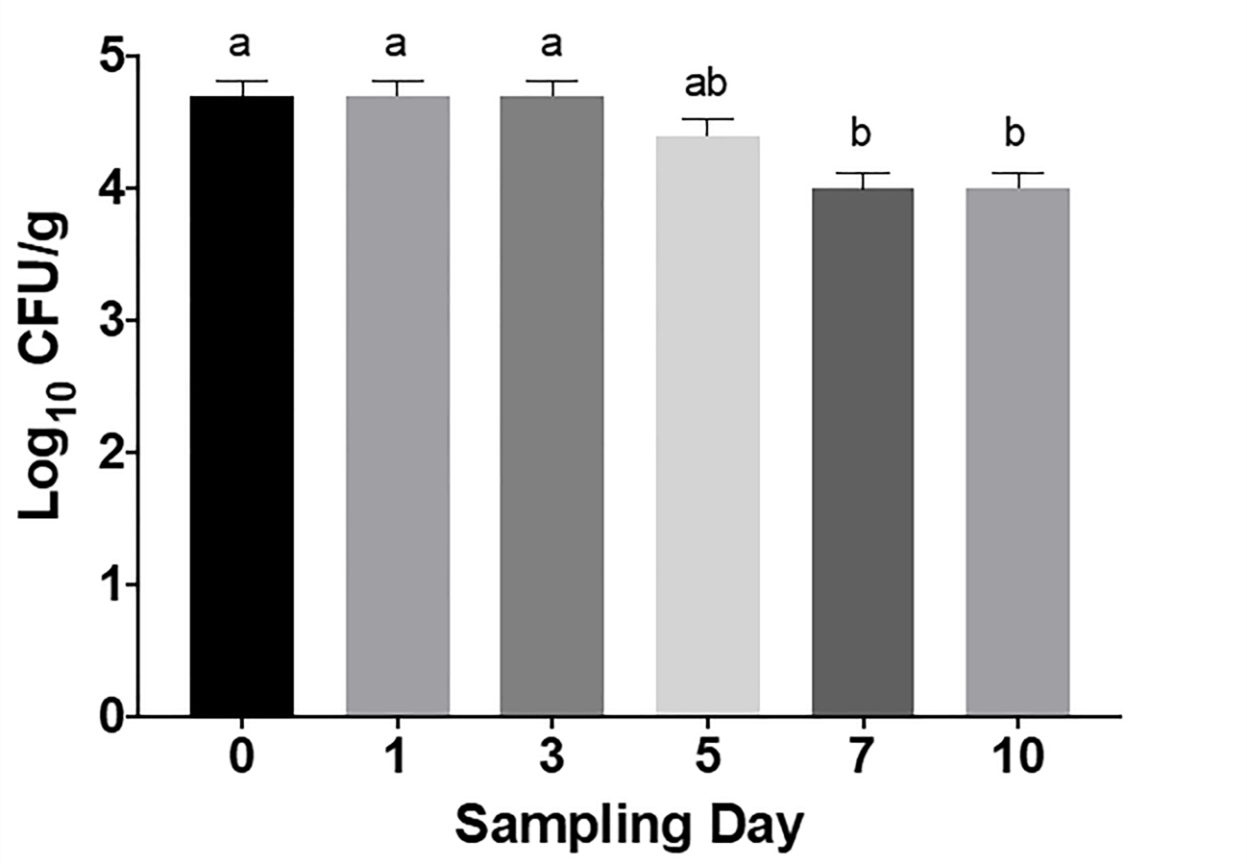

Figure 4.7. *E. coli* O157:H7 populations on fresh-cut romaine lettuce subjected to postharvest wash interventions and sampled throughout 10-day storage period at 7°C (*P*<0.0001) by plating on Sorbitol MacConkey Agar (CT-SMAC).

The main effect of sampling day was statistically significant ($P<0.0001$). The sampling day x treatment interaction was not significant ($P=0.9421$); thus, data for each day are not shown according to treatment.

Error bars represent the standard error of the mean.

[a,b,c] Observations with a common superscript do not differ statistically at the $P\leq0.05$ threshold.

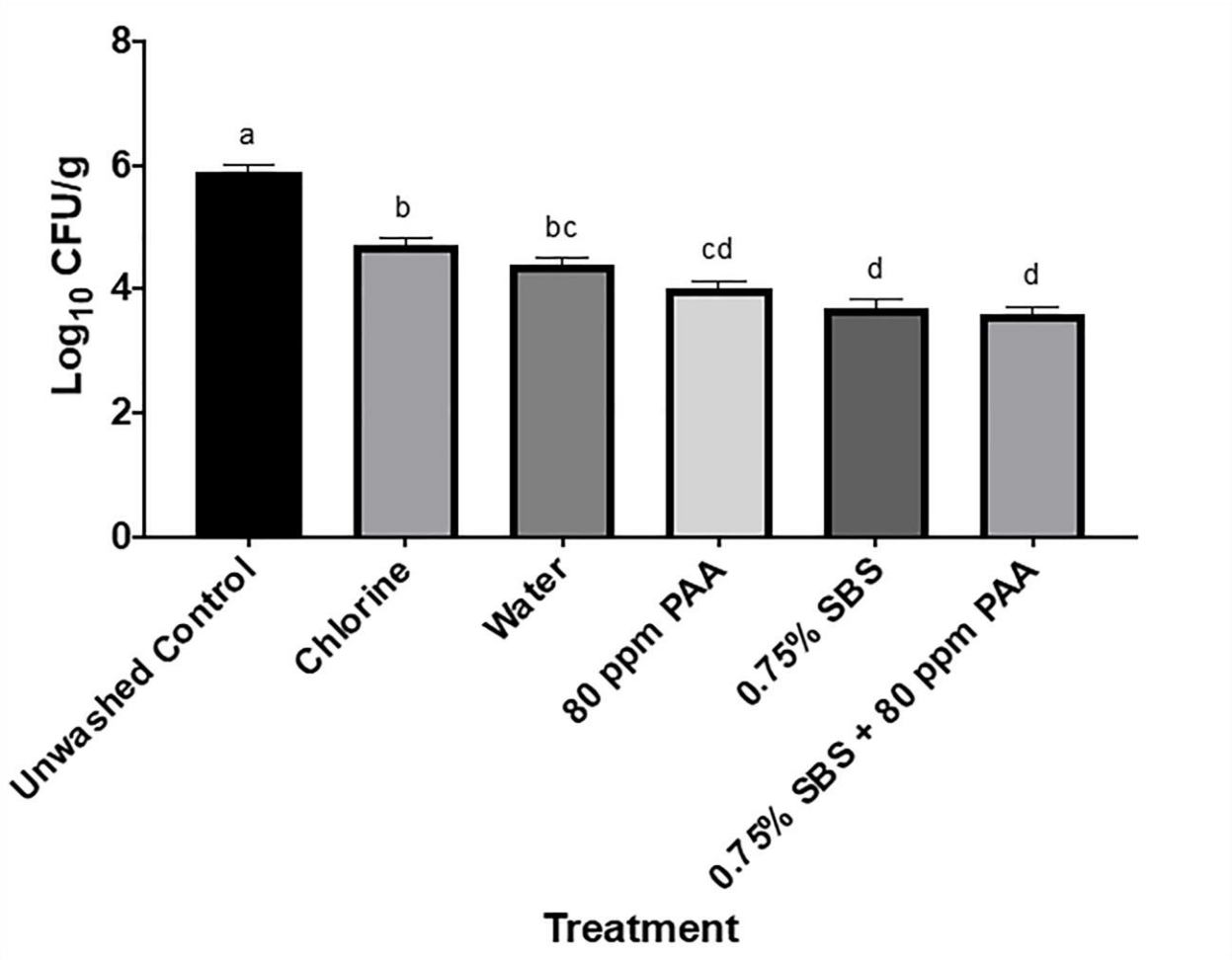

Figure 4.8. *E. coli* O157:H7 populations on fresh-cut romaine lettuce subjected to postharvest wash interventions and sampled throughout a 10-day storage at 7ºC by plating on Sorbitol MacConkey Agar (CT-SMAC).

The main effect of treatment was statistically significant ($P<0.0001$). The sampling day x treatment interaction was not significant ($P=0.9421$); thus, data for each treatment are not shown according to sampling day.

Error bars represent the standard error of the mean.

[a,b,c,d] Observations with differing superscripts are statistically different at the $P \leq 0.05$ threshold.

Chapter 5 - Policy Considerations for Food Safety Technologies Explored in this Dissertation

5.1. Introduction

This dissertation presents novel food safety technologies and their resulting benefits. Probiotics (Chapter 2), genetically enhanced feed (Chapter 3), and postharvest produce wash interventions (Chapter 4) offer food safety innovation within agricultural settings, specifically targeting the foodborne pathogen *E. coli* O157:H7 in beef and leafy greens.

Chapter 2 evaluated the inclusion of the probiotic microorganism *Megasphaera elsdenii* in cattle finishing diets for potential anti-*E. coli* O157:H7 effects. This probiotic did not significantly reduce the prevalence of *E. coli* O157:H7, yet it represents a novel approach to controlling foodborne pathogens pre-harvest. Chapter 3 explored addition of Enogen® feed corn—genetically enhanced to maximize starch availability—in cattle finishing diets with respect to its effect on *E. coli* O157:H7 carriage in cattle. The observed reduction in the odds of *E. coli* O157:H7 prevalence in cattle fed diets containing Enogen® feed corn demonstrated the food safety potential of this variety of genetically enhanced corn. Chapter 4 presented the effect of washing fresh-cut romaine lettuce with various concentrations and combinations of sodium bisulfate, peroxyacetic acid, chlorine, and water to reduce *E. coli* O157:H7. All postharvest wash treatments reduced this pathogen in comparison to an unwashed control, with minimal deleterious effects to postharvest quality. Additionally, sodium bisulfate, followed by sodium bisulfate and peroxyacetic acid in combination, produced the greatest observed reduction of *E. coli* O157:H7.

The benefits of the food safety technologies presented in this dissertation go further; they add additional weapons to combat transnational public health threats, including antimicrobial resistance. The aspiration of this chapter, along with the dissertation as a whole, is to contribute beneficial research to the scientific community while providing next steps for the incorporation of these research methods into the European Union and United States marketplaces. An encapsulation of the policy considerations and regulatory milieu for both countries are provided, in the context of the food safety technologies presented herein. The author hopes the scientific and regulatory considerations presented provide a framework for future research methodologies addressing complex, transnational public health threats, such as *E. coli* O157:H7 in agriculture (revealed in Chapters 2-4), and antimicrobial resistance.

5.1.1. The Challenge of Antimicrobial Resistance

The need exists for discovery and development of novel alternatives to antimicrobial drugs (i.e., antibiotics) in the field of animal agriculture. Analogous to the dangers of *E. coli* O157:H7 in agriculture (declared earlier in this dissertation; see Chapter 1), antimicrobial resistance (AMR) constitutes an apolitical, global health threat, unrestrained by diplomatic ideology and immune to political persuasion. AMR—recognized as one of the greatest public health dangers facing human and animal populations (Walker et al., 2009)—impacts thousands and, if left unchecked, threatens to eliminate future lifesaving antimicrobial use within the human and animal population. Urgently, the AMR problem (including multi-drug resistant bacteria and other pathogens who acquired resistance competence), necessitates a multidisciplinary, global response. The World Health Organization (WHO) estimates that 25,000 patients die within the European Union (EU) each year, due to AMR infections (European

Centre for Disease Prevention and Control, 2018). In the United States (U.S.), methicillin-resistant *Staphylococcus aureus* (MRSA) alone kills more Americans each year (~19,000) than HIV/AIDs, Parkinson's, emphysema, and homicide combined (Klevens et al., 2007). Generally, AMR refers to a microorganism's ability to evade the drugs designed to destroy them via the acquisition of genes or DNA mutations (Aarestrup et al., 2008). Transmission of AMR genes is not confined to a bacterial species or host (human, animal, environment, etc.) (Leverstein-van Hall et al., 2002; Schaberg & Zervos, 1986). The transnational reach of AMR, paired with its fundamentally complex nature, reinforces the urgency required to confront this public health danger.

Reports dating back to 1969 indicate concerns regarding the use of antibiotics for growth promotion purposes and transference of resistance to humans (Anadón, 2006). Echoed in 1997, this concern led to the development of the Special Advisory Committee on Antimicrobial Resistance (SACAR) in 2001 within the United Kingdom (Wise, 2007). The EU developed a formal approach towards combating AMR, including the development of a strategic action plan strengthening surveillance, multisectoral coordination, and antibiotic stewardship (Baku, 2011). Following the EU ban of antibiotics for growth promotion in 2006, subsequent lower rates of AMR were observed (Laxminarayan et al., 2013). Within the food sector, the European Food Safety Authority (EFSA) developed robust infrastructure for assessing emerging risk, established antibiotic monitoring, and imparted restrictions on all injudicious use of antimicrobials (Roca et al., 2015). Recently, the EU revised their strategic action plan, including a "one-health" approach (The European Commission, 2017). This revised action plan established the most comprehensive AMR policy to date.

The U.S. developed their first governmental "antibiotic policy" alongside the clinical introduction of penicillin. During WWII, prophylactic antibiotic use, aimed toward the prevention of bacterial infection of war wounds, led to the abandonment of sulfonamides and their replacement with penicillin (Gould & Meer, 2005). Sixty years later, the Centers for Disease Control and Prevention (CDC) released the first report quantifying the global health threat of AMR pathogens, with penicillin resistance considered a significant threat (Centers for Disease Control and Prevention, 2013). This report spurred the development of policy combating AMR, starting with U.S. Presidential Executive Order 13676, calling for establishment of a national task force (Obama, 2014). Subsequently, the National Action Plan directed federal agencies to increase multi-sector responses toward AMR (President's Council of Advisors on Science and Technology, 2015). In 2015, the U.S. Interagency Task Force released a 5-year National Action Plan, calling for strengthened surveillance efforts, upgraded diagnostics, increased research, and improved international collaboration and stewardship (President's Council of Advisors on Science and Technology, 2015). In 2017 the U.S. Food and Drug Administration (FDA) released Guidance for Industry #213 containing the Veterinary Feed Directive (VFD), legislation banning growth promotion use of antibiotics in food-producing animals and requiring veterinary prescription for use of all medically important antibiotics (Center for Veterinary Medicine, 2019a, 2019c). Policy analysts are in agreement with government opinion regarding the urgency of the AMR problem, proposing, in some cases, nonantibiotic interventions as effective alternatives (Hoelzer, 2020).

5.1.2. Technologies To Aid In the Comprehensive Fight Against AMR

5.1.2.1. Probiotics

For years, microorganisms have been supplemented in human and animal diets, hoping to harness their beneficial properties (Bernardeau & Vernoux, 2013). Growing concerns surrounding overreliance on antimicrobials in agriculture, along with the threat of increasing AMR, led to investigation into other potential management practices, or "antibiotic alternatives" (Centers for Disease Control and Prevention, 2019a; Simon, 2005). One such alternative includes the addition of probiotics, or live microorganisms which are administered at appropriate dosages to confer positive health benefits upon the host (Bernardeau & Vernoux, 2013; FAO/WHO working group, 2002; Fuller, 1992). Within the U.S., direct fed-microbials (DFM)—defined as "a source of live (viable) naturally-occurring microorganism"—refine the definition of a probiotic used within commercial agriculture production (Brashears & Guillen, 2014; Yoon & Stern, 1995). In the U.S., DFMs in animal feed represent a significant market, with an estimated value of $4.6 billion in 2019 (Markets and Markets, 2019). The EU defines these beneficial microbes as "feed additives" (Bernardeau & Vernoux, 2013). Administration of these microorganisms targets specific health benefits, including increased feed conversion and efficiency, resistance to colonization of pathogens, prevention of a disease state, or improved stress response (Gorsuch et al., 2019; McAllister et al., 2011). Researchers continue to explore the specific pre-harvest food safety applications of probiotic administration, with *Lactobacillus acidophilus* NP51 significantly reducing *E. coli* O157:H7 shedding in cattle by 48-80% (Stephens et al., 2007; Younts-Dahl et al., 2005). Similar to research on probiotics for human consumption, animal probiotics receive constant attention, with potential benefits to agriculture under exploration (Khan et al., 2016).

5.1.2.2. Genetically Enhanced Animal Feed

Another potential pre-harvest control for foodborne pathogens within agriculture includes the modification of animal diets. Demonstrated in 1998 by shifting cattle diets containing high grain, to a period of hay-based rations, reducing *E. coli* significantly (10^6 fold after 5 days; Diez-Gonzalez et al., 1998), diet modification is carried out in many different ways. Recently, inclusion of distiller's grains—a common high-protein byproduct of ethanol biofuel production—indicate an association with increased *E. coli* O157:H7 prevalence, depending on percentage added in cattle diets (Chaney et al., 2018; Jacob et al., 2008). Notably, orange peel and pulp—common citrus production byproducts—addition to animal diets produced observable reductions of *E. coli* O157:H7 in the gastrointestinal (GI) tract of ruminants (Callaway et al., 2011). Modification of animal diets—including the addition of genetically enhanced (GE) feed and regardless of method—constitutes a relatively understudied, yet important area of pre-harvest food safety research (Allen et al., 2013; G. R. Gibson & Roberfroid, 1995; Glenn R. Gibson et al., 2004).

5.1.2.3. Postharvest Produce Wash Interventions

Recently released U.S. foodborne illness surveillance data indicates row crop vegetables (such as leafy greens) as the primary food (46.3% of illnesses) implicated in all *E. coli* O157:H7 outbreaks between 2003-2017 (Interagency Food Safety Analytics Collaboration, 2019). Numerous outbreaks of *E. coli* O157:H7 have implicated romaine lettuce, with an outbreak in 2018 resulting in 210 cases, 96 hospitalizations, and 5 deaths (Centers for Disease Control and Prevention, 2018); foodborne illness outbreaks associated with fresh-cut vegetables are becoming increasingly common. Industrial leafy green production includes cutting, washing, drying, and packaging steps, frequently with the use of chlorine as a produce wash (Feliziani et

al., 2016; Salgado et al., 2014). Doubt surrounds chlorine's efficacy and safety, alluding to the toxic disinfection byproduct production following reaction of chlorine to organic matter (Joshi et al., 2013). This phenomenon influenced the ban of chlorine-based sanitizer use in Germany, Denmark, and other EU countries (Meireles et al., 2016). Chlorine use within fresh-cut applications is influenced by low cost and a wide spectrum of antimicrobial activity; yet, these factors do not outweigh its limited efficiency (Ramos et al., 2013; Weng et al., 2016; Yaron & Römling, 2014) and potential risk to those who handle it (Joshi et al., 2013; Meireles et al., 2016). Notwithstanding widespread chlorine use, efforts to find safer and more effective sanitizing agents will undoubtedly reduce dependence on this type of sanitizer.

5.1.2.4. Current Research

The scientific community demands high-quality, interdisciplinary research on safe and effective foodborne pathogen control methodologies. Suggested in the preceding chapters of this dissertation, application of "systems biology"—a concept used to describe the understanding of both the structure and dynamics of a system, equally (Breitling, 2010; Kitano, 2002)—is required when addressing complex health issues. For instance, Chapter 2 presents an investigation into a novel probiotic organism and its potential effect against *E. coli* O157:H7. This study requires analysis of the probiotic organism's interaction against *E. coli* with an understanding of its impact throughout the entire cattle gastrointestinal tract. Microbiome research necessitates this type of intricate and systematic thinking. The regulation and integration of this organism into the global food supply provide an additional veil of complexity worth exploring.

5.2. Policy Considerations For Each of the Technologies Studied in This Dissertation

The following sections provide a brief summary of the three technologies explored within this dissertation. The author provides a report of the main findings of chapters 2, 3, and 4 along with an overall recommendation of the next steps in researching the specified technologies (probiotics, genetically enhanced animal feed, and postharvest produce wash interventions). Following the research summary, a description of the regulatory framework and policy considerations for the technologies is provided. The research summary and policy consideration sections represent the broader application of the research described in this dissertation.

5.2.1. Impact of the Probiotic Organism *Megasphaera elsdenii* on *Escherichia coli* O157:H7 Prevalence in Finishing Cattle

5.2.1.1. Research Summary of *Megasphaera elsdenii*

Cattle producers feed *Megasphaera elsdenii* (ME), a lactic acid-utilizing bacterium present in the rumen of cattle, as a DFM to reduce lactic acid buildup within the rumen of grain-fed cattle, halting the disease state known as rumen acidosis (RA; L. Chen & Wang, 2016; Lianmin Chen et al., 2019). In this project, ME was fed as a DFM to determine the effect, if any, on *E. coli* O157:H7 prevalence in finishing cattle. Crossbred steer (n=448) were randomly assigned to a treatment diet containing 2 levels of ME, and following 117-126 days on feed, *E. coli* O157:H7 prevalence was measured by sampling animals twice via rectoanal mucosal swab sampling (RAMS; n=896), analyzed using standard laboratory methods.

This study demonstrated several noteworthy insights:

- Inclusion of ME in cattle finishing diets did not significantly reduce the prevalence of *E. coli* O157:H7
- A diet containing ME reduced the odds of *E. coli* O157:H7 prevalence by 50% during the first sampling period; conversely, a diet containing ME increased the odds of *E. coli* O157:H7 prevalence by 23% during the second sampling period, indicating the true influence of ME on *E. coli* O157:H7 carriage is not well defined
- In a companion study, investigation into the use of ME as a potential preventative agent of ruminitis and liver abscessation was evaluated along with performance and carcass characteristics. The study revealed a diet containing ME did not significantly impact feedlot performance, carcass weight, or liver abscess incidence (Veloso et al., 2019)
- **Recommendation**: From these results, conclusions on the food safety benefit of feeding cattle *M. elsdenii* as a DFM cannot be made; more research on use of *M. elsdenii* in this application is needed.

5.2.1.2. Policy Considerations for *Megasphaera elsdenii*

Although more research is needed, it is wise to anticipate the policy and approval context that probiotic technologies like ME face, or may face if found to be effective in a food safety context. As previously mentioned, the ban of antibiotics from the EU feed market in 2006 led to significant interest in research and development of novel microorganisms (Anadón, 2006; Bernardeau & Vernoux, 2013). Drug resistant bacteria constitute a tremendous public health threat, with recent advances in EU policy and legislation reflecting that concern (Alban, 2016). Following the ban of antibiotics in feed, adjustment of food safety policy by the European Commission included revised rules for labeling, marketing, and packaging feed additives (EC Regulation 1831/2003). Article 5 of this regulation describes general prerequisites:1) additives should have no adverse effect on animal health, human health, or the environment, 2) additives

shall not be presented in a manner misleading the user, and 3) additives shall not harm or mislead the consumer. Article 5 defines categorizations of feed additives, including 1) technological, 2) sensory, and 3) nutritional or zootechnical additives (Anadón, 2006). More specifically, zootechnical additives include those which enhance digestibility, stabilize gut flora, favor the GI environment, or provide other zootechnical benefit (Anadón, 2006; Bernardeau & Vernoux, 2013). Review of applications for a "novel food"—a newly developed or innovative food or food process—within the EU is governed by EC Regulation 285/97 and 2015/2283; the scientific risk assessment is completed by EFSA (Brodmann et al., 2017). Furthermore, the application requires 1) a description, 2) compositional data, 3) production process, 4) specifications, 5) proposed uses and levels, and 6) anticipated intake (Brodmann et al., 2017). Similar to the US GRAS status, the qualified presumption of safety (QPS) was introduced to harmonize the assessment and approval process (Markowiak & Śliżewska, 2018), with the intent to generate a system for microorganism used in feed and food production, applying to DFMs or "feed additives" as they are described in the EU (Dibner & Richards, 2005). QPS applications require the taxonomic characterization, a body of research on the application of the substance and potential pathogenicity, and a description of the end use (Koutsoumanis et al., 2020). Differing from GRAS, the EFSA requires QPS probiotic strains to not carry any transferable antimicrobial resistance (Sanders et al., 2010). The presence of AMR genes within a probiotic strain is a concern, as research demonstrates their ability to transfer to other bacterial species, possibly within the host gastrointestinal microbiome (Broaders et al., 2013).

Within the U.S., the Association of American Feed Control Officials (AAFCO) offers a process by which animal feeds and ingredients can be manufactured, labeled, and sold (CVM, 2020). AAFCO collaborates with state and federal agencies, including the FDA, to unify the

laws, regulations, and standards surrounding these products (FDA, 2019a). DFMs are regulated under the *Federal Food, Drug, and Cosmetic Act* (FDCA) and listed in 21 CFR § 573 and 579 unless given a Generally Recognized as Safe (GRAS) status (21 CFR § 170.30). DFM regulation depends upon the "intended use" of the product. Claims of disease mitigation, treatment, or prevention require the DFM to be considered a new animal drug and necessitate a new animal drug application (NADA) for approval (Office of Regulatory Affairs, 2019). If the DFM is labeled with claims that the product impacts the structure or function of the animal, the DFM is considered a drug, as the benefits are not derived from its nutritional characteristics (Office of Regulatory Affairs, 2019). DFM products labeled with an AAFCO-approved label statement, describing the live microorganism content, without any structure/function claims are regulated as a food and considered a food additive (Office of Regulatory Affairs, 2019). Food additives are intended to supply nutrients, aid processing and packaging, or alter the food's characteristics (Center for Veterinary Medicine, 2019b). Furthermore, GRAS status is given to substances that have adequately proven safe under its intended use conditions, with the responsibility being on the producer of the substance (Brodmann et al., 2017; FDA, 2020). Substances classified as GRAS by a safety assessment done my independent experts, are not considered food additives (FDA, 2019b) and require scientific procedures addressing the safety of the animal and human consuming the food-producing animal (FDA, 2020). In the case of a novel probiotic or DFM for use in animal populations, the first step toward approval for use, and subsequent regulation, should be completion of an Investigational Food Additive (IFA) file, or if the specific intended use and safety of the substance is known, either an animal food additive petition (FAP) or a GRAS notice (Center for Veterinary Medicine, 2020).

Currently, the FDA approved ME for use as a DFM in cattle (along with holding GRAS status), regulating ME as a feed additive and marketing as a lactic acid utilizer (AAFCO, 2019). No applications for use within the EU have been filed. Any effect of *M. elsdenii* on *E. coli* O157:H7 prevalence in cattle would not hinder the feed additive regulation; quite simply, it contributes to the body of research on this organism and demonstrates safety within the cattle host. As cattle are the primary asymptomatic reservoir of *E. coli* O157:H7 (Callaway et al., 2006), any effect on reducing ME prevalence does not impact its feed additive or regulatory status.

Navigating U.S. and EU regulation for microbial food additives provides a significant challenge. The Joint Food and Agriculture Organization (FAO) of the United Nations (UN)/World Health Organization (WHO) Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics attempted to consolidate the global standards for probiotic evaluation in 2001 (FAO/WHO working group, 2002). Within these guidelines lies a framework for the evaluation of probiotics and substantiation of their health claims. These recommendations include 1) identification of genus and species of probiotic strain, 2) *in vitro* testing to demonstrate probiotic effect, and 3) substantiation of health benefit of the agent with *in vivo* trials (Venugopalan et al., 2010). Standardization of probiotic regulatory guidelines—including a universally recognized definition—is needed to simplify research, development, manufacture, and marketing. Additionally, probiotics require extensive research and development, including interdisciplinary translational research emphasizing aspects of the host and microbe system. For instance, current research provides a framework for characterizing novel probiotic strains (Ayala et al., 2019). This research utilized techniques including whole genome sequencing, cell culture, and competitive broth exclusion assays to evaluate pre-harvest food safety applications of DFMs.

More specifically, the study evaluated the probiotic's: 1) ability to attach and colonize intestinal epithelial cells of the host, 2) susceptibility to antibiotics, 3) survivability and metabolic activity within the host, and 4) viability during delivery into the host (Ayala et al., 2019). Additional research on host microbiota interactions, physiology, and immunology components would help to fully characterize the safety and efficacy of potential probiotics and their resulting regulation. Moreover, determination of the intended use, host interactions, risk assessment, and post-market surveillance of the probiotic is required to establish a safe context for these technologies (Sanders et al., 2010).

5.2.2. Reduction of *Escherichia coli* O157:H7 in Finishing Cattle fed Enogen® Feed Corn

5.2.2.1. Research Summary regarding Enogen® Feed Corn

Enogen® Feed Corn (EFC) contains a GE α-amylase enzyme trait that improves starch digestion in feedlot cattle by maximizing starch availability (Erickson et al., 2018; Jolly-Breithaupt et al., 2019). Research suggests EFC improves energy availability and feed efficiency; however, the potential food safety impact of feeding feedlot cattle EFC has not yet been explored. It is possible that EFC may increase post-ruminal fermentation impacting the ability of foodborne pathogens—such as *E. coli* O157:H7—to colonize and persist within the hindgut. In this project, steers (n=960) were fed diets consisting of 2 levels of silage and 2 levels of corn (EFC or Control) for 62-93 days. *E. coli* O157:H7 prevalence was measured by sampling animals via rectoanal mucosal swabs (RAMS) samples, analyzed using standard laboratory methods.

The results of this study included the following:

- Addition of EFC, fed as grain in cattle finishing diets did not significantly reduce the prevalence of *E. coli* O157:H7

- EFC fed as silage in cattle finishing diets did not significantly reduce the prevalence of *E. coli* O157:H7

- A diet containing EFC fed as corn reduced the odds ratio of *E. coli* O157:H7 prevalence by 43.3% in comparison to a control corn diet

- In a companion study, investigation into the impact of feeding EFC—as corn silage and steam-flaked corn—on carcass characteristics and feedlot performance was evaluated, revealing diets containing EFC as silage resulted in less consumption of dry matter and an improvement in efficiency, with no observed effect of EFC on performance when fed as grain. Liver abscess incidence and carcass measurements were unaffected by grain or silage source (Baker et al., 2019)

- **Recommendation:** The present study suggests that feeding Enogen® feed corn may reduce *E. coli* O157:H7 carriage in cattle; however, the data are limited in scope and understanding, requiring further research to understand the mechanisms of this anti-*E. coli* effect.

5.2.2.2. Policy Considerations regarding Enogen® Feed Corn

Policies surrounding GE or genetically modified (GMO) crops for animal feed differ between the EU and U.S. The U.S. regulatory framework—known as the Coordinated Framework for the Regulation of Biotechnology (51 Federal Register[FR] 23302, 57 FR 22984)—consists of oversight by three major agencies: the USDA's Animal and Plant Health Inspection Service (APHIS), the FDA, and the Environmental Protection Agency (EPA). The FDA ensures safety and proper labeling of GMO animal feed while the EPA is responsible for the pesticides often produced by these GE crops (USDA, 2011). Under the Plant Protection Act (7 USC § 7701 *et seq.*) APHIS has primary responsibility for regulating GMOs in regard to their

plant and environmental impact. This multi-agency approach ensures a thorough investigation into the safety of new GMO technologies. Currently, 92% of corn, 94% of soybeans, and 94% of cotton in the U.S. are GMOs (USDA ERS, 2019), representing a significant influence on U.S. economic and agriculture sectors. Enogen® corn—containing a thermostable alpha-amylase enzyme enhancing ethanol production—was approved in 2007 by the FDA for use as food, animal feed, and the production of food products or ingredients, and in 2011, was approved by the USDA for commercial cultivation (USDA, 2011). The FDA concluded that the Enogen® corn was not "materially different in composition, safety, or any other relevant parameter" from other corn on the market (Pollack, 2011; USDA, 2011). Regardless of the potential improvements in ruminant digestion, feed efficiency gains, or anti-*E. coli* O157:H7 effects, the FDA regulates Enogen® corn as a food and animal feed (Erickson et al., 2018; M. Johnson et al., 2018; Jolly-Breithaupt et al., 2019).

Although approved, Enogen® corn is still met with objection, primarily from the North American Millers' Association (General Mills, ConAgra Mills, etc.) who claim Syngenta's corn has contaminated their corn via cross-pollination or unintentional mixing within grain elevators (Pollack, 2011). The current approval status for Enogen® corn in human food, animal feed, and commercial farming operations help soften these objections; however, effects on food industry corn quality, and potential disruption of export to countries where Enogen® corn is not approved, warrant attention. Differing opinions among those within the food and agriculture sectors surrounding genetically enhanced crops—regardless of regulatory approval or scientific risk assessment—appear inevitable.

Approval of Enogen® corn for use in the EU has had limited success, contrary to its regulatory acceptance within the U.S. GMO food and feed governance by EFSA resides within EC Regulation 1829/2003. This regulation establishes criteria based on potential risk and risk assessment harmonization, providing labeling and marketing requirements. Syngenta's application (EFSA-GMO-UK-2006-34)—in compliance with EC Regulation 503/2013 requirements including relevant research, purpose, scope, monitoring plan, labeling proposal, and detection method—was rejected in June 2013, the EU's report stating Syngenta had not provide key information to allow a full risk assessment to take place (EFSA, 2013). The EFSA GMO panel was unable to complete a risk assessment and could not conclude on the safety of this corn variety on human and animal health (Glass, 2013). In 2019, the GMO panel analyzed additional information related to the EFSA-GMO-UK-2006-34 application, concluding the agronomic and phenotypic characteristics of the forage and grain "do not give rise to food and feed safety, and nutritional concerns when compared to non-GMO maize" (p. 12, Naegeli et al., 2019). Syngenta supplied substantial scientific information regarding the safety of dried distillers' grains with solubles (DDGS; a common byproduct of ethanol production), with hopes to export this animal feed ingredient to the EU. The GMO panel performed an allergenicity assessment and concluded that DDGS from Enogen® corn "does not raise concerns when compared to DDGS from non-GMO corn" (p. 3, Naegeli et al., 2019). Currently, Enogen® corn is not approved for use in animal feed or human food but is approved for its byproduct, DDGS.

In summary, regulatory approaches toward GMOs differ significantly between the US and EU. Within these approaches lies complex regulatory administration, multi-agency oversight, and globalization of agricultural trade, all influencing the decision—whether the GMO in question is safe for use within a country's jurisdiction. At the heart of EU GMO regulation lies

the precautionary principle (Article 7 of EC 178/2002), with regulatory decisions on GMO safety requiring a high burden of proof prior (Alban, 2016). The U.S. approach is similar, yet different, with emphasis on risk assessment of GMOs vs their non-GMO counterparts (Bodiguel & Cardwell, 2010; Lau, 2015). Similar to the regulatory disputes which began in 1981 between the US and EU concerning hormone-treated beef, the U.S. has historically taken an approach focused on probability (likelihood), differing from the EU approach utilizing the precautionary principle and focusing on the mere possibility of a hazard or unwanted outcome occurring (R. Johnson, n.d.; J. Kastner, 2018; J. J. Kastner & Pawsey, 2002). Even with differences amid regulatory approaches, approval outcomes of GMO crops in the U.S. and EU are made based on the same scientific criteria from the same body of research. With this in mind, contributing high quality, unbiased research supports regulators in making the most informed decisions.

5.2.3. Sodium Bisulfate and Peroxyacetic Acid Reduce the Foodborne Pathogen *Escherichia coli* O157:H7 When Applied as a Postharvest Wash on Romaine Lettuce

5.2.3.1. Research Summary on SBS and PAA postharvest wash interventions

Fresh-cut romaine lettuce has recently been recognized as a significant vehicle of foodborne illness bacteria, with several outbreaks of *E. coli* O157:H7 in this commodity encouraging the need for novel interventions (Centers for Disease Control and Prevention, 2019b). Potential interventions include the use of peroxyacetic acid (PAA) and sodium bisulfate (SBS) as postharvest wash sanitizers. In this project, a) quality of fresh-cut romaine and b) anti-*E. coli* O157:H7 activity were evaluated following application of various triple-wash treatments: 1) municipal tap water, 2) 80±5 ppm PAA, 3) 0.75% SBS, 4) 80±5 ppm PAA + 0.75% SBS, 5) chlorine (free chlorine; 10 ppm wash 1, 20-30 ppm wash 2 & 3), and 6) an unwashed control.

Quality was evaluated using subjective measurements including overall visual quality, phytoxicity, and browning/discoloration and objective color measurements of the treated lettuce surfaces. For the microbiological analysis, fresh-cut romaine was inoculated with $1x10^6$ CFU/g of *E. coli* O157:H7, dried, subjected to a triple-wash treatment, stored at 7°C, and enumerated on days 0, 1, 3, 5, 7, 10, 12, and 14 to measure treatment efficacy against *E. coli* O157:H7 on romaine product.

The results of this study can be summarized as follows:

- Quality analysis revealed treatment type had no significant effect on color
- Quality analysis found no impact of treatment type on phytotoxicity score
- Treatment produced no observable impact on browning and discoloration of romaine product
- A significant treatment effect was observed on overall visual quality; yet, not below the salable threshold established for romaine lettuce using the quality scale
- Microbiological analysis revealed a significant treatment effect, with all treatments significantly reducing *E. coli* O157:H7 populations compared to the unwashed control
- SBS+PAA produced the greatest reduction of *E. coli* O157:H7 (2.3 log CFU/g) compared to the unwashed control, followed by SBS (2.2 log CFU/g), and PAA (1.9 log CFU/g)
- SBS, PAA, and SBS+PAA washes were not more effective than one another, but were all more effective than the industry standard (chlorine)
- **Recommendation:** This study suggests SBS, PAA, or SBS in combination with PAA, applied as a postharvest wash water intervention, are more effective against *E. coli* O157:H7 than the industry standard chlorine, with minimal deleterious effects on fresh-cut romaine quality.

5.2.3.2. Policy Considerations for use of Sodium Bisulfate and Peroxyacetic Acid

The U.S. FDA, alongside AAFCO, approved SBS in 1997 as a general-use feed additive used in poultry feed and granted SBS GRAS status (21 CFR § 175.105) in 1998 (Knueven, 2001; Organic Materials Review Institute, 2015). SBS is also an EPA registered pesticide, fungicide, herbicide, and microbicide (EPA registration 33907-3, EPA PC codes 073201 and 873201). In the EU, SBS—described as sodium hydrogen sulphate (SHS)—obtained approval for use as a food additive (EC Regulation 231/2012 and EU Regulation 2015/1416) under the E number E514ii. Recently, the Panel on Food Additives and Flavorings (FAF) completed a re-evaluation of SHS with concerns regarding safety of use as a food additive; however, the EFSA assessment concluded sulphates (including SHS) represent a "natural constituent of human, animals, and plants and is present in all biological materials, including foodstuffs" with no safety concern when used at approved doses (p. 3, EFSA Panel on Food Additives and Flavourings (FAF) et al., 2019). SBS meets international safety requirements set forth by the Food Chemicals Codex (FCC)—a compendium of international standards for food ingredients (Organic Materials Review Institute, 2015). Generally, SBS appears to be well-researched and globally accepted for use on or within food and feed products.

Regulatory approval of PAA (also known as peracetic acid) in the U.S. continues the theme of complexity, similar to SBS, involving multiple federal agencies involved in human, animal, and environmental safety. Focusing solely on the postharvest produce application of PAA, the FDA approved this substance for two uses: 1) as a secondary direct food additive permitted in food (21 CFR § 173) and 2) as an indirect food additive, sanitizer, or production aid (21 CFR § 178). PAA is legally classified as a pesticide, thus a memorandum of understanding (MOU 225-73-8010) between the EPA and FDA exists, streamlining the regulatory efficiency of drugs and pesticides that could adulterate food and feed (Organic Materials Review Institute,

2016). Approved concentrations and application of PAA is codified in 21 CFR § 173 and 178 along with specifications for use in organic applications in rinse water (7 CFR § 205.605b). The EU granted approval for use of PAA as a food and feed disinfectant (EC Regulation 1935/2004), with provisions for use in leafy-greens still under review (Banach et al., 2015). EFSA has evaluated and approved PAA for use in poultry processing, as it has produced significant reductions of *E. coli* and coliforms with no environmental risks (EFSA Panel on Biological Hazards, 2014). Presently, it appears that the EU requires more data to perform a sound risk assessment on the safety of PAA use as a postharvest treatment in fresh-cut leafy greens. The research presented in Chapter 4 of this dissertation, provides evidential support for completion of a PAA risk assessment by the EU.

In the last 3 years, *E. coli* O157:H7 leafy greens outbreaks resulted in a combined 464 cases, 215 hospitalizations, and 6 deaths (Centers for Disease Control and Prevention, 2019c). Foodborne illness outbreaks within this commodity are becoming increasingly common, representing a significant public health threat. The EU observed 8,161 cases of shiga toxin-producing *E. coli* (STEC) in 2018, with strong evidence to support 5 foodborne related outbreaks, 1 of which relating to "vegetables, juices, and other products thereof" (EFSA & ECDC, 2019). At first glance it would appear that in recent years the EU has been less susceptible to STEC outbreaks within leafy greens; however, the U.S. exported € 307, € 367, and € 311 million euros of vegetables (fresh, chilled, and dried) to the EU during 2016, 2017, 2018, respectively (European Commission, 2019). Furthermore, in 2018 the U.S. exported $510.3 million of lettuce (18.2% of all global lettuce exports), second only to Spain (Workman, 2019). The U.S. *E. coli O157:H7* outbreaks in leafy vegetables significantly influence global public health and trade. With U.S. outbreaks of foodborne pathogens in leafy greens becoming

increasingly commonplace, the export of these products to countries within the EU will no doubt render them susceptible to the same danger. Produce outbreaks impact the multilateral trading system and shed light on the need for additional weapons to combat transnational public health threats.

5.3. Concluding Remarks

The author acknowledges that the three technologies (probiotics, genetically enhanced animal feed, and postharvest wash water interventions) described in this dissertation are not perfect solutions to food safety nor AMR problems; yet, they do offer promise (see chapters 2, 3, and 4). If that promise is one day fully realized, the pioneers or owners of the technologies will face complex policy, regulatory, and risk analysis contexts. This chapter explored these very policy and regulatory considerations.

Although many issues are anticipated, the author suggests the following approval and regulatory challenges, perceives the following conclusions, and recommends the following actions:

- Regulatory approval of *Megasphaera elsdenii*—and other probiotics for use in animals—requires a complete characterization of the microorganism's safety and health benefit(s) within the host, susceptibility to antibiotics, and intended use.

- In order to perform a comprehensive risk assessment along with post-market surveillance research to establish continued safe use, additional research on the probiotic's impact on the host microbiome is needed.

- Policies surrounding genetically enhanced animal feed—similar to Enogen® feed corn—differ between U.S. and EU regulatory bodies, with emphasis on the "precautionary principle" in the EU and "risk assessment" in the U.S.

- Postharvest wash interventions—including sodium bisulfate and peroxyacetic acid—require a defined application for use (sanitizer, production aid, food additive, etc.) and demonstration of safety for that use, to avoid adulteration of food and feed products.

- Regardless of the governing body and regulatory approval process, reproducible, peer-reviewed research is needed to assist regulators in making informed decisions.

Overall, both U.S. and EU food safety regulatory and intervention-approval systems have made tremendous strides in the safeguarding of domestic and global food systems; however, transnational public health threats persist. The technologies presented do not provide an ultimate solution, rather, they illustrate additional weapons for use against these threats.

The use of probiotics in animal feed to control pathogens remains poorly understood, with varying layers of complexity throughout the regulatory process limiting adoption into commercial practice. Dr. Steve Traylor, regulatory manager at MS Biotec which produces the DFM Lactipro advance product, indicated that this *M. elsdenii* product "does not fit well in most global regulatory schemes for animal feeds, veterinary health products, or animal remedy laws and regulations." Dr Traylor said, "we are at the mercy of each regulatory body on how they want to classify the product", referring to the process and necessary requirements for submitting applications for approval to one authority over another (Traylor, 2020). DFM approval for use within the U.S.U. and EU requires characterization of the microbe's impact on human and animal populations along with the greater environmental influence—a process needing decades of research. Although this technology holds promise, regulatory approval requires a tremendous amount of data in order to perform an appropriate safety assessment. This consideration may impede those eager to use this technology. Genetically enhanced feed, although seen in Chapter 2 to be effective against foodborne pathogens, seem to be the most difficult technology to adopt into a commercial application. This difficulty stems from a fundamental difference in how the

EU and U.S. regulate this type of technology, thus the approval process comes with significant regulatory hurdles. The final technology, postharvest produce wash interventions, appears well researched, with regulatory approval of these treatments underway. Although the findings in chapter 4 seem optimistic, the produce safety community should avoid overreliance on any single intervention, as their efficacy is not absolute.

João Vale De Almeida, Ambassador of the EU to the United Nations, spoke of the globalization of trade and technological advancement, stating "So much has happened, in so little amount of time, that has affected so many people" (Vale de Almeida, 2019). Present at Ambassador Almeida's speech at the European Horizons Conference, the author participated in a transatlantic collaboration, mingling with policy experts and delivering a policy talk. Ambassador Almeida's speech, the author's policy presentation, and this final dissertation chapter all resonate the same message: globalization of people, animals, and food have shaped the interconnectedness we experience today; however, one-health threats like AMR or *E. coli* O157:H7 endanger the privilege of safe and secure food. Access to safe food is not simply important for the global food market but represents a fundamental human right (Food and Agriculture Organization of the United Nations, 2013). It is the duty of those who research and regulate within the food sector to protect this right. Researchers must collaborate with those who regulate, and vice versa. The development of new food safety technologies ought to be celebrated. The author hopes this dissertation celebrates the promise of novel food safety technologies while capturing the scientific, regulatory, and approval-related issues surrounding them.

5.4. References

AAFCO. (2019). *Association of American Feed Control Officials 2019 Official Publication* (No. 2019; p. 387). Association of American Feed Control Officials. https://www.aafco.org/Publications

Aarestrup, F. M., Wegener, H. C., & Collignon, P. (2008). Resistance in bacteria of the food chain: Epidemiology and control strategies. *Expert Review of Anti-Infective Therapy; London*, *6*(5), 733–750. http://dx.doi.org/10.1586/14787210.6.5.733

Alban, L. (2016). Regulatory Issues Associated with Preharvest Food Safety: European Union Perspective. *Microbiology Spectrum*, *4*(5). https://doi.org/10.1128/microbiolspec.PFS-0003-2014

Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., & Casey, T. A. (2013). Treatment, promotion, commotion: Antibiotic alternatives in food-producing animals. *Trends in Microbiology*, *21*(3), 114–119. https://doi.org/10.1016/j.tim.2012.11.001

Anadón, A. (2006). WS14 The EU ban of antibiotics as feed additives (2006): Alternatives and consumer safety. *Journal of Veterinary Pharmacology and Therapeutics*, *29*(s1), 41–44. https://doi.org/10.1111/j.1365-2885.2006.00775_2.x

Ayala, D. I., Cook, P. W., Franco, J. G., Bugarel, M., Kottapalli, K. R., Loneragan, G. H., Brashears, M. M., & Nightingale, K. K. (2019). A Systematic Approach to Identify and Characterize the Effectiveness and Safety of Novel Probiotic Strains to Control Foodborne Pathogens. *Frontiers in Microbiology*, *10*, 1108. https://doi.org/10.3389/fmicb.2019.01108

Baker, A., Veloso, V. de A., Baker, A., & Barros, L. (2019). 343 Feedlot performance and carcass characteristics of steers fed diets containing steam-flaked grain and corn silage from Enogen® Feed Corn. *Journal of Animal Science*, *97*(Supplement_2), 137–137. https://doi.org/10.1093/jas/skz122.243

Baku, A. (2011, September 12). *European Strategic Action Plan on Antibiotic Resistance*. Regional Commitee for Europe.

Banach, J. L., Sampers, I., Van Haute, S., & van der Fels-Klerx, H. J. I. (2015). Effect of Disinfectants on Preventing the Cross-Contamination of Pathogens in Fresh Produce Washing Water. *International Journal of Environmental Research and Public Health*, *12*(8), 8658–8677. https://doi.org/10.3390/ijerph120808658

Bernardeau, M., & Vernoux, J.-P. (2013). Overview of differences between microbial feed additives and probiotics for food regarding regulation, growth promotion effects and health properties and consequences for extrapolation of farm animal results to humans. *Clinical Microbiology and Infection*, *19*(4), 321–330. https://doi.org/10.1111/1469-0691.12130

Bodiguel, L., & Cardwell, M. (2010). *The Regulation of Genetically Modified Organisms: Comparative Approaches*. Oxford University Press. https://www.oxfordscholarship.com/view/10.1093/acprof:oso/9780199542482.001.0001/acprof-9780199542482

Brashears, M. M., & Guillen, L. M. (2014, February 5). Direct-Fed Microbials as Preharvest Interventions in Cattle Production. *Food Safety Magazine*. https://www.foodsafetymagazine.com/magazine-archive1/februarymarch-2014/direct-fed-microbials-as-preharvest-interventions-in-cattle-production/

Breitling, R. (2010). What is Systems Biology? *Frontiers in Physiology*, *1*. https://doi.org/10.3389/fphys.2010.00009

Broaders, E., Gahan, C. G. M., & Marchesi, J. R. (2013). Mobile genetic elements of the human gastrointestinal tract: Potential for spread of antibiotic resistance genes. *Gut Microbes*, *4*(4), 271–280. https://doi.org/10.4161/gmic.24627

Brodmann, T., Endo, A., Gueimonde, M., Vinderola, G., Kneifel, W., de Vos, W. M., Salminen, S., & Gómez-Gallego, C. (2017). Safety of Novel Microbes for Human Consumption: Practical Examples of Assessment in the European Union. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01725

Callaway, T., Carroll, J. A., Arthington, J. D., Edrington, T. S., Rossman, M. L., Carr, M. A., Krueger, N. A., Ricke, S. C., Crandall, P., & Nisbet, D. J. (2011). *Escherichia coli* O157:H7 populations in ruminants can be reduced by orange peel product feeding. *Journal of Food Protection*, *74*(11), 1917–1921. https://doi.org/10.4315/0362-028X.JFP-11-234

Callaway, T., Edrington, T. s., Brabban, A. d., Keen, J. e., Anderson, R. c., Rossman, M. l., Engler, M. j., Genovese, K. j., Gwartney, B. l., Reagan, J. o., Poole, T. l., Harvey, R. b., Kutter, E. m., & Nisbet, D. j. (2006). Fecal Prevalence of *Escherichia coli* O157, *Salmonella*, *Listeria*, and Bacteriophage Infecting E. coli O157:H7 in Feedlot Cattle in the Southern Plains Region of the United States. *Foodborne Pathogens and Disease*, *3*(3), 234–244. https://doi.org/10.1089/fpd.2006.3.234

Center for Veterinary Medicine. (2019a, April 16). *CVM GFI #213 New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food-Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GFI #209*. U.S. Food and Drug Administration. http://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-213-new-animal-drugs-and-new-animal-drug-combination-products-administered-or-medicated-feed

Center for Veterinary Medicine. (2019b). Food Additive Petitions for Animal Food. *FDA*. http://www.fda.gov/animal-veterinary/development-approval-process/food-additive-petitions-animal-food

Center for Veterinary Medicine. (2019c). *Veterinary Feed Directive (VFD)*. Food and Drug Administration. http://www.fda.gov/animal-veterinary/development-approval-process/veterinary-feed-directive-vfd

Center for Veterinary Medicine. (2020). Investigational Food Additive (IFA) Files. *FDA*. http://www.fda.gov/animal-veterinary/ingredients-additives/investigational-food-additive-ifa-files

Centers for Disease Control and Prevention. (2013). *Antibiotic Resistance Threats in the United States, 2013* (No. 1; p. 114). https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf

Centers for Disease Control and Prevention. (2018, June 28). *Multistate Outbreak of E. coli O157:H7 Infections Linked to Romaine Lettuce (Final Update)*. https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html

Centers for Disease Control and Prevention. (2019a). *Antibiotic Resistance Threats in the United States, 2019*. 148.

Centers for Disease Control and Prevention. (2019b, November 23). *Outbreak of E. coli Infections Linked to Romaine Lettuce | E.coli Infections | November 2019 | E. coli | CDC*. https://www.cdc.gov/ecoli/2019/o157h7-11-19/index.html

Centers for Disease Control and Prevention. (2019c, December 9). *Reports of Selected E. coli Outbreak Investigations*. https://www.cdc.gov/ecoli/outbreaks.html

Chaney, W. E., Maloney, R., Johnson, B. J., Brooks, J. C., Brashears, M. M., & Loneragan, G. H. (2018). Corn-Based Distillers' Grains in Diets for Feedlot Cattle Are Associated with the Burden of *Escherichia coli* O157 in Feces. *Foodborne Pathogens and Disease*, *15*(7), 398–405. https://doi.org/10.1089/fpd.2017.2395

Chen, L., & Wang, H. (2016). Advances in the metabolism and regulation of lactic acids in the rumen. *Pratacult. Sci.*, *33*, 972–980.

Chen, Lianmin, Shen, Y., Wang, C., Ding, L., Zhao, F., Wang, M., Fu, J., & Wang, H. (2019). *Megasphaera elsdenii* Lactate Degradation Pattern Shifts in Rumen Acidosis Models. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00162

CVM. (2020, February 12). *Ingredients & Additives*. FDA. http://www.fda.gov/animal-veterinary/animal-food-feeds/ingredients-additives

Dibner, J. J., & Richards, J. D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*, *84*(4), 634–643. https://doi.org/10.1093/ps/84.4.634

Diez-Gonzalez, F., Callaway, T. R., Kizoulis, M. G., & Russell, J. B. (1998). Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science (New York, N.Y.)*, *281*(5383), 1666–1668. https://doi.org/10.1126/science.281.5383.1666

EFSA. (2013, June 20). *Insufficient data stop EFSA from concluding on safety of GM maize 3272*. European Food Safety Authority. http://www.efsa.europa.eu/en/press/news/130620

EFSA, & ECDC. (2019). *The European Union One Health 2018 Zoonoses Report* (No. 17; p. 276). European Food Safety Authority. https://www.ecdc.europa.eu/sites/default/files/documents/zoonoses--EU-One-Health-2018-Zoonoses-Report.pdf

EFSA Panel on Biological Hazards. (2014). Scientific Opinion on the evaluation of the safety and efficacy of peroxyacetic acid solutions for reduction of pathogens on poultry carcasses and meat. *EFSA Journal*, *12*(3), 3599. https://doi.org/10.2903/j.efsa.2014.3599

EFSA Panel on Food Additives and Flavourings (FAF), Younes, M., Aquilina, G., Castle, L., Engel, K.-H., Fowler, P., Fürst, P., Gürtler, R., Gundert-Remy, U., Husøy, T., Mennes, W., Moldeus, P., Oskarsson, A., Shah, R., Waalkens-Berendsen, I., Wölfle, D., Boon, P., Crebelli, R., Di Domenico, A., … Frutos Fernandez, M. J. (2019). Re-evaluation of sulphuric acid and its sodium, potassium, calcium and ammonium salts (E 513, 514 (i), 514 (ii), 515 (i), 515 (ii), 516 and 517) as food additive. *EFSA Journal*, *17*(10), e05868. https://doi.org/10.2903/j.efsa.2019.5868

Erickson, G., Martin, D., & Watson, E. (2018, October 2). *Improving Profic Potential Through Rations With More Available Energy*. https://www.drovers.com/webinars/improving-profit-potential-through-rations-more-available-energy

European Centre for Disease Prevention and Control. (2018, November 15). *Surveillance of antimicrobial resistance in Europe 2017*. European Centre for Disease Prevention and Control. http://ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-resistance-europe-2017

European Commission. (2019). *Agri-Food Trade Statistical Factsheet European Union—USA* (p. 8). https://ec.europa.eu/info/sites/info/files/food-farming-fisheries/farming/documents/agrifood-usa_en.pdf

FAO/WHO working group. (2002). *Guidelines for the Evaluation of Probiotics in Food*. FAO/WHO, London, ON. https://scholar.google.com/scholar_lookup?title=Guidelines%20for%20the%20evaluation%20of%20probiotics%20in%20food.%20London%2C%20Ontario%2C%20Canada%3A%20Food%20and%20Agriculture%20Organization%20of%20the%20United%20Nations%20and%20World%20Health%20Organization%20Working%20Group%20Report&publication_year=2002&author=FAO%2FWHO

FDA. (2019a, July 10). *MOU 225-07-7001*. FDA. http://www.fda.gov/about-fda/domestic-mous/mou-225-07-7001

FDA. (2019b). Food Additive Petitions for Animal Food. *FDA*. http://www.fda.gov/animal-veterinary/development-approval-process/food-additive-petitions-animal-food

FDA. (2020, January 17). *Generally Recognized as Safe (GRAS) Notification Program*. FDA. http://www.fda.gov/animal-veterinary/animal-food-feeds/generally-recognized-safe-gras-notification-program

Feliziani, E., Lichter, A., Smilanick, J. L., & Ippolito, A. (2016). Disinfecting agents for controlling fruit and vegetable diseases after harvest. *Postharvest Biology and Technology*, *122*, 53–69. https://doi.org/10.1016/j.postharvbio.2016.04.016

Food and Agriculture Organization of the United Nations. (2013). *The Human Right to Adequate Food in the Global Strategic Framework for Food Security and Nutrition—A Global Consensus*. United Nations. http://www.fao.org/3/a-i3546e.pdf

Fuller, R. (1992). History and development of probiotics. In R. Fuller (Ed.), *Probiotics: The scientific basis* (pp. 1–8). Springer Netherlands. https://doi.org/10.1007/978-94-011-2364-8_1

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *The Journal of Nutrition*, *125*(6), 1401–1412. https://doi.org/10.1093/jn/125.6.1401

Gibson, Glenn R., Probert, H. M., Loo, J. V., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutrition Research Reviews*, *17*(2), 259–275. https://doi.org/10.1079/NRR200479

Glass, D. (2013, July 25). European food approval still elusive for Enogen corn. *Advanced Biotechnology for Biofuels*. https://dglassassociates.wordpress.com/2013/07/25/european-food-approval-still-elusive-for-enogen-corn/

Gorsuch, J., LeSaint, D., VanderKelen, J., Buckman, D., & Kitts, C. L. (2019). A comparison of methods for enumerating bacteria in direct fed microbials for animal feed. *Journal of Microbiological Methods*, *160*, 124–129. https://doi.org/10.1016/j.mimet.2019.04.003

Gould, I. M., & Meer, J. W. M. van der. (2005). *Antibiotic policies theory and practice*. New York : Kluwer Academic/Plenum.

Hoelzer, K. (2020, February 24). *New Studies Can Help Inform Antibiotic Stewardship in Animal Agriculture*. PEW Charitable Trusts. https://pew.org/32lG6Fo

Interagency Food Safety Analytics Collaboration. (2019). *Foodborne illness source attribution estimates for 2017 for Salmonella, Escherichia coli O157, Listeria monocytogenes, and Campylobacter using multi-year outbreak surveillance data, United States* (p. 14).

Jacob, M. E., Fox, J. T., Drouillard, J. S., Renter, D. G., & Nagaraja, T. G. (2008). Effects of Dried Distillers' Grain on Fecal Prevalence and Growth of *Escherichia coli* O157 in Batch Culture Fermentations from Cattle. *Applied and Environmental Microbiology*, *74*(1), 38–43. https://doi.org/10.1128/AEM.01842-07

Johnson, M., Spore, T., Montgomery, S., Weibert, C., Garzon, J., Hollenbeck, W., Wahl, R., Watson, E., & Blasi, D. (2018). Syngenta Enhanced Feed Corn (Enogen) Containing an Alpha Amylase Expression Trait Improves Feed Efficiency in Growing Calf Diets. *Kansas Agricultural Experiment Station Research Reports*, *4*(1). https://doi.org/10.4148/2378-5977.7543

Johnson, R. (n.d.). *The U.S.-EU Beef Hormone Dispute*. 38.

Jolly-Breithaupt, M. L., Harris, M. E., Nuttelman, B. L., Burken, D. B., MacDonald, J. C., Luebbe, M. K., Iragavarapu, T. K., & Erickson, G. E. (2019). Effects of Syngenta Enogen Feed Corn containing an α-amylase trait on finishing cattle performance and carcass characteristics. *Translational Animal Science*, *3*(1), 504–512. https://doi.org/10.1093/tas/txy121

Joshi, K., Mahendran, R., Alagusundaram, K., Norton, T., & Tiwari, B. K. (2013). Novel disinfectants for fresh produce. *Trends in Food Science & Technology*, *34*(1), 54–61. https://doi.org/10.1016/j.tifs.2013.08.008

Kastner, J. (2018, October 1). *Module 3: What role does risk assessment play in the multilateral trading system?* [Lecture]. DMP 816: Trade and Agricultural Health, Kansas State University.

Kastner, J. J., & Pawsey, R. K. (2002). Harmonising sanitary measures and resolving trade disputes through the WTO–SPS framework. Part I: A case study of the US–EU hormone-treated beef dispute. *Food Control*, *13*(1), 49–55. https://doi.org/10.1016/S0956-7135(01)00023-8

Khan, R. U., Naz, S., Dhama, K., Karthik, K., Tiwari, R., Abdelrahma, M. M., Alhidary, I., & Zahoor, A. (2016). *Direct-Fed Microbial: Beneficial Applications, Modes of Action and Prospects as a Safe Tool for Enhancing Ruminant Production and Safeguarding Health*. https://doi.org/10.3923/ijp.2016.220.231

Kitano, H. (2002). Systems Biology: A Brief Overview. *Science*, *295*(5560), 1662–1664. https://doi.org/10.1126/science.1069492

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., Fridkin, S. K., & Active Bacterial Core surveillance (ABCs) MRSA Investigators. (2007). Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *JAMA*, *298*(15), 1763–1771. https://doi.org/10.1001/jama.298.15.1763

Knueven, C. J. (2001). *Sodium bisulfate as acidulant in foods* (European Union Patent No. EP1104245A1). https://patents.google.com/patent/EP1104245A1/en#citedBy

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., Cesare, A. D., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G.,

Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Escámez, P. S. F., Maradona, M. P., … Herman, L. (2020). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 11: Suitability of taxonomic units notified to EFSA until September 2019. *EFSA Journal*, *18*(2), e05965. https://doi.org/10.2903/j.efsa.2020.5965

Lau, J. (2015, August 9). *Same Science, Different Policies: Regulating Genetically Modified Foods in the U.S. and Europe*. Science in the News. http://sitn.hms.harvard.edu/flash/2015/same-science-different-policies/

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., … Cars, O. (2013). Antibiotic resistance—The need for global solutions. *The Lancet Infectious Diseases*, *13*(12), 1057–1098. https://doi.org/10.1016/S1473-3099(13)70318-9

Leverstein-van Hall, M. A., Box, A. T. A., Blok, H. E. M., Paauw, A., Fluit, A. C., & Verhoef, J. (2002). Evidence of Extensive Interspecies Transfer of Integron-Mediated Antimicrobial Resistance Genes among Multidrug-Resistant *Enterobacteriaceae* in a Clinical Setting. *The Journal of Infectious Diseases*, *186*(1), 49–56. https://doi.org/10.1086/341078

Markets and Markets. (2019, April). *Probiotics in Animal Feed Market by Livestock, Source, Form, and Region—Global Forecast 2025*. https://www.marketsandmarkets.com/Market-Reports/probiotics-animal-feed-market-85832335.html?gclid=CjwKCAiAvonyBRB7EiwAadauqQeDMGT55u-SoJSS1AptQDXv4_LkxqUnwDfao_-N8QsVqA_0Ao3R4xoChKwQAvD_BwE

Markowiak, P., & Śliżewska, K. (2018). The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathogens*, *10*(1), 21. https://doi.org/10.1186/s13099-018-0250-0

McAllister, T. A., Beauchemin, K. A., Alazzeh, A. Y., Baah, J., Teather, R. M., & Stanford, K. (2011). Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. *Canadian Journal of Animal Science*, *91*(2), 193–211. https://doi.org/10.4141/cjas10047

Meireles, A., Giaouris, E., & Simões, M. (2016). Alternative disinfection methods to chlorine for use in the fresh-cut industry. *Food Research International*, *82*, 71–85. https://doi.org/10.1016/j.foodres.2016.01.021

Naegeli, H., Bresson, J.-L., Dalmay, T., Dewhurst, I. C., Epstein, M. M., Firbank, L. G., Guerche, P., Hejatko, J., Moreno, F. J., Mullins, E., Nogué, F., Rostoks, N., Serrano, J. J. S., Savoini, G., Veromann, E., Veronesi, F., Álvarez, F., Ardizzone, M., Lanzoni, A., … Neri, F. M. (2019). Statement complementing the EFSA Scientific Opinion on application (EFSA-GMO-UK-2006-34) for authorisation of food and feed containing, consisting of and produced from genetically modified maize 3272. *EFSA Journal*, *17*(11), e05844. https://doi.org/10.2903/j.efsa.2019.5844

Obama, B. (2014, September 18). *Executive Order 13676—Combating Antibiotic-Resistant Bacteria*. Whitehouse.Gov. https://obamawhitehouse.archives.gov/the-press-office/2014/09/18/executive-order-combating-antibiotic-resistant-bacteria

Office of Regulatory Affairs. (2019, May 8). *CPG Sec. 689.100 Direct-Fed Microbial Products*. U.S. Food and Drug Administration. http://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-689100-direct-fed-microbial-products

Organic Materials Review Institute. (2015). *Sodium Bisulfate Technical Evaluation Report* (Report for the USDA Organic Program, p. 17). https://www.ams.usda.gov/sites/default/files/media/Sodium%20Bi%20report%202015.pdf

Organic Materials Review Institute. (2016). *Peracetic Acid Technical Evaluation Report* (Report for the USDA Organic Program, p. 22). https://www.ams.usda.gov/sites/default/files/media/Peracetic%20Acid%20TR%203_3_2016%20Crops%20Final.pdf

Pollack, A. (2011, February 11). Enogen, Corn Modified for Use in Ethanol, Is Approved. *New York Times*. https://www.nytimes.com/2011/02/12/business/12corn.html?_r=1&src=busln

President's Council of Advisors on Science and Technology. (2015). *National action plan for combating antibiotic-resistant bacteria*. The White House. https://www.cdc.gov/drugresistance/pdf/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf

Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P., & Silva, C. L. M. (2013). Fresh fruits and vegetables—An overview on applied methodologies to improve its quality and safety. *Innovative Food Science & Emerging Technologies*, *20*, 1–15. https://doi.org/10.1016/j.ifset.2013.07.002

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D., Gyssens, I., Heuer, O. E., Kahlmeter, G., Kruse, H., Laxminarayan, R., Liébana, E., López-Cerero, L., MacGowan, A., Martins, M., Rodríguez-Baño, J., Rolain, J.-M., … Vila, J. (2015). The global threat of antimicrobial resistance: Science for intervention. *New Microbes and New Infections*, *6*, 22–29. https://doi.org/10.1016/j.nmni.2015.02.007

Salgado, S. P., Pearlstein, A. J., Luo, Y., & Feng, H. (2014). Quality of Iceberg (Lactuca sativa L.) and Romaine (L. *sativa L. var. Longifolial*) lettuce treated by combinations of sanitizer, surfactant, and ultrasound. *LWT - Food Science and Technology*, *56*(2), 261–268. https://doi.org/10.1016/j.lwt.2013.11.038

Sanders, M. E., Akkermans, L. M. A., Haller, D., Hammerman, C., Heimbach, J., Hörmannsperger, G., Huys, G., Levy, D. D., Lutgendorff, F., Mack, D., Phothirath, P., Solano-Aguilar, G., & Vaughan, E. (2010). Safety assessment of probiotics for human use. *Gut Microbes*, *1*(3), 164–185. https://doi.org/10.4161/gmic.1.3.12127

Schaberg, D. R., & Zervos, M. J. (1986). Intergeneric and interspecies gene exchange in gram-positive cocci. *Antimicrobial Agents and Chemotherapy*, *30*(6), 817–822.

Simon, O. (2005). *Micro-Organisms as Feed Additives – Probiotics*. 7.

Stephens, T., Loneragan, G., Chichester, L., & Brashears, M. (2007). Prevalence and enumeration of *Escherichia coli* O157 in steers receiving various strains of *Lactobacillus*-based direct-fed microbials. *Journal of Food Protection*, *70*(5), 1252–1255. https://doi.org/10.4315/0362-028x-70.5.1252

The European Commission. (2017). *The European One Health Action Plan against Antimicrobial Resistance*. https://ec.europa.eu/health/amr/sites/amr/files/amr_action_plan_2017_en.pdf

Traylor, S. (2020, March 10). *Regulatory Questions* [E-mail]. https://outlook.office.com/mail/deeplink?version=2020031603.12&popoutv2=1&leanbootstrap=1

USDA. (2011). *Syngenta Seeds, Inc. Alpha-Amylase Maize Event 3272: Final Environmental Assessment* (SYN-E3272-5). https://www.aphis.usda.gov/brs/aphisdocs/05_28001p_fea.pdf

USDA ERS. (2019). *Recent Trends in Genetically Engineered Crop Adoption*. https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-ge-adoption.aspx

Vale de Almeida, J. (2019, April 5). *European Student Conference—Opening Keynote*.

Veloso, V. de A., Horton, L., Baker, A., Aperce, C., & Drouillard, J. (2019). 333 Effects of ruminally-protected lysine and *Megasphaera elsdenii* on performance and carcass characteristics of finishing cattle. *Journal of Animal Science*, *97*(Supplement_2), 134–134. https://doi.org/10.1093/jas/skz122.238

Venugopalan, V., Shriner, K. A., & Wong-Beringer, A. (2010). Regulatory Oversight and Safety of Probiotic Use. *Emerging Infectious Diseases*, *16*(11), 1661–1665. https://doi.org/10.3201/eid1611.100574

Walker, B., Barrett, S., Polasky, S., Galaz, V., Folke, C., Engström, G., Ackerman, F., Arrow, K., Carpenter, S., Chopra, K., Daily, G., Ehrlich, P., Hughes, T., Kautsky, N., Levin, S., Mäler, K.-G., Shogren, J., Vincent, J., Xepapadeas, T., & Zeeuw, A. de. (2009). Looming Global-Scale Failures and Missing Institutions. *Science*, *325*(5946), 1345–1346. https://doi.org/10.1126/science.1175325

Weng, S., Luo, Y., Li, J., Zhou, B., Jacangelo, J. G., & Schwab, K. J. (2016). Assessment and speciation of chlorine demand in fresh-cut produce wash water. *Food Control*, *60*, 543–551. https://doi.org/10.1016/j.foodcont.2015.08.031

Wise, R. (2007). An overview of the Specialist Advisory Committee on Antimicrobial Resistance (SACAR). *Journal of Antimicrobial Chemotherapy*, *60*(suppl_1), i5–i7. https://doi.org/10.1093/jac/dkm151

Workman, D. (2019, May 1). *Top Lettuce Exports by Country*. World's Top Exports. http://www.worldstopexports.com/top-lettuce-exports-by-country/

Yaron, S., & Römling, U. (2014). Biofilm formation by enteric pathogens and its role in plant colonization and persistence. *Microbial Biotechnology*, *7*(6), 496–516. https://doi.org/10.1111/1751-7915.12186

Yoon, I. K., & Stern, M. D. (1995). Influence of direct-fed microbials on ruminal microbial fermentation and performance of ruminants—A Review -. *Asian-Australasian Journal of Animal Sciences*, *8*(6), 533–555. https://doi.org/1995.8.6.533

Younts-Dahl, S., Osborn, G., Galyean, M., Rivera, D., Loneragan, G., & Brashears, M. (2005). Reduction of *Escherichia coli* O157 in finishing beef cattle by various doses of *Lactobacillus acidophilus* in direct-fed microbials. *Journal of Food Protection*, *68*(1), 6–10. https://doi.org/10.4315/0362-028x-68.1.6

Appendix A - SAS Code for Analysis of *Megasphaera elsdenii* Impact on *E. coli* O157:H7 Prevalence in Feedlot Cattle

```
PROC IMPORT OUT= WORK.set1
            DATAFILE= "&path\2018-01 E coli_stat.csv"
            DBMS=CSV REPLACE;
     GETNAMES=YES;
     DATAROW=2;
     GUESSINGROWS=3000;
RUN;
data dat1.MegaSph;set set1;
if Ecoli=100 then resp=1;
else if Ecoli=0 then resp=0;
if EarTag in (57, 137,309,269,332,468) then resp=.;
if EarTag in (12) and week=2 then resp=.;/*See the e-mail on
06/25/2019*/
run;
proc sort data=dat1.MegaSph;by lactipro lysine pen;
proc means data=dat1.MegaSph noprint ;by lactipro lysine pen;
var resp;
output out=pensize n=pensize;
where week=1;
run;

proc freq data=pensize;
table lactipro*lysine*pensize/norow nocol nopercent;
run;

proc freq data=dat1.MegaSph;
table lactipro*lysine*block/norow nocol nopercent;
where week=1;
run;
ods rtf file="&path\sasoutput.doc" style=monochromeprinter;
proc tabulate data=dat1.MegaSph;
class week lactipro lysine;
var resp;
table lysine*lactipro, week=' '*resp=' '*(n sum='Number of Pos.
Animals'*f=8.0 mean='Prevalence Rate'*f=percent8.1);
run;


proc glimmix data=dat1.MegaSph ;
```

```
class pen eartag block lactipro lysine week;
model resp= week lactipro lysine lactipro*lysine week*lactipro
week*lysine week*lactipro*lysine /link=logit d=b ddfm=none s;
random intercept/subject=eartag;
random block pen;
lsmeans week*lactipro/ilink;
estimate 'wk1' lactipro -1 1 week*lactipro -1 0 1 0/exp cl;
estimate 'wk2' lactipro -1 1 week*lactipro 0 -1 0 1/exp cl;
ods output lsmeans=lsm1 estimates=est;
run;

proc glimmix data=dat1.MegaSph ;
class pen eartag block lactipro lysine week;
model resp= week lactipro lysine lactipro*lysine week*lactipro
week*lysine week*lactipro*lysine /link=logit d=b ddfm=none s;
random intercept/subject=eartag;
random block pen;
lsmeans week*lactipro*lysine/ilink;
ods select lsmeans;
ods output lsmeans=lsm2;
run;
data lsm2;format mu percent8.1 comb $30.;set lsm2;
if lysine=0 and lactipro=0  then  comb='0g Lysine;  0 ml Lactipro
';
if lysine=0 and lactipro=20 then  comb='0g Lysine;  20 ml Lactipro';
if lysine=40 and lactipro=0 then  comb='40g Lysine;0 ml Lactipro    ';
if lysine=40 and lactipro=20 then comb='40g Lysine;20 ml Lactipro';
run;
goptions vsize= 4 in hsize=5 in;
proc sort data=lsm2;by lysine lactipro week;
symbol1 i=j color=black line=1 value=plus;
symbol2 i=j color=black line=1 value=circle;
axis1 order=1 to 2 by 1 offset=(25 25) minor=none;
axis2 order=0 to 0.2 by 0.1 offset=(2 2) minor=(n=5) label=(a=90
'Prevalence Rate');
proc gplot data=lsm2;by lysine;
plot mu*week=lactipro/haxis=axis1 vaxis=axis2;
run;
proc sort data=lsm2;by comb week;
symbol1 i=j color=black line=1 value=plus;
symbol2 i=j color=black line=1 value=circle;
symbol3 i=j color=red line=1 value=plus;
symbol4 i=j color=red line=1 value=circle;
axis1 order=1 to 2 by 1 offset=(25 25) minor=none;
```

```
axis2 order=0 to 0.25 by 0.05 offset=(2 2) minor=(n=4) label=(a=90
'Prevalence Rate');
legend1 label=(' ') repeat=2 shape=symbol(2,1);
proc gplot data=lsm2;
plot mu*week=comb/haxis=axis1 vaxis=axis2 legend=legend1;
run;
ods rtf close;
```

Appendix B - SAS code for Analysis of Enogen® Feed Corn Impact on *E. coli* O157:H7 Prevalence in Feedlot Cattle

```
PROC IMPORT OUT= WORK.set1
            DATAFILE= "C:\Users\qkang\OneDrive - Kansas State
University
\Sara Gragg\Enogen\Adrian Cattle EC Results final_Stat.csv"
            DBMS=CSV REPLACE;
     GETNAMES=YES;
     DATAROW=2;
     GUESSINGROWS=1000;
RUN;
proc format;
value $sd 'A'='June 12'
      'B'='June 28'
       'C'='July 13';
run;
data dat1.Enogen;format sample_date $sd.;set set1;
if E_coli='1' then resp=1;
else if E_coli='0' then resp=0;
else if E_coli=' ' then resp=.;
else if E_coli='Removed' then resp=.;
run;

proc sort data=dat1.Enogen;by treatment studypen;
proc means data=dat1.enogen noprint missing;by treatment studypen;
var resp;
output out=pensize n=pensize;
run;

proc freq data=pensize;
table treatment*pensize/norow nocol nopercent;
run;
ods rtf file="&path\sasoutput.doc" style=monochromeprinter;
proc tabulate data=dat1.Enogen;
class sample_date silage corn;
var resp;
table silage*corn, sample_date=' '*resp=' '*(n sum='Number of Pos.
Animals'*f=8.0 mean='Prevalence Rate'*f=percent8.1);
run;

proc tabulate data=dat1.Enogen;
```

```
class sample_date silage corn;
var resp;
table corn*silage, sample_date=' '*resp=' '*(n sum='Number of Pos.
Animals'*f=8.0 mean='Prevalence Rate'*f=percent8.1);
run;

proc logistic data=dat1.Enogen descending;
class silage corn sample_date/param=glm;
model resp=silage corn silage*corn;
strata sample_date;
exact 'Interaction' silage*corn;
run;
proc logistic data=dat1.Enogen descending;
class silage corn sample_date/param=ref;
model resp=silage corn ;
strata sample_date;
*lsmeans silage corn/ilink;
exact 'Silage' silage/cltype=exact alpha=0.05 estimate=odds;
exact 'Corn' corn/cltype=exact alpha=0.05 estimate=odds;
run;
ods rtf close;
```

Appendix C - SAS Code for Sodium Bisulfate and Peroxyacetic Acid Effect on Phytotoxicity, Browning, and Overall Visual Quality Analyses in Fresh-Cut Romaine Lettuce

```
proc print data=phyto;
title 'Phytotoxicity DataLines';
run;

proc mixed data=phyto;
class rep treatment day score;
model score=rep;
title 'Phytotoxicity Analysis of Rep Effect';
run;

proc mixed data=phyto;
class rep treatment day score;
model score=treatment day treatment*day;
title 'Phytotoxicity';
run;

proc mixed data=phyto;
class rep treatment day score;
model score=treatment day treatment*day;
LSMEANS day / pdiff cl adjust=tukey;
title 'Phytotoxicity by Day';
run;
proc print data=browning;
title 'Browning';
run;

proc mixed data=browning;
class rep treatment day score;
model score=rep;
title 'Browning Analysis of Rep Effect';
run;

proc mixed data=browning;
class rep treatment day score;
model score=treatment day treatment*day;
title 'Browning Significant Effects';
run;
```

```
proc mixed data=browning;
class rep treatment day score;
model score=treatment day;
title 'Browning Significant Effects w/out Interaction';
run;

proc mixed data=browning;
class rep treatment day score;
model score=treatment day;
LSMEANS treatment day/pdiff cl adjust=tukey;
title 'Browning LSMEANS for treatment and day';
run;
proc print data=OVG;
title 'Overall Visual Quality';
run;

proc mixed data=OVG;
class rep treatment day score;
model score=rep;
title 'OVG Analysis of Rep Effect';
run;

proc mixed data=OVG;
class rep treatment day score;
model score=treatment day treatment*day;
title 'Analysis of Significant OVG Effects';
run;

proc mixed data=OVG;
class rep treatment day score;
model score=treatment day treatment*day;
title 'Analysis of Significant OVG Effects';
run;

proc mixed data=OVG;
class rep treatment day score;
model score=treatment day;
LSMEANS treatment day / pdiff cl adjust=tukey;
title 'LSMEANS of Treatment and Day for OVG';
run;
```

Appendix D - SAS Code for Sodium Bisulfate and Peroxyacetic Acid Effect on Color Visual Quality Analysis in Fresh-Cut Romaine Lettuce

```
proc mixed data=color;
class rep treatment day parameter response;
model response=rep;
title 'Color Analysis of Rep Effect';
run;

proc mixed data=color;
class rep treatment day parameter response;
model response=treatment day parameter parameter*day treatment*day;
title 'Color Analysis';
run;

proc mixed data=lightness;
class rep treatment day response;
model response=treatment day treatment*day;
title 'L Analysis';
run;

proc mixed data=A;
class rep treatment day response;
model response=treatment day  treatment*day;
title 'A Analysis';
run;

proc mixed data=B;
class rep treatment day response;
model response=treatment day  treatment*day;
title 'B Analysis';
run;

proc mixed data=Chroma;
class rep treatment day response;
model response=treatment day  treatment*day;
title 'Chroma Analysis';
run;

proc mixed data=hue;
```

```
class rep treatment day response;
model response=treatment day  treatment*day;
title 'hue Analysis';
run;

proc mixed data=color;
class rep treatment day parameter response;
model response=treatment day parameter parameter*day treatment*day;
title 'Color Analysis by Day and by Parameter';
run;
```

Appendix E - SAS Code for Analysis of Sodium Bisulfate and Peroxyacetic Acid Effect on *E. coli* O157:H7 in Fresh-Cut Romaine Lettuce

```
proc print data=romaine;
title 'Romaine SBS/PAA';
run;

proc mixed data=romaine;
class rep treatment day count;
model count=rep;
title 'Analysis of Rep Effect';
run;

proc mixed data=romaine;
class rep treatment day count;
model count=treatment day treatment*day
title 'Count Significant Effects'
run;

proc mixed data=romaine;
class rep treatment day count;
model count=treatment day;
title 'Count Significant Effects without interaction';
run;

proc mixed data=romaine;
class rep treatment day count;
model count=treatment day treatment*day;
LSMEANS day treatment / pdiff cl;
title 'Count LSMEANS for day and treatment without tukey';
run;

proc mixed data=romaine;
class rep treatment day count;
model count=treatment day treatment*day;
LSMEANS day treatment treatment*day / pdiff cl adjust=tukey;
title 'Count LSMEANS for day and treatment with tukey';
run;
```