

DISSERTATION

EXPLORATION OF NOVEL ANTIMICROBIALS AND COMPARATIVE GENOMIC
ANALYSIS FOR THE CONTROL OF FOODBORNE PATHOGENS

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ABSTRACT

EXPLORATION OF NOVEL ANTIMICROBIALS AND COMPARATIVE GENOMIC ANALYSIS FOR THE CONTROL OF FOODBORNE PATHOGENS

For decades, the meat industry faced emerging and evolving pathogenic microorganisms. To control foodborne pathogens in meat products, it is common practice to use chemical antimicrobials, such as lactic acid and peroxyacetic acid. However, it is acknowledged that use of antimicrobials could potentially lead to emergence of resistant pathogens. Therefore, there has been a great interest in developing novel antimicrobials to overcome the current challenge of antimicrobial resistance. Here, we conducted two studies to investigate two types of potential antimicrobials that might be used by the meat industry: plant-derived antimicrobials, and programmable sequence-specific CRISPR-Cas9 antimicrobials. Furthermore, another study was conducted to better understand the detailed mechanisms involved in the acquisition or loss of virulence genes and the particular genotypic traits associated with the presence or absence of the virulence genes using comparative genomic analysis.

In the first study, we compared three newly sequenced *E. coli* O157:H7 strains with different *stx* profiles from cattle fecal samples, C1-010 (*stx1*-, *stx2c*+), C1-057 (*stx*-) and C1-067 (*stx1*-, *stx2a*+), as well as five foodborne outbreak strains and six *stx*-negative strains from NCBI database, in order to expand our understanding of the exchange of *stx* genes and other genomic traits between typical and *stx*-negative *E. coli* O157:H7 isolated from farm animals. Phylogenomic analysis showed that strains C1-010 and C1-057 were grouped with other six *stx*-

negative strains, while strain C1-067 was grouped with five foodborne outbreak strains, and the closest relationship existed with strain Sakai causing a large outbreak in Japan. Since strains C1-010, C1-057 and C1-067 were grouped into different clusters, these three *E. coli* O157:H7 from a cattle feedlot with different virulence factors may exhibit different levels of pathogenicity. Additionally, we compared presence or absence of Red recombination genes (*exo*, *bet* and *gam*) and their repressor gene *cI* in the *stx*-negative prophages located at *stx* insertion sites in seven *stx*-negative *E. coli* O157:H7 strains and in the *stx*-carrying prophages in three selected *stx*-positive *E. coli* O157:H7 strains. All *stx*-carrying prophages contained both the three Red recombination genes and its repressor *cI*. Presence of repressor CI in *stx*-carrying prophages could inhibit the activity of Red homologous recombination during lysogenic phase and, as a consequence, stability of the *stx* genes in *stx*-positive *E. coli* O157:H7 could be increased. On the other hand, most of the *stx*-negative prophages at *stx* insertion sites carried only three Red recombination genes but the repressor *cI* was absent. In the absence of the repressor *cI* in *stx*-negative prophages, the consistent expression of the Red recombination genes during the lysogenic phase might result in more frequent gene exchange, potentially contributing to acquisition of *stx* gene. To our knowledge, this is the first report to describe potential functions for Red recombination genes and repressor *cI* associated with the maintaining of *stx* gene in *stx*-carrying prophages and acquisition of *stx* gene in *stx*-negative prophages. Furthermore, comparative pathway analysis showed that eight unique copies of genes involved in metabolic pathways were found in *stx*-positive (*stx1*+, *stx2a*+) *E. coli* O157:H7 strains Sakai and C1-067. One unique copy of metabolic pathway gene was found in seven *stx*-negative one *stx*-positive (*stx1*-, *stx2c*+) *E. coli* strains. Functions of these nine copies of genes were associated with glycerol metabolism, increasing survival, and improving iron uptake ability. Presence of these

metabolic pathway genes in *stx*-positive *E. coli* O157:H7 may increase competitiveness in complex environment such as cattle feedlot, compared with *stx*-negative *E. coli* O157:H7 which did not contain these metabolic pathway genes. This analysis may explain the facts that *stx*-positive *E. coli* O157:H7 is more prevalent than *stx*-negative *E. coli* O157:H7 in cattle. Additionally, due to the results of phylogenomic analysis and gene arrangements around the specific IS629, we suggested that *stx*-positive (*stx1*+, *stx2a*+) *E. coli* O157:H7 may be evolved from O55:H7 by different evolutionary pathways, compared with *stx*-positive (*stx1*-, *stx2c*+) and *stx*-negative *E. coli* O157:H7 strains. The findings of this study may help with the development of control strategies to reduce pathogenic *E. coli* O157:H7 in food production.

In the second study, we developed a plasmid-mediated and a phage-mediated system to deliver the Shiga toxin-specific CRISPR-Cas9 lethal modification into foodborne STEC, to targeting the *stx* genes for sequence-specific removal of foodborne STEC. Their killing efficacies were also evaluated. For the plasmid-mediated CRISPR-Cas9 lethal modification, we designed three gRNAs targeting to *stx1* at different locations and two gRNAs targeting to *stx2* at different locations. The plasmid-mediated CRISPR-Cas9 lethal modification with single gRNAs targeting either *stx1* or *stx2* reduced population of STEC by 0.38 to 2.64 log CFU/reaction. This result showed that the killing efficacies of the CRISPR-Cas9 lethal modification could be varied when different gRNAs were used, and that killing efficacies could be optimized by designing gRNAs which targeted specific genes in pathogens. Additionally, the plasmid-mediated CRISPR-Cas9 lethal modification with two gRNAs targeting both *stx1* and *stx2* achieved higher cell population reductions (3.20 log CFU/reaction reduction). This result indicated that the CRISPR-Cas9 lethal modification could be programmed to target multiple sequence locations on the chromosomes of target bacteria, to maximize killing efficacy. Alternatively, in recent years, bacteriophage has

been used as a vector to deliver CRISPR-Cas9 lethal modification for sequence-specific killing of target bacterial cells. In this study, for phage-mediated CRISPR-Cas9 lethal modification, we constructed a phagemid carrying the CRISPR-Cas9 system with two gRNAs targeting both *stx1* and *stx2*. This constructed phagemid was packaged into the helper phage M13K07, and killing efficacy was evaluated in fresh rumen fluid environment. At MOI of 25 and 0.25, phage-mediated CRISPR-Cas9 lethal modification reduced STEC cell populations by 2.52 and 2.74 log CFU/reaction, respectively. These results indicated that phage-mediated CRISPR-Cas9 lethal modification could achieve sequence-specific killing of target pathogens in a complex microbial environment. Overall, our study provided proof-of-concept evidence that plasmid- and phage-mediated CRISPR-Cas9 lethal modification could be programmed to achieve sequence-specific removal of target pathogens by targeting virulence genes of choice, suggesting that CRISPR-Cas9 lethal modification could potentially be used to control foodborne pathogens in meat production and processing environment.

In the third study, we conducted three experiments to evaluate inhibitory and bactericidal effects of nine herb extracts against five strains of *Listeria monocytogenes in vitro*. In experiment 1, each of herb extracts 2, 4, 5, 8 exhibited inhibitory effects against five strains of *L. monocytogenes* individually at 37°C in Mueller-Hinton broth (MHB). The MIC values of those four herb extracts ranged between 5 to 50 mg/mL. In experiment 2, herb extract 4, which showed the lowest MIC value (5 mg/mL), reduced populations of five strains of *L. monocytogenes* by 0.38 to 0.91 log CFU/mL after 30 min treatment at 37°C in MHB, indicating that herb extract 4 may not be expected to be used as an antimicrobial agent for the purpose of reducing *L. monocytogenes* within a short period of time. In experiment 3, herb extracts 2, 4, 5, 8 at the concentration of 1.56 mg/mL and herb extracts 2, 4, 5 at the concentration of 0.78 mg/mL

individually inhibited the growth of a five-strain *L. monocytogenes* cocktail at 12°C. At a concentration of 3.13 mg/mL, those four herb extracts reduced cell populations by 2.2 to 1.6 mg/mL at 11 days. Herb extracts 2, 4, 5 and 8 could be potentially developed into food preservatives for controlling foodborne *L. monocytogenes*.

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Chapter One

Literature Review

Despite decades of research, strict regulations and enforcements worldwide, foodborne illness is still a global public health concern. The Center for Disease Control and Prevention (CDC) estimated annual number of illnesses (9.4 million), hospitalizations (55,961) and deaths (1,351) caused by 31 known foodborne pathogens in the United States (Scallan et al., 2011). In addition, United States Department of Agriculture (USDA) estimated the annual economic burden of illnesses that imposed over \$15.5 billion (Hoffmann, Macculloch, & Batz, 2015). The top five foodborne pathogens resulting in hospitalization included *Salmonella enterica* (35%), *Norovirus* (26%), *Campylobacter spp.* (15%), *Toxoplasma gondii* (8%) and Shiga-toxin producing *Escherichia coli* (STEC) O157 (4%), contributing 88% of total foodborne illnesses resulting in hospitalization (Scallan et al., 2011).

In late 1970, an outbreak of *Salmonella enteritidis* caused 2119 illnesses and 11 deaths in the United States (Louis et al., 1988). Unlike previous outbreaks which were caused by cracked or soiled eggs, this epidemic of Salmonellosis was associated with shell eggs. Researchers have recognized that healthy food animals and food products could be reservoirs of human pathogens. Agricultural practices and food processing operations were enhanced to improve food safety. In 1993, an outbreak of *E. coli* O157:H7 infection due to consumption of undercooked ground beef resulted in 501 illnesses, 151 hospitalizations, and three deaths (Griffin, 1998). In 1996, USDA Food Safety and Inspection Service (FSIS) established the Hazard Analysis Critical Control Point (HACCP) Final Rule (FSIS, 1996). The goal of this new HACCP program is to prevent, eliminate or reduce potential biological, physical, or chemical food safety hazards from meat products. The HACCP programs were implemented by the year of 1998 for large establishments

(500 or more employees), the year of 1999 for small establishments (20-500 employees) and the year of 2000 for very small establishments (less than 20 employees or less than \$2.5 million annual sales) (Boyle, 2006).

For decades, the meat industry faced emerging and evolving pathogenic microorganisms. To control foodborne pathogens in food products, meat industry implements multiple-hurdle interventions. One of multiple hurdle intervention is to apply antimicrobials to carcasses, such as lactic acid and peroxyacetic acid. However, it is acknowledged that use of antimicrobials could potentially lead to emergence of resistant pathogens. Therefore, there has been a great interest in developing alternative antimicrobials to overcome the current challenge of antimicrobial resistance. In this review, we will discuss two types of antimicrobials, including the plant-derived antimicrobial and the programmable and sequence-specific CRISPR-Cas9 lethal modification.

Since the 2010's, whole genome sequencing of bacterial pathogens has been widely used in both research laboratories and public health practices. Whole genome sequencing can be used to identify the pathogens, providing information about the specific pathogens that cause outbreak. With the valuable information about pathogens, scientists can develop potential interventions to reduce the outbreak pathogens from spreading. Furthermore, evolutionary history of pathogens can be revealed by comparing the genomes of the pathogenic isolates with their related non-pathogenic isolates. In this review, we will discuss the methods and tools for the comparative genomic analysis in pathogenic bacteria.

1.1. Plant-derived antimicrobial

In recent times, plant extracts have gained great interests in meat industry. The essential oils (EOs), which are obtained from aromatic and medicinal plant materials, are alternative natural antimicrobial agents given the strong antimicrobial activities (Bozin, et al., 2006; Mimica-Dukić, et al., 2003). In fact, plant extracts have been suggested to be used as antioxidant and preservatives in food products, to incorporate into food packaging materials, and as medicinal products for human and livestock (Turek & Stintzing, 2013). In this review, we will discuss the history of medicinal plants, the applications of plant-derived antimicrobials in meat production, and the mechanisms of plant extracts against pathogenic bacteria.

1.1.1. History of uses of medicinal plants

It is estimated that 250,000 to 500,000 species of plants are present on Earth, whereas only a relatively small percentage of these plants can be used for medicinal purposes (Bose, 1958). According to the World Health Organization, more than 80% of the world's population uses traditional herb medicine primarily for their healthcare needed (Organization, 2002). Use of herbal medicines is common throughout human history. In China, the Classic of Shen Nung's *Materia Medica* is said to be the first medicinal book that describes the properties and functions of 365 herbs during Xia dynasty more than 4,000 year ago (Lu & Lui, 1998). Hippocrates (460 BC – 370 BC), who is referred as the Father of Medicine, recognized 300 to 400 medicinal plants (Schultes, 1978) . Dioscorides (40 AD – 90 AD) wrote a five-volume book titled *De Materia Medica* ("On Medical Material"), which became the prototype of modern pharmacopeias (Ullmann, 2007). In addition, the Bible describes approximately 30 herbal plants

for medicinal uses (Cowan, 1999). Although more medicinal plants have been recognized with the development of human history, most documents of plant pharmaceuticals were lost or destroyed (Stockwell, 1988).

1.1.1.1. Plant-derived antimicrobial agents in China

The concepts relating to Traditional Chinese Medicine (TCM) has been well accepted in Asian and Western countries. The term TCM describes a number of disciplines of practices originated in China, including herbalism, food cures, acupuncture and manipulative therapy (Lu, 1991). This review will focus on three types of common TCM plants and their antimicrobial activities.

The genus *Angelica* comprises about 90 species which are widely distributed in Asia, Europe and North America. A total of 45 *Angelica* species are distributed in China, and 32 of those species are endemic (Feng et al., 2009). Those endemic species have been used in the TCM for many years. Traditionally in China, the *Angelica sinensis* has been used to treat various diseases, including gynecological diseases, apoplexia, constipation, malaria, chills, fever, and hemorrhoids (Sowndhararajan, et al., 2017). Previous studies reported that the EOs from *Angelica* species exhibited antibacterial and antifungal activities. For example, The EO of *Angelica glauca* inhibited the growth of bacteria *E. coli* and *S. aureus* with the MICs of 141.3 and 159.3 µg/mL, respectively, and inhibited the growth of fungi *Microsporum canis* with the MIC of 178.1 µg/mL (Irshad, et al., 2011). In addition, a traditional Chinese herb pair which is constituted of *Angelica sinensis* and *Sophora flavescens* (1:1 in dry powder weight) inhibited growth of the pathogenic *E. coli*, *S. aureus* and *Shigella* Castellani and Chalmers with the MIC

of 1.25 µg/mL. However, use of *Sophora flavescens* solely can only inhibit *E. coli* with the MIC of 5 µg/mL but not *S. aureus* and *Shigella* Castellani and Chalmers, and none of these three pathogenic bacteria can be inhibited by *Angelica sinensis* solely (Han & Guo, 2012).

Astragalus is an herb that is originated from Northern China. For many years, it has been used to strengthen the immune system, manage fatigue, and treat diarrhea and respiratory infections (Tan & Vanitha, 2004). A previous study reported that the extract of astragali root increased the proportion of the total B cells and activated B cells and could enhance macrophage activation in mice. However, the antibody production inducer, IL-6 was suppressed (Song, et al., 2000). These results suggested that *Astragalus* could potentially strengthen the human immune system. *Astragalus* also displayed antibacterial activities against multiple types of pathogens. The whole plant ethanolic extracts of *Astragalus angulosus* can inhibit the growth of *S. epidermidis*, *E. coli* and *P. aeruginosa* with the MICs of 12.78, 204.50 and 102.20 mg/mL respectively *in vitro*. In addition, flower water extracts of *Astragalus angulosus* can achieve 67.7% biofilm eradication at the concentration of 0.2 mg/mL (Kanaan et al., 2017).

In TCM, *Scutellaria* is often used to cleanse toxins in the body. Traditionally, it has been used to treat fevers, colds and vomiting of blood. Flavonoids are a group of the active compounds which can be extracted from *Scutellaria* roots with high concentrations (~35%) (Tan & Vanitha, 2004). Previous studies reported that flavonoids exhibited antibacterial properties. Apigenin and luteolin, which are the two types of flavonoids from *Scutellaria barbata*, can inhibit the growth of 15 methicillin-resistant *S. aureus* (MRSA) strains and 5 methicillin-sensitive *S. aureus* (MSSA) strains (MIC, 3.9–15.6 µg/ml for apigenin and 62.5–125 µg/ml for luteolin) (Sato et al., 2000). However, this study also indicated that apigenin and luteolin do not exhibited inhibitory effects against other 10 bacterial species. In addition, linalool, which is a

type of flavonoids from *Scutellaria albida*, can inhibit the growth of *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* (Skaltsa, et al., 2000). These results demonstrate that the inhibitory effects against bacteria species can be varied based on the species of *Scutellaria*.

1.1.1.2. Traditional Indian medicine

Ayurveda is a traditional medicine system in India since 1,500 BC. The traditional Indian doctors use nutrition, yoga, exercise, medicines and surgical techniques to treat and prevent diseases (Kayne & Kayne, 2010). Indian medicine has spread to the most Asian regions, including China, Indonesia and Japan (Kayne & Kayne, 2010). The Ayurvedic medicine is heavily dependent to the herbal medicine products. In fact, more than 3,000 types of Indian plants have been recognized for their medicinal value (Dubey, Kumar, & Tripathi, 2004). In this review, we will discuss the two typical Ayurvedic plants and their antimicrobial activities against pathogens.

Cassia occidentalis L. is an Ayurvedic plant which is used in traditional Indian medicine to cure various diseases, such as malaria and filarial disease. Extracts from *C. occidentalis* were found to exhibit antimicrobial effects against various bacterial species. For example, the ethanol extract of *C. occidentalis* flower was shown to inhibit *E. coli* (inhibition zone 8 mm) and *P. vulgaris* (inhibition zone 10 mm) (Yadav et al., 2010). In addition, the metabolite-rich fraction anthraquinones, the active chemical from *C. occidentalis*, showed stronger inhibition of *E. coli* (inhibition zone 22 mm), *P. vulgaris* (inhibition zone 20 mm) and *S. aureus* (inhibition zone 22 mm) (Jain, et al., 1998). In addition, the mechanisms of the antimicrobial nature of *C. occidentalis* was investigated. Extracts of *C. occidentalis* has been observed to contribute the

leakage of sodium and potassium ion from bacterial cells, resulting to cell deaths. On *P. aeruginosa*, water extract and ethanol extract resulted to release 2.66 ppm and 13.3 ppm of Na ions respectively, whereas water and ethanol extracts led to release 9.28 and 49.98 ppm of K ions respectively (Oladunmoye, Adetuyi, & Akinyosoye, 2007). Sodium and potassium ions are critical for osmotic balances of the cells. The leakage of Na and/or K can potentially lead to the cell lyses and cell death (Yadav et al., 2010).

The history of *Withania somnifera* comes from the Ayurvedic system and this plant is known as “Prince of Herbs” in Ayurveda for its medicinal properties (Ali, et al., 2017; Teli, et al, 2014). Traditionally, *W. somnifera* has been used to treat various diseases and disorders, including rheumatoid arthritis pain, asthma, antibacterial, anticancer and antipyretic (Khare, 2008; Kirtikar & Basu, 1980). A more recent study revealed that each of three types of methanol extracts from *W. somnifera* roots, fruits and leaves displayed antimicrobial effects against five bacterial species, including *E. coli*, *S. typhi*, *C. freundii*, *P. aeruginosa* and *K. pneumoniae* (Alam et al., 2012). The leaf extracts exhibit the most effective antimicrobial activities within three types of extracts. With 200 µL of 5 mg/mL, the leaf extracts display the highest inhibitory effect against *S. typhi* (inhibition zone, 32 mm) and the lowest inhibitory effect against *K. pneumoniae* (inhibition zone, 19 mm). In addition, with 100 µL of 2 mg/mL of methanolic leaf extracts, sizes of inhibition zones for MRSA and MSSA were 21 mm and 22 mm respectively (Bisht & Rawat, 2014). These findings suggested that extracts of *W. somnifera* possess significant antimicrobial activities against both Gram-negative and Gram-positive pathogens.

1.1.2. Applications of plant-derived antimicrobial agents in meat industry

Some of the most serious meat safety challenges are associated with foodborne pathogen contamination in meat products. To control foodborne pathogens in meat products, the meat industry uses multiple-hurdle interventions. Use of chemical for preservatives and antimicrobials, such as sodium acetate, sodium lactate and various nitrites, is one of multiple-hurdle interventions to minimize pathogens. However, it is acknowledged that uses of chemical compounds have increased the consumer concerns and created a demand for “natural” and “minimally processed” food. Plants have been used as flavoring material and preservatives since ancient times. Due to their antimicrobial and natural properties, the use of plant-derived antimicrobial agents has increased in popularity.

Plants are widely used in processed meat products as flavoring materials. Some plants and spices can also potentially serve as natural antimicrobial agents in meat products. For example, clover, cinnamon, pimento, origanum and thyme are common and flavorful spices that are widely used in processed meat products. Essential oils of each of these five spices also exhibited inhibitory effects against *L. monocytogenes* (Aureli, Costantini, & Zolea, 1992). However, the applications of plant antimicrobial compounds must target specific pathogens in a particular product since the sensitivity to different plant antimicrobial compounds varies in Gram-positive and Gram-negative bacteria. In general, Gram-positive were more sensitive to some essential oils and herb extracts than Gram-negative (Chorianopoulos et al., 2004; Mangena & Muyima, 1999; Ouattara, et al., 1997). However, the *Ocimum sanctum* extract was found to be equally effective against Gram-negative bacteria (*E. coli*, *S. typhimurium* and *P. aeruginosa*) and Gram-positive bacteria (*S. aureus*) (Mishra & Mishra, 2011). Furthermore, gram-negative pathogens, *V. parahaemolyticus* and *S. typhimurium*, were more sensitive to eugenol than Gram-

positive *S. aureus* (Karapinar & Aktuğ, 1987). In addition, environmental conditions, such as pH and temperature, must be verified for a particular food product when applying plant-derived antimicrobials in the food product (Hintz, Matthews, & Di, 2015).

In addition to add plant-derived antimicrobial compounds directly to the food products, in recent years novel technology incorporates plant-derived antimicrobials into packaging materials. Since the growth of microorganisms primarily occurs on the surface of a packaged meat product, this technology takes advantage of concentrating antimicrobial compounds on the surface of the meat product. Studies have successfully applied plant-derived antimicrobial compounds into packaging materials. For example, a previous study incorporated thymol or carvacrol into polymer films, in order to determine their antimicrobial effects against five species of bacteria, including *E. coli* O157:H7, *S. aureus*, *L. innocua*, *S. cerevisiae* and *A. niger* (Guarda, et al., 2011). Both thymol and carvacrol active packaging materials inhibited all five species of bacteria, with the MIC of 125-250 ppm and 75-375 ppm respectively. In addition, another study tested the antimicrobial activities of soy edible films incorporated with 5% thyme, oregano, or a mixture of 5% thyme and 5% oregano essential oils against *S. aureus* and *P. aeruginosa* *in vitro* and on fresh ground beef patties (Sung et al., 2013). The soy edible films incorporated with thyme, oregano, or the mixture exhibited inhibitory effects against *S. aureus*, but none of these active packaging materials could inhibit the growth of *P. aeruginosa*. On the other hand, thyme, oregano and the mixture showed inhibitory effects against *P. aeruginosa*. These findings suggest active packaging materials with plant-derived antimicrobial compounds may exhibited inhibitory effects against pathogens. Therefore, active packaging materials incorporated with plant-derived antimicrobials should be validated and used to control specific pathogens in a particular meat product.

Plant-derived antimicrobials are strictly controlled by legislation in different countries. In the U.S., all food additives require premarket approval by FDA unless the substance is generally recognized as safe (GRAS). Most plant-derived antimicrobials and essential oils are categorized as GRAS. Under Title 21 of the Code of Federal Regulations (CFR) 170.3 and 170.30, the general recognition of safety for a substrate is based on the historical use of this substrate in foods by significant number of consumers, and/or proved by adequately scientific procedures. The existing GRAS plants are published in the Title 21 Part 182 of the Code of Federal Regulations. On 2016, Food and Drug Administration (FDA) issued a GRAS Final Rule (81 FR 54959). An individual can submit a GRAS notification to FDA that a substance is GRAS and it is used in a particular food product under specific conditions. FDA evaluates whether the submitted GRAS notification has sufficient basis.

1.1.3. Mechanisms of plant extracts against pathogenic bacteria

The antimicrobial activities of plant extracts and EOs is dependent on their chemical composition and the quantities of each chemical components. In general, the hydrophobic EOs can penetrate the bacterial cell membrane and lead to the alterations of structure and functionality of cell membrane (Nazzaro, et al., 2013), resulting to increase the cell permeability and to leak cell constituents and ultimately lead to cell deaths (Faleiro, 2011). In this review, we will focus on discussing five mechanisms against pathogenic bacteria.

1.1.3.1. Disturbance of cell wall and cell membrane

From the studies of antimicrobial effects of oregano, thyme EOs, carvacrol and thymol, researchers recognized that the components of those plant extracts could result to the release of

lipopolysaccharides from Gram-negative bacteria, leading to the increases of cell membrane permeability and ATP loss, and ultimately inhibiting bacterial growth or causing cell deaths (Stephane et al, 2005; Lambert, et al., 2001; Nguefack, et al., 2004; Oussalah, et al., 2006). For example, a previous study investigated the cellular damages of *P. aeruginosa* and *Staph. aureus* caused by the *Origanum compactum* EO (Bouhdid, et al., 2009). The EO treatment at its MIC level led to the changes of cell membrane permeability of *P. aeruginosa*. On the other hand, for the *S. aureus*, the cell membrane permeability was not changed with the EO treatment at its MIC value. However, when the EO concentration increased to 1.5x of the MIC, the cell membrane permeability of *Staph. aureus* was reduced. The differences between the two bacteria are mainly due to the different composition of cell membrane and cell wall.

In addition to inhibit bacterial growth or cell death, plant extracts can possibly reduce or inhibit the production of toxins from the pathogenic bacteria. A previous study indicated that the use of carvacrol against *B. cereus* significantly reduced the production of diarrheal toxin compared with the control samples (Ultee & Smid, 2001). In addition, the use of oregano at 15 µL/mL completely inhibited the enterotoxin production of *S. aureus* (de Souza, et al., 2010). The inhibition and reduction of toxin production can be explained by the changes of cell membrane permeability. The treatments of those EOs reduced the permeability of cell membrane, resulting to the leakage of cytoplasm and structural modification of cells, and subsequently possibly lead to energy limitation and ultimately reduce or inhibit toxin production. Furthermore, the attachment of the EOs to the cells can potentially disturb phospholipid bilayer in cell membrane and consequently reduce the secretion of toxin to the environment or the hosts (Okubo, et al., 1989).

1.1.3.2. Reduction of ATP production

In the bacteria, ATP is produced at both in the cell wall and in the cytosol by glycolysis (Faleiro, 2011). As discussed above, plant extracts can disrupt cell membrane, resulting in leakage of cytoplasm and reduction of ATP production. A previous study showed that the mustard EO at the MIC value (0.2%, v/v) caused losses of intracellular ATP against both *E. coli* O157:H7 strain EDL933 and *Salmonella enterica* subsp *enterica* serovar Typhi (Turgis, et al., 2009). Other previous studies indicated that the decreases of intracellular ATP were observed in the oregano EO treatments against *L. monocytogenes* at the concentrations of 0.020% and 0.025% (w/v) (Caillet & Lacroix, 2006) and against *S. aureus* at the concentrations of 0.010% and 0.013% (w/v) (Caillet, et al., 2009). In addition to the leakage of cytoplasm, the disruption of cell wall and cell membrane can cause the loss of inorganic phosphate, limiting ATP production (Oussalah et al., 2006; Turgis et al., 2009). Collectively, the decrease of the intracellular ATP production can potentially cause energy limitation and lead to cell death.

1.1.3.3. pH reduction

The reductions of intracellular pH in bacterial cells have been observed when cells were exposed to plant extracts. A previous study showed that the intracellular pH of *E. coli* O157:H7 and *S. enterica* typhi reduced from 6.23 to 5.20 and from 6.59 to 5.44 respectively when the bacterial cells were exposed to the mustard EO at the concentrations of their MIC values (Turgis et al., 2009). Another study indicated that the Chinese cinnamon EO at the concentration of 0.025% (v/v) reduced the intracellular pH of *E. coli* O157:H7 from 7.25 ± 0.20 to 5.16 ± 0.05 ,

whereas the Spanish oregano EO at the concentration of 0.025% (v/v) decreased the intracellular pH of *E. coli* O157:H7 from 7.25 ± 0.20 to 6.68 ± 0.37 (Oussalah et al., 2006).

Plant extracts and EOs can disrupt the cell membrane, leading to the reduction of the membrane permeability. A subsequent release of intracellular protons can occur (Turgis et al., 2009). When the intracellular pH tends to decline, the cytoplasmic buffering capacity of protons will increase, tending to maintain the intracellular pH in the neutral range (Booth, 1985). Nonetheless, when the release of intracellular proton exceeds the cytoplasmic buffering capacity of proton, the intracellular pH starts to decrease. The maintenance of intracellular pH is important for many cellular processes, including DNA transcription, protein synthesis, and enzyme activity (Breeuwer, et al., 1996). Exposing plant extracts and EOs to bacterial cells contribute to impair pH homeostasis and reduce intracellular pH, resulting to disturb the vital cellular functions.

1.1.3.4. Reduction of the percentage of unsaturated fatty acids

Due to the hydrophobic nature, plant extracts can alter the percentage of unsaturated fatty acid and the structures of fatty acids in bacteria (Burt & Reinders, 2003). Two previous studies showed that the treatments with EO compounds at the concentrations lower than their MIC values could decrease the percentages of total unsaturated fatty acids of the cell membrane (Di Pasqua et al., 2007; eath & Rock, 2004). The decreased proportion of unsaturated fatty acids leads to the increases of proportion of saturated fatty acid in the cell membrane lipid bilayer, resulting in a loss of membrane fluidity and a consequent disruption of cell membrane. As

discussed above, the disruption of cell membrane can result to the leakage of intracellular constitutions, energy limitations and pH decline, ultimately causing cell death.

1.1.3.5. Inhibition of quorum sensing

Quorum sensing (QS) is a bacterial process of cell-to-cell communication that regulate the gene expression in response to the cell population density. The QS is involved in a number of bacterial activities, including virulence factor expression, biofilm formation, mating, motility and stress resistance (Bassler, Greenberg, & Stevens, 1997; Kjelleberg & Molin, 2002; Nazzaro et al., 2013). Plant extracts have been reported to show anti-QS activity in previous studies (Bassler et al., 1997; Daniels, et al., 2004; Niu, et al., 2006; Khan, et al., 2009). At the concentration of 0.2%, 0.4%, 0.8% and 1.6% (v/v), the clove EO reduced the swarming motility of 40%, 56%, 68% and 78% respectively in *Pseudomonas aeruginosa* strain PAO1 (Khan, et al., 2009). The swarming motility is involved in biofilm formation and cell translocation which are regulated by the QS in strain PAO1 (Daniels, et al., 2004). Hence, the clove EO was expected to inhibit the QS activity in strain PAO1. In addition, at the concentrations of 60 μ M and 100 μ M, cinnamaldehyde reduces 55% bioluminescence in *V. harveyi* strain BB886 and 60% bioluminescence in *V. harveyi* strain BB170 (Niu, et al., 2006). The gene expression relevant to the bacterial bioluminescence is dependent on the QS activity (Bassler et al., 1997). Therefore, cinnamaldehyde can possibly inhibit the QS activity in *V. harveyi*.

1.2. The CRISPR-Cas lethal modification

In recent years, novel programmable and sequence-specific tools, called the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system, were developed as a genome editing tool by cleaving the target DNA sequences. However, studies found that the cleavage of DNA sequences without repairing in bacteria cells is lethal (Gomaa et al., 2014; Bikard & Barrangou, 2017). In this review, we will focus on the CRISPR-Cas lethal modification that target and kill specific bacteria of choice, especially the Type II CRISPR-Cas9.

1.2.1. History of CRISPR-Cas systems

In 1987, the unusual repeat sequences separated by non-repeating sequences in a palindromic pattern was firstly reported in *E. coli* (Ishino, et al., 1987). In 2002, the acronym CRISPR was proposed to describe the interrupted repeat sequences. Later, the CRISPRs were found in more than 40% of bacteria and 90% of archaea (Mojica, et al., 2005).

The function of the CRISPR remained unknown until the significant discovery in 2002. A group of genes adjacent to the CRISPR locus was found, and those genes were named as the CRISPR-associated system, or Cas (Jansen, et al., 2002). Later in 2005, studies reported that the non-repeating CRISPR spacer sequences were derived from the bacteriophage DNA, indicating that the CRISPR could possibly associate to the adaptive immune system in prokaryotes (Bolotin, et al., 2005; Mojica et al., 2005; Pourcel, Salvignol, & Vergnaud, 2005). In 2007, this hypothesis was proven (Barrangou et al., 2007). Next, the CRISPR was revealed to associate the genomic memory of other invading foreign DNA such as plasmid and DNA fragments, and Cas

proteins served as guided endonucleases to which scan and cleave the invading foreign DNA (Brouns et al., 2008). More recently, the CRISPR-Cas systems were classified into six types based on the types of *cas* genes (Makarova et al., 2015).

1.2.2. Biological mechanism of the Type II CRISPR-Cas9 system

While Type I, III, IV, V and VI CRISPR systems require multiple varied Cas proteins, the Type II CRISPR-Cas9 system only require one Cas9 protein to scan, bind and cleave the target DNA sequence (Makarova et al., 2011). Figure 1 shows the details of Type II CRISPR-Cas9 system. The Type II CRISPR-Cas9 system consists of three components: the trans-activating RNA (tracrRNA) sequence, the Cas genes, and the CRISPR repeat and spacer sequences, and these three components are subsequently transcribed or translated into tracrRNA, Cas9 protein and pre-crRNA respectively (Chylinski, et al., 2014). During the post-transcriptional processing, the RNase III cleaves the pre-crRNA at the repeat sequences and processes the pre-crRNA into crRNA. The crRNA, tracrRNA and Cas9 protein form a crRNA-guided complex which can target the specific DNA sequence complementary to the crRNA sequence (Jiang & Doudna, 2017). The crRNA:tracrRNA:Cas9 complex scans the DNA in the cell and search the protospacer adjacent motif (PAM). The PAM is a short DNA sequence which is adjacent to the target DNA sequence. When the PAM is recognized, the crRNA:tracrRNA:Cas9 complex unwinds the double stranded DNA and scan the 10-12 nucleotides following the PAM (Szczelkun et al., 2014). If the target DNA sequence matches the complementary crRNA sequence, the Cas9 protein cleaves the target DNA leading to a double stranded break (DSB) (Jinek et al., 2012).

The Type II CRISPR-Cas9 system is an adaptive immune system that is discovered in prokaryotic cells. However, it can also be programmed to target specific sequences for the purposes of gene editing. In 2012, the first report showed that the CRISPR-Cas9 system was programmed to introduce the DSBs in target DNA sequences (Jinek et al., 2012). In this report, they designed a single RNA molecule, called guide RNA (gRNA), which combined crRNA and tracrRNA. Experimental results indicated that the gRNA could guide the Cas9 protein to the specific DNA sequence. Based on this study, the CRISPR-Cas9 system was simplified into only two components: gRNA and Cas9 protein.

1.2.3. Use of Type II CRISPR-Cas9 as sequence-specific lethal modification

The irreparable chromosomal lesions caused by the Cas9 endonuclease is lethal, most likely due to the introduction of double stranded DNA break on the target gene (Gomaa et al., 2014; Jiang, et al., 2013). This observation reveals the possibility of using the CRISPR-Cas9 system as the sequence-specific lethal modification, in order to eliminate one or more target bacterial species within a complex bacterial population.

The main challenge of using CRISPR-Cas9 lethal modification is the delivery to the target bacterial population. As discussed above, the CRISPR-Cas9 system consists of two components, including gRNA and Cas9 protein. However, delivery of RNA and protein to the bacterial cells can be very difficult. Instead, *cas9* gene and the DNA sequences transcribing gRNA could be delivered to target bacterial cells by phagemid, using bacteriophages as vectors. For example, the phage Φ NM1 was used to deliver a constructed phagemid containing *cas9* gene and its gRNA, in order to target the kanamycin resistant gene and eliminate pathogenic *S.*

aureus. (Bikard et al., 2014). Another study used the phage M13 to deliver the reprogrammed CRISPR-Cas9 lethal modification targeting three antibiotic resistant genes (*bla_{NDM-1}*, *bla_{SHV-18}*, and *gyr_{AD87G}*) to the antibiotic resistance *E. coli* strains. The Cas9 endonuclease, which was guided by gRNA, cleaved the antibiotic resistant genes, resulting to the *E. coli* cell death (Citorik, Mimee, & Lu, 2014).

Phages have been proved to be a type of vector for the delivery of CRISPR-Cas9 lethal modification as described above. However, efficacy of phage infections to the target bacteria are questioned. The host range of most phage species are limited, indicating that delivering the CRISPR-Cas9 lethal modification to multiple bacterial species can be a challenge (Pursey, et al., 2018). In addition, in pre-harvest environment of meat production, the use of only one type of phage could cause the developing of phage-resistant foodborne pathogens (Bach, et al., 2003). According to Bach et al. (2003), host bacterial cells carrying prophages in lysogenic state resisted the infection by the second same or similar types of phages. Delivering the programmed CRISPR-Cas9 system into bacterial cells relies on the phage infection. Therefore, the efficacy of delivering CRISPR-Cas9 antimicrobial to the target cells could be limited when target bacteria resist to the phage infection (Pursey, et al., 2018). To overcome these challenges, multiple phages carrying the reprogrammed CRISPR-Cas9 lethal modification can be combined into a phage cocktail, in order to expand its host range to target more pathogen species as well as reduce the chance of developing phage-resistant pathogens.

Another challenge is the evolution of resistance to the CRISPR-Cas9 antimicrobials in bacteria. As discussed, the CRISPR-Cas9 antimicrobials can lead to sequence-specific killing of bacteria by targeting the genes of interests. However, point mutations in the sequences of targeted genes allow bacteria escape the recognition of the gRNA-Cas9 complex (van Houte et

al., 2016). In addition, resistance to the CRISPR-Cas9 antimicrobials can be associated to the inactivation of the CRISPR-Cas9 loci. Previous studies indicated that mutations or deletions of *cas* genes and deletions of spacer sequences could occur during phage delivering, leading to the inactivation of the CRISPR-Cas9 antimicrobial (Bikard, et al., 2012; Jiang, et al., 2013). Furthermore, resistance to the CRISPR-Cas9 antimicrobials can also be related to the anti-CRISPR (*acr*) genes. The *acr* genes encode proteins that inactivate the CRISPR-Cas9 system. Three ACR proteins, AcrIIC1, AcrIIC2 and AcrIIC3, are the first discovered inhibitor proteins that inactivate the Type II-C CRISPR-Cas9 activity in *Neisseria meningitidis* (Pawluk et al., 2016). Later, four ACR proteins, AcrIIA1, AcrIIA2, AcrIIA3 and AcrIIA4, were found to inhibit the Type II-A CRISPR-Cas9 activity in *Listeria monocytogenes* (Rauch et al., 2017). To overcome the evolved resistance to the CRISPR-Cas9 antimicrobials in bacteria, the gRNA can be designed to target various sequences from multiple genes, in order to avoid point mutations in the sequences of targeted genes (Bikard & Barrangou, 2017). In addition, for the resistance caused by the ACR proteins, since inactivation of different subtypes of the CRISPR-Cas9 system requires matched ARC proteins, using multiple subtypes of CRISPR-Cas9 variants simultaneously can potentially reduce the chance of the ARC proteins inhibiting CRISPR-Cas9 antimicrobials (Purse et al., 2018). Overall, the CRISPR-Cas9 antimicrobial offers an excellent potential alternative to antibiotics. However, currently it is expensive for large-scale production, and its use in the food industry remains a question.

1.3. Comparative genome analysis in pathogenic bacteria

With the development of the next-generation sequencing technology, high throughput sequencing of bacterial genomes is fast and cost-effective. With whole genomic sequencing data,

comparative genomic analysis can be used to investigate the topics relating to bacterial phylogeny and evolution, such as outbreak investigation (Snitkin et al., 2012), and the evolution of antimicrobial resistance (AMR) genes (Holt et al., 2012). In this review, we will discuss the methods and available tools for comparative genome analysis of bacteria.

1.3.1. Sequence alignment

DNA sequence alignment is usually the first step to understand the genetic relationship among bacteria for comparative genome analysis. For most researchers, it is important to visualize these DNA sequence alignments. In addition, the aligned sequences can be used for downstream comparative genomic analysis. In this review, we will discuss three free software tools: BLASTn (Basic Local Alignment Search Tool nucleotide), MEGA-X, and BRIG (BLAST Ring Image Generator).

In 1990, the original version of BLAST was released (Altschul, et al., 1990). Later, the original BLAST program was upgraded and extended to a family of programs, which can be used depending on the types of sequences (e.g. DNA, RNA or proteins). For example, BLASTn (nucleotide BLAST) can estimate the sequence similarity and coverage by searching a query sequence against a target sequence. In addition, BLASTn allows users search query sequences against the National Center for Biotechnology Information (NCBI) database, in order to find the most similar sequences in the database. The BLAST programs can be used either on local computer or can be accessed on the web server hosted by the NCBI.

MEGA-X is a free software that integrates various bioinformatic tools for comparative genome analysis (Kumar, et al., 2018). An advantage of the MEGA-X is that it can perform

multiple sequence alignments of whole genomes by two programs, ClustalW and MUSCLE, with FASTA format sequences. The alignments from multiple sequences can be further used to identify single nucleotide polymorphisms (SNP), which is suitable for downstream phylogenetic analysis.

BRIG is a user-friendly tool that generate comparative genomic image by comparing multiple sequences against one reference sequence (Alikhan, et al., 2011). Prior to use this program, query and reference sequences need to be converted into FASTA format. Subsequently, sequence alignments were performed between the reference sequence and each of query sequences by BLASTn which is incorporated in BRIG. The output image shows similarity between the reference sequence and each of query sequences as a set of concentric rings. BRIG can display the similarity of sequences in multiple genomes simultaneously, and the degree of similarities are shown in different colors. Therefore, it can be useful to compare the presence, absence or variation of genes, prophages and transposable elements in multiple genomes against the reference genome.

1.3.2. Identification of genes of interests and CRISPR locus

Comparative genomic analysis allows researchers determine the presence, absence, and mutation of genes of interests between bacterial genomes (e.g. AMR genes and virulence genes). One typical method is to manually create a set of gene sequences and aligned against each of bacterial genomes. NCBI operates a public genomic database that provides most of gene sequences that are freely accessible. However, manually aligning each gene against the bacterial

genomic sequences are time consuming. In this review, we outline some specialist tools to address this question.

The ResFinder is a free available online database which allows users to identify AMR genes from the submitted sequences (Zankari et al., 2012). The alignments of gene sequences against bacterial genomes are performed by BLAST, and the output shows the resistance genes which are found in the submitted sequences. Alternative, NCBI developed an up-to-date tool, called AMRFinder, which identifies AMR genes using either protein annotation or nucleotide sequences (Feldgarden et al., 2019). Recently, the AMRFinder has been updated to an online-based version called AMRFinderPlus. The AMRFinderPlus is used in the Pathogen Detection pipeline, and the identified AMR genes are displayed in NCBI's Isolate Browser. This allows users access the AMR genes of all bacterial genomes in the NCBI database. In addition, a local version of AMRFinderPlus is available on Linux platform.

By comparing the virulence genes between bacterial genomes, researchers can analyze the evolutionary history of pathogenesis. VirulenceFinder is an online-based tool to identify virulence genes in nucleotide sequences. The database contains only known virulence genes and did not include either housekeeping genes or hypothetical protein genes. Therefore, VirulenceFinder optimizes the examination of only virulence genes. However, VirulenceFinder is limited to identify the virulence genes within four species, including *Listeria*, *S. aureus*, *E. coli* and *Enterococcus*. Alternatively, the virulence factor database (VFDB) is an online up-to-date virulence gene database since 2004 (Liu, et al., 2019). Currently the VFDB includes the virulence genes from 32 genus. In addition, a recent updated has integrated VFAnalyzer, which automatically and systematically identifies potential virulence genes in complete and draft bacterial genomes, into the VFDB.

CRISPR-Cas system in bacteria provides acquired immunity by creating double-stranded breaks against foreign DNA such as bacteriophages, DNA fragment and plasmid. Comparing the CRISPR-Cas locus can potentially reveal the phylogenomic relationship between bacteria isolates. The CRISPR-Cas++ is an online-based toolbox that incorporates five programs for the identification of CRISPR locus (Couvin et al., 2018). CRISPRCasFinder identifies direct repeats (DR) and spacer sequences, and *cas* genes in whole genome sequences, while CRISPRCasMeta identifies the CRISPR locus in the metagenomes. The outputs can be further compared with the NCBI database using CRISPRCasdb-BLAST. In addition, users can browse the CRISPR locus in the CRISPRCasdb and view the taxonomy tree in the CRISPRCasdb-Taxo.

1.3.3. Phylogenetic analysis

Phylogenetic analysis is used to investigate the phylogenetic relationships of genetically related group of bacteria by comparison of protein sequences, gene sequences, and genomic sequences. Tettelin et al., 2005 purposed the concept of pan-genome that consists of core genome and accessory genome. The core genome represents the genes that present in all strains of a species, such as housekeeping genes. The accessory genome refers to the genes that present in two or more strains but not in all strains of a species, such as the AMR genes, virulence genes and other genes that can be acquired in specific environments. The output of pan-genome analysis can be used for SNP identification and constructing phylogenetic tree.

PanSeq is an online tool that determines both core and accessory genome among a collection of sequences submitted by the users (Laing et al., 2010). The output includes the SNPs within shared core genome and the SNPs for the presence and/or absence of accessory genome.

In addition, PanSeq includes a Loci Selector tool and a Novel Region Finder. The Loci Selector can determine the most variable and discriminatory loci among the SNPs of pan-genome. The Novel Region Finder can discover the genomic regions that is unique to a sequence or a group of sequences.

Although PanSeq can determine the pan-genome and the SNPs within pan-genome, it cannot perform downstream analysis. Alternatively, PGAweb is a web-based tool that provides a systemically phylogenetic analysis (Chen et al., 2018). PGAweb is composed of two module, PGAP and PGAP-X. More specifically, PGAP performs five comparative genomic analysis, including orthologous clustering, pan-genome profiling, sequence variation analysis, species phylogeny, and gene functional classification. PGAG-X supports four analytic functions: genome-wide sequence alignment, orthologous clustering, pan-genome profiling, and sequence variation analysis. In other word, both two modules can perform pan-genome analysis and SNP identification. PGAG generates phylogenetic tree based on the SNPs in core gene clusters, while PGAG-X visualizes the genomic sequence alignments and orthologous gene distribution that represents the degrees of conservation for each gene.

Both PanSeq and PGAweb need complete reference genomes for SNP identification. One limitation of these two tools is that they require complete or nearly complete draft genomes, indicating that these two tools cannot apply to the whole genome data with raw reads or contig sequences. The kSNP incorporates multiple analytical programs for phylogenetic analysis without the requirement of reference genomes on Linux platform (Gardner, Slezak, & Hall, 2015). Before SNP identification, the optimum k-mer is determined by Kchooser. In kSNP, the k-mer is defined as the length of the flanking sequence including the SNP at the central base. Subsequently, kSNP automatically performs SNP identification and SNP annotation. The newest

version kSNP3.1 supports to identify SNPs that were within chromosomes, plasmids or other replicons. The output SNP matrix can be used to generate phylogenetic tree by kSNP or other programs, such as MEGA-X (Kumar et al., 2018) and RAxML (Stamatakis, 2014).

1.3.4. Predictions of genomic islands and prophages

Mobile genetic elements (MGEs) are a heterogeneous group of molecules that can move around within a genome, or that can be transferred between bacterial cells. MGEs includes plasmids, bacteriophages, genomic islands (GI), chromosomal cassettes, and integrative and conjugative elements (Juhas et al., 2009; Malachowa & DeLeo, 2010; Smyth & Robinson, 2009). Among these MGEs, GIs and bacteriophages are important in the evolution of bacteria, influencing pathogenicity, AMR, symbiosis and fitness, and adaptation in bacteria (Brüssow, et al., 2004; Dobrindt, et al., 2004). In this review, we will focus on discussing the tools and methods to predict GIs and prophages in bacterial genomes.

The GI are originally discovered as the pathogenicity island (PAI), a type of MGE that harbored virulence genes and contributed to the evolution of pathogens (Neuhard, 1987). Later, DNA fragments that are similar with PAIs have been found in non-pathogenic bacteria (Dobrindt et al., 2004). These DNA fragments are defined as GIs that contribute to the evolution of both pathogenic and non-pathogenic bacteria through horizontal gene transfer. GIs are large DNA fragments ranging from 10 kb to 200 kb that are often integrated into tRNA clusters (Juhas et al., 2009) and can differ from the rest of the chromosome in terms of GC content and codon usages (Everitt et al., 2014; Juhas et al., 2009). Based on these features, bioinformatic researchers can

identify GIs and compare genes of interests harbored in the GIs with whole genome sequencing data.

IslandViewer is a web-based tool to predict and visualize GIs in bacterial genomes (Bertelli et al., 2017). Users can simply submit the sequences in Genbank format to the webserver to predict GIs. The updated version, IslandViewer 4, can predict GIs from three methods, including IslandPick, SIGI-HMM and IslandPath-DIMOB. The output shows the predicted GIs and the pathogen-associated genes within the predicted GIs, such as virulence genes, AMR genes and adhesion genes.

Bacteriophage is another type of MGE that primarily contribute the evolution of bacteria (Brüssow et al., 2004; Sváb, et al., 2015; Tozzoli et al., 2014). Bacteria acquires new genes by transduction, transposition, transformation, and lysogenization of prophages (Brüssow et al., 2004). By comparing the genes harbored in prophages within a given set of bacterial isolates, researcher can reveal the evolutionary relationship between bacterial isolates. For example, Ferdous et al., 2015 analyzed the *stx* phage insertion sites in the *stx*-negative *E. coli* O157:H7 and STEC O157:H7 from human patients. The result showed that the *wrbA* (insertion site for the typical *stx2a*-carrying prophage) and *sbcB* (insertion site for the typical *stx2c*-carrying prophage) were unoccupied by prophages in the *stx*-negative *E. coli* O157:H7 strains. Such unoccupied *stx* insertion sites indicates that typical *E. coli* O157:H7 can potentially lose *stx* genes due to loss of *stx*-carrying prophages. Conversely, *stx*-negative *E. coli* can also possibly gain *stx* genes by acquiring one or more *stx*-carrying prophages (Mauro & Koudelka, 2011).

Phage Search Tool Enhanced Release (PHASTER) is a web server for rapid prophage identification and annotation within bacterial genomes (Arndt et al., 2016). Users can simply

submit raw sequences in FASTA format or annotated sequences in GenBank format to the server. The identified prophages were evaluated for their completeness (intact, questionable or incomplete prophage) based on the completeness score. The output shows an interactive graph and a table that summarize the locations of identified prophages and the genes harbored in those identified prophages. In addition, PHASTER provides a pre-calculated genome database that lists all the genomes from NCBI that have analyzed with PHASTER.

1.3.5. Identification of orthologous genes and generation of biological pathways

Current technologies allow researchers extract meaningful information from biological experiments. Such information can be practical under specific conditions, but it may not necessarily represent biological knowledge in general. For example, iron uptake-associated genes can be identified by a genome-wide association study, but the entire iron uptake mechanism involving a complex signaling pathway may not be uncovered by analyzing one or more individual iron uptake-associated genes. In comparative genomic analysis, researchers can conduct pathway analysis approaches that compare genes and gene products between bacterial isolates, in order to predict the phenotypes associated with specific pathways. In this review, we will focus on discussing two databases associated with functional analysis, COG (Clusters of Orthologous Groups of proteins) and KEGG (Kyoto Encyclopedia of Genes and Genomes). In addition, we will introduce an online-based system for the analysis of biological pathway genes, Integrated Microbial Genomes & Microbiomes (IMG/M).

The COG database was first created in 1997 by NCBI (Tatusov, Koonin, & Lipman, 1997) and it is keep growing over the years (Galperin, et al., 2015). Over 20 years, the COG

database has been a popular tool for microbial genome annotation and comparative genomic analysis due to several significant features (Galperin, et al., 2019; Galperin et al., 2015). First, each COG represents a family of curated genes, which allows relatively reliable recognition of potential orthologs (genes are from a common ancestor and encode proteins with the same functions in different species) and paralogs (genes are evolved by duplication and encode proteins with similar but not identical functions) in the bacterial genomes. Second, the COG approaches can separate closely related paralogs in different COGs, while other databases may assign these paralogs to the same gene family. Third, COG approaches can be used to evaluate genome quality. For example, NCBI's prokaryotic genome annotation pipeline use the COG approaches to check presence of certain universal genes in submitted genomes, such as the genes encoding ribosomal proteins, translation system components and RNA polymerase subunits (Tatusova et al., 2016). The submitted genomic sequences may have low quality if they are missing the universal genes that should present in a specific species, and these low-quality genomes may be rejected by NCBI database. Fourth, the COG system assigns all COGs in prokaryotic cells to one of 26 functional categories. Four of them (B, Y, W, Z) are also found primarily in eukaryotic cells, allowing compare the COGs between prokaryotic and eukaryotic cells. Last, COG approaches annotate genes in given genomes by assigning COG names, which can be used for downstream comparative genomic analysis.

Although COG database is an important role for microbial genomic analysis due to significant features, it has not been updated since 2014. In addition, it does not provide pathway maps consisting of all genes and gene products associated with specific pathways. Alternatively, KEGG is established as a reference pathway database by organizing experimental biological knowledge from published literatures. KEGG database was established in 1995 (Ogata, et al.,

1998), and it has been expanded significantly over the years to support bioinformatics. Currently KEGG contains 18 up-to-date main databases that are characterized into four categories: (1) system information (PATHWAY, BRITE and MODULE); (2) genomic information (ORTHOLOGY, GENOME, GENES and SSDB); (3) chemical information databases (COMPOUND, GLYCAN, REACTION, RCLASS and ENZYME); and (4) health information databases (NETWORK, VARIANT, DISEASE, DRUG, DGROUP and ENVIRON) (Kanehisa, et al., 2019).

KEGG PATHWAY provides pathway maps representing molecular interaction/reaction network diagrams. A basic pathway map includes boxes (KO identifier or K number representing key genes or gene products), circles (other molecules such as chemical compounds) and lines (reactions). To generate pathways, gene sequences need to be annotated by assigning KO identifiers (K numbers) using BlastKOALA (Kanehisa, Sato, & Morishima, 2016). The annotated sequences can be used to reconstruct pathways by KEGG Mapper. Both BlastKOALA and KEGG Mapper are available online on KEGG database website.

KEGG approaches provide through information for pathway analysis, but it does not directly support comparative genomic analysis between bacterial isolates. Here, we introduce the online-based tool IMG/M system for functional analysis (Chen et al., 2018). IMG/G automatically updates genome sequence data from NCBI GenBank. Users can also submit annotated sequences in GenBank format or unannotated sequences in FASTA format through IMG submission system. The validated genome sequences are deposited into the Genomes Online Database. Subsequently, orthologous genes are assigned to protein family based on five functional terms, including, KO (Kanehisa et al., 2019), COG (Galperin et al., 2019), Pfam (Finn et al., 2016), InterPro (Jones et al., 2014) and TIGRfam (Haft et al., 2012). Next, proteomes in

genome sequences are associated with two biological pathway databases, including KEGG (Kanehisa et al., 2019) and MetaCyc (Caspi et al., 2020). IMG/M analyzes multiple microbial genomes in three dimensions: genomes, genes and functional annotations (pathways and terms). A comparative genomic analysis study can start with multiple sequence alignments between different but phylogenetically related genomes, in order to find similar and unique genes associated with biological functions. Next, these genes can be mapped to biological pathways, and subsequently predicted phenotypes.

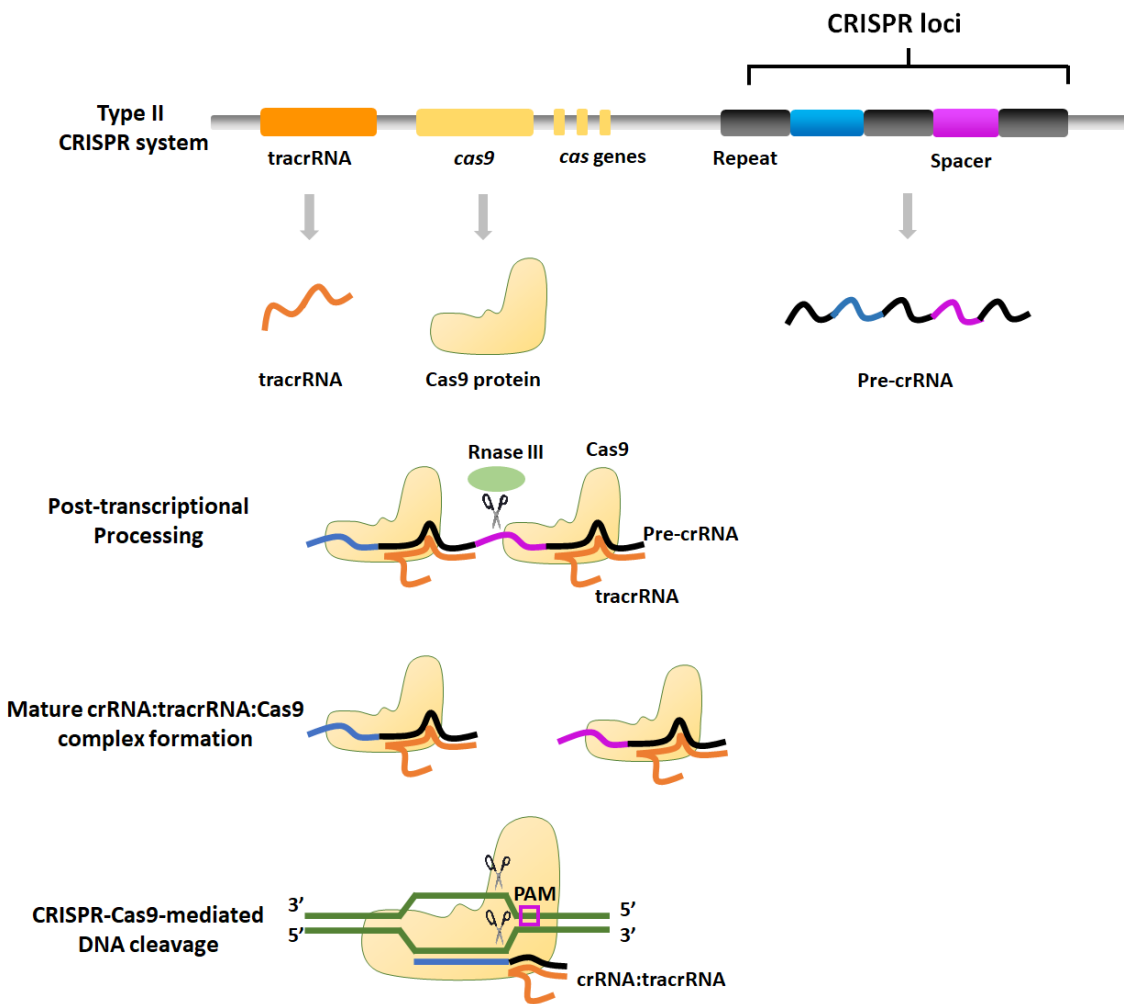


Figure 1.1. Details of the Type II CRISPR-Cas9 system along with the RNA and protein products.

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Chapter Two

Comparative Whole Genome Analysis of *Escherichia coli* O157:H7 Isolates from Feedlot Cattle to Identify Genotypes Associated with Presence and Absence of *stx* Genes

Summary

A comparative whole genome analysis was performed on three newly sequenced *Escherichia coli* O157:H7 strains with different *stx* profiles, previously isolated from feedlot cattle (C1-010 [*stxI*-, *stx2c*], C1-057 [*stx*-] and C1-067 [*stxI*+, *stx2a*+]), as well as five foodborne outbreak strains and six *stx*-negative strains from NCBI. Phylogenomic analysis showed that strains C1-010 and C1-057 were grouped with the other six *stx*-negative strains, while strain C1-067 was grouped with the five foodborne outbreak strains. Furthermore, the *stx*-carrying and *stx*-negative prophages in *stx* insertion sites were identified in ten selected *E. coli* O157:H7 strains. All *stx*-carrying prophages contained both the three Red recombination genes (*exo*, *bet*, *gam*) and their repressor *cI*. On the other hand, the majority of the *stx*-negative prophages at *stx* insertion sites carried only the three Red recombination genes but their repressor *cI* was absent. In the absence of the repressor *cI*, the consistent expression of the Red recombination genes in prophages might result in more frequent gene exchanges. As the result, *stx*-negative *E. coli* O157:H7 could potentially acquire *stx* genes. Furthermore, comparative pathway analysis showed that two *stx*-positive (*stxI*+, *stx2a*+) *E. coli* O157:H7 strains contained eight copies of unique genes involved in metabolic pathways, while seven *stx*-negative and one *stx*-positive (*stxI*-, *stx2c*+) *E. coli* O157:H7 strains carried one copy of unique metabolic pathway gene. Functions of these nine copies of genes are associated with glycerol metabolism,

increase of survival and improvement in iron uptake ability. Seven of the eight copies of metabolic pathway genes presented only in *stx*-positive (*stx1+*, *stx2a+*) *E. coli* O157:H7 strains were found to be located at the downstream of an insertion sequence (IS) 629. Gene arrangements around this specific IS629 were compared between 10 *stx*-positive and -negative *E. coli* O157:H7 strains and their ancestor *E. coli* O55:H7. The O55:H7 strain RM12579 carried eight copies of metabolic pathway genes (*modD*, *fepC*, *fecD*, *fbpA*, *dhaM*, *dhaL*, *dhaK*, and *dhaR*). Compared with O55:H7, two *stx*-positive (*stx1+*, *stx2a+*) O157:H7 strains carried seven metabolic pathway genes and an additional IS629 but lost *dhaR*. All seven *stx*-negative O157:H7 strains and one *stx*-positive (*stx1-*, *stx2c+*) strain remained the *dhaR* and harbored an additional IS629, but it lost seven pathway genes. Presence of these metabolic pathway genes in *stx*-positive *E. coli* O157:H7 may increase competitiveness in complex environment such as cattle feedlot, compared with *stx*-negative *E. coli* O157:H7 which did not contain these metabolic pathway genes. This analysis may explain the facts that *stx*-positive *E. coli* O157:H7 is more prevalent than *stx*-negative *E. coli* O157:H7 in cattle. Additionally, due to the results of phylogenomic analysis and gene arrangements around the specific IS629, we suggested that *stx*-positive (*stx1+*, *stx2a+*) *E. coli* O157:H7 may be evolved from O55:H7 by different evolutionary pathways, compared with *stx*-positive (*stx1-*, *stx2c+*) and *stx*-negative *E. coli* O157:H7 strains. The findings of this study may help with the development of control strategies to reduce pathogenic *E. coli* O157:H7 in food production.

Introduction

Foodborne pathogenic *Escherichia coli* O157:H7 causes more than 96,000 cases of diarrheal illness and 3,200 hospitalizations annually in the United States (Scallan et al., 2011). This pathogen can cause severe gastrointestinal illness, including bloody diarrhea, which may progress to more serious illness such as hemolytic uremic syndrome (HUS) and even death (Karmali, 2004). The *eae* (encoding intimin) and *stx* (encoding Shiga toxin) harbored in foodborne pathogenic *E. coli* O157:H7 strains are central to the pathogenesis of HUS (Paton & Paton, 1998). In addition to causing HUS, Shiga toxin produced by *E. coli* O157:H7 can enhance the adherence to epithelial cells and colonization in mice intestines (Robinson et al., 2006). Compared to Shiga toxin-producing *E. coli* O157:H7 that cause severe illness, *stx*-negative *E. coli* O157:H7 strains do not produce Shiga toxin. Though they may cause symptoms like diarrhea, they are not generally associated with HUS, even though they still carry virulence factors such as *eae* and *bfpA* genes (Black et al., 2010; Ferdous et al., 2015; Ochoa & Contreras, 2011).

For decades, conventional molecular methods such as polymerase chain reaction (PCR) and Sanger sequencing have provided valuable information on the potential mechanisms of acquisition or loss of the *stx* genes in the chromosome of *E. coli* O157:H7 cells. A previous PCR-based study found that *stx*-negative *E. coli* O157:H7 could acquire the *stx* genes in food animals (bovine and avian) and in feedlot environments by lysogenization of *stx*-carrying prophages into the *stx* insertion sites in bacterial genomes (Wetzel & LeJeune, 2007). Additionally, in humans, *stx*-positive *E. coli* O157:H7 may lose their *stx*-carrying prophages and, as a result, be converted into *stx*-negative *E. coli* O157:H7 during the infection process (Bielaszewska et al., 2007; Ferdous et al., 2015; Käppeli et al., 2011).

In addition to the *stx* genes, studies have also been conducted to compare antimicrobial resistance of *stx*-positive and *stx*-negative *E. coli* O157:H7 isolates. A recent study compared antibiotic resistance genes in 19 *stx*-positive and eight *stx*-negative *E. coli* O157:H7 isolates from cattle (Um et al., 2018). These *E. coli* O157:H7 isolates were screened, using a triplex real-time PCR assay, for 14 resistance genes including *bla*_{TEM}, *strA-strB*, *addA1*, *tet(A)*, *tet(B)*, *sulI*, *sulII*, *sulIII*, *cmlA*, *catI*, *catII*, *catIII*, *floR*. None of the eight *stx*-negative *E. coli* O157:H7 strains carried any antibiotic resistance genes, while one of the *stx*-positive strains contained several antibiotic resistance genes including *bla*_{TEM}, *strA-strB*, *tet(A)*, *sulIII*, and another *stx*-positive strain harbored a single *tet(A)* gene (Um et al., 2018). Another study compared the resistance to nine antibiotics in 12 *stx*-positive and 17 *stx*-negative *E. coli* O157:H7 isolates from bovine, caprine, and ovine milk in Greece using the disc diffusion method (Solomakos et al., 2009). The nine antibiotics evaluated included amoxicillin + clavulanic acid, ampicillin, cefachlor, cefuroxime, chloramphenicol, gentamicin, streptomycin, tetracycline, and trimethoprim + sulfamethoxazole. On average, the 12 *stx*-positive isolates were resistant to higher numbers of antibiotics (six of nine) than those of the 17 *stx*-negative isolates (four of nine) (Solomakos et al., 2009). These two studies suggest that the acquisition or loss of the *stx* genes between *stx*-positive and *stx*-negative *E. coli* O157:H7 may also result in changes in other phenotypic traits, such as antibiotic resistance.

The conventional molecular methods used to compare *stx*-positive and *stx*-negative *E. coli* O157:H7 are limited by their capabilities to study only certain selected DNA sequences from the bacterial genome, such as individual genes and/or short DNA fragments. With the development of next generation sequencing technology and whole genome sequencing (WGS), researchers are now able to compare and analyze DNA sequences at the level of the entire

bacterial genome. A whole genome comparison study of *E. coli* O157 isolates from patients and cattle indicated that *stx*-negative *E. coli* O157:H7/NM isolates shared 22 virulence genes, the locus of enterocyte effacement region, and plasmids with *stx*-positive *E. coli* O157:H7 isolates (Ferdous et al., 2015). Another study used timed phylogeny analysis of whole genome sequences to investigate the evolution of a clinical *stx*-positive *E. coli* O157:H7 strain (Byrne et al., 2018). From this analysis, the authors reported that *stx2a*-carrying *E. coli* O157:H7 had evolved from *stx*-negative *E. coli* O157:H7 by acquiring a *stx2a*-carrying prophage, and the *stx*-negative *E. coli* O157:H7 had evolved from a *stx2c*-carrying *E. coli* O157:H7 by losing a *stx2c*-carrying prophage (Byrne et al., 2018). In addition, a recent study used phylogenetic analysis to plot plasmids according to their phylogenetic positions in 15 *E. coli* O157:H7 and O157:NH strains that emerged stepwise (Nyong et al., 2020). By comparing *stx*-positive and *stx*-negative *E. coli* O157:H7 isolates from patients, cattle feces and swine feces, they observed a stable evolutionary relationship between the host chromosomes and their respective plasmids. This previous study suggested the coevolution of plasmids and the chromosome in *E. coli* O157:H7/NH.

Cattle are one of the primary reservoirs of both *stx*-positive and *stx*-negative *E. coli* O157:H7 (Keen et al., 2006; Kuhnert et al., 2005; Orden et al., 2002; Sanchez et al., 2010). While pathogenic *stx*-positive *E. coli* O157:H7 are frequently associated with severe disease in humans, food animals are often asymptomatic carriers because they lack vascular Shiga toxin receptors (Ferens & Hovde, 2011; Pruimboom-Brees et al., 2000). Previous studies conducted to compare the whole genome sequences of *stx*-positive and *stx*-negative *E. coli* O157:H7 have mainly focused on human isolates. Similar such studies with *stx*-positive and negative *E. coli* O157:H7 isolates of food animal origin are limited.

The current study was conducted to better understand the detailed mechanisms involved in the acquisition or loss of *stx* genes and the particular genotypes associated with the presence or absence of *stx* genes in *E. coli* O157:H7 isolated from feedlot cattle. The feedlot cattle *E. coli* O157:H7 strains included in the present study were from a previously conducted study (Carlson et al., 2009). The PCR results demonstrated that these *E. coli* O157:H7 strains displayed different *stx* profiles. The majority of the *E. coli* O157:H7 isolates from the Carlson et al. (2009) study carried both or either *stx1* or *stx2* genes, but some of the *E. coli* O157:H7 isolates were *stx*-negative (i.e., neither *stx1* nor *stx2* genes present). In the present study, we conducted comparative genomic analysis of three isolates with different *stx* profiles to investigate their virulence genes, genes involved in acquisition or loss of *stx* genes, and genes involved in metabolic pathways. Furthermore, the WGS data of these three strains were compared with those of five foodborne outbreak *E. coli* O157:H7 strains and six *stx*-negative *E. coli* O157:H7 strains from animal and food sources. Comparative whole genome analysis of *E. coli* O157:H7 strains with different *stx* gene profiles obtained from animal and food source will expand our understanding of the mechanisms of exchange of *stx* genes and other genes associated with the exchange of *stx* gene between *stx*-positive and *stx*-negative *E. coli* O157:H7. The results from this study may help develop control strategies to reduce pathogenic *E. coli* O157:H7 in food production.

Materials and Methods

Strain selection and whole genome sequencing

Three *E. coli* O157:H7 strains were selected for this study, including C1-057 (*stxI*-, *stx2*-), C1-010 (*stxI*-, *stx2c*+) and C1-067 (*stxI*+, *stx2a*+) . All three strains were previously isolated from fecal samples collected from cattle in a commercial feedlot (Carlson et al., 2009). For the *stx*-negative *E. coli* O157:H7 strain C1-057, we have previously reported its draft genome sequence (NCBI accession No. LAZO01000000; Yang et al., 2016). Following publication of the draft genome sequence, further sequencing was conducted by an external laboratory (University of Washington), using the Pacbio RSII system. A complete, gapless chromosome and a plasmid of this strain was deposited into NCBI with new NCBI accession No. (chromosome CP035366.1; plasmid CP035367.1). Additionally, the two *stx*-positive *E. coli* O157:H7 strains, C1-010 and C1-067, were sequenced via the Pacbio Sequel system. The two *stx*-positive *E. coli* O157:H7 strains were activated from frozen glycerol stocks (-80°C) by two transfers in tryptic soy broth (TSB; Difco, Becton Dickinson, Spark, MD) at 37°C for 24 h. The genomic DNA of each isolate was extracted using a QIAGEN Genomic DNA kit (QIAGEN, Redwood City, CA). The extracted genomic DNA was digested with the restriction enzyme *EcoRI* (New England Bioland, Ipswich, MA). Gel electrophoresis was performed to evaluate the DNA patterns of the digested genomic DNA. Smearing of DNA patterns showed that the extracted genomic DNA was fragmented by the restriction enzyme, indicating that no methylation occurred in the extracted genomic DNA.

The extracted genomic DNA samples without methylation were shipped to Genewiz, Inc (San Francisco, CA) for WGS. The Pacbio Sequel system was used to produce the raw reads with mean genome coverages of 477x for strain C1-010 (*stxI*-, *stx2c*+) and 497x for strain C1-067 (*stxI*+, *stx2a*+) . The lengths of raw reads were between 3 to 40 kb. The raw reads of strains C1-010 and C1-067 were subsequently *de novo* assembled into contigs separately by Canu

(version 1.6). When fully assembled, the genomes of strains C1-010 and C1-067 consisted of 13 and 12 scaffolds, respectively. To identify plasmids, DNA sequences of pO157 (NCBI accession No. NC_002128) and pOsak1 (NCBI accession No. AB011548) of *E. coli* O157:H7 strain Sakai were used as a reference for Basic Local Alignment Search Tool (BLAST) analyses (Altschul et al., 1990). Strain Sakai was used as it is a well-studied foodborne pathogenic *E. coli* O157:H7 strain that was responsible for a large outbreak in Japan (Hayashi et al., 2001). Chromosome and plasmid sequences of the three newly sequenced strains were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016). The sequences of strains C1-057, C1-010 and C1-067 were deposited in the NCBI database and their NCBI accession numbers are listed in Table 1.

In addition to the three newly sequenced *E. coli* O157:H7 strains, we also included five foodborne outbreak *E. coli* O157:H7 strains and six *stx*-negative *E. coli* O157:H7 strains obtained from the NCBI database (Table 1). These strains were selected to represent the different *stx* profiles, and their genomes were compared to the three newly sequenced strains. The *stx* gene profiles, origins, and NCBI accession numbers of all 14 *E. coli* O157:H7 strains used in this study are listed in Table 1.

Phylogenomic analysis

A phylogenomic analysis was performed on all 14 *E. coli* O157:H7 strains listed in Table 1. The phylogenomic tree was generated based on the single nucleotide polymorphisms (SNP) matrix between the *E. coli* O157:H7 strains using kSNP3.1 (available at <https://sourceforge.net/projects/ksnp/files/>) (Gardner, et al., 2015). The kSNP3.1 is a non-

reference-based SNP analysis software suite that incorporates multiple SNP utility programs on a Linux platform. The parameter k-mer size was determined by Kchooser, a utility program incorporated in kSNP3.1. Chromosome FASTA files of the 14 strains obtained from the NCBI database were imputed in kSNP3.1 to generate the SNP matrix. The SNP matrix output was subsequently used to construct a maximum likelihood (ML) phylogenetic tree by MEGA-X (available at www.megasoftware.net) with the parameter set at 2,000 bootstraps (Kumar et al., 2018).

Identification of prophages

Based on results from the phylogenomic analysis, nine *E. coli* O157:H7 strains were selected for prophage identification. Phage Search Tool Enhanced Release (PHASTER) (available at www.phaster.ca) is a web-based service for rapid identification and annotation of prophage sequences within bacterial genomes (Arndt et al., 2016). Prophages in the newly sequenced strains, C1-057 (*stxI*-, *stx2*-), C1-010 (*stxI*-, *stx2c*+) and C1-067 (*stxI*+, *stx2a*+) and the six aforementioned *stx*-negative strains (Table 1) were identified by PHASTER. GenBank files of these strains were uploaded to the PHASTER server for identifications of intact, questionable, and incomplete prophages harbored in each of the strains. In addition, strain Sakai containing 18 prophages was used as the *stx*-positive *E. coli* O157:H7 reference strain in this analysis (Hayashi et al., 2001).

Identification of *stx* insertion sites and Red recombination genes

DNA sequences of six previously reported *stx* insertion sites (*wrbA*, *sbcB*, *yehV*, *argW*, *yecE* and Z2577) were extracted from the whole genome sequence of *E. coli* O157:H7 strain Sakai (Eppinger et al., 2011; Hayashi et al., 2001; Ohnishi et al., 2002; Yokoyama et al., 2000). The *stx* insertion sites in the three newly sequenced strains and six *stx*-negative strains from NCBI were identified by screening the DNA sequences of the six *stx* insertion sites from strain Sakai against the chromosomal sequences of each of the nine *E. coli* O157:H7 strains using BLASTn (Johnson et al., 2008). Each of the six *stx* insertion sites in all nine *E. coli* O157:H7 strains was manually checked to determine if these *stx* insertion sites were occupied by any prophages (*stx*-carrying or *stx*-negative) identified by PHASTER.

DNA sequences of three Red recombination genes (*exo*, *bet* and *gam*) and Red operon repressor gene *cI* were extracted from the whole genome sequence of *E. coli* O157:H7 strain Sakai (Hillyar, 2012). Each of these DNA sequences were screened against the *stx*-carrying and *stx*-negative prophages that occupied the *stx* insertion sites using BLASTn (Johnson et al., 2008), in order to investigate if any of the prophages harbored Red recombination genes and/or the repressor gene *cI*.

Unique genes associated with metabolic pathways

The whole genome sequences of *E. coli* O157:H7 strain C1-057 (*stx1*-, *stx2*-) and strain Sakai were compared by DNASTAR® MegAlign Pro 14 to identify large specific sequence regions (SSRs) (>10 kb) that were located in either strain C1-057 or Sakai. All the genes within these SSRs were mapped against the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database using the Integrated Microbial Genomes & Microbiomes (accessible at

<https://img.jgi.doe.gov/>) (Kanehisa, et al., 2016; Chen et al., 2018). In addition to the SSRs, we also aligned the sequence regions (10 kb) from both upstream and downstream of each SSRs between strains C1-057 and Sakai. Genes that were only present in either strain C1-057 or Sakai in these sequence regions were identified and mapped against the KEGG pathway database. Next, those genes, from both SSRs and sequence regions close to each SSR, with a hit to the genes in the KEGG pathways were extracted and combined into one FASTA file. Furthermore, we mapped the extracted FASTA file containing sequences of the unique pathway genes found in strains C1-057 and Sakai against the genomes of the newly sequenced strains, C1-067 (*stx1+*, *stx2+*) and C1-010 (*stx1-*, *stx2c+*), and the six *stx*-negative strains from the NCBI database to investigate if the unique pathway genes were present in these strains.

We identified an IS629 and seven copies of pathway genes from one of SSRs in strain Sakai. Gene arrangements around this specific IS629 were compared between O55:H7 strain RM12579 (NCBI accession No. CP003109.1) and 10 O157:H7 strains used in this study.

Results

General features of the three newly sequenced *E. coli* O157:H7 strains

Table 2 shows the general features of the three newly sequenced *E. coli* O157:H7 strains previously isolated from feedlot cattle fecal samples. As already described, each of these three strains had different *stx* profiles. In the current study, the gapless chromosome of the *stx*-negative strain, C1-057 (5.45 Mb), was obtained. The chromosomes of the two *stx*-positive strains, C1-010 (*stx1-*, *stx2c+*) (5.57 Mb) and C1-067 (*stx1+*, *stx2a+*) (5.56 Mb), were composed of 12 and 11 scaffolds, respectively. In addition, the plasmid identification revealed that strains C1-057,

C1-010, and C1-067 each contained one pO157-like plasmid with sequence similarity of 97% (97.5 kb), 97% (91.9 kb) and 99% (92.7 kb) to the pO157 (92.7 kb) in strain Sakai, respectively. The DNA sequences of these three strains have been deposited to the NCBI (see Table 2 for their NCBI accession No.).

Phylogenomic analysis

SNP analysis was performed to evaluate the phylogenomic relationship among the 14 *E. coli* O157:H7 strains (Figure 1). The optimal length of k-mer was determined as 21 by Kchooser. A total of 3,581 SNP was identified from the 14 strains. Figure 1 shows the ML phylogenomic tree of the 14 *E. coli* O157:H7 strains. Overall, C1-057 (*stxI*-, *stx2*-) and C1-010 (*stxI*-, *stx2c*+), together with the six *stx*-negative *E. coli* O157:H7 strains from the NCBI database, were grouped into Cluster 1, while C1-067 (*stxI*+, *stx2c*+) and the five foodborne outbreak strains were grouped into Cluster 2. More specifically, strains C1-057 and C1-010 were clustered in Cluster 1a with three *stx*-negative strains from the USA and one *stx*-negative strain from Malaysia. Strain C1-067 grouped into Cluster 2b with three of the foodborne outbreak strains (Sakai, EDL933, Xuzhou21), and had the closest relationship with strain Sakai.

Prophage identification

Prophage identification, using PHASTER, was conducted on 10 of the 14 *E. coli* O157:H7 strains. The 10 strains included all the strains that grouped in Cluster 1 (i.e., C1-010 [*stxI*-, *stx2c*+] , C1-057 [*stx*-] and six other *stx*-negative strains), and strains C1-067 (*stxI*+, *stx2a*+) and Sakai from Cluster 2b since they had the closest phylogenomic relationship. The

results of the identified prophages are shown in Table 3. Overall, 17 to 27 prophages were identified on the chromosome sequences of the selected *E. coli* O157:H7 strains. These prophages contained genome sizes ranging from 6.3 to 124.9 kb.

Since both strains Sakai and C1-067 (*stxI*+, *stx2a*+) were grouped into Cluster 2b (Figure 1), prophages in strain C1-067 (isolated from feedlot cattle) were compared with the prophages in strain Sakai. The chromosome of *E. coli* O157:H7 strain Sakai contains 18 prophages (Sp1-Sp18) (Hayashi et al., 2001). Sequence alignment between strains Sakai and C1-067 demonstrated that strain C1-067 contained all 18 identical prophages harbored in strain Sakai (data not shown).

Since strains C1-057 (*stxI*-, *stx2*-) and C1-010 (*stxI*-, *stx2c*+) from feedlot cattle were grouped into Cluster 1 (Figure 1), prophages in strain C1-057 were compared with the prophages in strain C1-010. Strains C1-057 and C1-010 shared 10 intact, 1 questionable, and 2 incomplete prophages. Furthermore, strains C1-057 and C1-010 contained unique prophages that were not present in strains C1-010 and C1-057, respectively. Strain C1-057 harbored four unique intact and one unique incomplete prophage, while strain C1-010 had four unique intact and one unique questionable prophage. In addition, we found 1, 5, and 3 intact, questionable, and incomplete prophages, respectively, in strain C1-010 that shared similar sequences with the intact prophages in strain C1-057. However, these prophages in strain C1-010 were shorter than the corresponding intact prophages in strain C1-057. This result might be due to gaps in the sequences of strain C1-010.

A 14.2 kb intact prophage was only found in each of the strains from Cluster 1 (C1-010 [*stxI*-, *stx2c*+] and seven *stx*-negative strains), but not in the two strains within Cluster 2b (Sakai and C1-067). PHASTER results indicated that this prophage was the most similar to a known

phage P4 (11.6 kb, NCBI accession No. NC_001609.1) with 61% query coverage and 97.4% identity. It should be noted that when phage P4 infects *E. coli* cells, it has three known modes of propagation: (1) it can integrate within the host chromosome as a prophage; (2) it can replicate as a plasmid; or (3) it can replicate itself rapidly in its lytic cycle (Briani et al., 2001). Phage P4 contained 19 genes. The P4-like prophage in the strains from Cluster 1 contained 25 genes, and 10 of which were identical to the genes present in phage P4. It is reasonable to presume that these 10 genes may be involved in one or more of three modes of propagation.

Comparison of *stx* insertion sites occupied with prophages

The chromosome of the 10 *E. coli* O157:H7 strains selected for prophage identification were further examined for six types of previously-described *stx* insertion sites (*wrbA*, *sbcB*, *yehV*, *argW*, *yecE* and Z2577). All 10 strains carried all six *stx* insertion site genes. As shown in Table 4, three of the *stx* insertion sites of *wrbA*, *yehV*, and *sbcB* are occupied with, while the other three *stx* insertion sites of *argW*, *yecE* and Z2577 remained accessible and unoccupied.

Among the 10 selected *E. coli* O157:H7 strains, strains Sakai and C1-067 (*stx1+*, *stx2a+*) belonging to Cluster 2b carried two identical *stx*-carrying prophages: one *stx1*-carrying prophage at the *yehV* *stx* insertion site and one *stx2a*-carrying prophage at the *wrbA* *stx* insertion site. All strains from Cluster 1a, except for strain C1-010 (*stx1-*, *stx2c+*), harbored an identical *stx*-negative prophage at the *yehV* *stx* insertion site. The two strains from Cluster 1b and strain C1-010 carried an identical *stx*-negative prophage at the *yehV* *stx* insertion site, while strain C1-010 contained a *stx2c*-carrying prophage, and the two strains from Cluster 1b contained *stx*-negative prophages with varied DNA sequences at the *sbcB* *stx* insertion site.

For all 10 selected *E. coli* O157:H7 strains, one specific type of prophage with varied DNA sequences was observed at the *yehV stx* insertion site. The *stxI*-carrying prophage Sp15 (50.5 kb) was present in strains Sakai and C1-067 (*stxI*+, *stx2a*+). An intact *stx*-negative prophage (47.6 kb), named Sp15-like, in all Cluster 1 strains had 72% query coverage and 95% identity to prophage Sp15. It is worth noting that a 10.2 kb DNA fragment without the *stx* genes of the prophage Sp15-like in the strains from Cluster 1 was replaced by a 12.8 kb DNA fragment containing the genes of *stxIA* and *stxIB* of the *stxI*-carrying prophage Sp15 present in strains Sakai and C1-067 (Figure 2).

At the *sbcB stx* insertion site (Figure 3), prophages similar to phage 1717 (62.1 kb, NCBI accession No. FJ188381.1) but with varied sizes and varied query coverage and identities were found in the *stx2c*-carrying strain C1-010 belonging to Cluster 1a and the two *stx*-negative strains from Cluster 1b. A *stx2c*-carrying prophage (61.7 kb), named 1717-like-A, in strain C1-010 had 98% query coverage and 99% identity to phage 1717. A *stx*-negative prophage (22.0 kb, 34% query coverage and 99% identity to phage 1717), named 1717-like-B, and another *stx*-negative prophage (30.0 kb, 47% query coverage and 99% identity to phage 1717), named 1717-like-C, were found in the *stx*-negative strains CV261 and NZRM3614, respectively. Sequence comparisons demonstrated that a 29.2 kb DNA fragment containing the *stx2c* gene was present in prophage 1717-like-A in strain C1-010 belonging to Cluster 1a, but this DNA fragment was absent in the *stx*-negative prophages 1717-like-B and -C in strains CV261 and NZRM3614 from Cluster 1b, respectively.

Comparison of the Red recombination genes and their repressor gene *cI*

The prophages located at the three *stx* insertion sites were further investigated for the presence/absence of Red homologous recombination genes (*exo*, *bet*, and *gam*) and/or their repressor gene *cI* (Table 4). This analysis showed that three types of *stx*-carrying prophages (*stx2a*-carrying prophage Sp5, *stx1*-carrying prophage Sp15, and *stx2c*-carrying prophage 1717-like-A) contained both the three Red recombination genes and their repressor *cI*. Depending on the strain, *stx*-negative prophages had three situations: (1) prophage 1717-like-C in one strain contained both the three Red recombination genes and their repressor *cI*; (2) prophage Sp15-like in eight strains harbored only the three Red recombination genes but the repressor *cI* was absent; (3) prophage 1717-like-B in one strain does not contained neither any of the Red recombination genes nor the repressor *cI*. In addition, table 5 summarized functions three Red homologous recombination genes.

Genes involved in metabolic pathways located at SSRs and sequence regions closed to SSRs

Sequence alignment between strain Sakai (*stx1*+, *stx2a*+) and C1-057 (*stx1*-, *stx2*-) was performed to identify the SSRs in strains Sakai and C1-057. We found that there were nine SSRs and six SSRs uniquely present in strains Sakai and C1-057, respectively. A comparative metabolic pathway analysis using the KEGG database was conducted to find if any genes involved in metabolic pathways were located within these SSRs and sequence regions closed to these 15 SSRs. We found that for strain Sakai, eight copies of genes involved in metabolic pathways were located at two of the nine SSRs in strain Sakai. These eight copies of metabolic pathway genes included: *sodC*, *modD*, *fepC*, *fecD*, *fbpA*, *dhaM*, *dhaL*, and *dhaK*. However, unique pathway genes were not found in the sequence regions closed to the 15 SSRs. On the other hand, for strain C1-057, although unique pathway gene was not found in any of six SSRs in

strain C1-057, one copy of unique pathway gene (*dhaR*) was present in the sequence region corresponding downstream of one SSR in strain Sakai (Figure 4A and 4B). Therefore, strain Sakai contained eight copies of unique pathway genes, while strain C1-057 harbored one copy of unique pathway.

The two other *stx*-positive strains, C1-067 (*stxI*+, *stx2a*+) and C1-010 (*stxI*-, *stx2c*+) and six *stx*-negative strains were screened for presence/absence of the nine copies of pathway genes found in strain Sakai (*stxI*+, *stx2a*) and C1-057 (*stxI*-, *stx2*-). Strain C1-067 contained eight copies of unique pathway genes found in strain Sakai, but it did not carry the pathway gene from *stx*-negative strain C1-057. Strain C1-010 and six *stx*-negative strains only carried the pathway gene (*dhaR*) from strain C1-057, but not other eight copies of pathway genes from strain Sakai.

Protein functions of the nine copies of unique metabolic pathway genes in strains Sakai (*stxI*+, *stx2a*+) and C1-057 (*stxI*-, *stx2*-) were listed in Table 6. For the eight copies of unique pathway genes in strain Sakai, one copy of pathway gene *sodC* was located at prophage Sp10 (SSR). Seven copies of the pathway genes were located at the downstream of an insertion sequence (IS) 629 (SSR). Interestingly, for the unique pathway gene *dhaR* in strain C1-057, it was also found at the downstream of this specific IS629. Previous studies reported that O55:H7 was the ancestor of *stx*-positive and -negative *E. coli* O157:H7 (Byrne et al., 2018; Leopold et al., 2009; Shaikh & Tarr, 2003). We further compared the gene arrangements around this specific IS629 between *E. coli* O55:H7 strain RM12579 and the 10 *stx*-positive and -negative O157:H7 strains (Figure 4A and 4B). The gene alignment result indicated that sequence regions around this IS629 were highly related between O55:H7 and O157:H7 strains. However, O55:H7 strain RM12579 contained the seven copies of pathway genes described above and an additional *dhaR*,

but the IS629 was absent. The O157:H7 strains Sakai and C1-067 (*stx1*+, *stx2a*+) carried the IS629 and the seven copies of pathway genes, but *dhaR* was absent. The O157:H7 strain C1-010 (*stx1*-, *stx2c*+) and seven *stx*-negative strains harbored the IS629 and *dhaR*, but seven copies of pathway genes were absent.

Discussion

In this study, we performed a whole genome analysis of three *E. coli* O157:H7 strains with different *stx* profiles that were previously isolated from feedlot cattle (Carlson et al., 2009). Phylogenomic analysis of the three strains together with 11 other *E. coli* O157:H7 strains showed that these three strains were grouped into different clusters. The *stx*-negative strain C1-057 was grouped with four *stx*-negative strains in Cluster 1a. Strain C1-010, although *stx2c* positive, was grouped with five *stx*-negative strains in Cluster 1a. Strain C1-067, carrying both *stx1* and *stx2a* genes, was grouped into Cluster 2b with strain Sakai (*stx1*+, *stx2a*+) . These results suggested that the three *E. coli* O157:H7 from feedlot cattle may exhibit different levels of pathogenicity.

Wetzel & LeJeune (2007) reported that *stx*-negative *E. coli* O157:H7 could potentially be converted to *stx*-positive *E. coli* O157:H7 in cattle, swine, and avian production systems. A previous evolutionary model suggested that *stx*-positive *E. coli* O157:H7 could have evolved stepwise from an ancestor *E. coli* O55:H7, following the steps of 1) acquiring *stx2*-carrying prophages, 2) losing the O55 *rfb-gnd* gene, acquiring the O157 *rfb-gnd* gene, and losing the ability to ferment sorbitol, and 3) acquiring *stx1*-carrying prophage (Leopold et al., 2009; Shaikh & Tarr, 2003). However, the exact mechanisms involved in the acquisition and/or loss of the *stx*

genes in *E. coli* O157:H7 strains are not fully understood. A greater understanding of mechanisms involved in the acquisition or loss of *stx* genes could aid in the development of control strategies to reduce or eliminate highly pathogenic *E. coli* O157:H7 strains in cattle.

In addition to acquiring the *stx*-carrying prophage to become *stx*-positive *E. coli* O157:H7 strains, the results of this study suggested a potential alternative mechanism that could result in acquisition of *stx* genes via the Red homologous recombination system. In the current study, at the *yehV* *stx* insertion site, we observed a *stx2a*-carrying prophage Sp5 in *stx*-positive strains Sakai and C1-067 (*stx1*+, *stx2a*+) and a similar prophage but without the *stx* gene (Sp15-like) in seven *stx*-negative strains. The difference between these two prophages is that a 10.2 kb DNA fragment (without *stx*) in Sp15-like prophage was replaced by a 12.8 kb DNA fragment (with *stx1A* and *stx1B*).

This DNA fragment replacement in prophages resulting in the acquisition of *stx* genes might be mediated by the Red homologous recombination system. Other studies have investigated the contribution of the Red homologous recombination system to frequent gene exchanges within prophages (Johansen, et al., 2001; Smith et al., 2012). According to Yu et al. (2000), the Red homologous recombination system, which is usually found in lambda prophages, consists of three Red recombination genes, *exo*, *bet* and *gam*, and the expression of these genes is regulated by their repressor CI (Table 5). The binding of repressor protein CI to the promoter pL in lambda prophages inhibits the expression of the Red recombination genes (Yu et al., 2000).

We compared the presence or absence of Red recombination genes and the repressor gene *cI* between *stx*-negative and -positive prophages (Figure 2 and Figure 3). Overall, all *stx*-carrying prophages contained both the three Red recombination genes and their repressor *cI*, while the majority of the *stx*-negative prophages at *stx* insertion sites carried only the three Red

recombination genes but not the repressor *cI* gene. Our findings are consistent with the findings of previous studies conducted to examine *stx2*-carrying prophages in *stx*-positive *E. coli* O157:H7 strains (Smith et al., 2012; Tozzoli et al., 2014). These studies also demonstrated that both the three Red recombination genes and their repressor gene *cI* were present in *stx2*-carrying prophages (Smith et al., 2012; Tozzoli et al., 2014).

As previously mentioned, the presence of repressor CI in *stx*-carrying prophages could inhibit the activity of Red homologous recombination and consequently, the stability of the *stx* genes in *stx*-positive *E. coli* O157:H7 could be increased. On the other hand, the majority of the *stx*-negative prophages at *stx* insertion sites in this study carried only the three Red recombination genes but their repressor *cI* was absent. In the absence of the repressor *cI* in *stx*-negative prophages, the consistent expression of the Red recombination genes may result in more frequent gene exchange, potentially contributing to the acquisition of *stx* genes. Overall, our results indicate that the presence or absence of repressor *cI* might result in different levels of activity of Red recombination in *stx*-positive and *stx*-negative prophages. For the *stx*-carrying prophages containing both the Red recombination genes and their repressor *cI*, the potential of losing *stx* genes is relatively low due to the inhibition of the activity of Red homologous recombination by their repressor CI. However, for the *stx*-negative prophages containing only Red recombination genes but not repressor *cI*, the potential of acquiring *stx* genes is relatively high due to the consistent expression of Red recombination genes, resulting in frequent gene exchanges. To our knowledge, this is the first report to suggest possible functions of Red recombination genes and their repressor relating to the stability and acquisition of *stx* genes. Further studies are required to verify this proposed function associated with Red recombination

genes and their repressor *cI* in more *stx*-negative and *stx*-positive *E. coli* O157:H7 strains under laboratory conditions and in the cattle environment.

The acquisition or loss of *stx* genes may also result in changes in other genotypic traits between *stx*-positive and *stx*-negative *E. coli* O157:H7, such as antibiotic resistance genes (Colavecchio et al., 2017; Garcia-Aljaro et al., 2009). In this study, we identified the genes that are associated with metabolic pathways in the SSRs (>10 kb) between *stx*-positive and *stx*-negative *E. coli* O157:H7, in order to reveal if any of these unique pathway genes were associated with the acquisition or loss of *stx* genes. Our results show that *stx*-positive strains Sakai and C1-067 (*stx1*+, *stx2a*+) harbor eight copies of unique pathway genes (*sodC*, *modD*, *fepC*, *fecD*, *fbpA*, *dhaM*, *dhaL*, and *dhaK*). In comparison, strains C1-010 (*stx1*-, *stx2c*+) and seven *stx*-negative strains carried one unique pathway gene (*dhaR*). Among these nine copies of unique pathway genes, gene *sodC* might contribute to the increased survival of *stx*-positive *E. coli* O157:H7 in complex microbial community (Battistoni, 2003). Genes *fepC*, *fecD* and *fbpA* could potentially be associated with improved iron uptake ability (Staudenmaier et al., 1989; Chen et al., 1993; Raymond et al., 2003). Genes *dhaK*, *dhaL*, *dhaM*, and *dhaR* are associated with glycerol metabolism (Bächler et al., 2005). Compared with *stx*-negative *E. coli* O157:H7 strains, *stx*-positive strains contained more unique genes associated with metabolic pathways. These unique metabolic genes may potentially lead to increase competitiveness of *stx*-positive strains in complex environment such as cattle feedlot.

The *sodC* gene encodes copper/zinc-superoxide dismutase (Cu-Zn SOD) which catalyzes the dismutation of the superoxide radical ($O_2^{\cdot -}$) into hydrogen peroxide (H_2O_2) (Fridovich, 1995). *sodC* is considered a virulence gene since the Cu-Zn SOD protects bacteria from exogenous sources of superoxide. A previous study indicated that Cu-Zn SOD protected *E. coli* strains from

superoxide generated by macrophages and enhanced bacterial survival (Battistoni, 2003). We found one copy of *sodC* harbored on the chromosomes of all 10 strains used in this analysis. However, an additional copy of *sodC* harbored in the Sp10 prophage was found only in Sakai and C1-067 (*stxI*+, *stx2a*+) but was absent in strains C1-057 (*stxI*-, *stx2*-), C1-010 (*stxI*-, *stx2c*+) and the six *stx*-negative strains from the NCBI database. The second copy of *sodC* may provide additional protection from the toxicity of superoxide radical. Thus, the *stx*-positive strains Sakai and C1-067 (may be more resistant to exogenous superoxide radical than strains C1-057, C1-010 and the six *stx*-negative strains from the NCBI database.

The *fepC*, *fecD* and *fbpA* genes encode proteins that are involved in three separate iron uptake systems, respectively. The *fepC*, from the ferric enterobactin transport system FepABCDG, encodes an ATP-binding protein transport which provides energy to assist iron uptake from the environment (Raymond, Dertz, & Kim, 2003). The *fecD*, from the ferric citrate transport system FecABCDE, encodes a ferric citrate transporter permease which assists to uptake diferric dicitrate through the cytoplasmic membrane (Staudenmaier et al., 1989). The *fbpA*, from the iron uptake gene cluster *fbpABC*, encodes ferric substrate-binding protein (Chen et al., 1993). Although this TE did not harbor any of the intact iron uptake gene cluster, the presence of genes *fepC*, *fecD* and *fbpA* in strains Sakai and C1-067 (*stxI*+, *stx2a*+) can potentially increase iron uptake ability compared with strains C1-057 (*stxI*-, *stx2*-), C1-010 (*stxI*-, *stx2c*+) and the six *stx*-negative strains from the NCBI database.

E. coli can metabolize glycerol via two microaerobic pathways: the dihydroxyacetone kinase (DAK) pathway and the glycerol pathway (Durnin et al., 2009). DhaK, DhaL and DhaM, encoded by the *dha* operon, are proteins to phosphorylate DHA in the DAK pathway. The *dha* operon is controlled by DhaR, a transcriptional activator (Bächler et al., 2005). All four proteins

are essential for the DAK pathway. Based on the O serotype and *stx*-profiles, we observed four *dha* operons (Figure 4B): (1) *E. coli* O55:H7 strain RM12579 harbored an intact *dha* operon consisting of *dhaK*, *dhaL*, *dhaM*, and *dhaR*; (2) *E. coli* O157:H7 *stx*-positive strains Sakai and C1-067 (*stx1*+, *stx2a*+) harbored *dhaK*, *dhaL*, and *dhaM*, but the *dhaR* gene was absent; (3) *E. coli* O157:H7 *stx*-positive strain C1-010 (*stx1*-, *stx2c*+) harbored only *dhaR* but not *dhaK*, *dhaL*, or *dhaM*; (4) Seven *E. coli* O157:H7 *stx*-negative strains harbored a same *dha* operon as strain C1-010. Additionally, both two types of disruptions of *dha* systems inactivated the metabolism of glycerol via the DAK pathway. Durnin et al. (2009) reported that the deletion of *dhaK* led to a reduction in the efficacy of glycerol utilization by approximately 50%, resulting in a reduction in cell growth rate by 70%, in a minimal medium supplemented with glycerol as the sole carbon source. Another study also indicated that adding three levels of crude glycerin (0, 4 and 8%) in corn silage diets decreased prevalence levels of *E. coli* O157 to 4.4, 3.2 and 1.8% in fecal samples from growing cattle, respectively ($P < 0.01$) (Aperce et al., 2013). Our results provided evidence that disruption of the DAK pathway in both *stx*-positive and *stx*-negative *E. coli* O157:H7 strains from this study might contribute to a decrease in the rate of glycerol metabolism, whereas other bacteria in microbial communities are able to utilize glycerol. Therefore, the disruption of DAK pathways can potentially decrease competitiveness and survival of *E. coli* O157:H7 in growing cattle fed diets including crude glycerin. Our results support the findings of previous studies at the gene level, further confirming that adding glycerin to cattle diets can potentially decrease the prevalence of *E. coli* O157:H7 in fecal samples from cattle.

Previous studies suggested that both *stx*-positive and *stx*-negative *E. coli* O157:H7 could have evolved from an ancestor *E. coli* O55:H7 (Byrne et al., 2018; Leopold et al., 2009; Shaikh & Tarr, 2003). Figure 4A and 4B compared the gene arrangements around the IS629 and these seven pathway genes between *E. coli* O55:H7 strain RM12579 and 10 *stx*-positive or -negative *E. coli* O157:H7 strains. Our results suggested that O55:H7 strain RM12579 carried eight copies of metabolic pathway genes (*modD*, *fepC*, *fecD*, *fbpA*, *dhaM*, *dhaL*, *dhaK*, and *dhaR*). Compared with O55:H7, the *stx*-positive O157:H7 strains Sakai and C1-067 (*stxI*+, *stx2a*+) carried seven metabolic pathway genes and an additional IS629 but lost *dhaR*. Seven *stx*-negative O157:H7 strains remained the *dhaR* and harbored an additional IS629, but it lost seven pathway genes. IS629 insertion was reported to lead the insertion and deletion of genes or genomic rearrangements of chromosomal flanking region in *E. coli* O157 strains (Ooka et al., 2009). Thus, we suggested that IS629 insertion may result that *stx*-positive *E. coli* O157:H7 strains Sakai and C1-067 lost *dhaR*. In addition, IS629 insertion may lead that seven *stx*-negative *E. coli* O157:H7 strains lost seven pathway genes.

Leopold et al. (2009) and Shaikh & Tarr, (2003) indicated *E. coli* O157:H7 was evolved from O55:H7 by switching O serotype. Additionally, Byrne et al. (2018) suggested that *stx*-negative *E. coli* O157:H7 was evolved from *stx2c*-carrying *E. coli* O157:H7, while *stx2a*-carrying *E. coli* O157:H7 was converted from *stx*-negative *E. coli* O157:H7. In our study, the phylogenomic analysis revealed that *stx*-positive and -negative *E. coli* O157:H7 strains were grouped into different clusters (Figure 1). Furthermore, as described above, two *stxI*- and *stx2a*-carrying *E. coli* O157:H7 strains shared same gene arrangements around the specific IS629. However, seven *stx*-negative *E. coli* O157:H7 strains and strain C1-010 (*stxI*-, *stx2c*+) had same gene arrangements which were distinct to the two *stxI*- and *stx2a*-carrying O157:H7 strains.

These evidences indicated that *stx2c*-carrying O157:H7 and *stx*-negative O157:H7 were more genetically closed compared with *stx2a*-carrying O157:H7. Therefore, *stx*-negative and *stx2a*-carrying O157:H7 may be evolved from the same ancestor O55:H7 by different evolutionary pathways.

Conclusion

In summary, our study firstly reported the potential functions of Red recombination genes and their repressor *cI* associating with the maintenance of *stx* genes in *stx*-carrying prophages and the acquisition of *stx* genes in *stx*-negative prophages. Prophage analysis revealed that all *stx*-carrying prophages in the *stx*-positive strains contained both the three Red recombination genes and their repressor *cI*. The presence of repressor *cI* in *stx*-carrying prophages could inhibit the activity of Red homologous recombination. Consequently, the stability of the *stx* genes in *stx*-positive *E. coli* O157:H7 could be enhanced. In comparison, most of the *stx*-negative prophages located at *stx* insertion sites in the *stx*-negative strains carried only the three Red recombination genes, but the repressor *cI* was absent. The absence of repressor *cI* could lead to the consistent expression of Red recombination genes. As the result, gene exchange might occur more frequently, potentially contributing to the acquisition of *stx* genes in the *stx*-negative prophages at *stx* insertion site. Furthermore, comparative pathway analysis indicated that *stx1*- and *stx2a*-carrying *E. coli* O157:H7 harbored unique genes involved in metabolic pathways that were absent in *stx*-negative *E. coli* O157:H7. The unique metabolic pathway genes may potentially lead to increase the competitiveness and survival of *stx*-positive *E. coli* O157:H7. This analysis may partially explain why *stx*-positive *E. coli* O157:H7 is more prevalent compared with *stx*-negative *E. coli* O157:H7 in cattle. Finally, our study provided the evidence that *stx*-negative and

stx2a-carrying O157:H7 may be evolved from the same ancestor O55:H7 by different evolutionary pathways. Our comparative genomic analysis deepened the understanding of the mechanisms associated with the acquisition or loss of *stx* genes and the particular genotypic traits associated with the presence or absence of the *stx* genes. The results from this study may help develop control strategies to reduce pathogenic *E. coli* O157:H7 in food production.

Table 2.1. The *stx* gene profiles, accession numbers, sources, and countries of the *Escherichia coli* O157:H7 strains used in this study.

Strain	<i>stx</i> profile		NCBI Accession No.	Source	Country	Reference
	<i>stx1</i>	<i>stx2</i>				
Newly sequenced <i>E. coli</i> O157:H7 strains						
C1-010	N*	<i>stx2c</i>	NZ_SCKH000000000	Cattle feces	USA	Carlson et al. (2009)
C1-057	N	N	CP035366.1	Cattle feces	USA	Carlson et al. (2009)
C1-067	<i>stx1</i>	<i>stx2a</i>	RICC01000000	Cattle feces	USA	Carlson et al. (2009)
Foodborne outbreak <i>E. coli</i> O157:H7 strains						
EC4115	N	<i>stx2a, stx2c</i>	NC_011353.1	Unknown	USA	Eppinger et al. (2011)
TW14359	N	<i>stx2a, stx2c</i>	NC_013008.1	Spinach	USA	Kulasekara et al. (2009)
Sakai	<i>stx1</i>	<i>stx2a</i>	NC_002695.2	Human, spinach	Japan	Hayashi et al. (2001)
EDL933	<i>stx1</i>	<i>stx2a</i>	CP008957.1	Ground beef	USA	Latif et al. (2014)
Xuzhou21	<i>stx1</i>	<i>stx2a</i>	NC_017906.1	Swine, cattle and chicken	China	Xiong et al. (2012)
<i>stx</i> -negative <i>E. coli</i> O157:H7 strains						
21B8	N	N	CP040309.1	Cattle	USA	Allue-Guardia et al. (2019)
NZRM3614	N	N	CP032793.1	Unknown	Austria	Allue-Guardia et al. (2019)
M7638	N	N	CP040313.1	Unknown	USA	Allue-Guardia et al. (2019)
F1 E4	N	N	CP040307.1	Cattle	USA	Allue-Guardia et al. (2019)
CV261	N	N	CP040316.1	Cattle	France	Allue-Guardia et al. (2019)
MA11	N	N	CP040314.1	Meat	Malaysia	Allue-Guardia et al. (2019)

*N: negative

Table 2.2. General genomic features of the three newly sequenced *Escherichia coli* O157:H7 strains with different *stx* profiles.

	<i>E. coli</i> O157:H7 strain C1-057	<i>E. coli</i> O157:H7 strain C1-010	<i>E. coli</i> O157:H7 strain C1-067
<i>stx</i> gene profiles	<i>stx1</i> -/ <i>stx2</i> -	<i>stx1</i> -/ <i>stx2c</i>	<i>stx1</i> / <i>stx2a</i>
Chromosome length (bp)	5,452,210	5,571,092	5,564,227
Chromosome contigs	1	12 scaffolds	11 scaffolds
Plasmid No.	1	1	1
Plasmid length (bp)	97,494	91,863	92,720
Coverage	100x	477x	497x
Total coding genes	5,154	5,410	5,378
Total tRNA	101	111	104
NCBI Accession number	CP035366	SCKH01000000	RICC01000000

Table 2.3. Different prophage contents, including intact, questionable, and incomplete prophages, predicted from the *Escherichia coli* O157:H7 strains belonging to Cluster 1 by PHASTER.

<i>E. coli</i> O157:H7 Strain	<i>stx</i> profile	Number of intact prophages (size)	Number of questionable prophages (size)	Number of incomplete prophages (size)
21B8	N*	10 (14.2-55.4 kb)	4 (10.7-49.6 kb)	3 (7.9-13.8 kb)
M7638	N	11 (14.2-110.5 kb)	4 (10.7-49.6 kb)	3 (7.9-13.8 kb)
C1-057	N	14 (14.2-112.0 kb)	1 (22.4 kb)	3 (6.3-13.8 kb)
F1 E4	N	11 (14.2-55.8 kb)	4 (10.7-49.6 kb)	3 (7.9-13.6 kb)
MA11	N	11 (14.2-114.6 kb)	4 (10.7-49.6 kb)	4 (7.9-16.4 kb)
C1-010	<i>stx2c</i>	15 (11.2-101.1 kb)	7 (7.9-50.9 kb)	5 (7.9-41.0 kb)
CV261	N	14 (14.2-124.9 kb)	3 (10.7-49.6 kb)	5 (9.6-29.0 kb)
NZRM3614	N	12 (14.2-110.4 kb)	1 (50.3 kb)	5 (6.4-24.4 kb)

*N: negative

Table 2.4. Comparison of prophages at six *stx* insertion sites in *Escherichia coli* O157:H7 strains Sakai, the three newly sequenced strains, and six *stx*-negative strains from the NCBI database.

<i>E. coli</i> O157:H7 Strain	<i>stx</i> profile	<i>stx</i> insertion sites					
		wrbA	sbcB	yehV	argW	yecE	Z2577
Cluster 1a							
21B8	-	-	-	Sp15-like (R)	-	-	-
M7638	-	-	-	Sp15-like (R)	-	-	-
C1-057	-	-	-	Sp15-like (R)	-	-	-
F1 E4	-	-	-	Sp15-like (R)	-	-	-
MA11	-	-	-	Sp15-like (R)	-	-	-
C1-010	<i>stx2c</i>	-	1717-like-A (RC) (<i>stx2c</i>)	Sp15-like (R)	-	-	-
Cluster 1b							
CV261	-	-	1717-like-B (NA)	Sp15-like (R)	-	-	-
NZRM3614	-	-	1717-like-C (RC)	Sp15-like (R)	-	-	-
Cluster 2b							
Sakai	<i>stx1, stx2a</i>	Sp5 (RC) (<i>stx2a</i>)	-	Sp15 (RC) (<i>stx1</i>)	-	-	-
C1-067	<i>stx1, stx2a</i>	Sp5 (RC) (<i>stx2a</i>)	-	Sp15 (RC) (<i>stx1</i>)	-	-	-

R: The prophage contains three Red recombination genes consisting of *exo*, *bet* and *gam*.

C: The prophage contains the gene *cI* which encodes the Red recombination repressor protein CI.

NA: The prophage does not contain neither any of Red recombination genes nor repressor *cI*.

Table 2.5. Genes involved in the Red homologous recombination system in lambda prophages.

Gene Symbol	Protein	Protein function
<i>exo</i>	Exo	Cleaving the 5'-end of dsDNA and form 3'-end overhangs
<i>bet</i>	Bet	Protecting the ssDNA generated by Exo and promoting its annealing to a complementary ssDNA target in <i>E. coli</i> cells
<i>gam</i>	Gam	Preventing the endogenous nucleases (RecBCD and SbcCD) from digesting foreign linear DNA that is introduced into the <i>E. coli</i> cells
<i>cI</i>	Repressor CI	Inhibiting the expression of genes <i>exo</i> , <i>bet</i> and <i>gam</i> in prophage

Table 2.6. The unique genes involved in metabolic pathways harbored in *stx*-positive *Escherichia coli* O157:H7 strains Sakai and C1-067 (*stx1*+, *stx2a*+) but were absent in seven *stx*-negative *E. coli* O157:H7 strains and strain C1-010 (*stx1*-, *stx2c*+

Gene Symbol	Protein	Protein function
<i>sodC</i>	Cooper/zinc-superoxide dismutase	Catalyzing the dismutation of the superoxide radical (O_2^-) into the hydrogen peroxide (H_2O_2). Protecting bacteria from exogenous sources of superoxide
<i>fepC</i>	ATP-binding protein	Providing energy to assist the iron uptake system, ferric enterobactin transport system
<i>fecD</i>	Ferric citrate transporter permease	Assisting to uptake diferric dicitrate through cytoplasmic membrane in the iron uptake system, ferric citrate transport system
<i>fbpA</i>	Ferric substrate-binding protein	Binding iron substrate that crosses the outer membrane
<i>dhaM</i>	Dihydroxyacetone kinase phosphotransferase	Transferring the phosphate to the dihydroxyacetone kinase. Dependent on the sugar phosphotransferase system, an energy transducing system involved in carbohydrate uptake and control of carbon metabolism. Negatively regulating <i>dhaKLM</i> operon
<i>dhaL</i>	Dihydroxyacetone kinase subunit	Containing ADP as cofactor for the double displacement of phosphate from DhaM to Dha. Positively regulating <i>dhaKLM</i> operon
<i>dhaK</i>	Dihydroxyacetone kinase subunit	The Dihydroxyacetone binding site. Negatively regulating <i>dhaKLM</i> operon
<i>dhaR</i>	Transcription activator of <i>dhaKLM</i> operon	Stimulating transcription of <i>dhaKLM</i> operon. Disruption of <i>dhaR</i> leads that the <i>dhaKLM</i> operon cannot be induced with Dha
<i>modD</i>	ModD	Unknown, downstream of the molybdate transporter operon

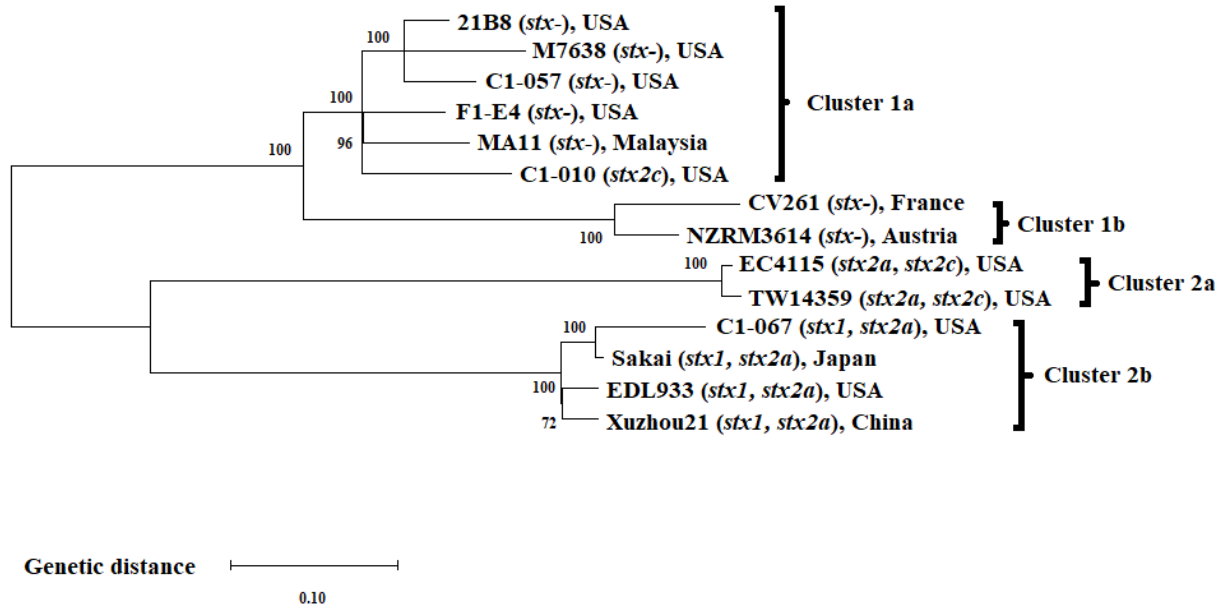


Figure 2.1. Maximum likelihood phylogenomic tree of 14 *Escherichia coli* O157:H7 strains. SNP matrix was generated from 3,581 SNP with 2,000 bootstraps and the phylogenomic tree was visualized using MEGA-X.

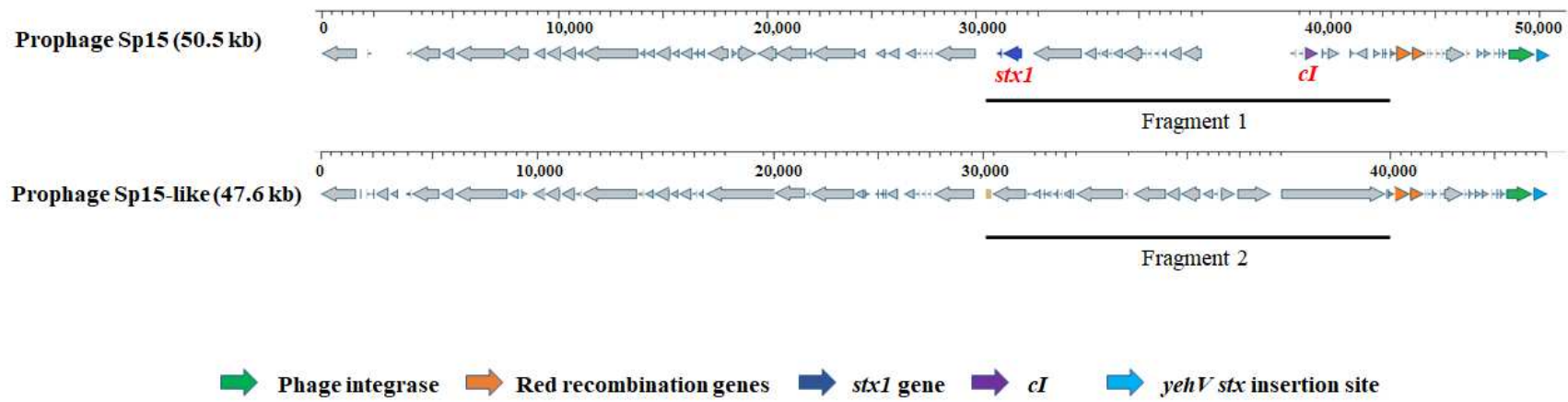


Figure 2.2. Prophage comparison at *yehV stx* insertion sites in *Escherichia coli* O157:H7 strains used in this analysis. The *stxI*-carrying prophage Sp15 was present in strains Sakai and C1-067 (*stxI*+, *stx2a*+). The *stx*-negative prophage Sp15-like was present in seven *stx*-negative strains and strain C1-010 (*stxI*+, *stx2*-). A 10.2 kb DNA fragment (Fragment 2) that does not contain the *stx* genes of the *stx*-negative prophage Sp15-like was replaced by a 12.8 kb DNA fragment (Fragment 1) of the *stxI*-carrying prophage Sp15 containing the genes *stxIA* and *stxIB*.

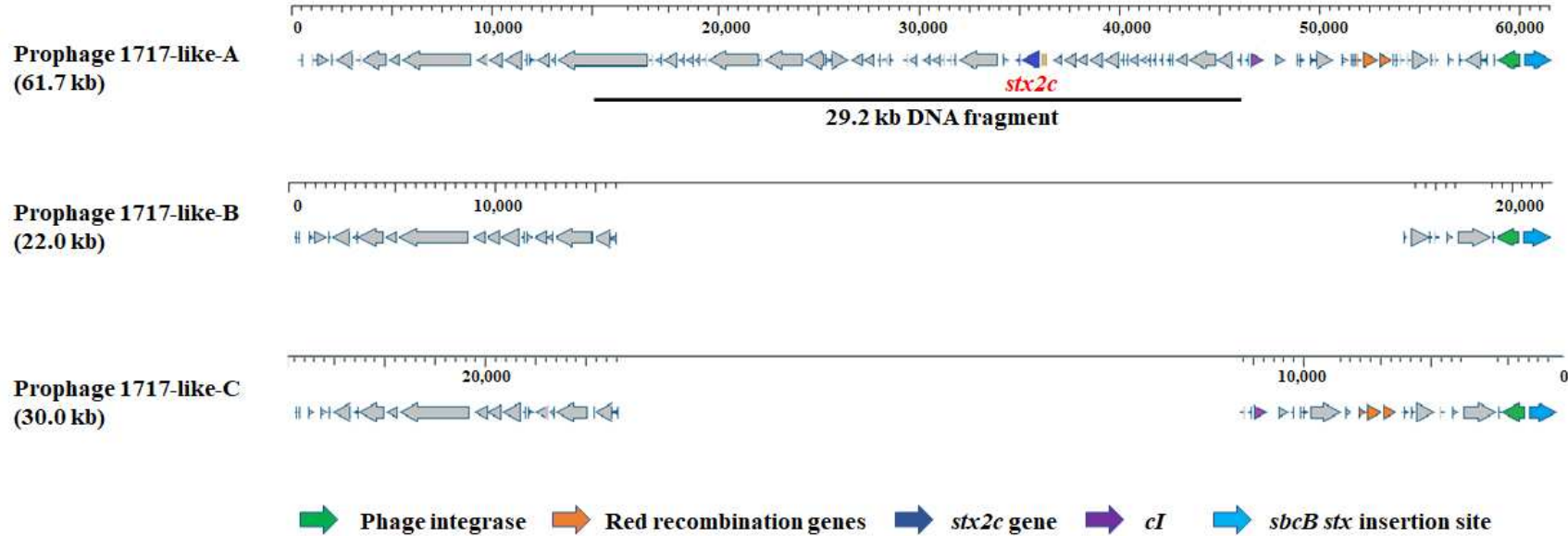


Figure 2.3. Prophage comparison at *sbcB stx* insertion sites in *Escherichia coli* O157:H7 strains used in this analysis. The *stx2c*-carrying prophages 1717-like-A was present in strain C1-010 (*stx1*⁻, *stx2c*). The *stx*-negative prophages 1717-like-B and C were present in strains CV261 and NZRM3614, respectively. A 29.2 kb DNA fragment containing the *stx2c* gene was present in prophage 1717-like-A in strain C1-010, but this DNA fragment was absent in the *stx*-negative prophages 1717-like-B and -C in strains CV261 and NZRM3614, respectively.

Figure 4A

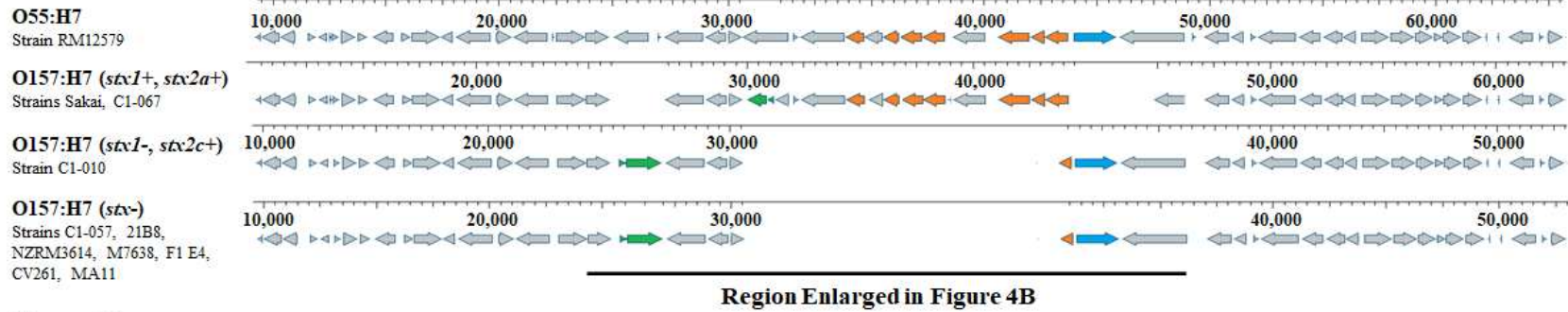


Figure 4B

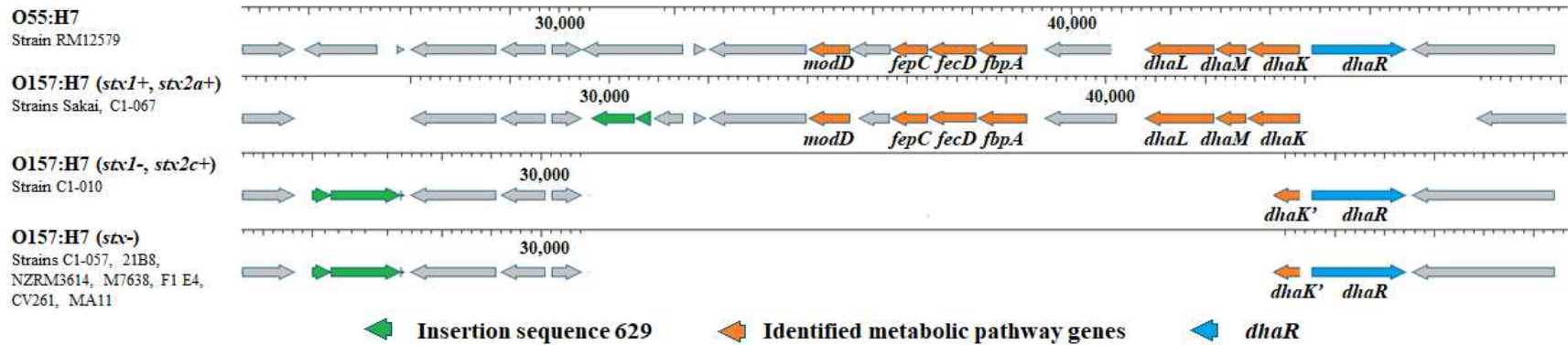


Figure 2.4. Gene arrangements around the specific IS629 and seven copies of genes involved in metabolic pathways between 10 *E. coli* O157:H7 strains used in this study and their ancestor *E. coli* O55:H7 strain RM12579.

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Chapter Three

Construct a Plasmid-mediated and a Phage-mediated System to Deliver CRISPR-Cas9 lethal modifications for Sequence-Specific Removal of STEC in Livestock Production

Summary

In this study, we conducted five experiments to develop a plasmid-mediated and a phage-mediated system to deliver Shiga toxin-specific CRISPR-Cas9 lethal modification for sequence-specific removal of foodborne pathogen Shiga toxin-producing *E. coli* (STEC). For the plasmid-mediated CRISPR-Cas9 lethal modification, we designed three gRNAs targeting *stx1* at different locations and two gRNAs targeting *stx2* at different locations. The plasmid-mediated CRISPR-Cas9 lethal modification with single gRNAs targeting either *stx1* or *stx2* reduced populations of STEC by 0.38 to 2.64 log CFU/reaction. This result showed that the cell reductions of the CRISPR-Cas9 lethal modification could be varied when different gRNAs were used, indicating that cell reductions of the CRISPR-Cas9 lethal modification could be optimized by the designing of gRNAs targeting to the specific genes in the pathogens. Additionally, the plasmid-mediated CRISPR-Cas9 lethal modifications with two gRNAs targeting both *stx1* and *stx2* achieved higher cell population reductions (3.20 log CFU/reaction). These results indicated that the CRISPR-Cas9 lethal modification could be programmed to target multiple sequence locations on the chromosomes of target bacteria, in order to maximize the cell reductions of CRISPR-Cas9 lethal modification. Alternatively, for the phage-mediated CRISPR-Cas9 lethal modification, we constructed a phagemid carrying the CRISPR-Cas9 lethal modification with two gRNAs targeting both *stx1* and *stx2*. This constructed phagemid was packaged into the helper phage M13K07, and the antimicrobial efficacy of this phage was evaluated in fresh rumen fluid environment. At MOI of 25 and 0.25, phage-mediated CRISPR-Cas9 lethal modification reduced

STEC populations by 2.52 and 2.74 log CFU/reaction, respectively. These results indicate that the phage-mediated CRISPR-Cas9 lethal modification could achieve sequence-specific destruction of target pathogens in a complex microbial environment. Overall, our study provided the proof-of-concept evidence that plasmid- and phage-mediated CRISPR-Cas9 lethal modification could be programmed to achieve sequence-specific removal of target pathogens by targeting selected genes in target pathogens. This indicates that the CRISPR-Cas9 lethal modifications could achieve sequence specific removal of pathogens, suggesting that it could potentially be developed into interventions to control foodborne pathogens in livestock production and processing environment.

Introduction

In bacteria, the CRISPR (Clustered, regularly interspaced, short palindromic repeats) and CRISPR associated (Cas) genes serve as the acquired immune system to eliminate foreign DNA such as plasmid and phage DNA (Barrangou et al., 2007; Bikard & Barrangou, 2017; Sorek, et al., 2013). Previous studies reported that the programmed CRISPR-Cas9 systems could be used for genome editing in both prokaryotic (Huang et al., 2016; Wang et al., 2016) and eukaryotic cells (Haapaniemi, et al., 2018; Osakabe et al., 2016). The Type II CRISPR-Cas9 system only requires one Cas9-gRNA complex protein to scan, bind and cleave the target DNA sequence (Makarova et al., 2011). The specificity of Cas9 endonuclease is guided by a designed guide RNA (gRNA) consisting of a trans-activating small RNA (tracrRNA) and CRISPR RNA (crRNA) harboring a spacer sequence that is complementary to the target gene (Bikard et al., 2014a; Brouns et al., 2008). In addition to the genome editing, CRISPR-Cas9 systems can be developed into sequence-specific lethal modification to eliminate the specific bacterial cells

harboring the genes of choices. The CRISPR-Cas9 lethal modification was reported that gRNAs could efficiently guide Cas9 to cleave all copies of target genes on the chromosomes in *Escherichia coli* (*E. coli*), inactivating the self-repairing via homologous recombination and resulting in cell death (Cui, L. and Bikard, D. 2016). Previous studies demonstrated that the plasmid- and phage-mediated CRISPR-Cas9 lethal modification can be programmed to target antibiotic resistance genes in *E. coli* (Bikard et al., 2014a; Citorik, et al., 2014; Yosef, et al., 2015) and to target methicillin-resistant genes in *Staphylococcus aureus* (Bikard et al., 2014a; Park et al., 2017) for sequence-specific cleavage of target genes.

Cattle are the one of the primary reservoirs of pathogenic *E. coli* O157:H7 (Ferens & Hovde, 2011). Pathogenic *E. coli* O157:H7 are often implicated in foodborne illness and are known to cause more than 96,000 cases of diarrheal illness and 3,200 hospitalizations annually in the United States (Scallan et al., 2011). In some cases, *E. coli* O157:H7 can causes severe illness, including hemolytic uremic syndrome (HUS). The *stx* gene (encoding Shiga toxin) harbored in pathogenic *E. coli* O157:H7 plays an important role in the pathogenesis of HUS (Paton & Paton, 1998). In this study, we constructed a plasmid-mediate system and a phage-mediate system to deliver programmed CRISPR-Cas9 lethal modification into the Shiga toxin-producing *E. coli* O157:H7 (STEC), in order to achieve the sequence-specific removal the *stx* gene and cell death. The objective of this study is to provide the proof-of-concept evidence that the programmed CRISPR-Cas9 lethal modification can selectively eliminate pathogens by targeting specific genes of choice. The results from this study may help to develop potential strategies to reduce pathogenic *E. coli* O157:H7 in livestock production.

Materials and Methods

Bacterial strains and growth conditions

Four *E. coli* O157:H7 strains, Sakai (*stxI*+, *stx2*+), C1-010 (*stx2*+), C1-158 (*stxI*+), and C1-057 (*stx*-) were used in this study. Strains C1-010, C1-158 and C1-057 were obtained from an initial sample population of 788 steer rectal samples, which represented various pulsed field gel electrophoresis types and *stx* profiles (described in Carlson et al., 2009). In addition, strain Sakai (ATCC® BAA460™) is a typical foodborne *E. coli* O157:H7 pathogen causing a large-scale outbreak in Japan. All strains were activated from 20% glycerol frozen stocks (-80 °C) by two transfers in tryptic soy broth (TSB) (Difco, Spark, MD) at 37 °C for 24 h and were subsequently subculture on tryptic soy agar (TSA) (Difco, Spark, MD) at 37 °C for 24h. Each activated strain was kept on TSA plates at 4 °C until uses.

The plasmid-mediated CRISPR-Cas9 lethal modification

We conducted three experiments to evaluate the potential uses of CRISPR-Cas9 lethal modification against STEC *in vitro*. In experiment 1 (Exp. 1), one gRNA targeting *stxI* was designed and cloned into the CRISPR plasmid (pCRISPR), to construct the pCRISPR containing gRNA targeting *stxI* (pCRISPR w/*stx1_1*). Subsequently, the pCRISPR w/*stx1_1* was delivered into the *E. coli* O157:H7 strain Sakai containing plasmid Cas9 (pCas9), in order to determine if the plasmid-mediated CRISPR-Cas9 lethal modification could reduce the population of STEC *in vitro*. In experiment 2 (Exp. 2), two additional gRNA targeting *stxI* at different locations (*stx1_2* and *stx1_3*) and two additional gRNA targeting *stx2* at different locations (*stx2_1* and *stx2_2*) were designed and cloned into pCRISPR, respectively. The pCRISPR with each of gRNA were introduced into strain Sakai containing pCas9 respectively, and their cell reductions were evaluated individually. In experiment 3 (Exp. 3), two gRNAs (*stx1_1* and *stx2_1*) were cloned into a single pCRISPR to construct a pCRISPR containing two gRNAs

(pCRISPR::stx1_1::stx2_1) that could target both *stx1* and *stx2*. The antimicrobial efficacy of the pCRISPR::stx1_1::stx2_1 was compared with the pCRISPR with single gRNA (pCRISPR w/stx1_1 and pCRISPR w/stx2_1).

Exp. 1: Determine the CRISPR-Cas9 antimicrobial against STEC *in vitro*

In this study, a two-plasmid platform, pCas9 and pCRISPR w/stx1_1, was used to construct the Shiga toxin-specific CRISPR-Cas9 lethal modification for specific destruction of STEC following the methods described in Jiang, et al., (2013) with modification. In bacterial cells, pCas9 could express the Cas9 protein constitutively, and pCRISPR with gRNA targeting *stx* genes could guide the Cas9 protein to the sequence-specific sites on *stx* genes and create double-stranded DNA breaks, resulting in cell death. Both pCas9 and original pCRISPR were purchased from Addgene, Inc. (Watertown, MA).

The gRNA was designed by screening *stx1* sequence from strain Sakai, where the target sequence was 20-nt and was followed by the protospacer adjacent motif sequence (5'-NGG-3', where N is any nucleobase). The gRNA stx1_1 was cloned into the pCRISPR in three steps. First, the pCRISPR was digested by the restriction enzyme BsaI (New England Biolabs, Ipswich, MA). Then, the digested pCRISPR was purified using a Monarch gel extraction kit (New England Biolabs, Ipswich, MA). Next, the gRNA stx1_1 was ligated with the digested pCRISPR to construct a new plasmid pCRISPR w/stx1_1. Finally, the sequence of the cloned region was confirmed by Sanger sequencing service provided by Genewiz, Inc (South Plainfield, NJ). In addition to the pCRISPR w/stx1_1, a pCRISPR containing an oligo (pCRISPR w/oligo) that

could not target any DNA sequence in *E. coli* O157:H7 strain Sakai was constructed as negative control.

The Shiga toxin-specific CRISPR-Cas9 lethal modification was introduced into *E. coli* O157:H7 strain Sakai that contained both *stx1* and *stx2*, by delivering pCas9 and pCRISPR w/stx1_1 sequentially via electroporation. First the pCas9 was delivered into the recipient Sakai cells, and then the pCRISPR w/stx1_1 was introduced into the recipient cells containing pCas9. More specifically, first *E. coli* O157:H7 strain Sakai cells were grown to a A_{600} of 0.6 to 0.8. Next, approximately 100 ng of pCas9 were transformed into bacterial cells using 0.1 cm Gene Pulser® cuvettes (Bio-Rad, Inc.) and the Electroporator 2510 (Eppendorf, Inc.) set at 1800 V. Recipient cells containing pCas9 were selected by plating onto the TSA + chloramphenicol (50µg/mL) (resistance in pCas9) plates. Subsequently, pCRISPR w/stx1_1 was introduced into the recipient cells containing pCas9 by electroporation using the same method. After electroporation, the transformed cells were plated onto the TSA + chloramphenicol (50µg/mL) (resistance in pCas9) + kanamycin (50µg/mL) (resistance in pCRISPR) plates and incubated for 24h at 37°C to enumerate surviving cells (n=2). In addition, pCRISPR w/oligo and original pCRISPR were introduced into the recipient cells containing pCas9 respectively as negative controls.

Exp. 2: Evaluate the antimicrobial efficacies of five CRISPR-Cas9 lethal modifications with single gRNAs for the sequence-specific removals of pathogenic *E. coli* O157:H7 strain Sakai

Two additional gRNAs targeting *stx1* at different locations (stx1_2 and stx1_3) and two gRNAs targeting *stx2* at different locations (stx2_1 and stx2_2) were designed and cloned into

pCRISPR separately using the methods described in Exp. 1. Next, pCas9 and each of the five of pCRISPR with different gRNAs (stx1_1, stx1_2, stx1_3, stx2_1, stx2_2) were sequentially and individually introduced into the *E. coli* O157:H7 strain Sakai by electroporation following the method described in Exp. 1. After electroporation, the transformed cells were plated onto the TSA + chloramphenicol (50µg/mL) (resistance in pCas9) + kanamycin (50µg/mL) (resistance in pCRISPR) plates and incubated for 24h at 37°C to enumerate surviving cells. Each of five pCRISPR with single gRNA was evaluated four times (n=4). Additionally, as negative control, original pCRISPR without any gRNA were also introduced into the recipient cells containing pCas9 as described above.

Exp. 3: Compare the antimicrobial efficacies between the CRISPR-Cas9 lethal modification with two gRNAs and the CRISPR-Cas9 lethal modifications with single gRNA for the elimination of pathogenic *E. coli* O157:H7 strain Sakai

As shown in Exp. 2 (Table 2), the CRISPR-Cas9 lethal modification with gRNA stx1_1 exhibited the greatest cell reduction within three gRNAs targeting *stx1*, while the CRISPR-Cas9 antimicrobials with gRNA stx2_1 showed the greatest reduction within two gRNAs targeting to the *stx2*. Therefore, in Exp. 3, these two gRNAs were selected and cloned into the pCRISPR to construct a new pCRISPR with two gRNAs that could target both *stx1* and *stx2* (pCRISPR w/stx1_1::stx2_1). To compare its sequence-specific antimicrobial efficacy with the CRISPR-Cas9 lethal modification containing single gRNA, the new constructed pCRISPR w/stx1_1::stx2_1, and pCRISPR w/stx1_1 and pCRISPR w/stx2_1 were introduced into the *E. coli* O157:H7 strain Sakai cells containing pCas9 by electroporation individually as described in Exp. 1. After electroporation, the transformed cells were plated onto the TSA + chloramphenicol

(50µg/mL) (resistance in pCas9) + kanamycin (50µg/mL) (resistance in pCRISPR) plates and incubated for 24h at 37°C. Following incubation, surviving cells were enumerated. The pCRISPR with two gRNAs and each of pCRISPR with single gRNA were evaluated four times (n=4). In addition, as negative control, the original pCRISPR without gRNA was delivered into the recipient cells containing pCas9 as described above.

The phage-mediated CRISPR-Cas9 lethal modification

In this study, we conducted two experiments to construct a phage-mediated CRISPR-Cas9 lethal modification which could be used to eliminate STEC by targeting both *stx1* and *stx2* genes. In experiment 4 (Exp. 4), we constructed a phagemid that could produce both Cas9 protein and two gRNAs targeting both *stx1* and *stx2* genes. Subsequently, the constructed phagemid was introduced into three *stx*-positive and one *stx*-negative *E. coli* O157:H7 strains via electroporation to evaluate its antimicrobial efficacy. In experiment 5 (Exp. 5), the constructed phagemid was packaged into the M13K07 phage. The antimicrobial efficacy of the M13K07 phage carrying the constructed phagemid was evaluated for its sequence-specific removal of pathogenic *E. coli* O157:H7 strain Sakai in the fresh cattle rumen fluid environment.

Exp. 4: Construct and evaluate the antimicrobial efficacy of a phagemid containing both *cas9* gene and two gRNAs targeting *stx1* and *stx2* genes for its Shiga-toxin removal of STEC strains

In Exp. 4, in order to construct the phagemid carrying two gRNAs targeting both *stx1* and *stx2* genes, a ~5-kb DNA fragment containing *tracrRNA*, *cas9*, *crRNA* and two 20-bp spacers from previously designed gRNAs (*stx1_1* targeting the *stx1* gene and *stx2_1* targeting the *stx2*

gene) were synthesized based on the genomic sequence of *Streptococcus pyogenes* M1GAS with modifications (Bikard et al., 2014a). These two spacers were chosen since they exhibited highest cell reductions of STEC in Exp. 2. More specifically, the gRNA stx1_1 showed the highest cell reductions of STEC within three gRNAs targeting *stx1* gene. The gRNA stx2_1 exhibited the highest cell reductions of STEC within two gRNAs targeting *stx2* gene. The synthesized DNA fragment was cloned into the phagemid vector pBluescript SK+ by Agilent Technologies, Inc. (Santa Clara, CA), to construct the pBluescript-F1 that carried two gRNAs both *stx1* and *stx2* genes (Figure 1a). In addition, a DNA fragment containing tracrRNA, *cas9*, crRNA but without any spacer sequence was synthesized and cloned into the vector pBluescript SK (+)(Agilent Technologies, Inc.) to construct a negative control phagemid, pBluescript-F2, that could not target any DNA sequences of *E. coli* O157:H7 (Figure 1b).

To evaluate the antimicrobial efficacy of the constructed phagemid pBluescript-F1 (with two gRNAs stx1_1 and stx2_1), this phagemid was separately introduced into four *E. coli* O157:H7 strains that had different *stx* gene profiles, including *stx*-positive strains Sakai (both *stx1*+ and *stx2*+), C1-010 (*stx2*+) and C1-158 (*stx1*+), and *stx*-negative strain C1-057, using the method described in Exp. 1. After electroporation, all transformed cells were plated onto TSA + ampicillin (50µg/mL) (resistance in pBluescript) to enumerate surviving cells (n=3). Furthermore, the phagemid pBluescript-F2 was individually introduced into those four *E. coli* O157:H7 strains as negative controls as described above.

Exp. 5: Evaluate the antimicrobial efficacy of phage-mediated CRISPR-Cas9 lethal modification for the Shiga toxin-specific removal of STEC in fresh cattle rumen fluid

In Exp. 5, the two constructed phagemids pBluescript-F1 and pBluescript-F2 were separately packaged into the helper phage M13K07 (New England Biolabs, Ipswich, MA) based on the company protocols with modifications. First, these two phagemids were separately introduced into the F-plasmid-containing (F^+) *E. coli* strain XL1-Blue MRF' (Agilent Technologies, Santa Clara, CA) by electroporation as described in Exp. 1. Next, the M13K07 helper phages packaged the two constructed phagemids separately from the transformant cells containing pBluescript-F1 or pBluescript-F2. Finally, a plaque-forming unit (PFU) assay was performed onto 2-YT agar (Invitrogen, Carlsbad, CA) plates to enumerate the number of plaques, in order to determine the concentrations of the helper phages M13K07 containing phagemid pBluescript-F1 (with two gRNAs *stx1_1* and *stx2_1*) or pBluescript-F2 (without gRNA).

The antimicrobial efficacy of phage-mediated CRISPR-Cas9 lethal modification was evaluated in fresh cattle rumen fluid. Prior to the experiment, the F^- strain Sakai obtained the F-plasmid by conjugation with the *E. coli* strains NEB 5-alpha F' Iq (New England Biolabs, Ipswich, MA) since M13K07 phage can only effectively infect F^+ *E. coli* cells. The F^+ strain Sakai cells were grown in Luria-Bertani (LB) broth (Difco, Spark, MD) + tetracycline (12.5 μ g/mL) (resistance in F-plasmid) to an OD600 of 1.0 before dilution in LB broth with 5 mM $MgCl_2$. Fresh rumen fluid was purchased from Bar Diamond Inc. (Parma, ID). The concentration of natural microbial flora was determined (10^7 CFU/reaction) prior to the experiment (data not shown). The diluted F^+ strain Sakai cells at the concentration of 10^6 CFU/reaction were inoculated into the fresh rumen fluid. Next, the inoculated fresh rumen fluid was infected by the phage M13K07 containing pBluescript-F1 (with two gRNAs targeting both *stx1* and *stx2*) and with phage M13K07 containing pBluescript-F2 (without gRNA) separately, at

two levels of multiplicity of infection (MOI): 25 and 0.25. After 1 h incubation, cells were plated onto the LB + tetracycline (12.5µg/mL) (resistance in F-plasmid) + ampicillin (50µg/mL) (resistance in pBluescript) plates to enumerate surviving cells (n=3).

Statistical Analysis

Microbiological were converted to log CFU/reaction prior to the statistical analysis. Data were subjected to analysis of variance and to compute least square means via the GLIMMIX procedure of SAS (SAS Studio Basic Edition 3.8, SAS Institute, Inc., Cary, N.C.). For both plasmid- and phage-mediated CRISPR-Cas9 lethal modification experiments, significant differences between means were determined with the significance at the $P < 0.05$.

Results

Sequence-specific destruction of STEC using a plasmid-mediated CRISPR-Cas9 lethal modification (Exp. 1)

Table 1 presents the number of surviving cells after electroporation of pCRISPR w/stx1_1, pCRISPR w/oligo and original pCRISPR into the recipient *E. coli* O157:H7 cells containing pCas9. When the pCRISPR w/stx1_1 was introduced into the recipient STEC cells containing pCas9, surviving cells were significantly ($P < 0.05$) lower (2.10 log CFU/reaction) compared to the negative control plasmids of pCRISPR w/oligo (4.24 log CFU/reaction) and original pCRISPR (4.13 log CFU/reaction). This suggests that introducing the Shiga toxin-specific CRISPR-Cas9 lethal modification to STEC cells could result in cleavage of *stx* on chromosome, causing death of STEC cells. However, surviving cells were not significantly

different ($P > 0.05$) when introducing the pCRISPR w/oligo and the original pCRISPR into recipient STEC cells containing pCas9. These results indicate that cloning an oligo that could not target any DNA sequence in STEC into the pCRISPR had minor impact on the number of surviving cells.

In Exp. 2, we compared the antimicrobial efficacies of five *stx* specific CRISPR-Cas9 lethal modification for the sequence-specific removal of the pathogenic *E. coli* O157:H7 strain Sakai (Figure 2). Among three CRISPR-Cas9 lethal modifications containing the gRNAs targeting the *stxI* gene (*stx1_1*, *stx1_2*, and *stx1_3*), no differences ($P > 0.05$) were found in cell reductions of the gRNA *stx1_1* (2.08 log CFU/reaction reduction) and the gRNA *stx1_2* (1.53 log CFU/reaction reduction). However, cell reduction of the gRNA *stx1_3* (0.38 log CFU/reaction reduction) was significantly lower ($P < 0.05$) than the cell reductions of gRNA *stx1_1* or gRNA *stx1_2*. In addition, for the cell reductions of gRNA *stx2_1* (2.64 log CFU/reaction) and gRNA *stx2_2* (2.23 log CFU/reaction), no significant differences ($P > 0.05$) were observed. Since gRNA *stx1_1* exhibited the greatest cell reduction within three gRNAs targeting the *stxI*, and gRNA *stx2_1* was observed with the greatest cell reduction with two gRNAs targeting the *stx2*, these two gRNAs were selected to be used in Exp. 3.

Table 2 showed the results of Exp. 3. The newly constructed pCRISPR w/*stx1_1::stx2_1* could target both *stxI* and *stx2*, leading to the simultaneous cleavage of the STEC chromosome at both of these two gene sites. When introducing the original pCRISPR into the recipient STEC cells containing pCas9, we observed that the surviving cells (4.71 log CFU/reaction) were significantly higher ($P < 0.05$) compared to the pCRISPR with two gRNAs (pCRISPR w/*stx1_1::stx2_1*) or the pCRISPR with single gRNAs (pCRISPR w/*stx1_1* or *stx2_1*). Furthermore, surviving recipient STEC cells transformed with pCRISPR with two gRNAs (1.51

log CFU/reaction) were significantly lower ($P < 0.05$) than the surviving recipient STEC cells containing pCRISPR with single gRNA (2.57 log CFU/reaction for pCRISPR w/stx1_1; 2.19 log CFU/reaction for pCRISPR w/stx2_1).

Antimicrobial efficacies of the phagemid pBluescript-F1 against three *stx*-positive *E. coli* O157:H7 strains

In Exp. 4, the constructed phagemid pBluescript-F1 (targeting both *stx1* and *stx2*) and pBluescript-F2 (without gRNA) was introduced into strains with different *stx* profiles (Figure 3). After introducing the pBluescript-F1 or pBluescript-F2 into each of STEC strains (Sakai, C1-010 and C1-158), number of surviving cells containing pBluescript-F1 (targeting both *stx1* and *stx2*) were significantly lower ($P < 0.05$) than the number of surviving cells containing pBluescript-F2 (without gRNA) within each strain. The cell reduction of strain Sakai (3.33 log CFU/reaction) containing two *stx* genes was greater than the cell reductions of strains C1-010 (2.08 log CFU/reaction) and C1-158 (2.37 log CFU/reaction) carrying single *stx* gene. In contrast, after introducing the pBluescript-F1 or pBluescript-F2 into the *stx*-negative strain C1-057, the number of surviving cells containing pBluescript-F1 (targeting both *stx1* and *stx2*) was not significantly different ($P > 0.05$) with the number of surviving cells containing pBluescript-F2 (without gRNA). This result indicated that phagemid pBluescript-F1 containing Shiga toxin-specific CRISPR-Cas9 lethal modification had little effect on survival of the bacterial cells without the *stx* gene.

Sequence-specific reduction of the phage-mediated CRISPR-Cas9 lethal modification to STEC in fresh cattle rumen fluid environment, with two levels of multiplicity of infection

In Exp. 5, the antimicrobial efficacy of the phage-mediated CRISPR-Cas9 lethal modification targeting *stx1* and *stx2* was evaluated for their Shiga toxin-specific removal of F+ strain Sakai in fresh cattle rumen fluid (Table 3). At an MOI of 25, surviving STEC cells infected by phage M13K07 carrying pBluescript-F1 (4.44 ± 0.10 log CFU/reaction) were significantly lower ($P < 0.05$) than surviving STEC infected by phage M13K07 carrying pBluescript-F2 (6.96 ± 0.10 log CFU/reaction). In addition, at an MOI of 0.25, surviving STEC cells infected by phage M13K07 containing pBluescript-F1 (2.70 ± 0.12 log CFU/reaction) were also significantly lower ($P < 0.05$) than surviving STEC infected by phage M13K07 containing pBluescript-F2 (5.44 ± 0.12 log CFU/reaction). These results indicated that M13K07 carrying pBluescript-F1 reduced the approximately 3 log CFU/reaction of STEC cells in fresh cattle rumen fluid at both MOI of 25 and 0.25.

Discussion

Antimicrobial efficacies of the plasmid-mediated CRISPR-Cas9 lethal modifications was determined by gRNAs

In Exp. 1 through 3, we demonstrated the Shiga toxin-specific removal of STEC strains using a plasmid-mediated CRISPR-Cas9 lethal modification. In Exp. 1, we observed an approximately 2 log fewer *stx*-carrying *E. coli* O157:H7 when the pCRISPR w/*stx1_1* was introduced into the recipient STEC cells containing the pCas9, compared to that of the original pCRISPR and pCRISPR w/oligo (Table 1). These results provided the evidence that the programmed plasmid-mediated CRISPR-Cas9 lethal modification containing the gRNA targeting the *stx* gene could destroy the *E. coli* harboring *stx* genes.

In Exp. 2, three gRNAs targeting *stx1* at different locations and two gRNAs targeting *stx2* at different locations were designed and cloned into pCRISPR, respectively. Our results demonstrate that antimicrobial efficacies of the CRISPR-Cas9 lethal modification targeting STEC could be affected when different gRNAs were used, suggesting that the designing of gRNAs were critical factors that determine antimicrobial efficacies of the CRISPR-Cas9 lethal modification targeting to STEC. Therefore, the antimicrobial efficacy of CRISPR-Cas9 lethal modification could be improved by optimizing the designing of gRNAs.

The CRISPR-Cas9 lethal modification could possibly be programmed with two or more gRNAs targeting different locations at chromosome, to potentially increased the antimicrobial efficacy to the target bacteria (Bikard et al., 2014b). Subsequently, in Exp. 3, the cell destruction efficacy of the CRISPR-Cas9 lethal modification with two gRNAs (*stx1_1* and *stx2_2*) was compared with the efficacies of the CRISPR-Cas9 lethal modification with single gRNA (*stx1_1* or *stx2_2*). Our results indicate that the CRISPR-Cas9 lethal modification could be programmed to target multiple sequence locations on the chromosomes of target bacteria, to maximize the cell destruction efficacy of CRISPR-Cas9 lethal modification.

In Exp. 1 through 3, we provided the evidence that STEC cells could be eliminated using the programmed plasmid-mediated CRISPR-Cas9 lethal modification by targeting the *stx* genes. However, introducing plasmids into target pathogenic cells by electroporation is not a practical method to deliver CRISPR-Cas9 lethal modification. Alternatively, bacteriophage has been used to deliver CRISPR-Cas9 lethal modification for sequence-specific destruction of target bacterial cells (Bikard et al., 2014b; Jiang, et al., 2013). For the following experiments, we exploited the phage-mediated CRISPR-Cas9 lethal modification to eliminate STEC in fresh cattle rumen fluid environment, and its antimicrobial efficacy was evaluated.

The phage-mediated CRISPR-Cas9 lethal modification can be used for sequence-specific removal of STEC in fresh cattle rumen fluid

We constructed a phagemid pBluescript-F1 that carried the CRISPR-Cas9 lethal modification with two gRNAs targeting both *stx1* and *stx2* genes that could be used to target STEC. Our results (Exp. 4) demonstrated significantly fewer ($P < 0.05$) surviving cells containing pCRISPR with two gRNAs than cells containing pCRISPR with single gRNA. These results indicate the constructed phagemid pBluescript-F1 could achieve the Shiga toxin-specific killing of STEC.

In Exp. 5, the phagemid pBluescript-F1 was packaged into the helper phage M13K07, and the antimicrobial efficacy of the phage with pBluescript-F1 was evaluated in fresh cattle rumen fluid. At MOI of 25 and 0.25, significant cell reductions were observed, from 6.96 ± 0.10 to 4.44 ± 0.10 log CFU/reaction, and from 5.44 ± 0.12 to 2.70 ± 0.12 log CFU/reaction, respectively. This result indicated that this phage-mediated CRISPR-Cas9 lethal modification achieved the Shiga toxin-specific destruction of STEC in fresh cattle rumen fluid, suggesting that cell destruction efficacies of this phage-mediated CRISPR-Cas9 lethal modification were not interfered by the fresh cattle rumen fluid at MOI of 25 and 0.25.

Our study suggests the antimicrobial efficacies of the phages carrying CRISPR-Cas9 lethal modifications were not interfered by fresh cattle rumen fluid. This indicated that the phage-mediated CRISPR-Cas9 lethal modification may be used to eliminate pathogens in complex microbial communities. We foresee that CRISPR-Cas9 lethal modification can be further developed as an intervention to reduce foodborne pathogens in livestock production. For

example, the phage-mediated CRISPR-Cas9 lethal modification can be potentially developed into sequence-specific phage spray antimicrobials to reduce the specific pathogens on carcasses. In addition, the phage-mediated CRISPR-Cas9 lethal modification can also be potentially developed as feed additives, to reduce the prevalence of specific pathogens that could cause disease to ruminant animals and improve animal health.

Challenge of using CRISPR-Cas9 lethal modifications

While our study demonstrated that antimicrobial efficiencies of the phage-mediated CRISPR-Cas9 lethal modification was great ($> 2 \log$ CFU/reaction in cell reduction, as shown in Exp. 5), a fraction of the transformed cells survived from the treatments. Resistance to the CRISPR-Cas9 lethal modification might be related to the mutations or deletions of the *cas9* gene and deletions of spacer sequences in the phagemid during phage infection, resulting to the inactivation of the CRISPR-Cas9 lethal modification (Bikard, et al, 2012; Jiang, et al., 2013). Furthermore, van Houte et al., (2016) indicated that target bacteria could escape the recognizing of gRNAs when point mutations occur on the targeted genes. To overcome the resistance to CRISPR-Cas9 lethal modification, multiple gRNAs targeting various sequences from multiple genes at different locations can be designed, to minimize the inactivation rate of the CRISPR-Cas9 lethal modification caused by the loss of spacers or the point mutations on the target genes.

In addition to the resistance to the CRISPR-Cas9 lethal modification, the efficacy of delivering CRISPR-Cas9 lethal modification could be limited when target bacteria resist to the phage infection (Pursey, et al., 2018). The potential strategy to overcome this challenge is to use

a phage cocktail containing multiple types of helper phages carrying the CRISPR-Cas9 lethal modification, to minimize the phage resistance by target bacteria.

Conclusion

Our study demonstrated that programmed plasmid- and phage-mediated CRISPR-Cas9 lethal modification could be used to eliminate foodborne pathogen STEC by targeting *stx1* and *stx2* genes on its chromosome. The plasmid-mediated CRISPR-Cas9 lethal modification with single gRNAs targeting either *stx1* or *stx2* reduced cell populations of STEC by 0.38 to 2.64 log CFU/reaction. This result showed that the antimicrobial efficacies of the CRISPR-Cas9 lethal modification could be varied when different gRNAs were used, indicating that antimicrobial efficacies of the CRISPR-Cas9 lethal modification could be optimized by the designing of gRNAs targeting specific genes in the pathogens. Additionally, the plasmid-mediated CRISPR-Cas9 lethal modification with two gRNAs targeting both *stx1* and *stx2* achieved a greater reduction (3.20 log CFU/reaction). This result suggests that the CRISPR-Cas9 lethal modification could be programmed to target multiple sequence locations on the chromosomes of target bacteria, to maximize the antimicrobial efficacy of CRISPR-Cas9 lethal modification. Furthermore, for the phage-mediated CRISPR-Cas9 lethal modification, M13K07 carrying pBluescript-F1 (targeting both *stx1* and *stx2*) reduced the approximately 3 log CFU/reaction of STEC cells compared with M13K07 carrying pBluescript-F2 (without gRNA) in fresh cattle rumen fluid at both MOI of 25 and 0.25. This indicated that phage-mediated CRISPR-Cas9 lethal modification may be used to eliminate pathogens in complex microbial environment. Overall, our study provided the proof-of-concept evidence that plasmid- and phage-mediated CRISPR-Cas9 lethal modification could be programmed to achieve sequence-specific removal of target

pathogens by targeting the genes of choice. The CRISPR-Cas9 lethal modification could potentially be developed into interventions for the purpose of controlling foodborne pathogens in livestock production and processing environment.

Table 3.1. Surviving cells (Log CFU/reaction) after electroporation of original pCRISPR, pCRISPR w/oligo, pCRISPR w/stx1_1 into the strain Sakai recipient cells containing pCas9 (n=2).

	Original pCRISPR	pCRISPR w/oligo	pCRISPR w/stx1_1
Log CFU/reaction	4.13 ^a	4.24 ^a	2.10 ^b

^{a-b} Least square means of bacterial counts with different letters are significantly different (P < 0.05)

Table 3.2. Surviving cells (Log CFU/reaction) after electroporation of pCRISPR with single or two gRNAs targeting *stx1* and *stx2* into the *E. coli* O157:H7 strain Sakai recipient cells containing pCas9 (n=4).

pCRISPR with different gRNA	Trail 1	Trial 2	Trail 3	Trail 4	Means \pm SE
Original pCRISPR	5.18	4.39	4.79	4.47	4.71 \pm 0.19 A
pCRISPR w/stx1_1	2.65	2.50	2.87	2.27	2.57 \pm 0.19 B
pCRISPR w/stx2_2	2.63	2.20	2.40	1.53	2.19 \pm 0.19 B
pCRISPR w/stx1_1::stx2_1	1.59	1.27	2.01	1.16	1.51 \pm 0.19 C

A-C: Least square means of bacterial counts with different upper-case letters are significantly different (P < 0.05)

Table 3.3. Surviving cells (log CFU/reaction) after infection of *E. coli* O157:H7 F+ strain Sakai cells in cattle rumen fluid containing 10^7 CFU/reaction natural microflora, with the phages carrying the pBluescript-F1 (targeting both *stx1* and *stx2*) and the negative control phages carrying pBluescript-F2 (without gRNA), at two levels of multiplicity of infection (n=3).

Phagemid	MOI	Log CFU/reaction			Mean \pm SE
		Trail 1	Trail 2	Trail 3	
pBluescript-F2 (without gRNA)	25	7.09	7.10	6.68	6.96 \pm 0.10A ^a
pBluescript-F1 (targeting <i>stx1</i> and <i>stx2</i>)	25	4.39	4.47	4.46	4.44 \pm 0.10B
pBluescript-F2 (without gRNA)	0.25	5.17	5.45	5.71	5.44 \pm 0.12a ^b
pBluescript-F1 (targeting <i>stx1</i> and <i>stx2</i>)	0.25	2.74	2.77	2.60	2.70 \pm 0.12b

^a Differing uppercase letters within a column are significantly different.

^b Differing lowercase letters within a column are significantly different.

Figure 1a

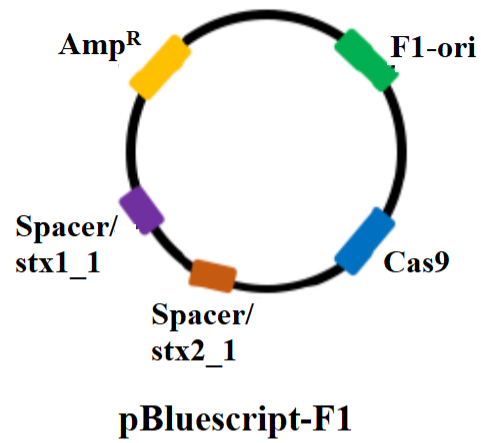


Figure 1b

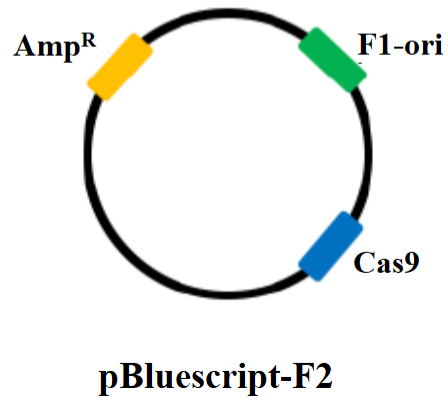


Figure 3.1. The constructed phagemids. pBluescript-F1 carries *tracrRNA*, *cas9*, *crRNA* and two 20-bp spacers that targeting both *stx1* and *stx2* genes (Figure 1a). pBluescript-F2 contains *tracrRNA*, *cas9*, *crRNA* but without any spacer sequence (Figure 1b).

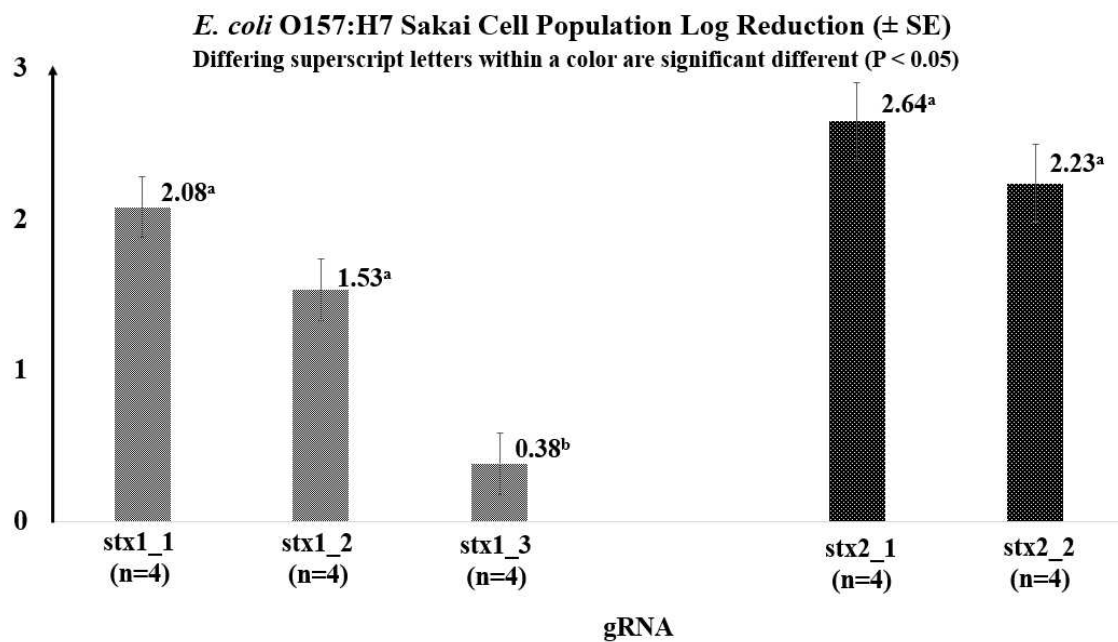


Figure 3.2. Log CFU/reaction reduction after electroporation of pCRISPR with different single gRNAs into the *E. coli* O157:H7 strain Sakai recipients cells containing pCas9. Different superscript letters within the gRNAs targeting same *stx* genes are significantly different ($P < 0.05$) (n=4).

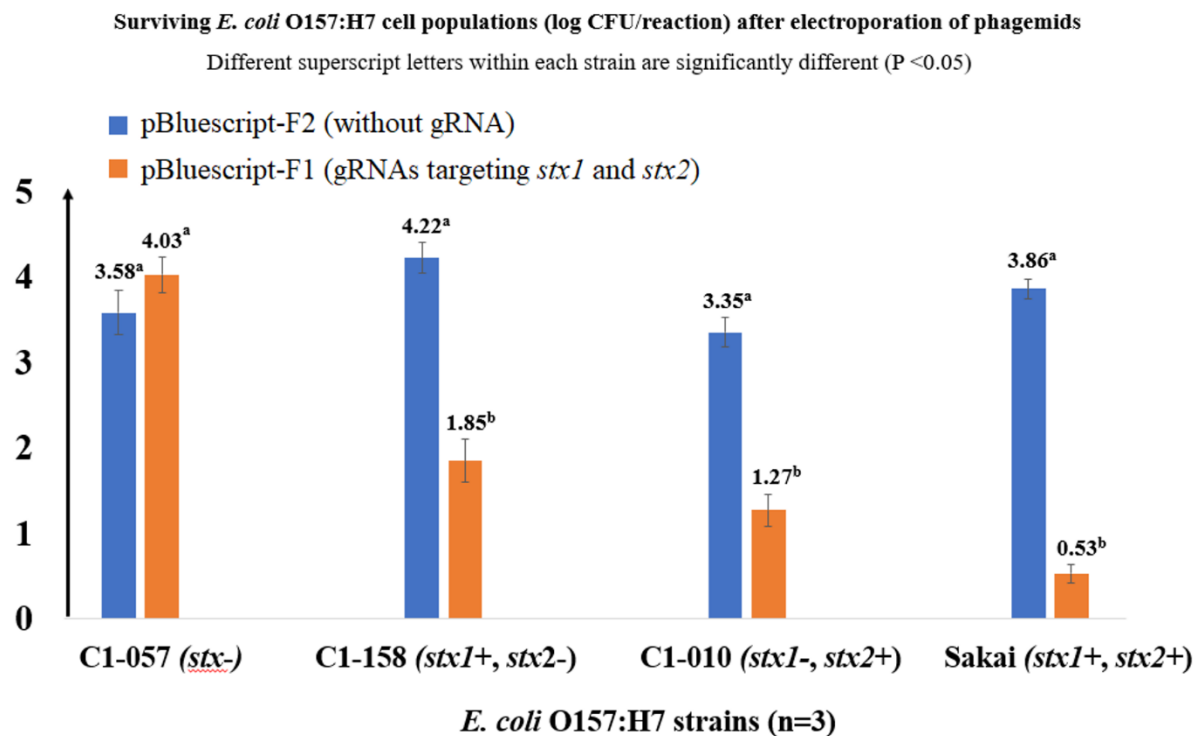


Figure 3.3. Surviving *E. coli* O157:H7 cell populations (log CFU/reaction) after electroporation of phagemid pBluescript-F1 (with two gRNAs targeting *stx1* and *stx2*) or pBluescript-F2 (without gRNAs) into the recipient cells containing pCas9. Different superscript letters within each strain are significantly different (P < 0.05). Four *E. coli* O157:H7 strains were used to represent different *stx* profiles: C1-057 (*stxI*-, *stx2*-), C1-158 (*stxI*+, *stx2*-), C1-010 (*stxI*-, *stx2*+), and Sakai (*stxI*+, *stx2*+) (n=3).

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Chapter Four

Antimicrobial Effects of Herb Extracts Against Foodborne Pathogen *Listeria monocytogenes* in vitro

Summary

Herb extract is a type of well-known natural antimicrobial from plants. Food Drug Administration recognized that most herb extracts as Generally Recognized as Safe for human consumption. The objective of this study is to conduct three experiments and to evaluate the inhibitory and bactericidal effects of nine herb extracts against five representative strains of *Listeria monocytogenes* in vitro. In the experiment 1, each of herb extracts 2, 4, 5, 8 exhibited inhibitory effects against five strains of *L. monocytogenes* individually at 37°C in Mueller-Hinton broth (MHB). The MIC values of those four herb extracts ranged between 5 – 50 mg/mL. In experiment 2, herb extract 4, which showed the lowest MIC value (5 mg/mL), reduced populations of *L. monocytogenes* in a range of 0.38 – 0.91 log CFU/mL after 30 min treatment at 37°C in MHB, indicating that herb extract 4 may not expected to be used as an antimicrobial agent for the purpose of reducing *L. monocytogenes* within a short period of time. In experiment 3, at concentrations of 1.56 and 0.78 mg/mL, herb extracts 2, 4, 5, 8 inhibited the growth of a five-strain *L. monocytogenes* cocktail individually at the abused refrigerator temperature of 12°C, except herb extract 8 at the concentration of 0.78 mg/mL. At a concentration of 3.13 mg/mL, those four herb extracts reduce cell populations in a range of 2.2 to 1.6 mg/mL at 11 days. Herb extracts 2, 4, 5 and 8 could be potentially developed into food preservatives for controlling foodborne *L. monocytogenes*.

Introduction

Listeria monocytogenes is a gram-positive foodborne pathogen that is widely distributed during food preparation, storage, and distribution. A variety of ready-to-eat (RTE) foods such as milk, cheeses, ice cream, raw meat, fresh vegetable and fruits may be contaminated with *Listeria monocytogenes* (FDA, 2003; Doyle & Buchanan, 2012). Consumption of foods contaminated with *L. monocytogenes* is linked to an increased risk of listeriosis. To control *L. monocytogenes* in food products, meat industry uses chemical preservatives such as sodium acetate, sodium lactate and various nitrites. However, it is acknowledged that uses of chemical antimicrobials have increased the consumer concerns and created a demand for “natural” and “minimally processed” food. As a result, there has been a great interest in natural antimicrobial agents.

Plant-derived extracts have been used since ancient times, especially in China (Shen, 1996) and India (Ahmad, Mehmood, & Mohammad, 1998; Chopra & Nayar, 1956). In addition to the uses as flavoring material, plant extracts and essential oils represent a natural alternative in the nutritional, pharmaceutical, and agricultural fields. Due to their antimicrobial properties, plant extracts have been suggested to be used as antioxidant and preservatives in food products, to incorporate into food packaging materials, plant and crop protectants against insect pests, and medicinal products for human and livestock (Turek & Stintzing, 2013). In recent times, plant extracts have gained great interests especially in food industry. Most plant extracts are classified as generally recognized as safe (GARS) by U.S Food and Drug Administration and are easily degradable in human body (Berahou et al., 2007; Ogbulie, et al., 2007). Previous studies have been proven that many spices and plant essential oils exhibited inhibition and/or bactericidal effects against *L. monocytogenes* in food products. For example, cinnamon essential oil and oregano reduced the growth rate of *L. monocytogenes* by 10% and 19% respectively in ham at

4°C (Dussault, Vu, & Lacroix, 2014). Thyme and clove essential oils reduced populations of *L. monocytogenes* in zero-fat beef hotdogs by 1.3 log CFU/g and 1.0 log CFU/g respectively with 5 min treatment at room temperature (21°C) (Singh, et al., 2003). The objective of this study is to evaluate potential inhibitory and bactericidal effects of nine herb extracts (HEs) against foodborne pathogenic *L. monocytogenes* in vitro in order to select natural antimicrobial agents for the control of foodborne *L. monocytogenes* in food products.

Materials and Methods

Experimental design

HEs can be used to inhibit the growth of foodborne pathogen and/or reduce pathogen populations. In this study, we conducted three experiments to evaluate their potential uses as antimicrobial agents in food products. In experiment 1 (Exp. 1), a minimum inhibitory concentration (MIC) study was conducted to compare inhibitory effects of each of nine HEs against each of five *L. monocytogenes* strains individually in Mueller-Hinton broth (MHB). In experiment 2 (Exp. 2), the HE with the lowest MIC was used to determine its reductions of each of five *L. monocytogenes* strains individually at 37°C for 30 min in MHB. In experiment 3 (Exp. 3), those HEs which could inhibit the *L. monocytogenes* growth in Exp. 1 were evaluated for their inhibitory effects against a five-strain *L. monocytogenes* cocktail in MHB up to 11 days at 12°C.

Bacterial strains and growth conditions

Five strains of *L. monocytogenes* which isolated from epidemics were used in this study and are listed in Table 1. According to Fugett et al., 2006, these five *L. monocytogenes* strains were selected from a total of 46 strains which represented a genetic diversity of ribotypes, pulsed-field gel electrophoresis types, serotypes, and lineages. In addition, these five strains are believed to cover the genetic diversity of human disease-associated *L. monocytogenes* and to provide a valuable tool for evaluating the effectiveness of antimicrobials to inactivate or inhibit *L. monocytogenes*. Therefore, we used these five genetically distinct strains of *L. monocytogenes* to evaluate inhibitory and bactericidal efficacies of nine HEs. All strains were activated from 20% glycerol frozen stocks (-80°C) by two transfers in tryptic soy broth (TSB) (Difco, Spark, MD) at 37°C for 24 h and were subsequently subcultured on tryptic soy agar (TSA) (Difco, Spark, MD) at 37°C for 24 h. Each activated strain was kept on TSA plates at 4°C.

Herb extracts preparation

A total of nine types of herbs were obtained in the form of powder. Each of nine herbs was extracted with sterile deionized water followed by the procedure of Nzeako, et al., (2006) with modification. The HEs were prepared before the day of experiment. Each of the HEs was made by combining 10 g of each herb powder with 90 mL of sterile deionized water, incubating in a water bath at 45°C for 30 minutes, and then boiling for 15 minutes. Each of the nine HEs was then cooled to room temperature and was centrifuged at 6000 x g for 15 minutes at room temperature (Thermo Scientific Sorvall Legend X1R Centrifuge, Am Kalkberg, Germany). The

supernatant of each HE was transferred into a 50 mL polypropylene tube and stored at 4°C until use next day.

Exp. 1: determining MICs of the HEs

Each strain of the *L. monocytogenes* listed in Table 1 was inoculated in TSB individually and was incubated at 37°C for 24 h. After the incubation, each strain was serially diluted in MHB (Difco, Spark, MD) to approximately 10^6 CFU/mL. Nine HEs were diluted with the sterile deionized water to six concentration levels: 100, 60, 30, 15, 10, 5 mg/mL. Five mL of each diluted strain was mixed with 5 mL of each diluted HEs in glass sterile test tubes, to make the final concentrations to be 50, 30, 15, 7.5, 5, 2.5 mg/mL for each HEs and approximately 5×10^5 CFU/mL for each strain. Negative control samples were prepared by combining 5 mL of each of nine diluted HEs with 5 mL of MHB separately to make the same final herb concentrations for each HE listed above but without inoculum. Positive control samples were prepared by combining 5 mL of each diluted strain with 5 mL of MHB separately to make same final bacteria concentrations for each diluted strain listed above but without any HE. All tubes were subsequently incubated at 37°C for 24 h. After 24 h incubation, all treatment and control samples were visually examined. The lowest herb concentration at which each treatment sample did not show turbidity were designated as the MIC. All tests were performed in two independent replication trails with three samples on each trail (n=6).

Exp. 2: reduction of *L. monocytogenes* cells treated with the HE 4

The HE 4 exhibited inhibitory effect against *L. monocytogenes* with the lowest MIC in Exp. 1. In this experiment, the HE 4 was determined for its reduction of *L. monocytogenes* cells. After the 24 h incubation, each strain was serially diluted in MHB to approximate concentration of 10^6 CFU/mL. The HE 4 was diluted in sterile deionized water to the concentration of 50 mg/mL. Two mL of each of diluted *L. monocytogenes* strains was combined with 2 mL of the diluted the HE 4 separately, to make a final concentration of 25 mg/mL of the HE and approximate 5×10^5 CFU/mL of each strain. The positive control samples were prepared by combining 2 mL of sterile deionized water and 2 mL of each of the five diluted strains separately, to make the same concentrations of each strain as the treatment samples but without HE 4. All treatment and control samples were incubated for 30 min at 37°C. Our preliminary data showed that the HE 4 exhibited the best reductions against each of five *L. monocytogenes* strains at 37°C (data not shown). In a previous published study, thyme and clover have been reported to reduce populations of *L. monocytogenes* after 5 min treatment in peptone water at room temperature (21°C) (Singh, et al., 2003). In our study, each of five strains were treated 30 min with HE 4, which was six times longer than Singh's study. After 30 min treatment, all treatment samples were immediately diluted with sterile deionized water to a concentration of 0.25 mg/mL for the HE 4, in order to terminate its further antimicrobial activity. Our preliminary study has shown at the concentration of 0.25 mg/mL, the HE 4 could not inhibit the growth of each of five *L. monocytogenes* strains (data now shown). Each of treatment and positive control samples was subsequently serially diluted in 0.1% buffered peptone water (BPW) and each diluted sample were then plated onto tryptic soy agar (TSA) with two duplications. The TSA plates were then incubated for 48 h at 37°C to enumerate the numbers of surviving *L.*

monocytogenes cells. All tests were performed in two independent replication trails with two samples on each trail (n=4).

Exp. 3: antimicrobial effects of HEs against *L. monocytogenes* cocktail at abused refrigerated temperature

In Exp. 1, HEs 2, 4, 5 and 8 which inhibited *L. monocytogenes* growth at or below 50 mg/mL concentrations. In this experiment, those four HEs were evaluated for their inhibitory effects at 12°C, which represents the abused refrigeration temperature. Each of the five *L. monocytogenes* strains listed in Table 1 was cultured in TSB separately for 24 h at 37°C. A five-strain *L. monocytogenes* cocktail was prepared prior to the study. A 10-mL volume of each 24 h grown culture was pooled and mixed in a 50 mL sterile falcon tube. After centrifugation at 6000 x g for 15 min at 4°C, the supernatant was removed. The cell pellet was washed once with a 10-mL volume of phosphate-buffered saline (PBS), and subsequently resuspended in 50 mL PBS. The *L. monocytogenes* cocktail was serially diluted in MHB to an approximate 5×10^2 CFU/mL concentration.

The HEs 2, 4, 5, and 8 were diluted in sterile deionized water to three levels of concentrations, 6.25, 3.13 and 1.56 mg/mL. Concentrations of HEs were determined based on the preliminary data (data not shown). The treatment samples were prepared by combining 2 mL of each of four diluted HEs and 2 mL of the diluted *L. monocytogenes* cocktail separately in glass test tubes, to make the final concentrations of each of the four HEs at three levels, 3.13, 1.56 and 0.78 mg/mL, and approximately 2.5×10^2 CFU/mL of the *L. monocytogenes* cocktail. The positive control samples were prepared by combining 2 mL of diluted *L. monocytogenes*

cocktail and 2 mL of sterile deionized water separately but without any HE. Surviving cells from control samples were enumerated immediately after inoculation (day 0). All treatment and control samples were incubated for up to 11 days at 12°C. All samples were serially diluted in 0.1% BPW and subsequently plated onto two duplicate TSA plates daily from day 1 to day 5, and every two days from day 7 to day 11. TSA plates were incubated for 48 h at 37°C to enumerate the numbers of surviving *L. monocytogenes* cells. Each treatment sample and control sample were performed in three independent replication trails with two samples on each trail (n=6).

Statistical analysis

Microbiological data were converted to log CFU/mL prior to the statistical analysis. Statistical analyses were conducted using analysis of variance via the glimmix procedure of SAS (SAS Studio Basic Edition 3.8, SAS Institute, Inc., Cary, N.C.). Least squares means were calculated and significant differences between means were detected at the $P < 0.05$ in the Exp. 2 and at $P < 0.001$ in the Exp. 3.

Results and Discussion

MICs of nine herb extracts

MIC is defined as the lowest concentration of an antimicrobial agent which prevents visible microbial growth under designed conditions (Andrews, 2001). In this study, the visible microbial growth was determined by comparing the turbidity between treatment samples and

control samples after 24 h incubation at 37°C. The MIC value for each of the nine HEs against each strain are shown in Table 2. Four HEs 2, 4, 5 and 8 inhibited the growth of the five *L. monocytogenes* strains at MIC values ranging from 5 to 50 mg/mL. The other five HEs 1, 3, 6, 7, 9 did not exhibited inhibition effects at up to 50 mg/mL. Based on the MIC values, the inhibitory effects of those four HEs were ranked from the strongest to weakest as follows: HE 4 (5 mg/mL) > HE 5 (15 mg/mL) > HE 2 (15-30 mg/mL) > HE 8 (50 mg/mL).

The sensitivity to different natural antimicrobials varies in some Gram-positive and Gram-negative bacteria. For example, studies have shown that Gram-positive *L. monocytogenes* were more sensitive to some essential oils and HEs than Gram-negative *E. coli* and *Salmonella enterica Enteritidis* (Chorianopoulos et al., 2004; Mangena & Muyima, 1999; Ouattara, et al., 1997); The *Ocimum sanctum* extract was found to be equally effective against Gram-negative bacteria (*E. coli*, *S. typhimurium* and *P. aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus*) (Mishra & Mishra, 2011); however, Gram-negative pathogens, *V. parahaemolyticus* and *S. typhimurium*, were more sensitive to eugenol than Gram-positive *S. aureus* (Karapinar & Aktuğ, 1987). As a result of Exp. 1, four out of nine HEs inhibited the growth of *L. monocytogenes*. Further studies can be conducted to evaluate and compare the antimicrobial effects of those nine HEs against other foodborne Gram-positive and Gram-negative pathogens.

Reduction of *L. monocytogenes* cells treated with HE 4

In Exp. 2, the HE 4 was chosen to evaluate its reductions of five *L. monocytogenes* strains individually at 37°C for 30 min treatment since HE 4 exhibited the strongest inhibition effect with the lowest MIC (5 mg/mL) in Exp. 1. After 30 min incubation with HE 4 at a

concentration of 25 mg/mL, differences ($P < 0.05$) of surviving cells between treatment samples and control samples were observed for each of five *L. monocytogenes* strains (Figure 1). Cell reductions of HE 4 against five *L. monocytogenes* strains were calculated: N1-227 (0.91 log CFU/mL), C1-056 (0.87 log CFU/mL), R2-499 (0.85 log CFU/mL), J1-177 (0.59 log CFU/mL), N3-013 (0.38 log CFU/mL).

In a previous published study, at the concentrations of 0.5 mL/L, essential oils of thyme and clover have been reported to reduce populations of *L. monocytogenes* from 7.2 to 1.8 log CFU/mL and from 7.1 to 1.2 log CFU/mL respectively after 5 min treatment in peptone water at room temperature (21°C) (Singh, et al., 2003). In addition, another study indicated that essential oil of origanum reduced populations of each of five *L. monocytogenes* strains in a range of 1-2 log CFU/mL after 30 min treatment in 0.9% saline solution at room temperature (Aureli, Costantini, & Zolea, 1992). In our study, Although HE 4 reduced less than 1 log CFU/mL for each strain, populations of surviving cells of each strain were significant ($P < 0.05$) after HE4 treatment compared with control samples. The result indicated that using HE 4 solely against *L. monocytogenes* might be less effective than essential oils of thyme, clover and origanum. However, there has been increased interests to the use natural antimicrobial agents in their combinations for controlling foodborne pathogens. The effects of the combined substances were observed to be greater than the sum of individual effects against *L. monocytogenes* in combinations of carvacrol/linalool (Bassolé et al., 2010) and oregano/rosemary (De Azeredo et al., 2011). HE 4 was expected to be used in combination with other compounds to increase antimicrobial effects.

Inhibitory effects and reductions of four herb extracts against *L. monocytogenes* cocktail at abused refrigerated temperature

Since HE 2, 4, 5 and 8 exhibited inhibitory effects against *L. monocytogenes* at 37°C in Exp. 1, we expected that those four HEs could inhibit *L. monocytogenes* growth at 12°C, which represented to the abused refrigerator temperature. We investigated the antimicrobial effects of HEs 2, 4, 5 and 8 at three concentration levels (3.13, 1.56, 0.78 mg/mL) against a five-strain *L. monocytogenes* cocktail. The initial populations of *L. monocytogenes* cocktail in control and all treatment samples were 2.3 log CFU/mL. For control samples without any HE, bacteria population rapidly increased from 2.3 log CFU/mL (day 0) to 8.4 log CFU/mL by 4 days, and then increased to 8.8 log CFU/mL by day 7. After 7 days, bacteria population did not have further increase in number. For treatment samples, the growth of *L. monocytogenes* during refrigerated storage was dependent on the type of herb and HE concentration. In general, compared with control samples, lower bacteria populations ($P < 0.001$) were observed in all treatments except for the HE 8 at the concentration of 0.78 mg/mL (Table 3 to 5).

At a concentration of 3.13 mg/mL (Table 3), HEs 2, 4, 5 and 8 reduced inoculated *L. monocytogenes* populations from 2.3 log CFU/mL to 0.2, 0.1, 0.7 and 0.5 log CFU/mL at day 11, respectively. Compared with positive control samples without any HE, each of four HEs had lower bacterial population ($P < 0.001$) on each day from day 1 to day 11. This result indicated that at the concentration of 3.13 mg/mL, all four HEs effectively reduced bacteria populations of *L. monocytogenes* cocktail at 12°C.

At the concentration of 1.56 mg/mL (Table 4), HE 2 reduced *L. monocytogenes* populations from 2.3 log CFU/mL to 0.2 log CFU/mL at day 11, which was 8.6 log CFU/mL lower ($P < 0.001$) than the control. Although counts of *L. monocytogenes* in the sample with HE

5 increased from 2.3 log CFU/mL to 4.0 log CFU/mL at day 11, it was still 4.8 log CFU/mL lower ($P < 0.001$) than the control. However, compared with the control, HE 4 and 8 were lower ($P < 0.001$) in bacteria populations only up to 5 days. Therefore, antimicrobial effects of those four HE at concentration of 1.56 mg/mL were ranked from the strongest to weakest as follows: HE 2 > HE 5 > HE 4 = HE 8.

Table 5 shows the inhibitory effects of each of the four HEs in MHB at the concentration of 0.78 mg/mL. Counts of the *L. monocytogenes* cocktail in the sample with HE 8 were not different ($P > 0.001$) from the control sample on each day from day 1 to day 11, indicating that HE 8 at a concentration of 0.78 mg/mL could not inhibit bacterial growth. Counts of samples with HE 4 or 5 increased from 2.3 log CFU/ mL to 6.7 and 6.4 log CFU/mL by day 4 respectively, which were lower ($P < 0.001$) than the control by about 2 log CFU/mL. After 4 days of incubation, the bacterial population of the sample with HE 4 were not different ($P > 0.001$) with the positive control sample on each day from day 5 to day 11. Although the sample with HE 5 did not show different ($P > 0.001$) in bacteria population with the positive control sample at day 5 and day 7, the population of HE 5 was 0.5 log CFU/mL lower ($P < 0.001$) than the control at day 9 and day 11. In addition, comparing with positive control samples, HE 2 slowed the microbial growth and reached to 5.6 log CFU/mL by day 5, which was lower than the controls for 3 log CFU/mL ($P < 0.001$). After 7 days of incubation, the bacterial population of the sample with HE 2 were not different ($P > 0.001$) with the control sample on each day from day 7 to day 11. In summary, at the concentrations of 0.78 mg/mL, HE 2 inhibited the microbial growth up to 5 days; HE 4 and 5 inhibited *L. monocytogenes* growth up to 4 days; HE 8 could not inhibit the microbial growth.

The demand for convenience foods such as RTE foods has increased in recent years. The majority of listeriosis cases are foodborne (Scallan et al., 2011) and linked to the consumption of RTE foods which are contaminated with *L. monocytogenes*. Due to the high mortality rate of listeriosis, the U.S. Department of Agriculture and the FDA labels *L. monocytogenes* as an adulterant of RTE foods. Examples of RTE foods that support the growth of *L. monocytogenes* are milk, high fat dairy products, soft unripened cheese, cooked and raw seafood, deli-type salads, sandwiches, fresh-cut vegetable and fruits (FDA, 2017) and the processed meat which is under refrigerator conditions (Donnelly, 2001). Although *L. monocytogenes* will continue to thrive at low temperature as 1.1 °C (Junttila, Niemelä, & Hirn, 1988), the storage temperature and duration of refrigerated storage before consumption are important factors which reduce the risks of foodborne listeriosis (Godwin, et al., 2007). The recommended refrigerator temperature is 40°F (4.4°C); however, abuse home refrigerator temperature can rise to more than 12°C (Godwin, et al., 2007; James & Evans, 1992).

Previous published studies indicated that the inhibitory efficacies of plant-derived antimicrobials may be affected by temperature (Campo, et al., 2000; TING & DEIBEL, 1991). The results from Exp. 1 showed that HEs 2, 4, 5 and 8 exhibited inhibitory effects against each of five *L. monocytogenes* strains at 37°C. However, in order to use those four HEs as food preservatives, they must be effective against *L. monocytogenes* under food storage conditions. In this experiment, inhibition efficacies of those four HEs were evaluated at 12°C which represented the abused refrigerator temperature. As discussed above, at concentrations of 1.56 and 0.78 mg/mL, HEs 2, 4, 5 and 8 inhibited growth of a five-strain *L. monocytogenes* cocktail at abuse refrigeration temperature of 12°C, except herb extract 8 at the concentration of 0.78 mg/mL. At a concentration of 3.13 mg/mL, these four HEs reduced cell populations in a range of

2.2 to 1.6 log CFU/mL at 11 days. In a previous study, thyme essential oil showed the inhibitory effect against *L. monocytogenes* cocktails at 10°C up to 12 days in minced beef (Solomakos, et al., 2008). HEs 2, 4, 5 and 8 were also expected to be developed into food preservatives for inhibiting and/or reducing foodborne *L. monocytogenes*. For example, those four HEs could be added to RTE foods as supplements or incorporated into food packaging materials to control *L. monocytogenes* growth. Further experiments should be conducted to determine the inhibitory effects and reductions of those four HEs in food products. In addition, since HEs carry specific odor, palatability of the food applied with HEs should be evaluated by sensory panel.

Conclusions

In summary, HEs 2, 4, 5 and 8 exhibited inhibitory effects against *L. monocytogenes* at 37°C in a range of MIC between 5 – 50 mg/mL. HE 4 reduced cell populations of each selected strain ranged between 0.38 – 0.91 log CFU/mL after 30 min treatment at 37°C. In addition, at concentrations of 1.56 and 0.78 mg/mL, HEs 2, 4, 5 and 8 inhibited growth of a five-strain *L. monocytogenes* cocktail at 12°C, except the HE 8 at the concentration of 0.78 mg/mL. At a concentration of 3.13 mg/mL, these four HEs reduced cell populations in a range of 2.2 to 1.6 log CFU/mL at 11 days. For their practical application, further experiments should be conducted to determine the inhibitory effects and reductions of those HEs in a variety of food products. In addition, palatability of the foods which applied with HEs should be evaluated by sensory panel.

Table 4.1. Bacterial strains used in the study (Fugett, et al., 2006).

Strain	Lineage	Serotype	Source
<i>L. monocytogenes</i>			
J1-177	I	1/2b	Human, sporadic
C1-056	II	1/2a	Human, sporadic
N3-013	I	4b	Food, epidemic
R2-499	II	1/2a	Human, epidemic
N1-227	I	4b	Food, epidemic

Table 4.2. Minimum inhibitory concentration of the nine herb extracts against five *L. monocytogenes* strains (n=6).

Strain	MIC values (mg/mL)								
	HE 1	HE 2	HE 3	HE 4	HE 5	HE 6	HE 7	HE 8	HE 9
<i>L. monocytogenes</i>									
J1-177	>50	30	>50	5	15	>50	>50	50	>50
C1-056	>50	30	>50	5	15	>50	>50	50	>50
N3-013	>50	15	>50	5	15	>50	>50	50	>50
R2-499	>50	15	>50	5	15	>50	>50	50	>50
N1-227	>50	15	>50	5	15	>50	>50	50	>50

HE: Herb extract

Table 4.3. Least squares means \pm standard deviation of *Listeria monocytogenes* cocktail populations in inoculated Mueller-Hinton broth with each of four herb extracts at concentration of 3.13 mg/mL or deionized water (control), stored at 12°C for 11 days (n=6).

Herb Extract	Bacterial populations (log CFU/mL) in days								
	0	1	2	3	4	5	7	9	11
Control	2.3 \pm 0.0	3.6 \pm 0.3 ^a	5.3 \pm 0.2 ^a	7.2 \pm 0.3 ^a	8.4 \pm 0.1 ^a	8.6 \pm 0.1 ^a	8.8 \pm 0.1 ^a	8.8 \pm 0.1 ^a	8.8 \pm 0.1 ^a
HE 2	-	2.0 \pm 0.1 ^b	1.2 \pm 0.3 ^c	0.9 \pm 0.3 ^b	0.7 \pm 0.3 ^b	0.4 \pm 0.3 ^b	0.2 \pm 0.3 ^c	0.1 \pm 0.2 ^c	0.2 \pm 0.3 ^b
HE 4	-	1.7 \pm 0.2 ^b	1.5 \pm 0.3 ^{bc}	1.1 \pm 0.4 ^b	0.9 \pm 0.5 ^b	0.8 \pm 0.7 ^b	0.9 \pm 0.1 ^{bc}	0.2 \pm 0.3 ^c	0.1 \pm 0.2 ^b
HE 5	-	2.3 \pm 0.1 ^b	2.2 \pm 0.0 ^b	2.1 \pm 0.1 ^b	1.9 \pm 0.1 ^b	1.8 \pm 0.1 ^b	1.5 \pm 0.1 ^b	1.3 \pm 0.1 ^b	0.7 \pm 0.2 ^b
HE 8	-	2.3 \pm 0.2 ^b	2.0 \pm 0.3 ^{bc}	1.8 \pm 0.5 ^b	1.5 \pm 0.7 ^b	1.5 \pm 0.6 ^b	1.1 \pm 0.6 ^{bc}	0.9 \pm 0.1 ^b	0.5 \pm 0.8 ^b

HE: Herb extract

^{a-c}: Means within a day that have a common lowercase letter are not significantly different ($P < 0.001$).

Table 4.4. Least squares means \pm standard deviation of *Listeria monocytogenes* cocktail populations in inoculated Mueller-Hinton broth with each of four herb extracts at concentration of 1.56 mg/mL or deionized water (control), stored at 12°C for 11 days (n=6).

Herb Extract	Bacterial populations (log CFU/mL) in days								
	0	1	2	3	4	5	7	9	11
Control	2.3 \pm 0.0	3.6 \pm 0.3 ^a	5.3 \pm 0.2 ^a	7.2 \pm 0.3 ^a	8.4 \pm 0.1 ^a	8.6 \pm 0.1 ^a	8.8 \pm 0.1^a	8.8 \pm 0.1^a	8.8 \pm 0.1^a
HE 2	-	1.9 \pm 0.1 ^c	1.1 \pm 0.4 ^c	1.1 \pm 0.1 ^c	0.8 \pm 0.2 ^c	0.8 \pm 0.1 ^c	0.3 \pm 0.5 ^c	0.1 \pm 0.2 ^c	0.2 \pm 0.4 ^c
HE 4	-	2.1 \pm 0.1 ^{bc}	2.2 \pm 0.4 ^{bc}	3.1 \pm 0.6 ^{bc}	4.3 \pm 0.9 ^b	5.9 \pm 0.9 ^b	8.4 \pm 0.2^a	8.4 \pm 0.1^a	8.4 \pm 0.2^a
HE 5	-	2.4 \pm 0.1 ^{bc}	2.5 \pm 0.1 ^{bc}	2.8 \pm 0.1 ^{bc}	2.9 \pm 0.1 ^{bc}	2.8 \pm 0.2 ^c	2.6 \pm 0.5 ^b	3.1 \pm 0.9 ^b	4.0 \pm 1.1 ^b
HE 8	-	2.7 \pm 0.2 ^b	3.4 \pm 0.6 ^b	4.5 \pm 1.2 ^b	4.6 \pm 1.2 ^b	5.2 \pm 1.0 ^b	7.8 \pm 0.9^a	8.6 \pm 0.1^a	8.6 \pm 0.1^a

HE: Herb extract

^{a-c}: Means within a day that have a common lowercase letter are not significantly different ($P < 0.001$).

Table 4.5. Least squares means \pm standard deviation of *Listeria monocytogenes* cocktail populations in inoculated Mueller-Hinton broth with each of four herb extracts at concentration of 0.78 mg/mL or deionized water (control), stored at 12°C for 11 days (n=6).

Herb Extract	Bacterial populations (log CFU/mL) in days								
	0	1	2	3	4	5	7	9	11
Control	2.3 ± 0.0	3.6 ± 0.3^a	5.3 ± 0.2^a	7.2 ± 0.3^a	8.4 ± 0.1^a	8.6 ± 0.1^a	8.8 ± 0.1^a	8.8 ± 0.1^a	8.8 ± 0.1^a
HE 2	-	2.3 ± 0.1 ^b	3.0 ± 0.1 ^b	3.7 ± 0.1 ^c	4.5 ± 0.1 ^c	5.6 ± 0.3 ^b	8.2 ± 0.1^a	8.6 ± 0.1^{ab}	8.6 ± 0.1^a
HE 4	-	2.6 ± 0.3 ^b	3.6 ± 1.0 ^{ab}	5.0 ± 1.0 ^{bc}	6.7 ± 0.7 ^b	7.8 ± 0.6^a	8.5 ± 0.2^a	8.5 ± 0.1^{ab}	8.7 ± 0.1^a
HE 5	-	2.6 ± 0.1 ^b	3.8 ± 0.2 ^{ab}	5.0 ± 0.0 ^{bc}	6.4 ± 0.2 ^b	7.8 ± 0.1^a	8.5 ± 0.2^a	8.3 ± 0.0 ^b	8.3 ± 0.1 ^b
HE 8	-	3.2 ± 0.3^{ab}	4.7 ± 0.3^{ab}	6.4 ± 0.2^{ab}	8.2 ± 0.2^a	8.5 ± 0.0^a	8.6 ± 0.2^a	8.6 ± 0.1^{ab}	8.8 ± 0.1^a

HE: Herb extract

^{a-c}: Means within a day that have a common lowercase letter are not significantly different ($P < 0.001$).

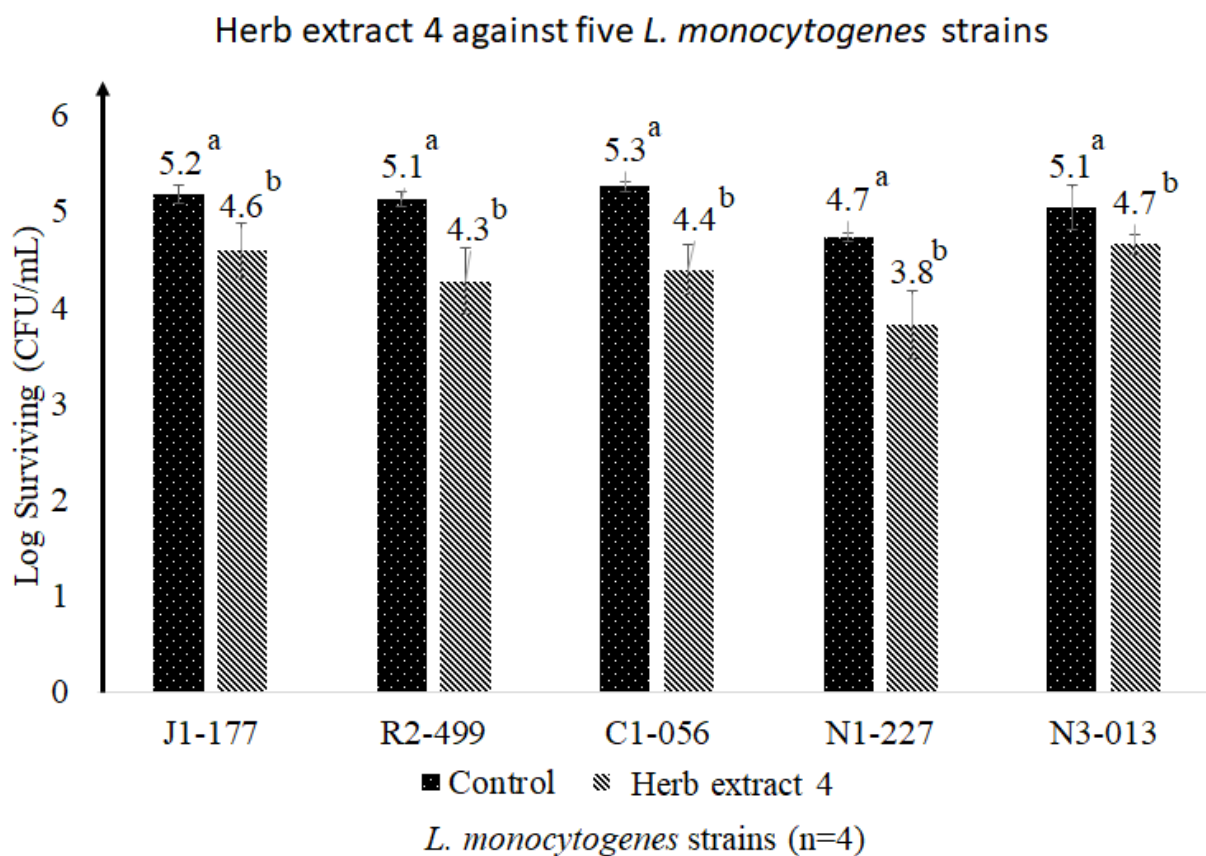


Figure 4.1. Number of surviving cells of five *Listeria monocytogenes* strains after treatment with herb extract 4 (25 mg/mL) for 30 min at 37°C (n=4).

^{a-b} Bars indicate standard deviations calculated from 4 replicates. Differing superscript letters within each strain are significantly different ($P < 0.05$).

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