Antimicrobial alternatives to control pathogens involved in liver abscesses, food safety and mastitis in cattle

by

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B.S., Purdue University, 2017

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
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Abstract

Antimicrobial agents are used to treat and prevent bacterial infections. In cattle, antimicrobials are most often used to control, treat, or prevent respiratory disease and liver abscesses in feedlot cattle and mastitis in dairy cows. Liver abscesses occur in feedlot cattle because of feeding an energy dense, high grain diet. The primary causative agent is *Fusobacterium necrophorum,* a ruminal bacterium that enters portal circulation to reach the liver and cause abscesses. Currently, in-feed tylosin is widely used to reduce the incidence of liver abscesses.

Mastitis, an economically important disease of dairy cows, is caused by a number of bacterial pathogens, mainly *Staphylococcus aureus, Streptococcus agalactiae, S. dysgalactiae, S. uberis, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Escherichia coli.* Beta-lactams, macrolides (erythromycin), and lincosamides are the common antimicrobials used for mastitis prevention and treatment. Additionally, foodborne illnesses are a major public health concern and food animals are major carriers of many foodborne pathogens. Three major foodborne bacterial pathogens that reside in the gut and are shed in the feces of cattle are Shiga toxin-producing *E. coli, Salmonella enterica, Campylobacter jejuni,* and *C. coli.* The most common antimicrobials used to treat *Salmonella* and *Campylobacter* infections in humans are fluoroquinolones, macrolides, cephalosporins, and diaminopyrimidine plus sulfa drug combinations.

Antimicrobial resistance (AMR) to antimicrobials, particularly to medically important antimicrobials, such as cephalosporins, fluoroquinolones, and macrolides, are a major public health concern. Antimicrobial resistance occurs in pathogens as well as in commensals. Because of a clear association between antimicrobial use and emergence of AMR, there are efforts to restrict and minimize the use of antimicrobials, and at the same time, if possible, replace with antimicrobial alternatives. In my studies, I have focused on the following three antimicrobial alternatives:

bacteriophages, clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated proteins (Cas), and sorghum grain phenolic extract. Therefore, the main objectives were to evaluate the potential of bacteriophages, CRISPR-Cas9, and phenolic extracts of sorghum grains to inhibit major pathogens of liver abscesses, mastitis, and foodborne illnesses associated with cattle.

Bacteriophages are viruses that infect bacteria and cause them to lyse (lytic bacteriophages) and are known for their host specificity. Therefore, we hypothesized that bacteriophages could be an effective tool to selectively target and eliminate or reduce the concentration of *F. necrophorum* in the rumen, thereby minimizing the chance for it to cross the ruminal epithelium to reach the liver to cause abscesses. Studies were conducted to isolate and characterize bacteriophages lytic to *F. necrophorum.* Pooled bovine ruminal fluids from slaughtered cattle at abattoirs and untreated city sewage samples were collected on five separate dates. A total of 68 *F. necrophorum* subsp. *necrophorum* strains of liver abscess origin were used to isolate bacteriophages. Aliquots of pooled ruminal fluid or sewage samples were incubated anaerobically overnight with lysine and subsp. *necrophorum* strains. The aliquots were then filtered, bacteria-free filtrates were spotted on lawns of the strains, and presence of plaques were considered as positive for phages. Presumptive bacteriophage plaques were harvested, and the viruses were purified by serial passaging on the susceptible bacterial strains. The bacteriophage isolation frequencies were compared between sample types, sampling dates, and *F. necrophorum* strains. The overall relative frequency of isolated bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* was 17.1 %. The frequency of bacteriophage isolation between collection dates ranged from 0 to 25.4 % for ruminal fluid, and from 13.7 to 32.0 % for sewage samples. The frequency of isolation of bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* from sewage samples was higher ($p < 0.0001$) than lytic

bacteriophages isolated from bovine ruminal fluid samples. Bacteriophages were isolated more frequently with certain *F. necrophorum* subsp. *necrophorum* strains than others. The characterization of the isolated bacteriophages included determination of host specificity, electron microscopic morphology, development of insensitivity, and optimal pH and temperature stability. All 25 bacteriophages were lytic to *F. necrophorum* subsp. *necrophorum* only and did not lyse subsp. *funduliforme* or any of the other bacterial species tested. Based on morphology determined by transmission electron microscopy, twenty bacteriophages belonged to *Siphoviridae, Myoviridae,* or *Podoviridae* families. Four bacteriophages with icosahedral heads and no tail and one with multiple tails could not be unclassified. In broth cultures, 11 bacteriophages inhibited *F. necrophorum* subsp. *necrophorum* up to 12 hrs, while the other 14 bacteriophages only slightly slowed the growth of the bacterium. *F. necrophorum* subsp. *necrophorum* became insensitive to all 11 lytic bacteriophages after 24 h. At a pH of 2.5, all 11 bacteriophages were inactivated immediately, while at a pH of 3.5, six bacteriophages were only partially inactivated upon initial exposure and five survived for 1 hr. Between pH 4.5- pH 8.5, no reduction in bacteriophage activity was observed. After 24 h of incubation at 30 °C, 40 °C, and 50 °C, lytic activities of the 11 bacteriophages were unaffected.

Bacteria use RNA-guided (gRNA) CRISPR-Cas systems to protect against foreign nucleic acids, particularly mobile genetic elements, including bacteriophages. Because CRISPR-Cas systems can be used to modify genetic material by precisely cutting target nucleotides, we hypothesized that the system could be used to inhibit *F. necrophorum* in the rumen or liver. The concept was first tested in *E. coli* with plasmids that had the *F. necrophorum rpsP* gene inserted. A 1.3- to 3.3-$\log_{10}$ reduction was observed when the *rpsP* gene was targeted from a plasmid in *E. coli*. The approach was then used to cleave the *fomA* gene that encodes an outer membrane protein

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Phenolic compounds have antimicrobial properties, and some specialty sorghum grain varieties are high in phenolic compounds. Therefore, sorghum grain phenolic extract may have the potential to be used as a natural antimicrobial alternative. The antimicrobial effects of sorghum phenolic extract on bacterial pathogens that cause bovine mastitis and human foodborne illnesses were determined. Bacterial pathogens tested included Shiga toxin-producing *Escherichia coli*, *Salmonella* Typhimurium, *Campylobacter jejuni*, *C. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *K. oxytoca*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Antibacterial activities of sorghum phenolic extracts at concentrations of 0, 100, 200, 500, 1,000, or 4,000 µg/mL were determined by agar well diffusion assay. Plates were incubated for 18-24 hours and the diameter of each zone of inhibition was measured. The results indicated that sorghum phenolic extract inhibited *Staphylococcus aureus*, *Enterococcus faecalis*, *Campylobacter jejuni*, and *Campylobacter coli*.

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Dedication

This long-time dream could not have come true without the support of my family, friends, and colleagues. I would like to devote my work to the people who stood by and supported me, even on the late nights and stressful days.

- My parents, Deana and Mike Hancock, and my husband, Luke for their unending love, support, and most of all, understanding.
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I could not have accomplished this dream without the help and confidence of these friends and family members.

Chapter 1 - Problems with Antibiotics in Cattle Production

Introduction

Antimicrobials are agents that either kill microbes or inhibit their normal functions such as reproduction or growth. Although antimicrobials are most often referred to as agents that impact bacteria, antimicrobials also include compounds that kill fungi, viruses, or protozoa. Antibiotics are a subset of antimicrobials that are active against various bacteria and are derived from microbial sources.

Antibiotics have been discovered and modified over the last century. One of the first antibiotics, Salvarsan, was discovered in 1909 by Ehrlich and was used to treat syphilis through oxidation caused by a mixture of arsenic bonded molecules (Taylor, 1942; Lloyd et al., 2005; Dougherty and Pucci, 2012). Fleming first published that *Penicillium* extracts had bacteriostatic properties against Gram-positive bacteria in 1929, but he could not isolate the antibacterial compound within the mold (Fleming, 2001; Dougherty and Pucci, 2012). In 1940, Ernst Chain was able to isolate and identify the compound, and by 1945, penicillin had proved to be a powerful antibiotic against Gram-positive bacteria in medicine (Clark, 1985; Dougherty and Pucci, 2012). A few years after the isolation of penicillin, in 1943, an antibiotic derived from soil bacteria and active against Gram-negative bacteria, streptomycin, was discovered in Waksman's lab (Dougherty and Pucci, 2012). In the years following, the discoveries of penicillin and streptomycin antibiotics were rapidly studied through various screening methods, and by the 1970s most major antibiotic classes had been discovered (Dougherty and Pucci, 2012).

As more natural antibiotics were being discovered, synthetic derivatives of these antibiotics were starting to be produced by pharmaceutical companies. These derivatives were made to have a broader spectrum compared to their natural predecessors. Today, third and fourth-generation

antimicrobials are used most often in medicine, and most are very broad-spectrum, working against Gram-positive and Gram-negative bacteria.

Antibiotic and antimicrobial use has rapidly increased since they were first approved in the 1940s to treat bacterial infections. Antimicrobials have been used in the United States and other countries to treat infections in both humans and animals for nearly a century. Food animal production has continually increased over time to feed a growing global population, and thus it has required an increased need for disease management (Mathew et al., 2007; Amachawadi, 2014). Furthermore, maximizing the efficiency of production at every step in the production chain is necessary for optimizing food animal production, and the use of antimicrobials provide an increase in production due to better animal health, improved feed efficiency, increased growth promotion, and reduced foodborne pathogens (Mathew et al., 2007; Amachawadi, 2014). There are four major uses of antimicrobials in cattle production (Landers et al., 2012):

1. Treatment (therapeutic)- treatment of clinically ill animals in the herd.
2. Control (metaphylactic)- treatment of clinically healthy animals in a herd with clinically ill animals.
3. Prevention (prophylactic)- treatment of clinically healthy animals to prevent disease.
4. Growth promotion- using antimicrobials in feed or water to promote growth or feed efficiency of animals. Not using antimicrobials for disease management, but for production purposes (FDA, 2012). Currently the use of human medically important antimicrobials is not allowed in food animal production for growth promotion in the United States (FDA, 2015).

For more than 60 years, antimicrobial use in livestock production has been routinely used to control and treat diseases, as well as for growth promotion (Amachawadi, 2014). However, antimicrobial resistance has become a major public health concern (FDA, 2012, 2015). Therefore, research is being conducted on antimicrobial alternatives for use in food animal production.

Antimicrobials in Cattle Production:

Antimicrobials are commonly used in food animal production to control, prevent, and treat diseases, and some are used for growth promotion. Most antimicrobials that are medically important in human medicine must be prescribed by a veterinarian for use in food animal production (FDA, 2015). Some of the commonly used antimicrobials in cattle production are as follows (Table 1.1) (7):

1. Aminoglycoside: Aminoglycosides are bactericidal, water-soluble, and are used to treat Gram-negative and Gram-positive bacteria (Farouk et al., 2015). They inhibit bacterial growth by binding to the 30S ribosomal subunit, thus inhibiting protein synthesis (Davies and Davis, 1968). The beef industry stopped using extra-label aminoglycosides in the early 2000s. However, some oral aminoglycosides are labeled to treat calf scours, an intestinal disease caused by various *Escherichia coli* strains. (Osaili et al., 2013; Liang et al., 2020).
2. Amphenicol: Amphenicols are broad-spectrum, fat-soluble, and used to treat bovine respiratory disease (2020d). Many are bacteriostatic but can be bactericidal against some bacterial species (Ullah et al., 2017; Assane et al., 2019). They kill bacteria by binding to the 50S ribosomal subunit, thus terminally inhibiting protein synthesis (James et al., 1962). Florfenicol is largely used to treat calf pneumonia, bovine respiratory disease, and foot rot (Salaheen et al., 2019; Yang et al., 2019).

3. Macrolide: Most macrolides are bacteriostatic, broad-spectrum, and fat-soluble (Mazzei et al., 1993). They inhibit bacteria by inhibiting protein synthesis by binding to the 50S ribosomal subunit (Mazzei et al., 1993). Macrolides are a medically important antimicrobial, so producers need to be careful not to misuse them (Aidara-Kane et al., 2018). In food animal production, tylosin is commonly used in the feed to reduce the incidence of liver abscesses in feedlot cattle (Nagaraja and Lechtenberg, 2007; Alicia et al., 2015; Müller et al., 2018; 2020c). The mode of action of tylosin is to inhibit *F. necrophorum*, which reduces the bacterium's opportunity to enter the portal blood via ruminal wall lesions and reach the liver. Liver abscesses are an animal health and welfare issue and cause producers substantial economic losses (Nagaraja and Lechtenberg, 2007; Tadepalli et al., 2009).
4. Beta-lactam: Beta-lactams are bactericidal and water-soluble. Their mode of action is to inhibit mucopeptide synthesis in the cell wall. Penicillin and Cephalosporin antimicrobials are used in food animal production to treat mastitis, foot rot, pneumonia, and bovine respiratory disease (2019; 2020f). Beta-lactam antimicrobials are medically important in human medicine (FDA, 2015; Aidara-Kane et al., 2018).
5. Fluoroquinolone: Fluoroquinolones are bactericidal and fat-soluble. They inhibit bacteria by impeding with bacterial DNA-gyrase, therefore preventing DNA synthesis. Fluoroquinolones can inhibit Gram-negative and Gram-positive bacteria by also inhibiting DNA type IV topoisomerase in conjunction with bacterial DNA gyrase (Oliphant and Green, 2002). In food production animals, fluoroquinolones are used to treat bovine respiratory disease (2020f; 2020a). Furthermore, quinolones are highly used in human

medicine, thus are medically important and must be used with care (Oliphant and Green, 2002).

6. Sulfonamide: Sulfamides are broad-spectrum and water soluble. They slow the growth of bacteria by inhibiting enzymes in the folic acid pathway. In food animal medicine, sulfadimethoxine is used to treat bovine respiratory disease, foot rot, shipping fever, and calf diphtheria (2020f; Papich, 2021).
7. Tetracycline: Tetracyclines are bacteriostatic and have broad-spectrum antimicrobial activity (Ian and Marilyn, 2001). They inhibit protein syntheses by binding to the 30S ribosomal subunit (Ian and Marilyn, 2001). Oxytetracycline is frequently used in the cattle industry to treat bovine respiratory disease, foot rot, pink eye, and leptospirosis (2019; 2020e; 2020f).
8. Antimycobacterial: Antimycobacterial antimicrobials are used to treat mycobacterium infections. They inhibit mycobacterium by inhibiting the synthesis of their cell walls (Ballell et al., 2005; Kulkarni et al., 2018). In cattle and humans, they are primarily used to treat tuberculosis.
9. Trimethoprim: Trimethoprim antimicrobials are similar to sulfonamides by also inhibiting the folic acid pathway. They do this by not allowing dihydrofolate to reduce to tetrahydrofolate via competitive inhibition (Skld, 2011). In the cattle industry, trimethoprim antimicrobials are primarily used to treat Listeriosis (Srinivasan et al., 2005).
10. Ionophores: Ionophore antimicrobials select against Gram-positive bacteria and protozoa by transporting ions across the lipid membrane of bacteria, thus disrupting intramolecular protein transport (Schelling, 1984; Duffield and Bagg, 2000). Ionophores can be used for growth promotion purposes as they are not medically important in human medicine.

(Holman et al., 2018; Scott et al., 2019). Monensin and Lasalocid are examples of ionophores used to improve feed efficiency for beef and dairy cattle (Schelling, 1984; Ensley, 2020). Monensin works by modulating feed intake and altering the rumen microbial population by decreasing protozoa and Gram-positive bacteria, thus, increasing Gram-negative bacteria in the rumen (Schelling, 1984). These changes benefit cattle health by stabilizing the ruminal pH and preventing acidosis (Schelling, 1984; Ensley, 2020).

Table 1.1. Cattle bacterial diseases and the major antimicrobial classes that are used to prevent or treat them (Constable et al., 2008; Cockcroft, 2015; Nagaraja, 2018; Thomson, 2019; 2020b).

Disease Type	**Disease**	**Major Causative Agent(s)**	**Major Antimicrobial Prevention/ Treatment Class(s)**
Respiratory	Calf Diphtheria	*Fusobacterium necrophorum*	Sulfonamides, β-lactams, Macrolides, Amphenicols
Respiratory	Calf Pneumonia	*Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida*	Quinolones, Amphenicols, β-lactams, Macrolides
Metabolic	Liver Abscess	*Fusobacterium necrophorum*	Macrolides

Table 1.1 continued…

Disease Type	**Disease**	**Major Causative Agent(s)**	**Major Antimicrobial Prevention/ Treatment Class(s)**
Enteric	Blackleg	*Clostridium chauvoei*	β-lactams
Enteric	Johnes	*M. paratuberculosis*	None
Enteric, Neurological	Listeriosis	*Listeria monocytogenes*	β-lactams, Macrolides, Florfenicol
Enteric	Calf Scour	*E. coli, Salmonella*	Tetracyclines, Cephalosporins, Aminoglycosides
Neurological	Anaplasmosis	*Anaplasma marginale*	Tetracyclines
Neurological	Botulism	*Clostridium botulinum*	None
Neurological, Reproductive	Leptospirosis	*Leptospira*	Tetracyclines, β-lactams, Macrolides
Neurological	Tetanus	*Clostridium tetani*	β-lactam
Reproductive	Brucellosis	*Brucella*	None
Reproductive	Vibriosis	*Campylobacter fetus*	β-lactam, Aminoglycosides, Amphenicols, Tetracyclines
Feet	Foot Rot	*Fusobacterium necrophorum*	Tetracyclines, β-lactams, Amphenicols, Macrolides

Table 1.1 continued…

Disease Type	Disease	Major Causative Agent(s)	Major Antimicrobial Prevention/ Treatment Class(s)
Eye	Infectious Bovine Keratoconjunctivitis	*Moraxella bovis*	β-lactams, Tetracyclines
Skin	Rain Scald	*Dermatophilus congolensis*	β-lactams
Skin (tongue)	Wooden Tongue	*Actinobacillosis lignieresii*	Tetracycline
Udder	Mastitis	*S. aureus, P. aeruginosa, M. bovis, Klebsiella*	Aminoglycosides, β-lactams, Tetracyclines

Antimicrobial Resistance in Cattle Production

Antimicrobial resistance occurs when a pathogen, or another microbe, can survive in the presence of an antimicrobial. This typically occurs through genetic modification of the bacteria so the bacteria can produce proteins to neutralize the antimicrobials, increase efflux pumps so antimicrobials are forced out of the microbe, or change the target of the antimicrobial, such as the membrane, so antimicrobials can no longer attach (Gallo, 2013; Gualerzi, 2013). The use of antimicrobials can select antimicrobial resistant pathogens and commensal bacteria that can serve as vectors that can transfer resistance genes to other pathogenic microbes (Holmes et al., 2016). This increase in antimicrobial resistance in human health has led to the need of improved antimicrobial stewardship programs by medical professionals (Gozdzielewska et al., 2020).

Modifications of bacteria leading to antimicrobial resistance

Bacteria can modify themselves to become resistant to various antimicrobials. These changes commonly occur through spontaneous mutation, horizontal gene transfer, or bacteriophage transfection (Nakata et al., 2010; Snyder, 2013; Volker et al., 2013; Donnelly et al., 2015). Most modifications occur in proteins, efflux pumps, or the bacterial membrane (Coates, 2012; Dougherty and Pucci, 2012; Gualerzi, 2013).

Proteins

Proteins leading to antimicrobial resistance can be inherited from bacteria by spontaneous mutations or through horizontal gene transfer (Nakata et al., 2010; Volker et al., 2013). These bacterial proteins cause resistance by blocking, inactivating, disabling, or digesting the antibiotic (Coates, 2012; Dougherty and Pucci, 2012). Most of these proteins are encoded on the chromosome or on a plasmid of the bacteria (Claypool et al., 2010; Tanner et al., 2017).

Efflux Pumps

Efflux pumps use energy to expel antimicrobials from the bacterial cell (Dougherty and Pucci, 2012). There are five major classes which can all be single or multi-compartment transporters that can work on Gram-positive and Gram-negative bacteria (Figure 1.1) (Dougherty and Pucci, 2012):

1. Major Facilitator (MF) superfamily- MF superfamily efflux pumps use proton motive force and are the largest characterized family (Fluman and Bibi, 2009). They are mostly plasmid-encoded and commonly cause tetracycline and chloramphenicol resistance in bacteria (Roca et al., 2009; Somprasong et al., 2021).
2. ATP-Binding Cassette (ABC) family- ABC family efflux pumps are found in bacteria and mammals and are known to have highly specific reactions (Locher,

2016). They are typically involved with nutrient uptake and toxin secretion but can be involved in antimicrobial resistance in bacteria (Davidson and Chen, 2004).

3. Resistance-Nodulation-Division (RND) family- RND family efflux pumps contribute to the broadest range of antimicrobial resistance in Gram-negative bacteria (Fernando and Kumar, 2013). They are overexpressed in many bacteria and have also played a role in bacterial virulence (Fernando and Kumar, 2013).
4. Small Multi-Drug Resistance (SMR) family- SMR family efflux pumps were initially discovered in *staphylococci* isolates (Dougherty and Pucci, 2012). They are plasmid-encoded and cause multi-drug resistance in bacteria (Handzlik et al., 2013; Andersen et al., 2015).
5. Multi-Drug and Toxic Compound Extrusion (MATE) family- MATE family efflux pumps are the most recent efflux pumps to be discovered and described (Dougherty and Pucci, 2012). They are chromosome encoded and cause multi-drug resistance in bacteria (Fàbrega et al., 2009; Andersen et al., 2015).

Efflux pumps in Gram-positive bacteria are typically antimicrobial class-specific and mediated by the MF superfamily. Some Gram-positive bacteria use the ABC, SMR, or MATE family efflux pumps (Handzlik et al., 2013). Gram-negative bacteria have more variety than Gram-positive bacteria in their efflux pump usage (Dougherty and Pucci, 2012; Andersen et al., 2015).

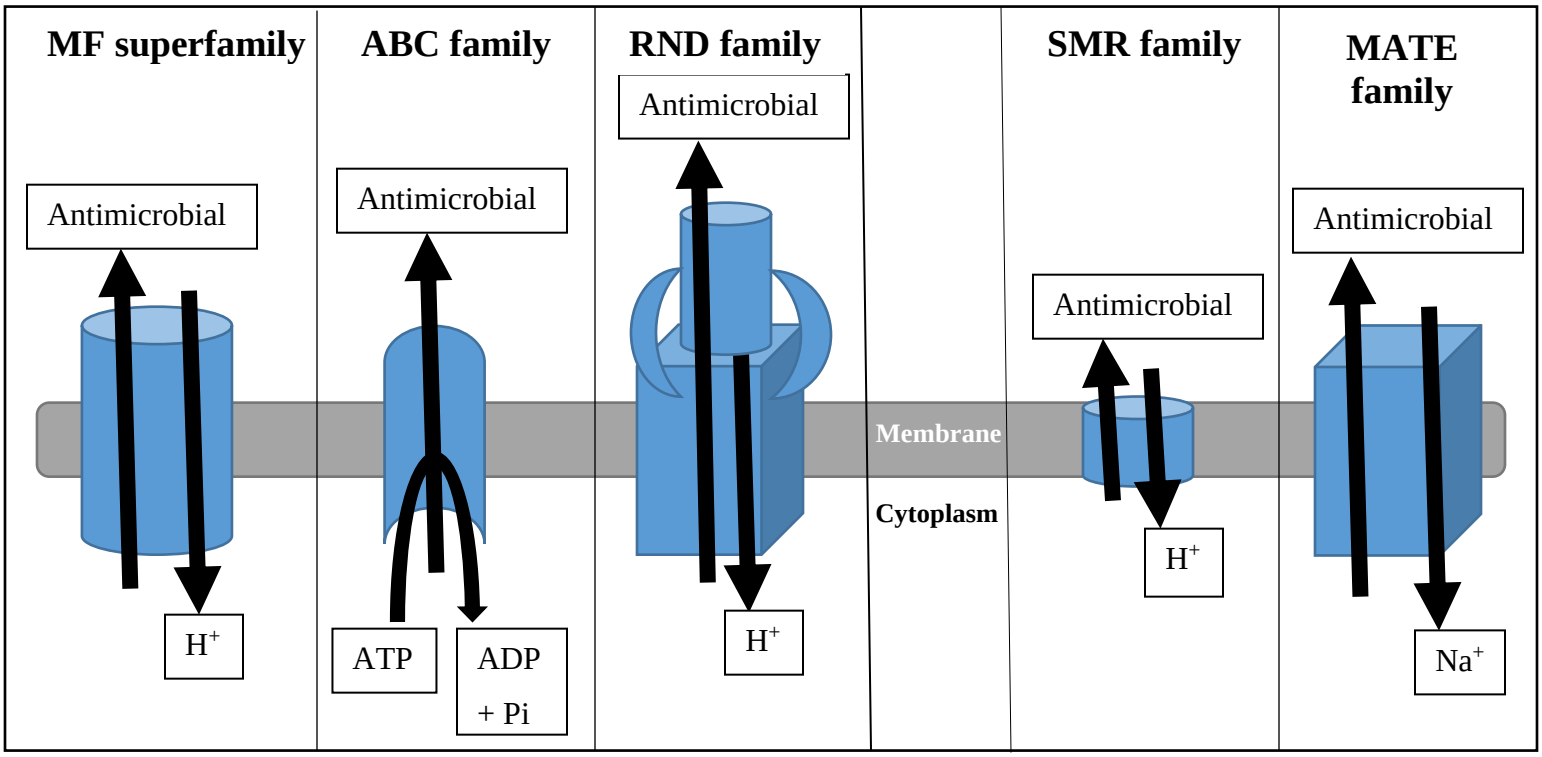

Figure 1.1. The differences between the five efflux pump families (Laura, 2006).

Membrane Modifications

Membrane modifications protect the bacteria from antimicrobials by not allowing the antimicrobials into the cell (Gualerzi, 2013). Bacterial membranes may alter their pore size or shape and may change their surface charges or produce surface proteins (Skld, 2011; Kawasaki, 2012). These modifications decrease the antimicrobial's likelihood of penetrating the bacterial membrane and entering the cell (Gualerzi, 2013).

Transfer of antimicrobial resistance

Bacteria can inherit and transfer antimicrobial resistance in three significant ways: horizontal gene transfer, vertical gene transfer, and bacteriophage transfection (Figure 1.2). Horizontal gene transfer allows bacteria to directly transfer genetic elements from one bacterium to another (46, 52). While vertical gene transfer is the transfer of genetic elements to the next generation of bacteria (48). Bacteriophage transfection can occur when lysogenic bacteriophages can inherit genetic elements and spread them from one bacterium to other bacteria (48, 49).

Horizontal gene transfer and lytic bacteriophages spread resistance genes to different bacterial species, while vertical gene transfer increases the number of a bacterial species carrying the resistance genes. When bacteria are pressured with long-term, low doses of antimicrobials, all these mechanisms select for bacteria with antimicrobial resistance (Gualerzi, 2013). This makes it possible for single- and multi-drug resistant genes to spread quickly between different bacterial species of bacteria.

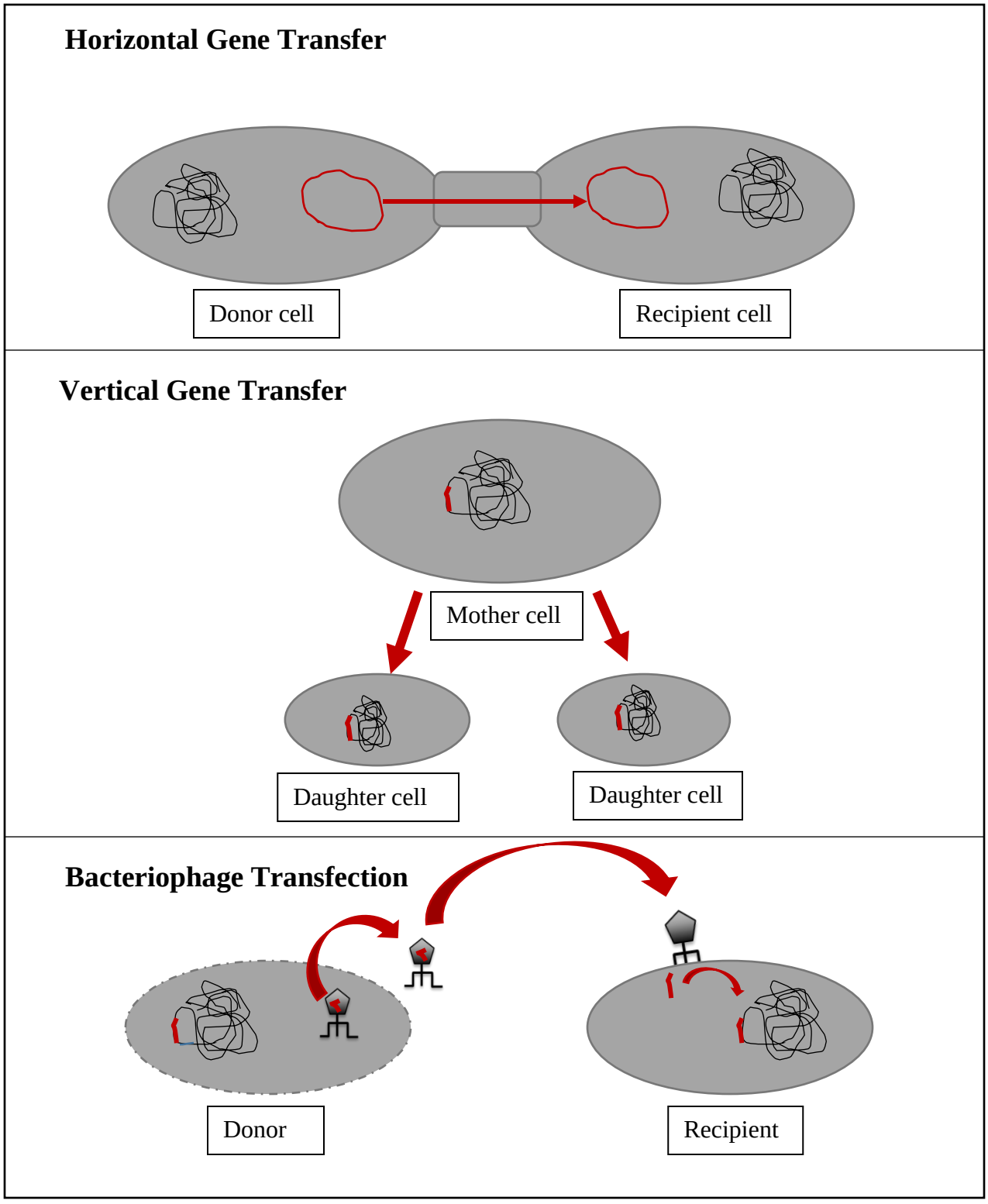

Figure 1.2. The differences between the three methods to transfer antimicrobial resistance.

Antimicrobial resistance in common cattle production antimicrobials

Antimicrobial resistance has become a major issue around the world in human and animal medicine (Aidara-Kane et al., 2018). An increase in resistance to medically important antimicrobials, such as macrolides, cephalosporins, and glycopeptides has led to public health concerns (Torio and Padilla, 2018; WHO, 2019). Some of the common antimicrobial resistance seen in antimicrobials that are used in cattle production are from plasmid-borne, chromosomal, efflux pump, or spontaneous mutations, as well as acetylation and methylation of proteins (Skld, 2011; Krause et al., 2016). For example, aminoglycosides can become resistant to antimicrobials by accumulating aminoglycoside-modifying enzymes from horizonal gene transfer, obtaining spontaneous mutations or methylations that inhibit aminoglycoside binding, or by acquiring RND family efflux pumps (Skld, 2011; Krause et al., 2016). Likewise, macrolide resistance is commonly caused by plasmid-borne genes which encode enzymes that methylate the ribosome's nucleotide sequence in bacteria (Skld, 2011). Macrolides also have overlapping binding sites, making it easy for multi-drug resistance to occur via homologous recombination in bacteria (Descheemaeker et al., 2000; Leclercq and Courvalin, 2002). Additionally, bacteria also develop resistance to amphenicols, Beta-lactams, quinolones, tetracyclines, sulfamides, antimycobacterials, and trimethoprim through horizontal gene transfer, efflux pumps, spontaneous mutations, methylation, acetylation, or enzymatic inactivation (Skld, 2011; Ma et al., 2014; Tang et al., 2014; Ou et al., 2015).

Antimicrobial effects on the microbiome

Overview of the cattle rumen microbiome

Cattle rely on the rumen microbiome to digest plants (Huws et al., 2018). The rumen microbiome is diverse and complex. It is comprised of bacteria, protozoa, fungi, bacteriophages,

and archaea (Wang et al., 2017). Bacteria and bacteriophages are the most prominent microbes in the rumen microbiome, compromising an estimate of 10^{11} cells/ gram and 10^{9} cells/ gram, respectively (Wright and Klieve, 2011; Bath et al., 2013; Patel et al., 2014). The microbes work symbiotically with each other to produce volatile fatty acids (VFAs) and microbial protein for cattle to use (Wright and Klieve, 2011; Bath et al., 2013). The most prominently made VFAs in the rumen are acetate, butyrate, and propionate (Bergman, 1990; Morvay et al., 2011). These VFAs provide the majority of substrates that offer the cattle energy to support animal maintenance and production (Bergman, 1990; Chaves et al., 2008).

Research on the human microbiome has led to discoveries involving its contribution to obesity, malnutrition, diabetes, multiple sclerosis, asthma, eczema, cancer, and other illnesses (Iizumi et al., 2017; Leong et al., 2018). From this, the assumption that the rumen microbiome is necessary for cattle health and performance can be made. Therefore, understanding the rumen microbiome is crucial for improving cattle performance, as it is likely that optimizing the rumen microbiome will enhance cattle health, growth, and efficiency.

Factors affecting rumen microbiome establishment in cattle

Diet

The cattle diet is believed to be the most influential factor affecting the rumen microbiome (Henderson et al., 2015). When comparing high forage and high starch diets, there are differences in the rumen microbiome. These differences include a change in the *Firmicutes* to *Bacteroidetes* ratio, decreased fibrolytic bacteria, and *increased Prevotella* (Ehsan et al., 2009; Fernando et al., 2010; Clemmons et al., 2019). In high starch diets, *Bacteroidetes* and *Proteobacteria* species increase in concentration (Fernando et al., 2010). The increase in these bacterial species is likely due to them being more efficient carbohydrate fermenters and their ability to survive at a lower

pH (Flint et al., 2012; Auffret et al., 2017). *Lactobacillus* species and *Streptococcus bovis*, both lactate-producing bacteria, are also commonly associated with high starch diets (Russell and Hino, 1985; Nagaraja and Titgemeyer, 2007). These bacteria contribute to cattle having a lower rumen pH when fed a high starch diet (Harlow et al., 2017). Furthermore, protozoa concentrations tend to increase in the rumen of cattle fed starch-based diets (Neto et al., 2017).

Methanogenic archaea and fungi are more abundant and diverse in the rumen when cattle are fed high forage diets (Mi et al., 2010). The greater diversity of methanogenic archaea likely contributes to the increased methane production observed from cattle fed high forage diets (Popova et al., 2011; Clemmons et al., 2019). Also contributing to the higher methane production is the increased acetate to propionate ratio seen in cattle fed forage-based diets (Hindrichsen et al., 2004; Clemmons et al., 2019). Moreover, studies have demonstrated that fungi favor stemmy forages over grains (Grenet et al., 1989).

Rumen Development

As cattle age, the rumen develops, and differences are seen in the rumen microbiome. Calves tend to have increased levels of Proteobacteria and Firmicutes (Jami et al., 2013). Over time the rumen develops which leads to bacterial population changes, one major change is that Bacteroidetes increases in concentration while Proteobacteria decreases (Jami et al., 2013). These differences are mainly due to calves being milk-fed, whereas adult cattle are fed solid feed (Clemmons et al., 2019). Furthermore, protozoa populations are altered as cattle age (Duarte et al., 2018).

Weaning age can also affect the rumen microbiome for a short period of time (Meale et al., 2017; Clemmons et al., 2019). When calves are weaned early, a rapid change is observed in the rumen microbiome while when calves are weaned slowly over time, the microbiome can gradually

adjust its microbial population (Meale et al., 2017). Once the microbiome is established after weaning, it does not seem to alter much as cattle age unless other factors, like diet change, occur (Meale et al., 2017).

Breed

There is not much known on the effects of cattle breed on the rumen microbiome. There is evidence to suggest that some breeds may contribute to host regulation of the rumen microbiome, but it is more likely that individual genetic variation and regulation plays a more significant role in rumen microbiome establishment (Hernandez-Sanabria et al., 2013).

Antimicrobial effects on the cattle rumen microbiome

The microbiome is a complex microbial system within many living organisms. This microbial system can be easily disrupted by diet, environment, and antimicrobial agents (Mazmanian and Round, 2009; Iizumi et al., 2017). Antimicrobial effects on the microbiome are an increasing area of study. In humans, antimicrobial use has been positively correlated with obesity, type 1 diabetes, asthma, and autoimmune diseases (Iizumi et al., 2017; Leong et al., 2018). When younger children are given antimicrobials, the correlation for these diseases increases (Iizumi et al., 2017), this association is likely due to the alterations that antimicrobials make on the microbiome. Furthermore, antimicrobial use has been shown to alter both the microbiome's richness and evenness, leading to decreased overall diversity of bacteria in human digestive tracts (Thomas et al., 2017; de Gunzburg et al., 2018).

Few studies have been performed on the rumen microbiome, but it can be inferred that antimicrobials alter the rumen microbiome from the human studies. Likewise, the rumen studies that have been completed support this inference (Thomas et al., 2017; Spirito et al., 2018). However, it seems that many of these alterations lead to increased animal performance and

productivity (Nagaraja and Chengappa, 1998; Spirito et al., 2018). For example, monesin has been demonstrated to decrease methane production and improve feed efficiency by altering the rumen microbiome so there is a decrease in the acetate-propionate ratio and more Gram-negative bacteria (Spirito et al., 2018). Furthermore, monesin has been reported to decrease methanogenic archaea and overall *Bacteroidales* concentrations while increasing *Prevotella* concentrations (Spirito et al., 2018). Nevertheless, additional studies should be performed on antimicrobials' effects on the rumen microbiome, especially in reference to young calves.

Effects of common control and preventative antimicrobials used in cattle production on the rumen microbiome

Tetracyclines

Tetracyclines are one of the most widely used antibiotic classes in animal production (Gavilán et al., 2015). While no studies were found on the effects of tetracyclines on the rumen microbiome, many were found for other animal species. For example, in chickens, tetracycline was demonstrated to decrease the prevalence of *Bifidobacteriales*, *Bacteroidales*, *Clostridiales*, *Desulfovibrionales*, *Burkholderiales*, and *Campylobacterales*; while increasing the prevalence of *Enterobacteriales* and *Lactobacillales* (Videnska et al., 2013). Furthermore, in mice, tetracycline use has been reported to reduce gut microbial diversity, increase *Firmicutes* concentrations, and decrease *Bacteroidetes* concentrations (Yin et al., 2015). From these studies, it raises the question if tetracycline use alters the rumen microbiome, but studies should be performed to confirm this statement.

Tylosin

Tylosin is a macrolide antimicrobial that is used to prevent liver abscesses (Nagaraja and Lechtenberg, 2007). Few studies have been performed on the effects of tylosin on the microbiome,

but the ones completed have shown no alterations in bacterial diversity (Nagaraja et al., 1999; Weinroth et al., 2019). Most of these studies look only at the diversity of the order or phylum of bacteria. Therefore, short- and long-term studies should be performed at the genus and species level to determine if any differences in the rumen microbiome are seen when using tylosin.

Ionophores

Rumensin is an ionophore that is added to cattle diets to increase productivity and monesin is added to cattle diets to increase productivity and decrease methane production (Nagaraja et al.; Potter et al.; Dinius et al., 1976; Dawson and Boling, 1983). When added to the diet, monensin alters the rumen microbiome, so more propionate is produced (Schelling, 1984). This increase in propionate production leads to increased energy for the animal to use (Dinius et al., 1976).

It has been reported that monesin alters the rumen microbiome by decreasing methanogenic archaea, *Rikenellaceae*, *Lachnospiraceae*, *Butyrivibrio*, *Pseudobutyrivibrio*, and *Incertae sedis* concentrations, while increasing *Coriobacteriaceae*, *Prevotellaceae*, *Cyanobacteria*, and *Oribacterium* concentrations (Schären et al., 2017). Likewise, monesin has been demonstrated to decrease the relative abundance of operational taxonomic units of bacteria in the rumen microbiome (Kim et al., 2014).

Antimicrobial Alternatives

A general overview of antimicrobial alternatives

Antimicrobial alternatives are substances with antimicrobial activity or the same effects as antimicrobials but are not antibiotics or their derivatives. They are generally used to replace antimicrobials that bacteria have formed resistance against. There has been a growing need to discover antimicrobial alternatives due to increasing antimicrobial resistance in disease-causing bacterial pathogens and for growth promotion purposes in the cattle industry (Pukrop, 2017). For

an antimicrobial alternative to be effective, it needs to have similar, or better, effects as the antimicrobial it is replacing (Amachawadi, 2014). There are three major categories of antimicrobial alternatives (Ghosh et al., 2019):

1. Natural: Antimicrobial alternatives that occur naturally in the environment.
2. Synthetic: Antimicrobial alternatives that are synthetically designed.
3. Biotechnology-based: Antimicrobial alternatives that use biological processes in their design.

Potential antimicrobial alternatives for use in the cattle industry

Plant Extracts

Two major categories of plant extracts are phenolic compounds and organic acids. Phenolic compounds are derived mostly from plant sources and can interact with proteins, slow degradation of ruminal dietary protein, enhance the bioavailability of amino acids, reduce fermentation waste products, improve growth efficiency, and are antioxidants (2018). They also have been shown to have antimicrobial effects, increase microbial protein, and decrease bloat severity (Jacela et al., 2013). Antimicrobial activity is believed to be caused by reducing pH, disrupting the bacterial membrane's permeability, or altering bacterial efflux pumps (Srivastava et al., 2014). Some examples of antimicrobial alternatives containing phenolic compounds are essential oils, sorghum extract, and herb extracts (Puupponen-Pimiä et al., 2001; Sieniawska et al., 2013; Omonijo et al., 2018; Pempek et al., 2018).

Essential oils are volatile and aromatic substances extracted from plants (Castillejos et al., 2007). Research has shown that they improve digestibility, immunity, and promote gut health in production animals (Brenes and Roura, 2010; Chitprasert and Sutaphanit, 2014; Omonijo et al., 2018). Furthermore, essential oils have antimicrobial, antioxidant, and anti-inflammatory activities

(Zhang et al., 2006). Their antimicrobial activity is caused by damaging the cell wall of bacteria, which leads to cell death (Xu et al., 2008; Yap et al., 2014). Another compound high in phenolics is sorghum extract, which is a plant-based compound that has potential as a natural antimicrobial alternative (Herald et al., 2012). It has also been shown to have many health and pharmaceutical benefits (Dykes and Rooney, 2006). Sorghum grains with increased concentrations of phenolic compounds have been associated with higher antioxidant activities, anticarcinogenic effects, and antimicrobial activities (Dykes et al., 2005; Guajardo Flores, 2008; Kil et al., 2009). Herbs are another common source of phenolic compounds. They are frequently used in diets to increase palatability, but they have also been shown to have antimicrobial effects (Cutter, 2000; Terranova et al., 2020).

Organic acids are another common plant extract. They are carboxylic acids that are thought to inhibit bacteria by altering rumen pH (Sheoran and Tewatia, 2017). When studied in ruminants, organic acids have been shown to improve rumen fermentation, maintain rumen pH, have a buffering effect in the rumen, decrease methane production, and lower harmful bacteria on the intestinal wall (Callaway and Martin, 1996; Khampa et al., 2006). Furthermore, organic acids have been demonstrated to improve cattle overall health and production (Kung et al., 1982; Khampa et al., 2006). Some examples of organic acids include aspartate, malate, and fumarate (Callaway and Martin, 1996).

Bacteriophages

Bacteriophages are viruses that infect bacteria (Dias et al., 2013). They are very selective to one species of bacteria; therefore, cocktails are often used to inhibit bacteria (Chan et al., 2013; Kakasis and Panitsa, 2019). However, bacteriophages are capable of transmitting antimicrobial resistance genes (Kakasis and Panitsa, 2019). There are two main types of bacteriophages, lytic

and lysogenic (Kakasis and Panitsa, 2019). Lytic bacteriophages propagate inside the cell, burst the cell, and release bacteriophage progeny to infect more bacteria (Saha and Mukherjee, 2019). However, lysogenic, or temperate, bacteriophages integrate into the bacterial DNA and are then called prophages (Selle et al., 2020). The prophages replicate with the bacterial DNA until the bacteria become stressed, and then the bacteriophages lyse and kill the host bacterial cell (Machuca et al., 2010).

Bacteriophages can also be genetically modified. These bacteriophages have been engineered to re-sensitize bacteria to antimicrobials or deliver molecules with antimicrobial activity (Libis et al., 2014). Furthermore, some bacteriophages have been engineered to be less specific and more virulent (Pires et al., 2016). Currently, several genetically modified bacteriophages are being investigated (Pires et al., 2016).

Additionally, endolysins, or lysins, are the enzymes bacteriophages use to lyse bacterial cells (2017). Lysins are typically heat-stable hydrolases that degrade bacterial peptidoglycan (Fischetti, 2008; Parisien et al., 2008). Research has been done on the antimicrobial activity of lysins extracted from the bacteriophage (Fischetti, 2008). These studies have shown greater antimicrobial activity against Gram-positive bacteria, but endolysins have also been shown to inhibit Gram-negative bacteria (Fischetti, 2018).

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), in conjunction with CRISPR-associated (Cas) proteins, have the potential to be used as an antibiotic alternative. CRISPR-Cas proteins are endonucleases that are naturally produced by bacteria and are used as a defense mechanism against foreign nucleic acids, such as bacteriophages (Makarova et al., 2006; Brouns et al., 2008; Josiane et al., 2010). There are many different CRISPR-Cas proteins, but

CRISPR-Cas9 is the most well studied. CRISPR-Cas9 is a class II CRISPR-Cas endonuclease that is guided by RNA and cleaves double-stranded DNA (Elitza et al., 2011; Jinek et al., 2012). Cleavage is caused by complementary base pairing of the target DNA with a programmable single guide RNA (sgRNA) followed by a necessary protospacer adjacent motif (PAM) sequence in the target DNA (Jinek et al., 2012; Shah et al., 2013; Doudna and Charpentier, 2014). The PAM sequence binding after complementary base pairing of the target DNA is necessary for the double-stranded cleavage of the target DNA (Gasiunas et al., 2012; Jinek et al., 2012; Sternberg et al., 2014). Furthermore, CRISPR-Cas9 has been shown to edit bacterial genomes; therefore, CRISPR-Cas9 paired with an efficient delivery method will potentially replace many antibiotics (Cong et al., 2013; Gomaa et al., 2014; Robert et al., 2014; Moreb et al., 2017). Due to CRISPR- Cas9's ability to efficiently cleave targeted DNA strands, the protein complex has the potential to cleave and disrupt a gene in targeted bacteria effectively.

A significant obstacle will be delivering the CRISPR- cas9 complex into bacteria. Two possible delivery methods are via nanoparticles and bacteriophages. Nanoparticles are microscopic particles with varying properties that benefit therapeutic use in the health industry (Anu Mary Ealia and Saravanakumar, 2017). Lipid and polymeric-based nanoparticles have been researched with CRISPR-Cas9 for therapeutic use in treating cancer and genetic disorders (Chen et al., 2017; Lee et al., 2018). The second delivery method is the use of bacteriophages, which are bacterial viruses that insert DNA into a bacterial host. Bacteriophages have two possible life cycles: lytic or lysogenic (Snyder, 2013). Lytic bacteriophages lyse bacterial cells, while lysogenic bacteriophages insert their DNA into bacterial cells' genome (Snyder, 2013). Lysogenic bacteriophages have been used to insert CRISPR-Cas9 into *Escherichia coli* and *Staphylococcus aureus in vitro* (David et al., 2014; Robert et al., 2014; Yosef et al., 2015). The main difference

between the two delivery methods is that nanoparticles do not have target specificity unless a modality is added to make them specific, and they can be easily modified, while most bacteriophages have species-specific specificity and can be more difficult to modify (Carl et al., 2003; Lino et al., 2018). Due to the specificity of CRISPR-Cas9, delivery to more than the target bacteria should not cause other, possibly beneficial bacteria to be affected as long as the gRNA is properly designed (Jinek et al., 2012; Cong et al., 2013).

Other antimicrobial alternatives

Probiotics

Probiotics are live cultures of microorganisms that work mainly by competitive exclusion and colonization, leading to increased beneficial microbes in the rumen and intestines (Ballou et al., 2019). Probiotics have been shown to increase cattle health and performance (Uyeno et al., 2015). Studies have demonstrated increased average daily gain, increased feed to gain ratio, and improved milk production in cattle fed probiotics (Uyeno et al., 2015; Dailidavičienė et al., 2018; Kelsey and Colpoys, 2018). However, probiotics can carry antimicrobial resistance genes and spread them to other bacteria (Egervärn et al., 2009).

Prebiotics

Prebiotics are feed ingredients digestible by microbes found in the rumen and intestines (Manning and Gibson, 2004). They are fed to selectively stimulate a more beneficial microbial community (Manning and Gibson, 2004; Ballou et al., 2019). Prebiotics enrich beneficial bacteria in the rumen and intestines, thus competitively inhibiting harmful bacteria (Callaway et al., 2014). By enriching beneficial bacteria, probiotics assist in improving cattle health and increasing efficiency (Callaway et al., 2014).

Microminerals

Microminerals are essential to health and growth of cattle. Some microminerals are used in higher concentrations in the diet to improve overall cattle health and increase growth promotion (Jacob et al., 2010). Moreover, high concentrations of microminerals can be cytotoxic to bacteria (Abou-Shanab et al., 2007). However, bacteria are becoming resistant to metals, and their resistance genes are associated with resistance genes to multiple antimicrobials (Amachawadi, 2014; 2017). Some examples are copper and zinc (Zhang et al., 2011).

Antimicrobial Peptides

Many organisms produce antimicrobial peptides (AMPs) to protect against pathogens (Wang et al., 2019). They interact with the bacterial cell membrane and cause it to disintegrate, leading to cell death (Powers and Hancock, 2003; Wang et al., 2019). Researchers have shown that they are typically broad-spectrum and are active against multidrug-resistant pathogens (Powers and Hancock, 2003; Barksdale et al., 2016). However, resistance to AMPs has been documented, but bacteria do not easily develop resistance (Gunn, 2001; Peschel and Vincent Collins, 2001; Wang et al., 2019).

Bacteriocins are AMPs produced by bacteria that inhibit competing bacteria, including antimicrobial-resistant bacteria (Meade et al., 2020). These have three classes. Class I, less than five kDa; Class II, between 5-10 kDa; and Class III, over 30 kDa (Meade et al., 2020). Many bacteriocins can inhibit bacteria by inhibiting peptidoglycan biosynthesis and translation, along with the typical mode of action of AMPs, cell membrane disintegration (Imke et al., 2001; Meade et al., 2020). Furthermore, bacteriocins can withstand harsh environmental conditions (Perez et al., 2014; Meade et al., 2020).

Predatory Bacteria

Predatory bacteria prey on a variety of Gram-negative bacteria (Stolp and Starr, 1963; Lambina et al., 1983). They attack other bacteria by attaching, entering the periplasmic space, consume nutrients from and replicate inside the bacterium they have entered, and eventually lyse the bacterium (Riccardo et al., 2015). *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are two commonly studied species of predatory bacteria (Riccardo et al., 2015). They have been demonstrated to inhibit multi-drug resistant Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae* (Dashiff et al., 2011).

Immune Modulators

Immune modulators enhance the immune system, making the host more resistant to pathogens (Amachawadi, 2014). They cause a passive immune response via administration of antibodies, cytokines, egg yolk, or dried plasma (Amachawadi, 2014; 2017). Immune modulators have been demonstrated to have growth-promoting effects similar to antimicrobials (Coffey and Cromwell, 1995; Chae et al., 1999).

Vaccines

Vaccines stimulate a protective immune response and are used to prevent diseases (2017). Vaccines are typically a dead or modified version of a pathogen that is injected into an animal. This causes the animal's immune system to create antibodies against the pathogen. Furthermore, studies have shown some vaccines to improve animal productivity and performance.

Synthetic Mimics of Antimicrobial Peptides (SMAMPs)

Synthetic mimics of antimicrobial peptides are designed to mimic the properties of antimicrobial peptides (Scorciapino and Rinaldi, 2012). There are three major approaches to designing SWAMPS (Ghosh et al., 2019). One approach is to use peptidomimetic oligomers which

have modifications in the peptide backbone of AMPs (Ghosh et al., 2019). Their antimicrobial activity mostly comes from the secondary structures they form. Currently, oligoureas, beta-peptides, alpha-AA peptides, oligoacyl-lysines, and peptoids are being studied as potential antimicrobial alternatives (Ghosh et al., 2019). Another method is to use synthetic antimicrobial polymers. These polymers have hydrophobic and cationic domains incorporated to give them antimicrobial activity (Huang et al., 2016; Konai et al., 2018). The third major approach is the use of small molecules. Small molecules are derivatives of larger molecules that are synthetically manipulated to mimic the properties of AMPs (Ghosh et al., 2019). Currently, brilacidin, AMC-109, ceradenins, binaphthyl-based dicationic compounds, xanthone derivatives, aryl-alkyl-lysines, biphenyl-based lysine derivatives, and norspermidine-based compounds are being studied as potential antimicrobial alternatives (Ghosh et al., 2019).

Innate Defense Regulatory (IDR) Peptides

Innate defense regulatory peptides have been designed with antiendotoxin and immunomodulatory activities but no antibacterial activity (Hilchie et al., 2013). Interestingly, IDR peptides have demonstrated the ability to protect mice from Gram-negative and Gram-positive bacterial infections and have completed clinical trials (Mansour et al., 2015; Ghosh et al., 2019).

Antibacterial oligonucleotides

Antibacterial oligonucleotides are designed to silence essential and antimicrobial resistance genes with antisense oligonucleotides like phosphorothioate oligodeoxynucleotides (Ghosh et al., 2019). They have demonstrated antimicrobial activity against various bacterial species, including *E. coli* and *Salmonella (Ayhan et al., 2016)*.

Nanoparticles

Nanoparticles have been shown to have antimicrobial properties at small doses (Durazzo et al., 2020). Nanoparticles have two significant modes of action, which are the production of oxygen-free radicals and disrupting membrane potential and integrity, although some nanoparticles inhibit bacteria by inhibiting enzymes, producing reactive nitrogen species, or inducing bacterial cell death (Beyth et al., 2015). Furthermore, nanoparticles can be used to deliver antimicrobials and antimicrobial alternatives to bacteria (Gao et al., 2018).

Inorganic and organic nanoparticles are the two major categories of nanoparticles (Beyth et al., 2015). Inorganic nanoparticles are comprised of metals and metal oxides (Beyth et al., 2015). These nanoparticles typically inhibit bacteria by producing oxygen-free radicals (Jin et al., 2009; Liu et al., 2009; Pelgrift and Friedman, 2013; Pati et al., 2014; Beyth et al., 2015). Some examples of inorganic nanoparticles with antimicrobial properties are silver, titanium oxide, zinc oxide, iron oxide, gold, copper oxide, magnesium oxide, nitric oxide, and aluminum oxide (Wei et al., 1994; Lellouche et al., 2009; Hamal et al., 2010; Zhao et al., 2010; Ansari et al., 2012; Carpenter and Schoenfisch, 2012; Lellouche et al., 2012; Schairer et al., 2012; Zhigang et al., 2012; Pelgrift and Friedman, 2013; Anghel et al., 2014; Ansari et al., 2014; De Simone et al., 2014; Majdalawieh et al., 2014; Palanikumar et al., 2014; Poulose et al., 2014; Beyth et al., 2015). Organic nanoparticles are less stable than inorganic molecules (Beyth et al., 2015). These are cationic molecules that have multiple mechanisms of action, most of which result in membrane disruption and cell death (Denyer and Stewart, 1998; Nurit et al., 2010; Huh and Kwon, 2011; Ibrahim et al., 2014; Beyth et al., 2015). Poly-ε-lysine, quaternary ammonia compounds, N-Halamine compounds, polysiloxanes, quaternary phosphonium groups, quaternary sulfonium groups, 5-Chloro-8-

hydroxy-quinoline, polycationic nanoparticles, and chitosan are examples of organic nanoparticles (Nonaka et al., 2003; Rabea et al., 2003; Sauvet et al., 2003; Beyth et al., 2015).

Antibiotic Inactivators

Antibiotic inactivators are genetically engineered enzymes that degrade antibiotics (Ghosh et al., 2019). These are being studied for use with bacterial infections, like *C. difficile*, where antibiotic use increases infection risk (Kokai-Kun et al., 2017). They work by degrading or inhibiting the antibiotic so the normal microflora can reestablish quicker and competitively exclude the infection-causing pathogenic bacterium (Therrien and Levesque, 2000; Kokai-Kun et al., 2019).

Conclusion

In conclusion, there are two main concerns with antimicrobial use in cattle production. The primary concern is antimicrobial resistance, while the lesser is alteration of the rumen microbiome. Antimicrobial resistance is a major threat to the health of both livestock and humans. However, microbiome alterations are a more significant concern in human medicine than in cattle production. This is because most alterations on the rumen microbiome benefit cattle productivity, while most alterations of the human microbiome can increase the risk for many diseases. These concerns have led to research on many different antimicrobial alternatives. Each alternative has its strengths and weaknesses. Some antimicrobial alternatives are on the market for use in cattle, while others are still being researched. Research on antimicrobial alternatives for use in cattle production must continue to improve health and increase production.

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Chapter 2 - General Introduction

Antimicrobial agents are used to treat or prevent bacterial infections. In recent years, there has been an increased emphasis on effective disease prevention and management in food animal production because of the need to feed a growing global population (Mathew et al., 2007; Amachawadi, 2014). In cattle, antimicrobials are used more often to treat or prevent respiratory disease complex and liver abscesses in feedlot cattle and mastitis in dairy cows.

Bovine respiratory disease (BRD) complex is of major economic concern to cattle producers because of morbidity and mortality (Bach, 2011; Teixeira et al., 2017; Dunn et al., 2018; Holschbach et al., 2020). Bacterial pathogens associated with BRD infections are *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, and *Mycoplasma bovis* (Apley, 2006; DeDonder, 2016). Macrolides, florfenicol, fluoroquinolones, tetracyclines and 3rd generation cephalosporins are commonly used antimicrobials for the treatment and prevention of BRD in cattle (USDA, 2018).

Liver abscesses are also of major economic concern in beef cattle production. In addition to liver condemnations in beef processing plants (Eastwood et al., 2017), liver abscesses cause decreased animal performance and lower carcass yield and quality (Nagaraja and Lechtenberg, 2007). The primary causative agent is *Fusobacterium necrophorum,* an anaerobic and Gram-negative, rod-shaped opportunistic pathogen that is part of the rumen microbiota (Langworth, 1977). The species is further categorized into two subspecies, *necrophorum* and *funduliforme*, which differ in a number of characteristics, including virulence. The subsp. *necrophorum* is more frequently involved in causing liver abscesses than subsp. *funduliforme* (Tadepalii et al. 2008). Currently, tylosin, a macrolide, is most commonly used in the feed to reduce the incidence of liver abscesses (Nagaraja and Lechtenberg, 2007).

Bovine mastitis is a major production disease of dairy cows with significant economic impact. Mastitis causes reduced milk yield and is caused by a number of bacterial pathogens, mainly *Staphylococcus aureus, Streptococcus agalactiae, S. dysgalactiae, S. uberis, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* (DeGraves and Fetrow, 1993; Hortet and Seegers, 1998; Gianneechini et al., 2002; Gröhn et al., 2004; Park et al., 2014). Beta-lactams, macrolides (erythromycin) and lincosamides (pirlimycin) are the common antimicrobials used for mastitis prevention and treatment (Ma et al., 2021).

Additionally, foodborne illnesses are a major public health concern and food animals are carriers for many food-borne pathogens. Three major foodborne bacterial pathogens of cattle are Shiga toxin-producing *E. coli*, *Salmonella enterica*, and *Campylobacter jejuni*. The most common antimicrobials used to treat *Salmonella* and *Campylobacter coli* food-borne infections are ciprofloxacin, cephalosporins, trimethoprim/sulfamethoxazole, and azithromycin (Switaj et al., 2015). However, antimicrobial use is not recommended for infections caused by Shiga toxin producing *E. coli* because antimicrobial use can increase the risk of hemolytic uremic syndrome (Wong et al., 2012).

Antimicrobial resistance to antimicrobials, particularly to medically important antimicrobials such as cephalosporins and macrolides, has become a major public health concern (FDA, 2012, 2015; Torio and Padilla, 2018; WHO, 2019). Antimicrobial resistance occurs when a bacterium changes and survives in the presence of an antimicrobial. The use of antimicrobials can select for resistant bacterial strains that can transfer resistance genes to other bacteria (Holmes et al., 2016). For example, Tenhagen et al. (2014) reported 631 antimicrobial resistant isolates of *S. aureus* from various cattle production areas. Isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* have similarly shown resistance to multiple

antimicrobials (Liu et al., 2013). Likewise, Karama et al. (2020) demonstrated that 52.3 % of 86 *Campylobacter* isolates were resistant to one or more antimicrobials and of those 32.5 % were multidrug resistant. Clindamycin, nalidixic acid, tetracycline, erythromycin, azithromycin, florfenicol, gentamicin, telithromycin, and ciprofloxacin were among the antimicrobials that the *Campylobacter* isolates become resistant to (Karama et al., 2020). Furthermore, *Salmonella* isolates have been demonstrated to be resistant to multiple antimicrobials (Foley and Lynne, 2008). Antimicrobial resistance to ceftiofur and ceftriaxone in *Salmonella* isolates is concerning because both antimicrobials are third generation, and broad-spectrum cephalosporins and ceftriaxone are used in treatment of food-borne *Salmonella* infections in humans (Douris et al., 2008; Switaj et al., 2015). Resistance genes for tetracyclines, fluoroquinolones, beta-lactams, macrolides, sulfonamides, lincosamides, phenicols, and aminoglycosides have been reported in bacterial pathogens (*P. multocida, M. haemolytica, H. somni*, and *M. bovis*) associated with BRD (O'Connor et al., 2010; D'Amours et al., 2011; Desmolaize et al., 2011; Kadlec et al., 2011; Katsuda et al., 2012; Michael et al., 2012; Alexander et al., 2013; Pardon et al., 2013).

Macrolides are medically important antimicrobials, therefore, antimicrobial resistance to macrolides is a major public health concern. Tylosin, a macrolide, is exclusively used in animals, but is structurally similar to erythromycin. Therefore, there is concern with the use of tylosin in animals, which is the reason for placing tylosin under veterinary oversite with the Veterinary Feed Directive (FDA, 2021). The influence of tylosin use on antimicrobial resistance to macrolides and potential spread to other bacteria through horizontal transfer, has been studied (Zaheer et al., 2013; Alicia et al., 2015; Müller et al., 2018). Recent studies on tylosin in the feed of feedlot cattle to prevent liver abscesses have investigated AMR of gut bacteria and *F. necrophorum*. These studies concluded that there was no evidence of macrolide resistance in *F. necrophorum* isolates and only

an occasional increase in macrolide-resistant fecal enterococci was detected (Nagaraja et al., 1999; Amachawadi et al., 2017; Schmidt et al., 2020). These results indicate minimal AMR impact of administering in-feed tylosin to prevent liver abscesses. Still, there is considerable interest in finding alternatives to replace tylosin because of the public health concern of using a medically important antibiotic in cattle diets. Tylosin alternatives that have been examined are direct-fed microbials (Huebner et al., 2019), oil seeds (Mir et al., 2008), egg yolk immunoglobulins (Stotz et al., 2021), essential oils (Elwakeel et al., 2013), and vaccines (Fox et al., 2009). Vaccines have not been able to replace tylosin in the feedlot industry because there has been minimal efficacy at reducing liver abscesses (Checkley et al., 2005). Likewise, direct-fed microbial products that use yeast have been shown to decrease severity, but not overall prevalence of liver abscesses in feedlot cattle (Shen et al., 2019).

Antimicrobial alternatives have also been researched on other common cattle pathogens, such as *E. coli, Salmonella,* and *S. aureus*. Some of these alternatives are antimicrobial peptides (Wang et al., 2019), innate defense regulatory (IDR) peptides (Mansour et al., 2015), antimicrobial oligonucleotides (Ayhan et al., 2016), nanoparticles (Beyth et al., 2015), bacteriophages (Kakasis et al., 2019), CRISPR-Cas (Cong et al., 2013), and plant extracts (2018) Bacteriophages are bacterial viruses that are typically specific to one bacterial species. They have been proven to greatly inhibit bacteria, but resistance is common and therefore cocktails are typically made to treat pathogenic bacteria (Ghosh et al., 2019; Saha and Mukherjee, 2019; Shlezinger et al., 2017). Furthermore, clustered regularly interspaced short palindromic repeats (CRISPR) in conjunction with CRISPR-associated proteins (Cas) have been demonstrated to reduce antimicrobial resistant bacteria and inhibit bacteria through cleaving bacterial DNA, leading to removal of plasmid carrying resistance genes or bacterial cell death (Beisel et al., 2014; Bikard et al., 2014; Buckner

et al., 2018). Plant extracts, such as essential oils and phenolic compounds, have also been shown to inhibit bacterial pathogens (Villalobos et al., 2016). For example, Kil et al. (2009) described the minimum inhibitory concentrations (MIC) for a phenolic compound on *S. aureus, Salmonella* Typhimurium, *K. pneumoniae,* and *E. coli* of 250, 500, 500, and 250 µg/mL, respectively,

Therefore, our main objectives were to evaluate antimicrobial alternatives to inhibit or reduce population density of *F. necrophorum* in the rumen, as well as inhibit foodborne pathogens and pathogens involved in bovine mastitis. The alternatives evaluated to inhibit or reduce *F. necrophorum* were bacteriophages and CRISPR-Cas, and sorghum phenolic compounds to control food borne pathogens and mastitic pathogens.

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Chapter 3 - Relative Isolation Rates of Bacteriophages Lytic to *Fusobacterium necrophorum* of Liver Abscess Origin from Ruminal Fluid and City Sewage Samples

Abstract

Fusobacterium necrophorum subsp. *necrophorum*, an anaerobic ruminal bacterium is the primary causative agent of liver abscesses in feedlot cattle. Currently, tylosin, a macrolide, is widely used as a feed additive to reduce the incidence of liver abscesses. Because of the public health implication associated with the use of a medically important antimicrobial in food animal production system, there is considerable interest in finding an antimicrobial alternative to control liver abscesses. Bacteriophages that specifically target and lyse *Fusobacterium* have the potential to replace tylosin for the control of liver abscesses. Our objective was to detect and isolate bacteriophages lytic to *F. necrophorum* subsp. *necrophorum*. Pooled bovine ruminal fluids from slaughtered cattle at abattoirs and pooled untreated city sewage samples were collected on five separate dates. A total of 68 *F. necrophorum* subsp. *necrophorum* strains, isolated from liver abscesses, were used to isolate bacteriophages. Aliquots of pooled ruminal fluid or sewage samples were incubated anaerobically overnight with lysine and subsp. *necrophorum* strains, filtered, bacteria-free filtrates were spotted on lawns of the strains, and presence of plaques were considered as positive for phages. Presumptive bacteriophage plaques were harvested, and the viruses purified by serial passaging on the susceptible bacterial strains. The bacteriophage isolation frequencies were compared between sample types, sampling dates, and *F. necrophorum* strains. The overall relative frequency of isolated bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* was 17.1 %. The frequency of bacteriophage isolation between collection dates ranged from 0 to 25.4

% for ruminal fluid, and from 13.7 to 32.0 % for sewage samples. The frequency of isolation of bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* from sewage samples was higher ($p < 0.0001$) than lytic bacteriophages isolated from bovine ruminal fluid samples. Bacteriophages were isolated more frequently with certain *F. necrophorum* subsp. *necrophorum* strains than others. Bacteriophages have the potential to be used as feed additives to reduce the population density of *F. necrophorum* subsp. *necrophorum* in the rumen, thereby reduce the incidence of liver abscesses.

Introduction

Liver abscesses are one of the major reasons for liver condemnations in beef cattle processing plants. According to the recent National Beef Quality Audit, abscesses accounted for 58 % of all condemned livers (Eastwood et al., 2017). Additionally, liver abscesses contribute to the economic loss to the cattle industry due to decreased animal performance and lower carcass yield and quality, as well as time and labor costs associated with handling liver abscesses at the slaughter plant (Nagaraja and Lechtenberg, 2007). *Fusobacterium necrophorum*, an anaerobic, Gram negative, rod-shaped opportunistic pathogen, is the primary causative agent of liver abscesses (Langworth, 1977). The bacterium is a member of the microbial community of the rumen and travels across the ruminal epithelium into the portal circulation to the liver to produce abscesses. Chronic ruminal acidosis in cattle fed high grain diets compromises ruminal epithelium and permits *F. necrophorum* to penetrate the ruminal wall to cause ruminal abscesses and rumenitis. From these abscesses, the bacterium enters the portal blood to reach the liver (Nagaraja and Chengappa, 1998). Two subspecies, *necrophorum* and *funduliforme*, are reported, which differ in colony and cell morphologies, growth patterns in broth, and more importantly, in virulence (Tadepalli et al., 2009).

The subsp. *necrophorum* is more virulent, hence, more frequently involved in liver abscesses than the subsp. *funduliforme* (Tadepalli et al., 2009).

Currently, the antibiotic tylosin is used in the feed to reduce the incidence of liver abscesses (Nagaraja and Lechtenberg, 2007). Use of in-feed tylosin is of concern because the antibiotic, although used exclusively in animals, belongs to the macrolide class of antimicrobials, a class considered as a medically important antimicrobial (FDA, 2019). Because of the public health concern associated with medically important antimicrobial use in feedlot cattle there is considerable interest in finding an alternative to replace tylosin use.

Bacteriophages are viruses that infect bacteria and are either lytic or lysogenic (Snyder, 2013). Originally, phages were investigated as potential therapeutic agents against human and animal infections (Waldor et al., 2005). In recent years, the therapeutic use of phages or phage therapy, has regained interest as an alternative to antimicrobial therapy (Czaplewski et al., 2016; Gadde et al., 2017; Fischetti, 2018). Bacteriophages have been used in food animal processing plants to control for foodborne pathogens (Bruce and James, 2004; Housby and Mann, 2009; Tang et al., 2009; Bhardwaj et al., 2015). Although *F. necrophorum* is a major bovine pathogen, there is only one report on the isolation of bacteriophages lytic to this bacterium (Tamada et al., 1985). However, bacteriophages have been isolated from other species of *Fusobacterium,* such as *nucleatum,* a human oral pathogen (Andrews et al., 1997; Machuca et al., 2010). Here, we report on the relative frequency of isolation of bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* from ruminal fluid and city sewage samples.

Materials and Methods

F. necrophorum Strains

A total of 68 *F. necrophorum* subsp. *necrophorum* strains, previously isolated from liver abscesses of beef cattle, were used for phage detection and isolation (Amachawadi et al., 2017). Of the 68 strains, 65 were used for bacteriophage isolation from ruminal fluid and 56 were used for isolation from city sewage samples. Fifty-three strains were used for both sample types, while 12 strains were used for isolation from ruminal fluid samples only and three were used for isolation from city sewage samples only. The strains, stored at -80°C, were streaked onto sheep blood agar plates (SBA; Remel Inc., Lenexa, KS) and incubated at 37° C for 48 h in an anaerobic glove box (Forma Scientific Inc., Marietta, OH). Single colonies of each isolate were inoculated into 10 ml pre-reduced (with 0.05 % cysteine HCl; Sigma-Aldrich, St. Louis, MO), anaerobically sterilized brain heart infusion broth (PRAS-BHI; Becton Dickinson, Sparks, MD) and incubated overnight at 37°C.

Collection of Ruminal Fluid and City Sewage Samples

Ruminal fluid samples were collected at a beef cattle packing plant in the Midwest on five different dates between July 2019 and November 2019. At each visit, over a period of 4 to 6 hours, 3 liters of pooled ruminal fluid samples from squeezed ruminal contents from cattle originating from at least five different feedlots were collected and transported in tightly capped thermos containers kept on ice to the laboratory. Sewage samples were collected at a wastewater treatment plant on five different dates between October 2019 and February 2020. At each visit, 3 liters of untreated sewage samples were collected and transported to the laboratory. Ruminal fluid and sewage samples were placed in anaerobic jars and kept in a refrigerator and processed the next day for bacteriophage isolation.

Isolation of Bacteriophages

One hundred milliliters of ruminal fluid or sewage sample was aliquoted into twelve 125 mL flasks and 0.73 g L-lysine (Sigma-Aldrich, Burlington, VT) and 3.7 g of dehydrated BHI powder (Beckton Dickinson) were added and mixed until dissolved (Russell, 2005). The flasks were placed in an anaerobic glove box and 1 mL of three to five randomly selected *F. necrophorum* strains were inoculated into each flask. The flasks were placed on a shaker for overnight incubation at 37 C. The following day, 1 mL from each flask was pipetted into an Eppendorf tube (Eppendorf AG, Hamburg, Germany) and centrifuged at 12,000 x g for 2 minutes. Five hundred microliters of each supernatant were then pipetted into two mL Spin-X centrifuge tubes (Corning Costar, Corning, NY) with a 0.22 µm pore size membrane filter and centrifuged at 12,000 x g for 2 minutes. Supernatant from the Spin-X tube was pipetted into a 0.22 µm syringe filter and filtered a second time. Twenty microliters of the filtrate were then diluted in 180 mL of sterile SM buffer (G-Biosciences, St. Louis, MO) in a 1.5 mL Eppendorf tube. Individual bacterial cultures were spread-plated on BAP with sterile cotton swabs to obtain bacterial lawns and 20 µL of the corresponding flask filtrates and dilutions were spotted onto each plate. The plates were placed agar side down in an anaerobic glove box and incubated at 37°C overnight. The next day, the bacterial lawns were checked for phage plaques (Figure 3.1a). The plaques were then harvested by pipetting the agar where the spot was with a transfer pipette so that the agar was pipetted into the transfer pipet and was transported into 500 µL sterile SM buffer in 1.5 mL Eppendorf tubes. This was allowed to elute overnight at 37°C in an anaerobic glove box. The following day, the buffer with harvested bacteriophages was centrifuged for 30 seconds at 12,000 x g and the supernatant was transferred to a sterile Spin- X tube with a 0.22 µm filter before being centrifuged at 12,000 x g for 2 minutes. The filtrates were serially diluted 10-fold in SM buffer and 100 µL of each dilution was mixed

with 100 µL of the corresponding *F. necrophorum* strain. This mixture was spread plated onto pre-labeled blood agar plates. Bacterial lawns were checked for the presence of single plaques and these single plaques were then harvested as described above (Figure 3.1b). If multiple plaque morphologies were observed, each morphologically different plaque was harvested separately. The filtrates were diluted two times as described above and each dilution was spotted onto a new lawn of the corresponding *F. necrophorum* strain. Bacterial lawns were checked for the presence of plaques and then harvested as previously described (Figure 3.1c). The following day, 100 µL of the bacteriophage isolate was then distributed into two cryovials, each containing 300 µl 20 % glycerol and stored in a -80 °C freezer.

Statistical Analysis

The frequency of *F. necrophorum* bacteriophage isolation was estimated as the proportion of samples testing positive for the presence of bacteriophage divided by the total number of *F. necrophorum* strains tested. Prevalence of bacteriophage was stratified by sample type, sampling day, and *F. necrophorum* strains used for isolation. Crude associations between frequency of sample type or sampling date were tested via the likelihood-ratio based Chi-square test or the Fisher's exact test. Descriptive statistics were obtained for each strain per sample type. The results were analyzed using STATA ver 16.1 (StataCorp, 2019 #747). The results of the bacteriophage isolation, presence or absence, were the outcome variable. Associations between the isolated bacteriophages and sample types or sampling days were tested with multivariable random effects logistic regression models in considering *F. necrophorum* strains identifier as potential clustering variable to account lack of independence associated with testing *F. necrophorum* strains for sample type on multiple sampling days. All possible two-way interactions between individual explanatory variables were assessed at significance level $p < 0.05$. The Wald type test was used to assess the

statistical significance of association between the outcome and each explanatory variable or two-way interaction between variables. An explanatory variable or two-way interaction between variables was considered significant at *p*-value of 0.05. Odds ratio (OR) and their respective 95 % confidence interval were used to evaluate the probability between outcome and explanatory variable in the model. Regression model diagnostics including normality of predicted values of the random variables (or best linear unbiased predictors-BLUPS) and Pearson and Deviance residuals for observation at the lower level (sample level) from the final multivariable models. Relative frequencies were determined by dividing the number of isolated bacteriophages by the total number of samples. The frequency of isolation was determined for sample collection dates, pooled ruminal and sewage samples, pooled total samples, and individual *F. necrophorum* strains.

Results

Overall, a total of 89 bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* were isolated from 5 different sampling days from both sample types and 65 strains of *F. necroporum* subsp. *necrophorum*. For ruminal fluid samples, the bacteriophage isolation frequency between collection dates ranged from 0 to 25.4 %, while the relative frequencies of bacteriophage isolation from sewage ranged from 13.7 to 32.0 % (Table 3.1). The frequency of isolation of bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* from city sewage samples was higher ($p < 0.0001$) than lytic bacteriophages isolated from bovine ruminal fluid samples. Furthermore, bacteriophages isolated from sewage had a 0.9 log odds increase compared to bacteriophages isolated from ruminal fluid ($p < 0.0001$). There were no differences between sewage collection dates ($p = 0.2909$), but differences between ruminal fluid collection dates were significant ($p = 0.0091$).

Bacteriophages were isolated more frequently with certain *F. necrophorum* subsp. *necrophorum* strains than others (Tables 3.2 and 3.3). Of the 65 strains used with ruminal fluid,

22 strains yielded bacteriophages and 43 did not. In the case of sewage samples, 26 strains did not yield bacteriophages, while 33 strains yielded bacteriophages, and three strains yielded bacteriophages at each collection date (Table 3.2). Overall, 40 strains of *F. necrophorum* yielded bacteriophages with either ruminal fluid or sewage sample in one or more of the sampling dates (Table 3.3). Thirteen strains yielded bacteriophages from both ruminal fluid and sewage samples, whereas 8 strains yielded bacteriophages from ruminal fluid and 17 strains yielded from sewage sample only (Table 3.4). The overall relative frequency of isolation of bacteriophages for three *F. necrophorum* subsp. *necrophorum* strains (2016-13-11, 2016-13-29, and 2016-13-69) were 6/10, 6/10, and 6/7 or 60 %, 60 %, and 85.7 %, respectively (Table 3.3). For two strains (2016-13-11 and 2016-13-29), the relative frequencies of isolating bacteriophages in bovine ruminal fluid and city sewage samples were 1/5 and 5/5 or 20 % and 100 %, respectively. The third strain (2016-13-69) had relative frequencies of isolating bacteriophages in bovine ruminal fluid and city sewage samples of 1/2 and 5/5 or 50 % and 100 %, respectively. The overall relative frequency of isolating bacteriophages from this *F. necrophorum* subsp. *necrophorum* strain (2016-13-69) was the highest at 5/7 (85.7 %). No *F. necrophorum* subsp. *necrophorum* strains had a relative frequency of isolating bacteriophages over 50 % from bovine ruminal fluid, while nine *F. necrophorum* subsp. *necrophorum* strains had relative frequencies of isolating bacteriophages over 50 % and three of these were 100 % from city sewage samples.

Discussion

Currently, the available method to control liver abscesses is with the use of antimicrobial feed additives. Five antimicrobial compounds have FDA approval for use in the feed to control liver abscesses in the US (Feed Additive Compendium, 2021). The most widely used antibiotic is tylosin, which belongs to the antimicrobial class, macrolide. (Nagaraja and Chengappa, 1998).

Tylosin is a narrow spectrum antibiotic, primarily effective against Gram-positive bacteria (Gingerich et al., 1977). However, the antibiotic is inhibitory to certain Gram-negative bacteria, including *F. necrophorum* (Tan et al., 1994; Lechtenberg et al., 1998). Although tylosin is used exclusively in animals, it is structurally similar to erythromycin, and antibiotics in the macrolides class are categorized as medically important because of their extensive use in human medicine (Scott et al., 2019). Therefore, there is growing scrutiny in the use of tylosin in animals, which is the reason for placing tylosin under the Veterinary Feed Directive with veterinary oversight (FDA, 2021). The impact of tylosin exposure on antimicrobial resistance of gut bacteria, particularly enterococci, which has the propensity for horizontal transfer of AMR genes, is of some concern (Zaheer et al., 2013; Alicia et al., 2015; Müller et al., 2018). In a recent study that measured the AMR of gut bacteria in feedlot cattle administered with in-feed tylosin to prevent liver abscesses, the conclusion was that the AMR impact was minimal and did not extend beyond occasional increase in macrolide-resistant fecal enterococci (Schmidt et al., 2020). Interestingly, there is no evidence of macrolide resistance in *F. necrophorum* isolated from liver abscesses of tylosin-fed cattle (Nagaraja et al., 1999; Amachawadi et al., 2017). Because of the public health concern associated with the use of a medically important antibiotic in cattle diets, there is considerable interest in finding alternatives to replace tylosin. Some of the alternatives that have been evaluated include vaccines (Saginala et al., 1997; Jones et al., 2004; Fox et al., 2009), essential oils (Meyer et al., 2009; Elwakeel et al., 2013; Samii et al., 2016), oil seeds (Mir et al., 2008), egg yolk immunoglobulins (Stotz et al., 2021) and direct-fed microbials (Huebner et al., 2019). Because virulence factors have been identified and a few have been well-characterized, there have been many efforts to develop an efficacious vaccine (Amachawadi and Nagaraja, 2016). Although a bacterin is commercially available (Fusogard®; Elanco Animal Health, Greenfield, IN), the

efficacy of the vaccine has been shown to be marginal and has not replaced tylosin use in the feedlot industry (Checkley et al., 2005). Feeding certain yeast-based commercial direct-fed microbial products (Natursafe or XPC, Diamond V, Cedar Rapids, IA) have been shown to reduce severity of liver abscesses compared to the control, although the proportion of total abscessed livers was not different (Shen et al., 2019). The likely mode of action is alleviation of ruminal acidosis by the yeast product (Shen et al., 2018).

Virulent or lytic bacteriophages are a potential alternative to antimicrobials to treat animal and human infections (Waldor et al., 2005). Phage therapy was investigated in the 1920s and 1930s, but interest was lost as antibiotics became available (Adams, 1959; Summers, 2012). In recent decades, there has been a renewed interest in phage therapy, as data show it could be an effective tool against bacterial infections in animal and human medicine (Joerger, 2003; Matsuzaki et al., 2005; Kutateladze and Adamia, 2010; Ghosh et al., 2019; Saha and Mukherjee, 2019). However, limited research has been done on phage therapy for pathogens in animals. The only study reported on isolation of bacteriophages lytic to *F. necrophorum* was by Tamada et al (1985). The study reported a single phage isolated and characterized from 54 bovine ruminal fluid samples (Tamada et al., 1985). The phage was lytic to both subspecies of *F. necrophorum.*

In the phage isolation procedure, we used an enrichment step that included incubation of ruminal fluid or sewage samples with lysine and *F. necrophorum* strains (3 to 5 strains per sample aliquot) in order to promote fusobacterial growth and enhance the chance of phage detection. *Fusobacterium necrophorum* utilizes lysine as an energy source and rapidly deaminates it to acetate, butyrate, and ammonia (Russell, 2005, 2006). Therefore, addition of lysine to ruminal fluid and sewage samples, enriches the fusobacterial population. A total of 68 strains were used in the enrichment step and because the collection dates for the two sample types were different, the

same number of strains were not used at each sampling time. It is evident that phages were detected more often with certain strains than others, which is not surprising because of known host specificity of phages (Szafrański et al., 2017; Zheng et al., 2019). A limitation of this study was that different collection dates had a varying number, between 47-59, of *F. necrophorum* subsp. *necrophorum* strains used. This was because some isolates did not grow on either blood agar or in BHI broth. The isolates that did not grow were random for each sampling date. Also, for logistical reasons, collections of bovine ruminal fluid and city sewage sample were conducted in different seasons.

The finding that phages were isolated from city sewage samples, in addition to ruminal fluid, was not unexpected. *Fusobacterium necrophorum* is also a member of the normal bacterial flora of the human throat, urogenital, and gastrointestinal tracts (Kristensen and Prag, 2000; Amess et al., 2007; Kuppalli et al., 2012). In humans, *F. necrophorum* is an opportunistic pathogen and causes pharyngotonsillitis, especially in adolescent and young adults, and in rare situations leads to an invasive infection called Lemierre syndrome (Kristensen and Prag, 2000; Amess et al., 2007; Eaton and Swindells, 2014; Holm et al., 2017). The strains of *F. necrophorum* isolated from humans are more often subsp. *funduliforme* than subsp. *necrophorum* (Tadepalli et al., 2008; Holm et al., 2017). A surprising finding in our study was the higher frequency of detection of phages from city sewage samples than bovine ruminal fluid. There was approximately a two-fold increase in the overall frequency of bacteriophage isolation lytic to *F. necrophorum* subsp. *necrophorum* from city sewage compared to ruminal fluid samples.

Conclusion

In summary, relative frequencies of bacteriophage isolation lytic to *F. necrophorum* subsp. *necrophorum* were higher in city sewage samples than bovine ruminal fluid.

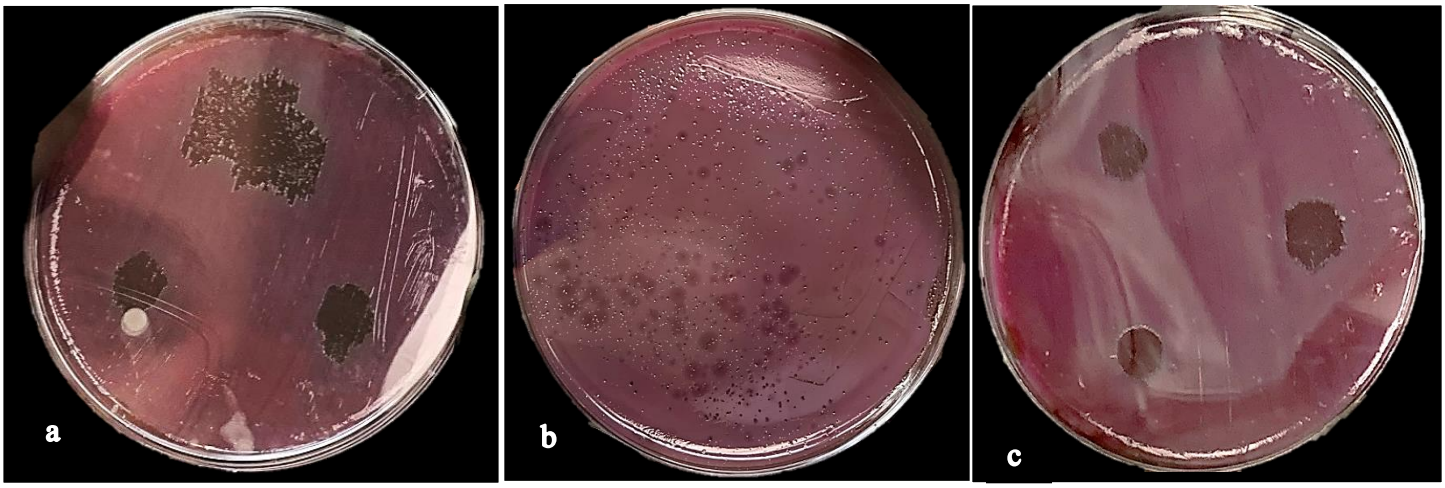

Figure 3.1. Isolation of bacteriophages lytic to *F. necrophorum subsp. necrophorum.*

First spots were made from rumen or sewage fluids on *F. necrophorum* lawns and bacteriophage plaques were observed (a). Then plaques were harvested, diluted, mixed with *F. necrophorum* culture, and spread plated onto blood agar. Single plaques were harvested of the same morphologies (b). These filtrates were then spotted onto lawns of *F. necrophorum* and these large plaques were used to harvest and save bacteriophages in -80 °C (c).

Table 3.1. Relative frequency of bacteriophage isolations from bovine ruminal fluid and city sewage samples with *Fusobacterium necrophorum* strains of liver abscess origin.

Sample	Sample collection date	No. positive isolated/no. of *F. necrophorum* strains	Relative frequency, %
Ruminal fluid	7/29/2019	15/59	25.4
	8/12/2019	9/54	16.7
	8/26/2019	2/53	3.8
	9/9/2019	0/51	0
	10/28/2019	3/47	6.4
City sewage[a]	10/21/2019	13/52	25.0
	12/2/2019	13/51	25.5
	12/9/2019	7/51	13.7
	12/16/2019	16/50	32.0
	2/17/2020	11/53	20.8

[a] Relative frequency of isolation was higher with city sewage sample compared to ruminal fluid ($p < 0.0001$).

Table 3.2. Frequency of bacteriophage isolations from bovine ruminal fluid and city sewage samples with *Fusobacterium necrophorum* strains of liver abscess origin.

Bovine ruminal fluid		City sewage	
No. of *F. necrophorum* strains	Frequency of phage isolation	No. of *F. necrophorum* strains	Frequency of phage detection
43	0	26	0
15	1	19	1
7	2	1	2
.	.	5	3
.	.	2	4
.	.	3	5

Table 3.3. Number of bacteriophages isolated from bovine ruminal fluid or city sewage samples with *Fusobacterium necrophorum* strains of liver abscess origin on different sampling dates.

F. necrophorum subsp. *necrophorum* strains[1]	Bovine ruminal fluid		City sewage	
	No. positive/sampling date	Relative Frequency, %	No. positive/sampling date	Relative Frequency, %
2016-13-12	1/4	25.0	.	.
2016-13-15	0/5	0	1/5	20.0
2016-13-17	0/4	0	1/3	33.3
2016-13-28	1/5	20.0	.	.
2016-13-58	0/2	0	1/4	25.0
2016-13-67	0/5	0	1/4	25.0
2016-13-68	1/4	25.0	0/5	0.0
2016-13-75	0/5	0	1/5	20.0
2016-13-77	0/5	0	1/5	20.0
2016-13-89	1/5	20.0	0/5	0
2016-13-91	0/5	0	1/5	20.0
2016-13-92	0/3	0	1/4	25.0
2016-13-102	1/5	20.0	0/5	0
2016-13-890	1/4	25.0	0/5	0
2016-13-8	1/5	20.0	1/5	20.0
2016-13-10	1/5	20.0	1/5	20.0
2016-13-14	1/5	20.0	1/3	33.3
2016-13-36	2/5	40.00	0/5	0
2016-13-46	2/5	40.0	0/5	0
2016-13-49	0/5	0	3/5	60.0
2016-13-62	1/5	20.0	1/4	25.0
2016-13-66	0/2	0	2/5	40.0
2016-13-76	1/4	25.0	1/5	20.0
2016-13-88	2/5	40.0	0/4	0
2016-13-148	0/5	0	1/5	20.0
2016-13-9	2/5	40.0	1/5	20.0
2016-13-10	0/4	0	1/5	20.0
2016-13-41	0/5	0	3/5	60.0
2016-13-54	0/5	0	3/5	60.0
2016-13-57	2/5	40.0	1/3	33.3
2016-13-61	2/5	40.0	1/5	20.0
2016-13-63	1/4	25.0	1/5	20.0
2016-13-83	0/4	0	3/5	60.0
2016-13-98	2/5	40.0	1/5	20.0

2016-13-882	0/1	0	3/5	60.0
2016-13-51	0/1	0	4/5	80.0
2016-13-84	.	.	4/5	80.0
2016-13-11	1/5	20.0	5/5	100.0
2016-13-29	1/5	20.0	5/5	100.0
2016-13-69	1/2	50.0	5/5	100.0

[1]Twenty-eight *F. necrophorum* stains are not listed because they were not inhibited by any bacteriophages.

Table 3.4. Number of bacteriophage isolated from bovine ruminal fluid or city sewage samples with *Fusobacterium necrophorum* of liver abscess origin.

	No. of bacteriophages isolated	
F. necrophorum strains	Bovine ruminal fluid	City sewage
2016-13-8, 2016-13-10, 2016-13-14, 2016-13-62, 2016-13-76, 2016-13-9, 2016-13-57, 2016-13-61, 2016-13-63, 2016-13-98, 2016-13-11, 2016-13-29, 2016-13-69	17	25
2016-13-12, 2016-13-28, 2016-13-68, 2016-13-89, 2016-13-102, 2016-13-890, 2016-13-36, 2016-13-46, 2016-13-88	12	0
2016-13-15, 2016-13-17, 2016-13-58, 2016-13-67, 2016-13-75, 2016-13-77, 2016-13-91, 2016-13-92, 2016-13-49, 2016-13-66, 2016-13-148, 2016-13-10, 2016-13-41, 2016-13-54, 2016-13-83, 2016-13-882, 2016-13-51, 2016-13-84	0	35

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Chapter 4 - Characterization of Bacteriophages Lytic to *Fusobacterium necrophorum* of Liver Abscess Origin

Abstract

Fusobacterium necrophorum subsp. *necrophorum,* an anaerobic ruminal bacterium is the primary causative agent of liver abscesses in feedlot cattle. Currently, tylosin, a macrolide, is commonly used as a feed additive to reduce the incidence of liver abscesses. Because of the public health implication associated with the use of a medically important antimicrobial in food animal production system, there is considerable interest in finding an antimicrobial alternative to control liver abscesses. Bacteriophages that specifically lyse and reduce population density of *F. necrophorum* subsp. *necrophorum* in the rumen have the potential to replace tylosin for the control of liver abscesses. Our objective was to characterize 25 bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* previously isolated from rumen fluid and sewage samples. The characterization included determination of host specificity, electron microscopic morphology, development of insensitivity and optimal pH and temperature stability. All 25 bacteriophages were lytic to *F. necrophorum* subsp. *necrophorum* only and did not lyse subsp. *funduliforme* or any of the bacterial species tested. Five bacteriophage morphological classifications belonging to *Siphoviridae, Myoviridae, Podoviridae,* four unclassified with icosahedral heads and no tail, and one with multiple tails was unclassified. In broth cultures, 11 bacteriophages inhibited *F. necrophorum* subsp. *necrophorum* up to 12 hrs, while the other 14 bacteriophages only slightly slowed the growth of the bacterium. *F. necrophorum* subsp. *necrophorum* became insensitive to all 11 lytic bacteriophages after 24 h. At a pH of 2.5, all 11 bacteriophages were inactivated immediately, while at a pH of 3.5, six bacteriophages were only partially inactivated upon initial

exposure and five survived for 1 hr. Between pH 4.5- pH 8.5, no reduction in bacteriophage activity was observed. After 24 h of incubation at 30 °C, 40 °C, and 50 °C, all 11 lytic activities bacteriophages were unaffected.

In conclusion, lytic phages were highly host specific, belonged to several morphological groups, and their pH and temperature requirements were compatible with ruminal conditions of feedlot cattle. However, the development of insensitivity of the susceptible strains could be problematic in the application of lytic phages to reduce population density of *F. necrophorum* subsp. *necrophorum* in the rumen.

Introduction

Fusobacterium necrophorum is the primary causative agent of liver abscesses in cattle (Langworth, 1977). The bacterium is an anaerobic, Gram-negative, rod-shaped opportunistic pathogen that is a member of the ruminal bacterial flora. Chronic acidosis causes ruminal epithelial lesions that facilitate the bacterium to travel to the liver by allowing it to enter the portal blood. Two subspecies, *necrophorum* and *funduliforme*, are described, which differ in cell and colony morphologies, growth patterns and virulence. The subsp. *necrophorum* is more frequently involved in liver abscesses, likely because it is more virulent than the subsp. *funduliforme* (Tadepalli et al., 2009).

The most recent National Beef Quality Audit Report estimates that liver abscesses cause 58 % of all condemned livers in beef cattle processing plants (Eastwood et al., 2017). Therefore, liver condemnation are of economic concern for the cattle producers and packers. More importantly, liver abscesses cause decreased animal performance and lower carcass yield, which contribute to economic loss to the cattle industry, along with increased time and labor costs at the slaughter plants (Nagaraja and Lechtenberg, 2007). Currently, to reduce the incidence of liver abscesses,

tylosin, a macrolide antibiotic, is used in the feed (Brown et al., 1975; Nagaraja and Lechtenberg, 2007). Tylosin's mode of action is to inhibit *F. necrophorum* and decrease ruminal concentration, which decreases the chance of the bacterium to enter the ruminal wall and subsequently reach the liver. Use of in-feed tylosin is of concern because macrolides are medically important antibiotics (FDA, 2015). Public health concerns associated with the use of medically important antimicrobials causing antimicrobial resistance have led to considerable interests in finding antimicrobial alternatives in food animal production systems (Aidara-Kane et al., 2018; Hoelzer et al., 2018).

Bacteriophages are viruses that can infect and lyse host bacterial cells (Adams, 1959). They have been used for many years as a powerful tool to study bacterial genetics, and because of their host specificity, they have been used in the identification and characterization of bacteria, called phage typing. Bacteriophages are relatively safe and have no effects on animal or human cells, therefore, they have been investigated as potential therapeutic agents against bacterial infections, even before the advent of antibiotics (Waldor et al., 2005). Antimicrobial resistance to medically important antimicrobials has increased in recent years, which has renewed interest in the use of antimicrobial alternatives, including therapeutic use of phages, or phage therapy (Czaplewski et al., 2016; Gadde et al., 2017). Food animal meat processing plants have used bacteriophages to reduce contaminations of food-borne pathogens (Bruce and James, 2004; Housby and Mann, 2009; Tang et al., 2009; Bhardwaj et al., 2015). Eighty-nine bacteriophages from ruminal content and city sewage samples lytic to *F. necrophorum* subsp. *necrophorum* of liver abscess origin have been isolated (Schnur et al., 2021). Here, the characterization of bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* of liver abscess origin is reported.

Materials and Methods

Bacteriophages

Eighty-nine bacteriophages lytic to *F. necropohorum* subsp. *necrophorum* were isolated from ruminal fluid and city sewage samples (Schnur et al., 2021). Of these, 29 were isolated from ruminal fluid and 60 were isolated from sewage samples. At the time of isolation, bacteriophages were frozen at -80 °C in two aliquots of 100 µL mixed with 300 µl 20 % glycerol. For the current study, 50 of the frozen bacteriophage stocks were randomly selected, thawed, spotted onto the *F. necrophorum* strain they were originally isolated from, and harvested as previously described by Schnur et. al. (2021). The fresh bacteriophage stocks were stored at 4 °C until further analysis was performed.

F. necrophorum strains

Thirty strains of *F. necrophorum* subsp. *necrophorum*, previously isolated from liver abscesses of beef cattle, were used to determine the host range (Amachawadi et al., 2017). The strains, stored at -80 °C, were streaked onto sheep blood agar plates (SBA; Remel Inc., Lenexa, KS) and incubated for 48 h at 37 °C in an anaerobic glove box (Forma Scientific Inc., Marietta, OH). Single colonies were inoculated into 10 ml pre-reduced (with 0.05 % cysteine HCl; Sigma-Aldrich, St. Louis, MO), anaerobically sterilized brain heart infusion broth (PRAS-BHI; Becton Dickinson, Sparks, MD) and incubated at 37 °C overnight.

Host specificity

Bacteriophages were prepared by making a lawn of an overnight culture of *F. necrophorum* on SBA with sterile cotton swabs and 20 µL of the frozen bacteriophage stock (Schnur et al., 2021). The plates were placed agar side down in a humidified anaerobic glove box and incubated at 37°C overnight. The next day, the bacterial lawns were checked for phage plaques. The plaques

were then harvested by pipetting the agar surrounding the plaque with a transfer pipette into 500 µL sterile SM buffer (G-Biosciences, St. Louis, MO) in 1.5 mL Eppendorf tubes and allowed to elute overnight. The buffer with harvested bacteriophages was centrifuged for 30 seconds at 12,000 x g to sediment agar pieces. The supernatant was then transferred to a sterile Spin- X tube with a 0.22 µm membrane filter (Corning Costar, Corning, New York) and centrifuged for 2 minutes at 12,000 x g. The filtrates were serially diluted 10-fold in SM buffer and each dilution was spotted onto a new lawn of the corresponding *F. necrophorum* strain. Bacterial lawns were checked for single plaques, which were then harvested as described above and stored at 4 °C. Next, individual bacterial cultures were spread-plated on SBA with sterile cotton swabs to obtain bacterial lawns and 20 µL of each bacteriophage were spotted onto each plate with 6 bacteriophages/ SBA. A total of 50 bacteriophages were spotted onto 30 strains of *F. necrophorum* subsp. *necrophorum* and 20 bacteriophages were tested against five *F. necrophorum* subsp. *funduliforme* strains, four *F. varium* strains, three *F. nucleatum* strains, one each of strains *F. equinum, F. ulcerans, F. gonidiaformans, F. russii, F. gastrosuis*, six *Escherichia coli* O157 strains, five strains of *Campylobacter coli*, and four strains of *C. jejuni*, and one strain each of *Salmonella typhimurium, Klebsiella pneumoniae, K. oxytoca, Staphylococcus aureus*, and *Pseudomonas auruginosa*. The plates were placed agar side down in an aerobic incubator, except for *Campylobacter* and *Fusobacterium* strains, which were placed in a microaerophilic incubator (5 % oxygen, 10% carbon dioxide and 85 % nitrogen) or in an anaerobic glove box, agar side down, respectively, and incubated at 37 °C overnight. The following day the plates were examined for phage plaques. The number of bacterial strains that each bacteriophage lysed was recorded and the 25 bacteriophages with the widest *F. necrophorum* host range were selected for further characterization.

Standardization of bacteriophage stock concentrations

Bacteriophage stocks were made as described by Bonilla et al. (2016). Ten microliters of filtrates from bacteriophages lytic to *F. necrophorum* were serially diluted ten-fold in 90 µL of sterile SM buffer) in a 96-well plate. One hundred microliters of an overnight culture of *F. necrophorum* subsp. *necrophorum* were then added to each well to mix with dilutions of bacteriophage. Two hundred microliters of the *F. necrophorum* and bacteriophage mixture were then spread-plated onto SBA for all the dilutions. The plates were incubated overnight at 37 °C in an anaerobic glove box and individual bacteriophage plaques were counted and the plaque forming units (PFU/ml) were determined. Each bacteriophage stock was diluted to a concentration of 10^4 and the concentration was confirmed by serial dilution and spread plating on SBA as described above and stocks were stored at 4 °C.

Electron microscopy

Individual *F. necrophorum* cultures were spread-plated on SBA with sterile cotton swabs to obtain lawns and 20 µL of bacteriophage stock were spotted onto plates with four spots of one individual bacteriophage per plate. The plates were placed agar side down in an anaerobic glove box and incubated at 37 °C overnight. The next day, a sterile transfer pipet was used to transfer bacteriophage plaques to 1.5 mL microcentrifuge tubes with 500 µL sterile double distilled water and allowed to elute overnight at room temperature. The eluted bacteriophages were centrifuged for 30 seconds at 12,000 x g, the supernatant was transferred to a sterile Spin-X tube with a 0.22 um filter (Corning Costar, Corning, NY), and was centrifuged for 2 minutes at 12,000 x g. Twenty microliters of bacteriophage filtrate were then pipetted onto a piece of parafilm and 20 µL of UranyLess EM Stain (Electron Microscopy Sciences, Hatfield, PA) was pipetted next to, but not touching, the bacteriophage filtrate on the same piece of parafilm. Fine tip forceps were then used

to place a carbon film, dull side down, on the bacteriophage filtrate. After 1 minute, the carbon film was removed with fine tip forceps, gently tapped against a tissue paper, and placed, dull side down, on the UranyLess EM stain for 1 minute. The carbon film was removed with fine tip forceps, gently tapped against a tissue paper, and placed in the carbon film carrier. The carrier was then brought to the Kansas State University Microscopy Facility for visualization under a CM 100 transmission electron microscope (FEI Company, Hillsboro, OR).

F. necrophorum inhibition curve by bacteriophages

One milliliter of overnight *F. necrophorum* subsp. *necrophorum* culture was inoculated into 10 mL of PRAS-BHI and incubated for 1 hour at 37 ℃. One mL of standardized bacteriophage stock was added to the culture and incubated at 37 °C for 48 h. Culture absorbance was measured at 600 nm at 0, 1, 2, 3, 4, 5, 6, 12, 24, and 48 hours of incubation

Bacteriophage insensitivity

Bacteriophage insensitivity was tested by inoculating 1 ml of overnight *F. necrophorum* subsp. *necrophorum* into 10 mL of PRAS-BHI and incubating for 1 hour at 37 °C. One mL of standardized bacteriophage stock was then added to the culture and incubated at 37 ℃ for 24 h. A cotton swab was used to make a lawn of overnight cultures on SBA, 10 μL of corresponding standard bacteriophage stock were pipetted onto the lawn in two spots and the plates were incubated overnight in an anaerobic glove box at 37 °C. The following day the plates were examined for bacteriophage plaques. If there were no plaques, the *F. necrophorum* culture was considered insensitive to the bacteriophage. Each phage was tested in duplicates and the testing was replicated three times. *Fusobacterium necrophorum* strains that had not been inoculated with phage were used as controls.

The overnight cultures that were insensitive were tested against all other lytic bacteriophages to determine their susceptibilities. This was done by inoculating 1 ml of overnight *F. necrophorum* subsp. *necrophorum* into 10 mL of PRAS-BHI and incubating for 1 hour at 37 °C. One mL of standardized bacteriophage stock was then added to the culture and incubated at 37 °C for 24 h. A cotton swab was used to make a lawn of overnight cultures on SBA, 10 µL of each virulent standard bacteriophage stock was pipetted onto a separate lawn in two spots, and the plates were incubated overnight in an anaerobic glove box at 37 °C. The following day, plates were examined for bacteriophage plaques. If there were no plaques, the *F. necrophorum* culture was considered insensitive to the bacteriophage. Each phage was tested in duplicate and the testing was replicated three times. *Fusobacterium necrophorum* strains that had not been inoculated with phage were used as controls.

pH tolerance

Bacteriophage pH tolerance was tested at pH values of 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 and for 0 (control), 30 min, 1, 6, and 24 hr for the 11 bacteriophages (Phages ϕ2019-6-16, ϕ2019-6-25, ϕ2019-6-30, ϕ2019-6-31, ϕ2019-6-32, ϕ2019-6-34, ϕ2019-6-36B, ϕ2019-6-38, ϕ2019-6-40, ϕ2019-6-46, ϕ2019-6-47). Briefly, BHI broth was adjusted to the desired pH values with the addition of 1 M NaOH or 1 M HCl, then sterilized through a 0.22 µm syringe filter prior to storage. Aliquots (450 µl) of the pH-adjusted BHI were transferred into a 0.22 µ Spin-X tube along with 50 µl of bacteriophage stock that had been previously standardized to a 1 x 10^{-4} titer. The Spin-X tubes were then centrifuged at 12,000 x g for 2 minutes at room temperature to filter sterilize the bacteriophage-BHI solution. At each designated time point, 10 µl aliquots of the pH-adjusted bacteriophage solution were "spotted" onto a blood agar plate with a lawn of *F. necrophorum* subsp. *necrophorum*. Inoculated plates were then incubated overnight at 37 °C in an anaerobic

glovebox before examining for evidence of phage activity. The plaques were scored as ++, +, and -, indicating a zone of complete lysis, zone of incomplete lysis, and no lysis, respectively (Figure 4.1). Bacteriophage pH stability testing, performed in duplicates, was done twice.

Temperature tolerance

Temperature tolerance of 11 bacteriophages was tested at 30 ℃, 40 ℃, and 50 ℃, at time points of 1, 24, and 48 hr of temperature exposure. Briefly, 200 µl of bacteriophage stock that had been previously standardized to a 1 x 10^{-4} titer was aliquoted into sterile 1.5 ml Eppendorf (Eppendorf, Germany) tubes. For the 30 ℃ and 40 ℃ exposure temperatures, the Eppendorf tubes were arranged into a tube rack and placed in incubators, while the 50 ℃ exposure group was submerged into a pre-heated water bath for the duration of the study. At the designated sampling time points, the bacteriophages were removed and allowed to return to room temperature. Ten microliters of phage were then spotted onto a SBA agar plate that had been streaked with an overnight culture of *F. necrophorum* subsp. *necrophorum* to obtain a bacterial lawn. Bacteriophages were immediately placed in incubators/water bath until the next sampling time point. Inoculated plates were incubated overnight at 37 ℃ in an anaerobic glovebox and checked for lysis zones the following day. The lytic zone was scored as described above. Temperature stability testing was performed in duplicates and replicated twice.

Results

Host specificity

Thirty of the bacteriophages lysed less than 10 *F. necrophorum* subsp. *necrophorum* strains, while 20 bacteriophages lysed over 10 *F. necrophorum* subsp. *necrophorum* strains (Table 4.1; Figure 4.2). The highest number of *F. necrophorum* subsp. *necrophorum* strains lysed by one bacteriophage was 18. Twenty bacteriophages with the lowest specificity against *F. necrophorum*

subsp. *necrophorum* strains showed no lysis against *F. necrophorum* subsp. *funduliforme, F. varium* strains, *F. nucleatum, F. equinum, F. ulcerans, F. gonidiaformans, F. russii, F. gastrosuis, E. coli* O157 strains, *C. coli, C. jejuni, S. typhimurium, K. pneumoniae, K. oxytoca, S. aureus,* and *P. aeruginosa*.

Electron microscopy and morphological features

Electron microscopy was performed on 25 bacteriophages. Six morphological types were observed, and based on the morphology, phages were assigned to viral families (Figure 4.3). Eleven bacteriophages had non-elongated, helical heads with long and thin tails. Theses phages were morphologically classified as phages belonging to the *Siphoviridae* family. Four bacteriophages had medium length tails and were classified as part of the *Myoviridae* family. Of the *Myoviridae* phages, one had an elongated helical head and the other three had non-elongated helical heads. Three bacteriophages had non-elongated helical heads with short tails and two had round heads with short tails. These bacteriophages were morphologically assigned to the *Podoviridae* family. Four bacteriophages had round heads with no visible tails and were unable to be classified at this time. One bacteriophage had an unusual morphology with three long tail fibers and a very large, round head and this bacteriophage could not be assigned to any known phage families (Table 4.2).

F. necrophorum inhibition curve and insensitivity

Bacteriophage lytic efficiencies were determined for 25 bacteriophages. In broth cultures, eleven bacteriophages inhibited *F. necrophorum* subsp. *necrophorum* for up to 12 hr (Table 4.3; Figure 4.4). The other 14 bacteriophages did not inhibit *F. necrophorum* subsp. *necrophorum* at any time points, but slightly slowed the growth of the bacterium in broth cultures. At 24 and 48 hr, none of the bacteriophages inhibited *F. necrophorum* subsp. *necrophorum*. Therefore, after 24

hours, *F. necrophorum* was considered insensitive against the 11 lytic bacteriophages, and to confirm insensitivity spot testing was performed. When the bacteriophages were spotted against their corresponding insensitive *F. necrophorum*, strain no lysis spots were detected (Figure 4.5). All virulent bacteriophages were spotted against each insensitive bacteriophage culture. After 24 hours of incubation, there were no spots on any of the insensitive *F. necrophorum* cultures when each of the virulent bacteriophages were spotted on a lawn (Figure 4.6). As a control, the *F. necrophorum* strains not inoculated with phage were spotted against this parent *F. necrophorum* strain and lysis spots were detected for all bacteriophages.

Effects of pH and temperature lytic activity of bacteriophages

At a pH of 2.5, all 11 bacteriophages were inactivated upon initial exposure with no lytic activity observed at any of the six sampling time points. Partial bacteriophage inactivation, noted as an incomplete zone of lysis on spotted blood agar plates, was immediate (0 hr) at pH 3.5. However, a total of five of the tested bacteriophages were able to survive initial exposure, of which two were able to survive up to 30 min of exposure and two were viable for up to 1 hr of exposure (Table 4.4). All bacteriophages were completely inactivated after 1 hour of exposure to pH 3.5. Between pH 4.5- pH 8.5, no reduction in bacteriophage activity was observed, with all 11 bacteriophages consistently producing complete zones of lysis at 24 hours.

The same 11 bacteriophages used for pH stability analysis were chosen to determine temperature stability over a 48-hour period. None of the bacteriophages showed any loss of viability after 48 hours of exposure to any of the three tested temperatures (30, 40 or 50 °C), demonstrated by complete zones of lysis on spotted blood agar plates.

Discussion

Currently, antimicrobial feed additives are used to control liver abscesses. The most widely used antibiotic is a macrolide antibiotic, tylosin (Nagaraja and Chengappa, 1998). Tylosin is a narrow spectrum antibiotic that is predominantly effective against Gram-positive bacteria (Gingerich et al., 1977). However, the antibiotic inhibits some Gram-negative bacteria, including *F. necrophorum* (Tan et al., 1994; Lechtenberg et al., 1998). Although tylosin is solely used in animals, it is similar to erythromycin and antibiotics in the macrolide class are categorized as medically important because of their widespread use in human medicine (Scott et al., 2019). Therefore, there is a growing public concern about the use of tylosin in animals (FDA, 2021). The effect of tylosin use on antimicrobial resistance is of some concern, but studies have shown it has little effect on AMR (Nagaraja et al., 1999; Zaheer et al., 2013; Alicia et al., 2015; Amachawadi et al., 2017; Müller et al., 2018; Schmidt et al., 2020). Because of the public health concerns regarding the use of a medically important antibiotic in cattle diets, there is considerable interest in finding alternatives to replace tylosin.

Phage therapy could be an effective tool against bacterial infections in animal and human medicine (Joerger, 2003; Matsuzaki et al., 2005; Kutateladze and Adamia, 2010; Ghosh et al., 2019; Saha and Mukherjee, 2019). Virulent or lytic bacteriophages are a possible antimicrobial alternative to treat animal and human infections (Waldor et al., 2005). However, limited research has been conducted on phages lytic to bacterial pathogens in cattle. There has only been one report of isolation of bacteriophages lytic to *F. necrophorum* and the phage, FnP1 was lytic to both subspecies of *F. necrophorum* (Tamada et al., 1985). Interestingly, the 25 bacteriophages examined in the current study were only lytic to *F. necrophorum* subsp. *necrophorum* and not to subsp. *funduliforme*. Similarly, the bacteriophage Fnpϕ02 was only lytic to *F. nucleatum* species

(Machuca et al., 2010). However, unlike the phages in the current study, Fnpϕ02 was lytic to multiple subspecies of *F. nucleatum*.

The characterized bacteriophages could be classified into three different phage groups or families: groups A, B, and C or the families *Myoviridae*, *Siphovirida*, and *Podoviridae*, respectively (Bradley, 1967; Hyman and Abedon, 2012). Of the 25 bacteriophages characterized, four were phenotypically part of the *Myoviridae* family, like the one other characterized bacteriophage lytic to *F. necrophorum*, phage FnP1 (Tamada et al., 1985). Unlike the 25 bacteriophages characterized, phage FnP1 was lytic to both *F. necrophorum* subsp. *necrophorum* and *fundilforme*. *Myoviridae* bacteriophages have contractile tails and icosahedral heads that can be elongated (Hyman and Abedon, 2012). One other fusobacterial *Myoviridae* bacteriophage has been characterized as lytic to *Fusobacterium symbiosis* (Foglesong and Markovetz, 1974). Eleven of the bacteriophages were *Siphoviridae* family phages. *Siphoviridae* bacteriophages have long, thin, and flexible tails that are not contractile with an icosahedral head (Hyman and Abedon, 2012). Hohne et al. (1997) discovered three *Siphoviridae* fusobacterial phages (fv 8501, fv 2377, and fv 2527) lytic to *Fusobacterium varium* from sewage plants. Two other *Siphoviridae* family fusobacterial bacteriophages (FNU1 and Fnpϕ02) have also been isolated from human oral samples that were lytic to *Fusobacterium nucleatum* (Machuca et al., 2010; Kabwe et al., 2019). Moreover, five bacteriophages were part of the *Podoviridae* family, characterized by an icosahedral head with a very short tail (Hyman and Abedon, 2012). Four characterized fusobacterial bacteriophages (Phage f277, fv 81-531/2, fv 83-554/3, and phage 277) lytic to *F. varium* are also classified as *Podoviridae* family phages (Bradley, 1967, 1972; Höhne et al., 1997). Four bacteriophages were tailless with a round head and could not be classified at this time because there are two families, *Cystoviridae* and *Plasmaviridae*, with this morphology (Hyman and

Abedon, 2012). There has been one other tailless fusoabacterial bacteriophage, ϕFonu2, that is lytic to *F. nucleatum* described, but it has an icosahedral head (Cochrane et al., 2016). One of the bacteriophages characterized, ϕ2019-6-20A, had a novel phenotypic morphology. This bacteriophage was similar to *Siphiloviridae* family bacteriophages, as it had long and thin tails. However, ϕ2019-6-20A had multiple tails and a round head, which is not seen in the *Siphiloviridae* family (Bradley, 1967; Hyman and Abedon, 2012).

Eleven bacteriophages inhibited *F. necrophorum* subsp. *necrophorum* for up to 24 h. All 11 bacteriophages showed lysis of bacteria after 1-2 hours after phage inoculation. Bacteriophages lytic to *F. nucleatum*, characterized by Machuca et al. (2010), had much slower bacterial lysis. These bacteriophages showed lysis 30 hours after phage inoculation. However, *the F. varium* bacteriophage 277 showed lytic activity around 20 minutes after phage inoculation (Bradley, 1972). After 24, *F. necrophorum* subsp. *necrophorum* became insensitive to all 11 of the bacteriophages, which is a major limitation for phage therapy (Tanji et al., 2004; Gu et al., 2012; Shlezinger et al., 2017). To overcome insensitivity, phage cocktails can be made with bacteriophages that use different bacterial membrane receptors (Tanji et al., 2004). To our knowledge, no phage cocktails have been made against *Fusobacterium* to combat bacteriophage insensitivity. However, Tanji et al. (Tanji et al., 2005) made a phage cocktail of three bacteriophages for use against *E. coli* O157:H7 and no insensitivity was observed, whereas bacterial insensitivity was seen when each of the bacteriophages were tested against *E. coli* O157:H7 separately. Furthermore, Gu et al. (2012) used a phage cocktail against *K. pneumoniae* to reduce bacteriophage insensitivity. Unfortunately, *F. necrophorum* subsp. *necrophorum* became insensitive to all 11 bacteriophages after 24 h inoculation with one bacteriophage, indicating that the 11 bacteriophages likely use the same bacterial membrane receptor to infect the bacterium.

Establishing the pH and temperature stabilities of the bacteriophages was important because they need to survive physiological environment of the rumen and storage conditions. The pH values chosen included a pH range representing the physiological pH fluctuations of the bovine rumen. If the rumen pH is below 5.6 for over 3-5 hours then the rumen is likely to be acidotic, which lead to lesions that allow *F. necrophorum* to travel from the rumen to the liver (Nagaraja and Chengappa, 1998; AlZahal et al., 2008). The phages became inactive against *F. necrophorum* subsp. *necrophorum* after 0 h and 0-1 h at pH's of 2.5 and 3.5, respectively. The bacteriophages were not inactivated by pH's 4.5-8.5, demonstrating their ability to survive in the physiological pH of cattle rumens. Moreover, the temperature of the rumen is around 39 °C and storage conditions in cattle production systems can be variable depending on location (Rutherford et al., 2019). Consequently, the activity of 11 bacteriophages was examined after 24 hours of exposure to 30 °C, 40 °C, and 50 °C. The bacteriophages were all active after 24 h exposure to each temperature.

Conclusions

In conclusion, 25 bacteriophages were characterized and classified into three bacteriophage families: *Myoviridae, Siphoviridae,* and *Podoviridae*. Five bacteriophages could not be classified. One unclassified bacteriophage had multiple, while the other four had no visible tails. The bacteriophages were specific to *F. necrophorum* subsp. *necrophorum* and 11 were inhibitory to the bacterium in broth cultures. However, after 24 hours of incubation, *F. necrophorum* subsp. *necrophorum* became insensitive to all phages and all of the phages likely use the same bacterial membrane receptor to infect the bacterium. Moreover, the bacteriophages were active after 24 hours of exposure to pH's of 4.5-8.5 and temperatures of 30 °C, 40 °C, and 50 °C.

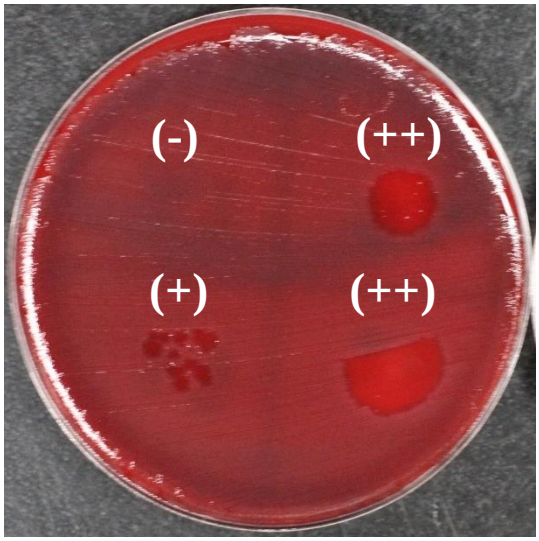


Figure 4.1. Bacteriophage plaques scored as negative (-) , partial (+) and complete lysis (++) on *Fusobacterium necrophorum* subspecies *necrophorum* lawn on blood agar.

Table 4.1. Host range of bacteriophages with strains of *Fusobacterium necrophorum* subsp. *necrophorum* of liver abscess origin.

Under 10 *F. necrophorum* strains with lysis spots		**Ten or more *F. necrophorum* strains with lysis spots**	
Phage ID	**No. of *F. necrophorum* strains**	**Phage ID**	**No. of *F. necrophorum* strains**
ϕ2019-6-1	2	ϕ2019-6-15	12
ϕ2019-6-2	2	ϕ2019-6-16	16
ϕ2019-6-3	2	ϕ2019-6-17	14
ϕ2019-6-4	2	ϕ2019-6-18	11
ϕ2019-6-5	2	ϕ2019-6-19	13
ϕ2019-6-6	2	ϕ2019-6-20	15
ϕ2019-6-7	2	ϕ2019-6-25	12
ϕ2019-6-8	7	ϕ2019-6-30	10
ϕ2019-6-9	2	ϕ2019-6-31	14
ϕ2019-6-10	2	ϕ2019-6-32	17
ϕ2019-6-11	2	ϕ2019-6-33	13
ϕ2019-6-12	2	ϕ2019-6-34	16
ϕ2019-6-13	1	ϕ2019-6-36	14
ϕ2019-6-14	2	ϕ2019-6-37	12
ϕ2019-6-21	3	ϕ2019-6-38	16
ϕ2019-6-22	2	ϕ2019-6-40	15
ϕ2019-6-23	2	ϕ2019-6-41	16
ϕ2019-6-24	2	ϕ2019-6-46	17
ϕ2019-6-26	8	ϕ2019-6-47	18
ϕ2019-6-27	8	ϕ2019-6-48	14
ϕ2019-6-28	6	-	-
ϕ2019-6-29	0	-	-
ϕ2019-6-35	2	-	-
ϕ2019-6-39	5	-	-
ϕ2019-6-42	9	-	-

ϕ2019-6-43	1	-	-
ϕ2019-6-44	0	-	-
ϕ2019-6-45	2	-	-
ϕ2019-6-49	0	-	-
ϕ2019-6-50	8	-	-

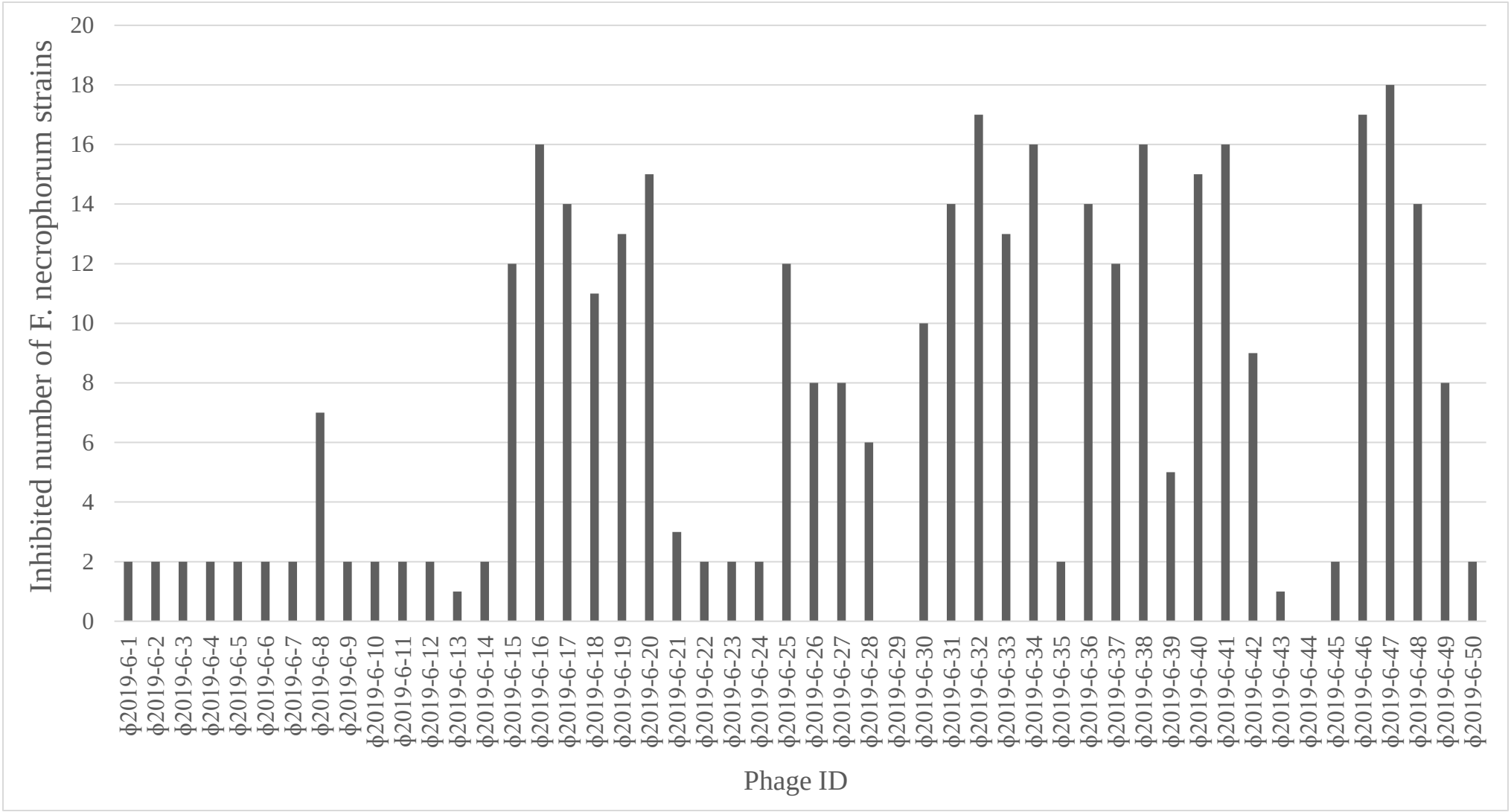
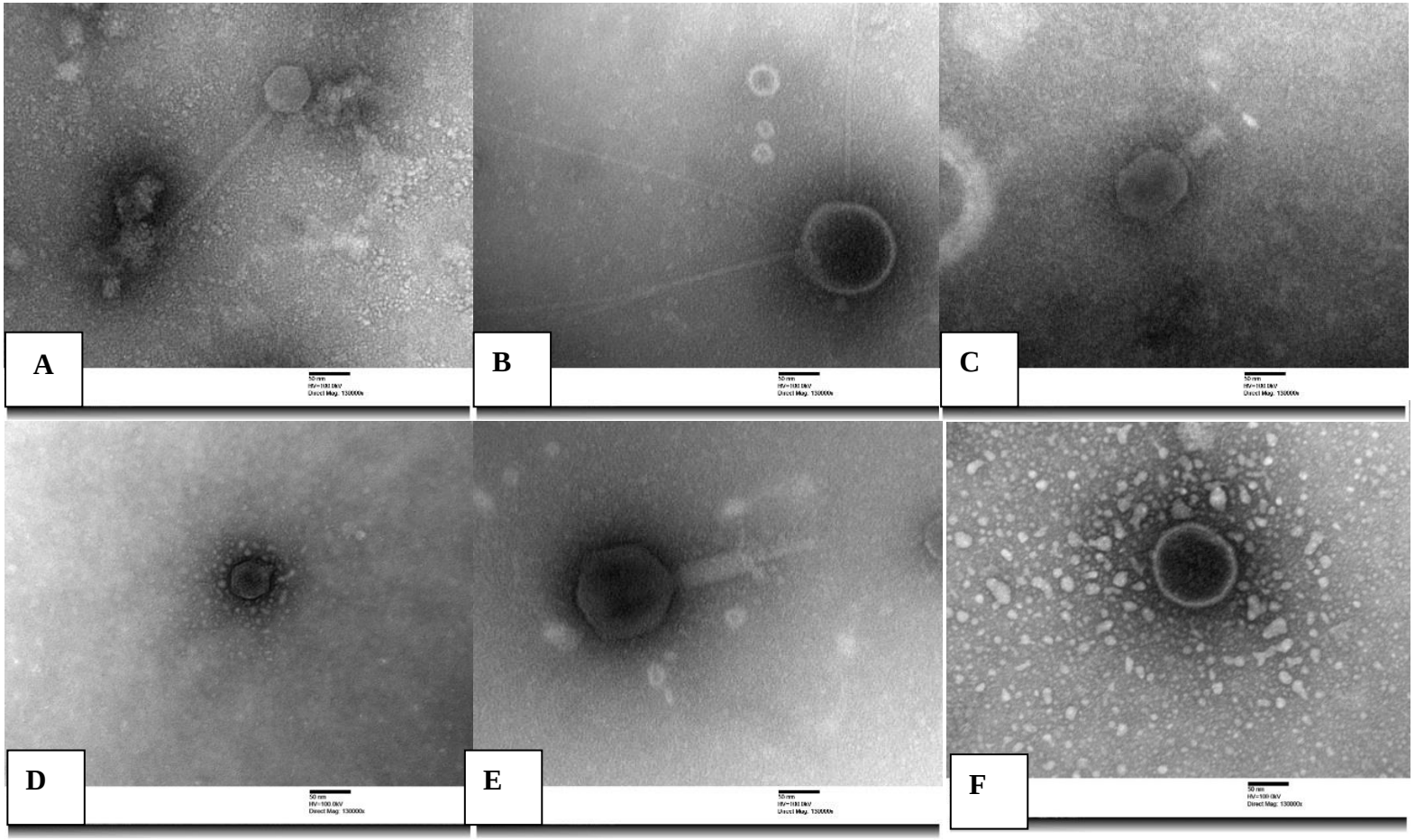


Figure 4.2. Host specificity of bacteriophages.



A. Phage ID ϕ2019-6-16. *Siphoviridae* family
B. Phage ID ϕ2019-6-20A. Unknown family
C. Phage ID ϕ2019-6-25A. *Myoviridae* family
D. Phage ID ϕ2019-6-30. *Podoviridae* family
E. Phage ID ϕ2019-6-33A. *Myoviridae* family
F. Phage ID ϕ2019-6-15A. Unknown family

Figure 4.3. Electron microscopic images of bacteriophages.

Table 4.2. Average measurements of bacteriophages from electron microscopic images.

Phage ID	Family Classification	Head width (nm)	Head height (nm)	Tail width 1 (nm)	Tail length 1 (nm)	Tail width 2 (nm)	Tail length 2 (nm)	Tail width 3 (nm)	Tail length 3 (nm)
ϕ2019-6-15A	Unknown	106.3	119.0						
ϕ2019-6-15B	Unknown	93.6	107.5						
ϕ2019-6-16	*Siphoviridae*	89.1	100.4	12.9	179.0				
ϕ2019-6-18A	*Podoviridae*	95.2	96.7	12.6	47.1				
ϕ2019-6-19A	Unknown	132.2	128.2						
ϕ2019-6-19B	*Podoviridae*	74.5	75.1	22.3	28.9				
ϕ2019-6-20A	Unknown	139.3	149.4	5.1	249.0	5.5	286.8	3.7	246.3
ϕ2019-6-20B	Unknown	139.6	147.8						
ϕ2019-6-25	*Myoviridae*	81.0	92.5	23.0/ 3.5[a]	55.8/ 33.6[a]				
ϕ2019-6-30	*Podoviridae*	51.6	53.9	8.4	11.0				
ϕ2019-6-31	*Siphoviridae*	63.2	74.2	11.0	206.6				
ϕ2019-6-32	*Siphoviridae*	57.2	55.5	7.2	177.7				
ϕ2019-6-33A	*Myoviridae*	93.1	96.5	27.4/ 8.4[a]	90.2/ 78.1[a]				
ϕ2019-6-33B	*Podoviridae*	59.8	56.9	50.1	30.4				
ϕ2019-6-34	*Myoviridae*	76.9	82.3	19.9/ 6.3[a]	54.9/ 62/5[a]				
ϕ2019-6-36A	*Podoviridae*	104.7	106.9	9.0	20.4				
ϕ2019-6-36B	*Siphoviridae*	133.3	140.1	9.6	133.8				
ϕ2019-6-37A	*Siphoviridae*	113.4	121.0	8.1	131.5				
ϕ2019-6-37B	*Siphoviridae*	42.5	55.8	7.8	194.2				
ϕ2019-6-38	*Siphoviridae*	57.5	61.8	11.1	185.7				
ϕ2019-6-40	*Siphoviridae*	42.3	47.5	7.4	155.2				
ϕ2019-6-41A	*Siphoviridae*	56.5	57.3	7.7	126.1				
ϕ2019-6-41B	*Siphoviridae*	96.6	96.4	24.8	143.0				
ϕ2019-6-46	*Siphoviridae*	97.1	90.9	26.8	165.9				
ϕ2019-6-47	*Myoviridae*	95.7	93.0	27.7/ 7.2[a]	55.8/ 38.8[a]				

[a] tail/ contracted sheath

Table 4.3. Lytic efficiencies of bacteriophages.

	Growth (Absorbance at 600 nm)									
Phage ID	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**12**	**24**	**48**
No phage	0.23	0.40	0.60	0.68	0.75	0.78	0.79	0.80	0.80	0.64
ϕ2019-6-15A	0.15	0.22	0.41	0.53	0.66	0.72	0.73	0.74	0.77	0.64
ϕ2019-6-15B	0.21	0.33	0.55	0.63	0.69	0.72	0.73	0.74	0.76	0.63
ϕ2019-6-16	0.16	0.14	0.06	0.05	0.04	0.04	0.04	0.08	0.63	0.64
ϕ2019-6-18A	0.19	0.32	0.57	0.66	0.70	0.68	0.67	0.69	0.67	0.57
ϕ2019-6-19A	0.13	0.21	0.39	0.51	0.63	0.70	0.70	0.62	0.74	0.60
ϕ2019-6-19B	0.13	0.20	0.37	0.48	0.62	0.69	0.71	0.73	0.74	0.60
ϕ2019-6-20A	0.12	0.18	0.36	0.47	0.61	0.68	0.70	0.72	0.73	0.53
ϕ2019-6-20B	0.12	0.18	0.36	0.47	0.61	0.71	0.73	0.75	0.76	0.60
ϕ2019-6-25	0.14	0.18	0.14	0.09	0.06	0.06	0.05	0.15	0.58	0.58
ϕ2019-6-30	0.23	0.08	0.03	0.03	0.03	0.03	0.04	0.08	0.65	0.64
ϕ2019-6-31	0.26	0.11	0.06	0.05	0.05	0.04	0.04	0.10	0.61	0.59
ϕ2019-6-32	0.12	0.15	0.06	0.05	0.04	0.04	0.04	0.08	0.66	0.67
ϕ2019-6-33A	0.13	0.18	0.36	0.47	0.61	0.67	0.65	0.62	0.67	0.54
ϕ2019-6-33B	0.19	0.31	0.56	0.64	0.68	0.68	0.68	0.71	0.71	0.56
ϕ2019-6-34	0.13	0.13	0.05	0.04	0.04	0.04	0.04	0.15	0.64	0.63
ϕ2019-6-36A	0.13	0.18	0.34	0.44	0.58	0.64	0.62	0.60	0.66	0.54
ϕ2019-6-36B	0.15	0.10	0.16	0.18	0.07	0.06	0.06	0.19	0.51	0.50
ϕ2019-6-37A	0.15	0.22	0.40	0.52	0.65	0.72	0.72	0.74	0.76	0.56
ϕ2019-6-37B	0.22	0.36	0.60	0.69	0.74	0.76	0.77	0.76	0.78	0.61
ϕ2019-6-38	0.14	0.14	0.05	0.04	0.04	0.04	0.04	0.10	0.62	0.63
ϕ2019-6-40	0.16	0.15	0.05	0.04	0.05	0.04	0.04	0.14	0.61	0.59
ϕ2019-6-41A	0.15	0.23	0.40	0.51	0.64	0.71	0.73	0.75	0.76	0.59
ϕ2019-6-41B	0.15	0.22	0.38	0.47	0.54	0.60	0.60	0.63	0.69	0.59
ϕ2019-6-46	0.16	0.20	0.07	0.05	0.05	0.04	0.04	0.09	0.58	0.59
ϕ2019-6-47	0.16	0.19	0.06	0.05	0.04	0.05	0.04	0.15	0.58	0.58

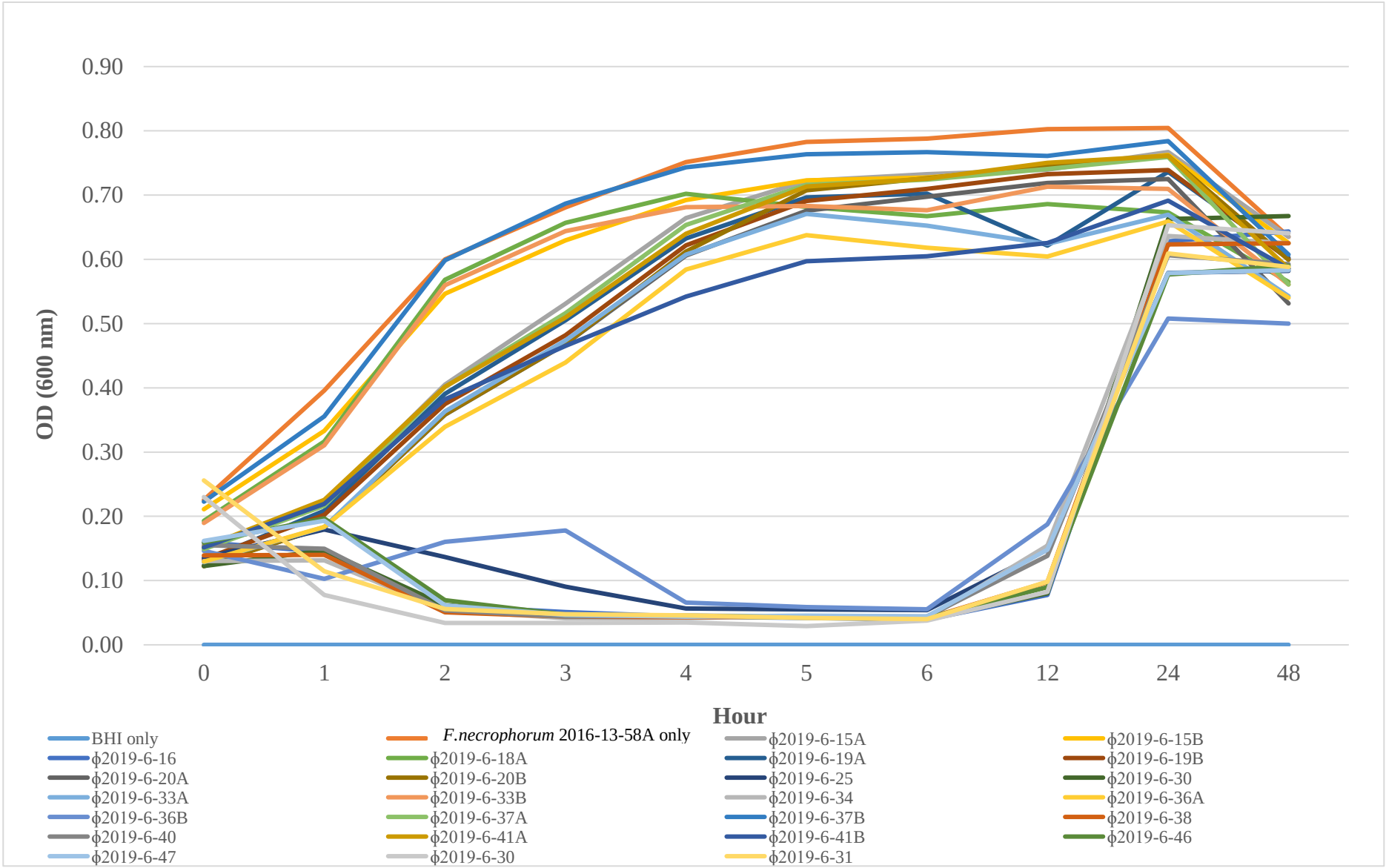


Figure 4.4. Average efficiencies of bacteriophages.

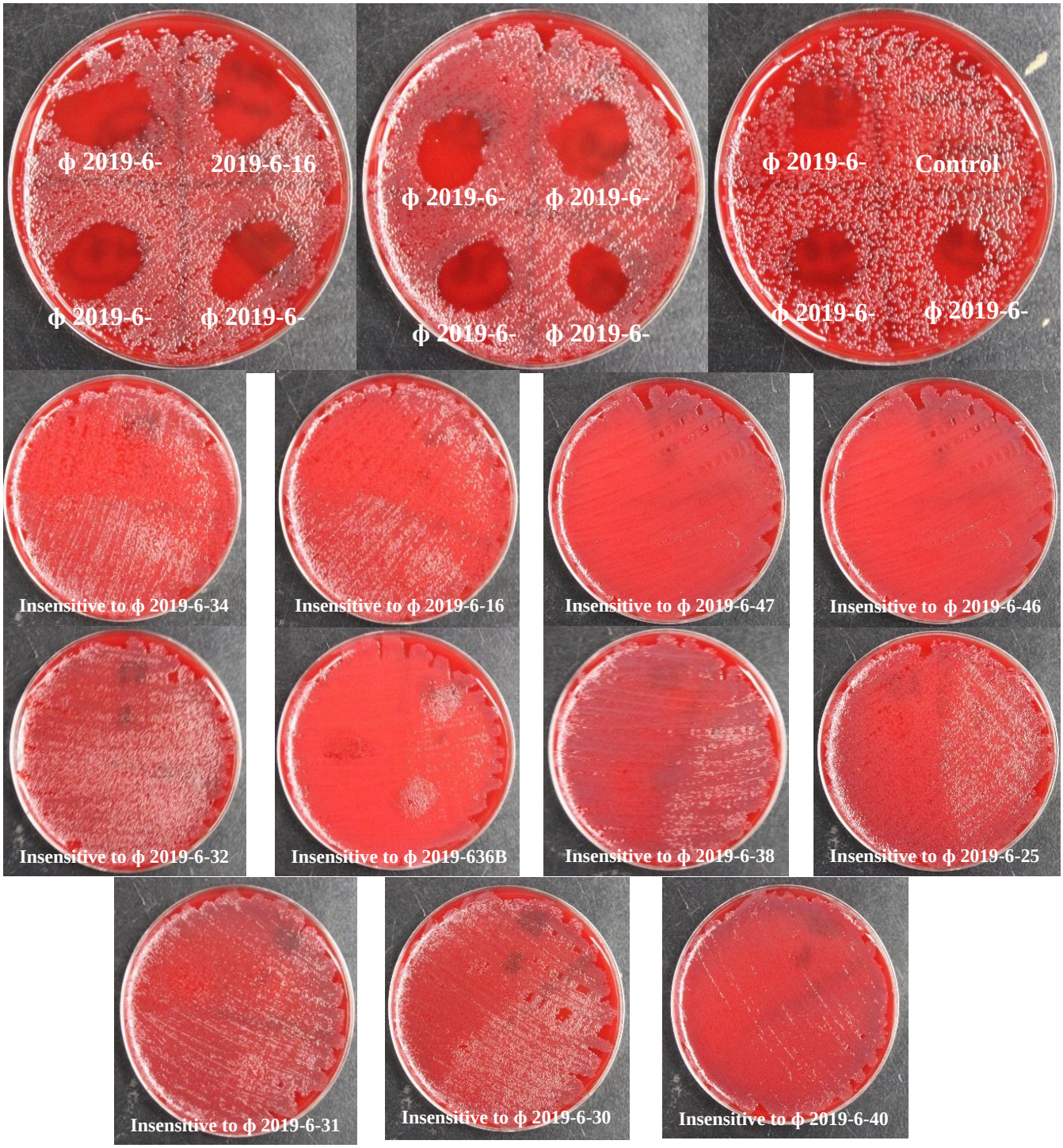


Figure 4.5. *Fusobacterium necrophorum* subsp. *necrophorum* insensitivity to bacteriophages after incubation in broth for 24 hours.

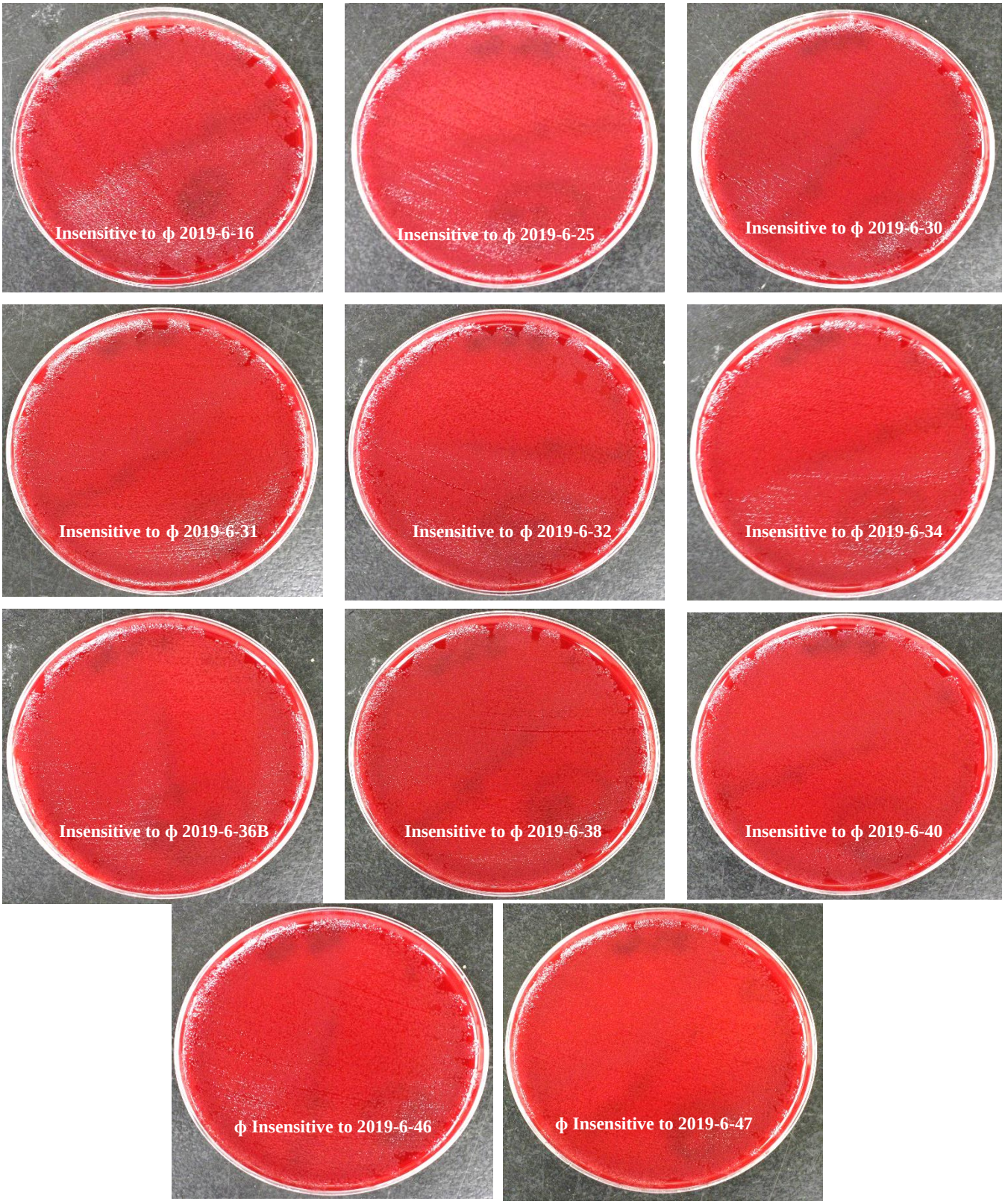


Figure 4.6. *Fusobacterium necrophorum* subsp. *necrophorum* insensitivity to bacteriophages after incubation in broth for 24 hours with bacteriophage ϕ2019-6-16.

Table 4.4. pH stability of bacteriophages.

Hour	Phage ID	pH						
		2.5	3.5	4.5	5.5	6.5	7.5	8.5
0	ϕ2019-6-16	-	-	++	++	++	++	++
	ϕ2019-6-25	-	+	++	++	++	++	++
	ϕ2019-6-30	-	-	++	++	++	+	++
	ϕ2019-6-31	-	-	++	++	++	+	++
	ϕ2019-6-32	-	-	++	++	++	++	++
	ϕ2019-6-34	-	-	++	++	++	++	++
	ϕ2019-6-36B	-	+	++	++	++	++	++
	ϕ2019-6-38	-	+	++	++	++	++	++
	ϕ2019-6-40	-	+	++	++	++	++	++
	ϕ2019-6-46	-	-	++	++	++	++	+
	ϕ2019-6-47	-	-	++	++	++	++	+
0.5	ϕ2019-6-16	-	+	++	++	++	++	++
	ϕ2019-6-25	-	+	++	++	++	++	+
	ϕ2019-6-30	-	-	+	++	++	++	+
	ϕ2019-6-31	-	-	++	++	++	++	++
	ϕ2019-6-32	-	-	++	++	++	++	++
	ϕ2019-6-34	-	-	++	++	++	++	++
	ϕ2019-6-36B	-	++	++	++	++	++	++
	ϕ2019-6-38	-	-	++	++	++	++	++
	ϕ2019-6-40	-	-	++	++	++	++	++
	ϕ2019-6-46	-	-	+	++	++	++	+
	ϕ2019-6-47	-	-	++	++	+	+	++
1	ϕ2019-6-16	-	-	++	++	++	++	++
	ϕ2019-6-25	-	-	+	++	++	+	+
	ϕ2019-6-30	-	-	++	++	++	++	++
	ϕ2019-6-31	-	-	++	++	++	++	++
	ϕ2019-6-32	-	-	++	++	++	++	++
	ϕ2019-6-34	-	-	++	++	++	++	++
	ϕ2019-6-36B	-	+	++	++	++	++	++
	ϕ2019-6-38	-	-	++	++	++	++	++
	ϕ2019-6-40	-	+	++	++	++	++	++
	ϕ2019-6-46	-	-	++	++	++	++	++
	ϕ2019-6-47	-	-	++	++	++	+	++
6	ϕ2019-6-16	-	-	++	++	++	++	++
	ϕ2019-6-25	-	-	++	++	++	++	++

	ϕ2019-6-30	-	-	++	++	++	++	++
	ϕ2019-6-31	-	-	++	++	++	++	++
	ϕ2019-6-32	-	-	++	++	++	++	++
	ϕ2019-6-34	-	-	++	++	++	++	++
	ϕ2019-6-36B	-	-	++	++	++	++	++
	ϕ2019-6-38	-	-	++	++	++	+	++
	ϕ2019-6-40	-	-	++	++	++	++	++
	ϕ2019-6-46	-	-	++	++	++	++	+
	ϕ2019-6-47	-	-	+	+	++	++	+
	ϕ2019-6-16	-	-	++	++	++	++	+
	ϕ2019-6-25	-	-	++	++	++	++	+
	ϕ2019-6-30	-	-	++	++	++	++	++
	ϕ2019-6-31	-	-	+	++	++	++	++
	ϕ2019-6-32	-	-	++	++	++	++	++
24	ϕ2019-6-34	-	-	++	++	++	++	++
	ϕ2019-6-36B	-	-	++	++	++	++	++
	ϕ2019-6-38	-	-	++	+	+	++	++
	ϕ2019-6-40	-	-	++	++	++	++	++
	ϕ2019-6-46	-	-	++	++	+	+	++
	ϕ2019-6-47	-	-	++	++	++	++	++

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Chapter 5 - CRISPR-Cas use on disease causing bacterial species in cattle

Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), in conjunction with CRISPR-associated (Cas) proteins, have the potential to be used as an antibiotic alternative and disease diagnostic tool in animal production. CRISPR-Cas proteins are endonucleases that are naturally produced by bacteria and are used as a defense mechanism against foreign nucleic acids, such as bacteriophages (Makarova et al., 2006; Brouns et al., 2008; Josiane et al., 2010). In short, CRISPR-Cas complexes cleave DNA or RNA and are typically guided by a short RNA strand (Elitza et al., 2011; Jinek et al., 2012a). It was first discovered in 1987 by Ishino in *E. coli* and in 2002 it started being called CRISPR (Jansen et al., 2002; Ishino et al., 2018). The CRISPR-Cas systems contain short non-repetitive sequences (protospacers) between repetitive sequences and 6-20 Cas protein genetic sequences (Shabbir et al., 2016).

There are three steps involved in DNA cleavage in bacterial CRISPR-Cas systems (Figure 5.1): adaptation, expression, and interference (Jackson et al., 2017). Cas1 and Cas2 proteins are involved in adaptation and acquisition of protospacers from foreign invaders (Wan et al., 2021). Once a bacterium has a protospacer, the genetic sequence is expressed as a guide RNA (gRNA) (Jackson et al., 2017). The complex then enters the interference stage, where the gRNA recognizes and hybridizes with complementary nucleic acid sequences and nucleic acid cleavage occurs.

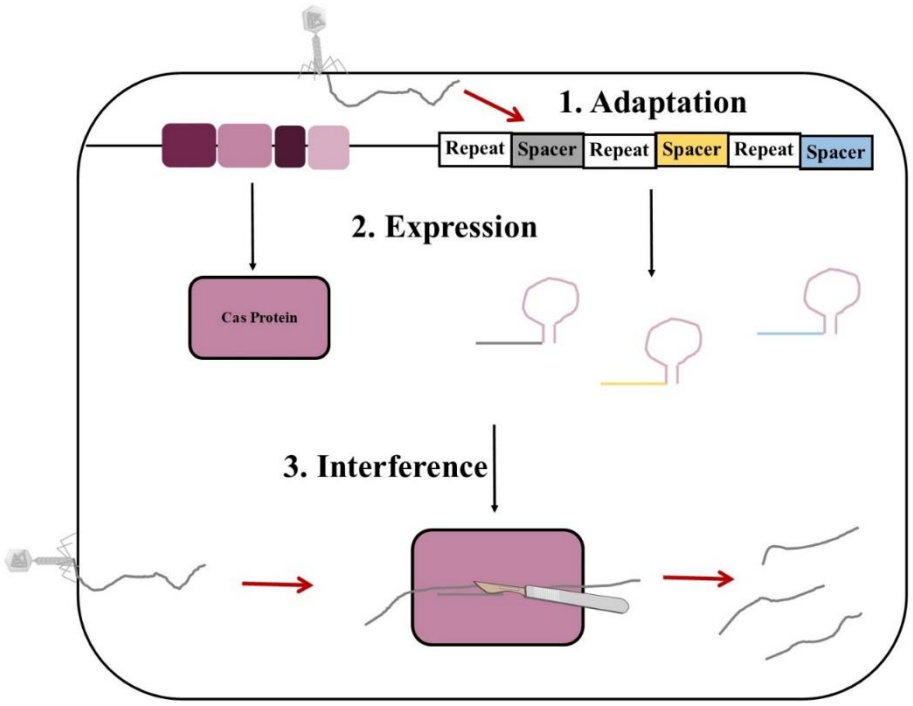

Figure 5.1. Overview of CRISPR-Cas systems: adaptation, expression, and interference.

Additionally, there is a necessary protospacer adjacent motif (PAM) sequence near the target nucleic acid sequence that unlocks the cutting mechanisms of the CRISPR-Cas system (Mojica et al., 2009; Jinek et al., 2012a; Shah et al., 2013; Doudna and Charpentier, 2014). The PAM sequence binding is essential for the cleavage of the target nucleic acid in many CRISPR-Cas systems (Gasiunas et al., 2012; Jinek et al., 2012a; Sternberg et al., 2014). There are two major classes of CRISPR-Cas systems (Table 5.1), Class 1 and 2, and six types of systems within the classes, I, II, III, IV, V, and VI (Makarova et al., 2015). Class 1 systems consist of types I, III, and IV, while Class 2 systems consist of types II, V, and VI (Chylinski et al., 2014; Makarova et al., 2015; Mohanraju et al., 2016).

Table 5.1. Classification of CRISPR-Cas systems. [a]

Class	Type	Subtype	Major Cas Nuclease	Microbial Origin	Common PAM Sequence(s)	Cutting Location	Cut structure	Type of nucleotide cut
1	I	I-A	Cas3 (Cascade)	*Archaeoglobus fulgidus*	CCN	5' of PAM	Unidirectional 5' of PAM	DNA
		I-B		*Clostridium kluyveri*	TTC, ACT, TAA, TAT, TAG, CAC			
		I-C		*Bacillus halodurans*	NTTC			
		I-U		*Geobacter sulfurreducens*	-			
		I-D		*Cyanothece* spp. 8802	-			
		I-E		*Escherichia coli* K12	ATG, AAG			
		I-F		*Yersinia pseudotuberculosis, Shewanella putrefaciena, Vibrio crassostreae*	GC, CG			
1	III	III-A	Cas10/ Cas7 (Csm complex)	*Staphylococcus epidermis*	Do not recognize	-	-	DNA/ RNA
		III-B	Cas10/ Cas7 (Cmr complex)	*Pyrococcus furiosus*	Do not recognize	-	-	DNA/ RNA
		III-C		*Methanothermobacter themautotrophicus*				
		III-D	Cas10/ Cas7 (Csm complex)	*Synechocystis sp.* 6803				
		III-E		*Candidatus Scalidua brodae*				
		III-F		*Thermoflexia bacterium*				
	IV	IV-A	Variable- Lacks many conserved *cas* genes and sometimes a CRISPR array (effector complex)	*Thioalkalivibrio sp K90mix*	AAG	-	-	-
		IV-B		*Rhodococcus joatii*	-			
		IV-C		*Thermoflexia bacterium*	-			

Table 5.1 continued…

Class	Type	Subtype	Major Cas Nuclease	Microbial Origin	Common PAM Sequence(s)	Cutting location	Cut structure	Type of nucleotide cut
2	II		Cas9	*Streptococcus thermophilus*	NGGNG	~ 3 bp 5' of PAM	Blunt-ended double-strand breaks	DNA
				Legionella pneumophila str. paris	-			
				Neisseria lactamica, Micrarchaeum acidiphilum	-			
				Francisella novicida	NGG			
				Staphylococcus aureus	NNGRRT			
				Neisseria meningitidis	NNNNGATT			
				Streptococcus pyogenes	NGG			
				Campylobacter jejuni	NNNNACAC			
	V	V-A	Cas12	*Francisella cf. novicida Fx1, Acidaminococcus sp.*	TTTN	3' of PAM	Sticky double-strand breaks	DNA
	V	V-B	Cas12	*Alicyclbacillus acidoterrestris, Planctomycetes bacterium*	TTT, TTA, TTC	3' of PAM	Sticky double-strand breaks	DNA
		V-C		*Oleiphilus spp.*				
		V-D		*Bacterium* CG09_39_24	TA			
		V-E		*Deltaproteobacteria bacterium*	TTCN			
		V-F		*Bacillus thuringiensis, Candidatus Microarachaeota archaeon, Gordonia otitidis, Cyanothece sp. PCC 8801, Rothia dentrocariosa*	-			
		V-G		-	-			RNA
		V-H		-	-			-
		V-I		-	-			DNA
		V-K		*Cyanothece sp. PCC* 8801	-			-

Table 5.1 continued…

Class	Type	Subtype	Major Cas Nuclease	Microbial Origin	Common PAM Sequence(s)	Cutting location	Cut structure	Type of nucleotide cut
2	VI	VI-A	Cas13	*Leptotrichia shahii*	PAM not required; some need a single base-specific protospacer flanking site (PFS) sequence	Promiscuous cleavage		RNA
		VI-B		*Provotella buccae, Bergeyella zoohelcum*				
		VI-C		*Fusobacterium perfoetens*				
		VI-D		*Ruminococcus bicirculans*				

[a] (Westra et al., 2013; Chylinski et al., 2014; Makarova and Koonin, 2015; Makarova et al., 2015; Makarova et al., 2017; Shmakov et al., 2017; Gleditzsch et al., 2019; Liu and Doudna, 2020; Makarova et al., 2020; Nidhi et al., 2021; Taylor et al., 2021)

Class I systems utilize multi-protein effector complexes and are divided into three types, I, III, and IV (Makarova et al., 2015). CRISPR-Cas types I and III mostly contain the Cas1 and Cas2 proteins (Makarova et al., 2017). Type I systems also contain the Cas3 protein and CRISPR arrays. The Cas3 protein is a helicase-nuclease and is responsible for DNA cleavage after PAM recognition in type I CRISPR-Cas systems (Sinkunas et al., 2011). Types III and IV do not always contain CRISPR arrays or adaption module genes (Makarova et al., 2017). Type III types do not require a PAM site in the target nucleic acid sequence and are able to cleave both DNA and ssRNA (Hale et al., 2009; Deng et al., 2013; Estrella et al., 2016). Furthermore, the Cas10 protein is responsible for DNA cleavage, while Cas7 proteins are responsible for ssRNA cleavage in type III systems (Samai et al., 2015; Kazlauskiene et al., 2016; Zhang et al., 2016). Type IV systems do not contain the Cas 1, Cas2, Cas3, or Cas10 proteins and are associated with Csf4, a DinG family nuclease (Liu and Doudna, 2020). Csf4 is necessary for interference in Type IV CRISPR-Cas systems, but its role is unclear (Crowley et al., 2019).

Class II systems utilize a single multidomain effector protein and are divided into three types: II, V, and VI. Type II CRISPR-Cas systems are the most studied system and use the Cas9 nuclease to make a blunt-end DNA double stranded break three base pairs upstream from the PAM site (Jinek et al., 2012b; Cong et al., 2013; Mali et al., 2013). Enhanced specificity Cas9 (eCas9) and high-fidelity Cas9 (Cas9-HF) are improved spCas9 systems that have less off target effects (Kleinstiver et al., 2015; Slaymaker et al., 2016). Furthermore, Type V CRISPR-Cas systems use the protein Cas12 to make a staggered DNA double stranded break with a 5' overhang and Type VI systems use Cas13 for promiscuous RNA cleavage (Zetsche et al., 2015; Meeske and Marraffini, 2018).

CRISPR-Cas systems have been modified to create dead Cas proteins (dCas) and Cas9 nickase (Cas9n) systems. Dead Cas proteins have been modified so they lack nuclease activity but are still able to bind to the target nucleic acid sequence (Brezgin et al., 2019). These proteins are used to deliver functionally active domains to specific locations in the genome (Brezgin et al., 2019). These domains may be used for transcriptional regulation, analyzing chromatin structure, base editing, epigenetic modeling, and studying chromatin interactions (Komor et al., 2016; O'Geen et al., 2017; Zhou et al., 2018; Brezgin et al., 2019). Furthermore, Cas9 has been manipulated so it causes single stranded breaks in DNA (Cong et al., 2013; Ran et al., 2013). This edited protein is called Cas9 nickase (Cas9n). Cas9n can be used to create more specific double stranded breaks with less off target effects than the original Cas9 because it requires the targeting of two nucleic acid sites on opposite strands at the same time (Ran et al., 2013).

Antimicrobial Alternative Uses of CRISPR-Cas Systems

Antimicrobial resistance has become a major issue around the world in human and animal medicine (Aidara-Kane et al., 2018). Antimicrobial resistance occurs when a pathogen, or another

microbe, can survive in the presence of an antimicrobial. This typically occurs through genetic modification of the bacteria so the bacteria can produce proteins to neutralize the antimicrobials, increase efflux pumps so antimicrobials are forced out of the microbe, or change the target structures so antimicrobials can no longer attach (Gallo, 2013; Gualerzi, 2013). The use of antimicrobials can select antimicrobial resistant pathogens and commensal bacteria that can serve as vectors that can transfer resistance genes to other pathogenic microbes (Holmes et al., 2016). An increase in resistance to medically important antimicrobials, such as macrolides, cephalosporins, and glycopeptides has led to public health concerns (Torio and Padilla, 2018; WHO, 2019).

Antimicrobial use of CRISPR-Cas has been studied on few cattle pathogens. However, its antimicrobial alternative potential has been demonstrated on *Stapylococcus aureus, Clostridium difficile, Eschericia coli, Salmonella enterica* and *Mycobacterium tuberculosis* (Bikard et al., 2014; Gomaa et al., 2014; Kiga et al., 2019; Li et al., 2019; Hao et al., 2020; Selle et al., 2020a). For example, Gomaa et. al. (Gomaa et al., 2014) used the endogenous type I-E CRISPR-Cas system in *E. coli* to selectively inhibit bacterial strains in mixed cultures. Bacteriophages and bacteria carrying plasmids transmissible by conjugation have been demonstrated to deliver CRISPR-Cas9 to *E. coli* which reduced antimicrobial resistance genes or inhibited growth of the bacterium (Citorik et al., 2014). Moreover, bacteriophage-delivered CRISPR-Cas13a inhibited *E. coli* and *S. aureus* by targeting chromosomal or plasmid DNA through its promiscuous ssRNA cleavage (Kiga et al., 2019). CRISPR-Cas9 has also been reported to destroy plasmids carrying Beta-lactamase resistance genes in *E. coli* (Yosef et al., 2015). Bikard et al. (2014) demonstrated the use of CRISPR-Cas9 in a mouse skin colonization model to kill *S. aureus*. Likewise, CRISPR-Cas9 nanocomplexes have been delivered into Methicillin- resistant *S. aureus,* which were

efficient at editing the *mecA* gene and inhibiting bacterial growth (Kang et al., 2017). Selle et al. (Selle et al., 2020b, a) demonstrated that bacteriophages engineered with CRISPR-Cas type I systems inhibited *C. difficile in vitro* and in a mouse model. *Salmonella enterica* has been inhibited by CRISPR-Cas9 (Hamilton et al., 2019). Carbapenem-resistant plasmids in *K. pneumonia* have also been cleaved by CRISPR-Cas9 (Hao et al., 2020). Furthermore, bacteriophages have been modified to insert gRNAs that interact with *M. tuberculosis'* endogenous CRISPR-Cas type III system to inhibit the bacterium (Carrigy et al., 2017; Li et al., 2019; Li and Peng, 2019). Interestingly, cattle have been modified with CRISPR-Cas9 to cause a genetic mutation that makes the cattle more resistant to *M. tuberculosis* (Gao et al., 2017).

There are many documented patents for CRISPR-Cas use with common bacterial pathogens in cattle and, specifically, as an antimicrobial alternative on cattle pathogens (Table 5.2). For example, the patent "Altering microbial populations and modifying microbiota" (patent number 10,624,349) uses CRISPR-Cas systems to alter *S. aureus, P. aeruginosa, C. difficile, E. coli, M. tuberculosis, S. pyogenes, S. agalactiae,* and more bacterial species in mixed cultures with a goal of altering antimicrobial resistant strains so they are inhibited or no longer resistant (Clube, 2020). Likewise, the patent "Sequence specific antimicrobials" (patent number 10,660,943) uses CRISPR-Cas9 to reduce antimicrobial resistant and virulent strains of *S. aureus* in mixed cultures consisting of *Staphylococcus, Clostridium, Bacillus, Salmonella, Helicobacter pylori, Neisseria gonorrhoeae, Neisseria meningitidis*, and *Escherichia coli (Bikard and Marraffini, 2020)*. These examples demonstrate the antimicrobial therapies involving CRISPR-Cas being developed and CRISPR-Cas' potential as an antimicrobial alternative.

Table 5.2. Patents involving CRISPR use with common bacterial pathogens in cattle [a]

Bacterial species	Number of Patents	Patent number	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Staphylococcus aureus	356	10,624,349	Altering microbial populations and modifying microbiota	Mastitis	Zoonotic, Food-borne
		10,584,344	Genetically modified bacteria and methods for genetic modification of bacteria		
Pseudomonas aeruginosa	207	10,624,349	Altering microbial populations and modifying microbiota	Mastitis	Zoonotic
		10,913,987	Bacteria identification and antibiotic susceptibility profiling device		
		10,584,344	Genetically modified bacteria and methods for genetic modification of bacteria		
		10,920,222	Treating and preventing microbial infections		
		10,760,065	Tuning microbial populations with programmable nucleases		
Clostridium difficile	120	10,624,349	Altering microbial populations and modifying microbiota		Zoonotic
		10,260,111	Method of detecting sepsis-related microorganisms and detecting antibiotic-resistant sepsis-related microorganisms in a fluid sample		
		10,920,222	Treating and preventing microbial infections		
		10,760,065	Tuning microbial populations with programmable nucleases		

Table 5.2 continued…

Bacterial species	Number of Patents	Patent number	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Clostridium novyi	11	10,933,102	Bacteria engineered to treat a disease or disorder	Infectious necrosis hepatitis	
		10,808,232	Dynamically- adaptive live therapeutic agents and methods of use thereof		
		10,920,222	Treating and preventing microbial infections		
		10,953,090	Selectively altering microbiota for immune modulation		
		10,610,104	Gastrointestinal tract detection methods, devices and systems		
Clostridium hemolyticum	None	-	-	Redwater	
Clostridium perfringens	92	10,913,934	Methods and systems for rapid detection of microorganisms using infectious agents		Zoonotic/ Food-borne illness
		10,752,906	Precise microbiota engineering at the cellular level		
Clostridium chauvoei	6	10,953,090	Selectively altering microbiota for immune modulation	Black leg	
		10,920,222	Treating and preventing microbial infections		
Clostridium septicum	5	10,610,104	Gastrointestinal tract detection methods, devices and systems	Malignant edema	
		10,953,090	Selectively altering microbiota for immune modulation		
		10,920,222	Treating and preventing microbial infections		

Table 5.2 continued…

Bacterial species	Number of Patents	Patent number	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Escherichia coli	750	10,624,349	Altering microbial populations and modifying microbiota	Calf scours, mastitis, cystitis, Neonatal mortality	Zoonotic, Food-borne
		10,913,987	Bacteria identification and antibiotic susceptibility profiling device		
		8,361,725	Detection and typing of bacterial strains		
		10,584,344	Genetically modified bacteria and methods for genetic modification of bacteria		
		10,260,111	Method of detecting sepsis-related microorganisms and detecting antibiotic-resistant sepsis-related microorganisms in a fluid sample		
		10,570,465	Method of improved identification of and antibiotic resistance of sepsis-related microorganisms		
		10,920,222	Treating and preventing microbial infections		
		10,760,065	Tuning microbial populations with programmable nucleases		
Klebsiella pneumoniae	99	10,506,812	Altering microbial populations and modifying microbiota	Mastitis	
		10,913,987	Bacteria identification and antibiotic susceptibility profiling device		

Table 5.2 continued…

Bacterial species	Number of Patents	Patent number	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Klebsiella pneumoniae	99	10,260,111	Method of detecting sepsis-related microorganisms and detecting antibiotic-resistant sepsis-related microorganisms in a fluid sample	Mastitis	
		10,570,465	Method of improved identification of and antibiotic resistance of sepsis-related microorganisms		
		10,920,222	Treating and preventing microbial infections		
		10,760,065	Tuning microbial populations with programmable nucleases		
Fusobacterium necrophorum	None	-	-	Liver abscesses, Foot rot	Zoonotic
Listeria monocytogens	None	-	-	Listeriosis, abortion	Zoonotic, Food-borne
Yersinia enterocolitica	26	9,248,175	Live-attenuated compositions for bacterial infections	Yersiniosis, pneumonia, abortions	Zoonotic, Food-borne
		10,752,906	Precise microbiota engineering at the cellular level		
		10,920,222	Treating and preventing microbial infections		
Yersinia pseudotuberculosis	26	10,920,222	Treating and preventing microbial infections	Shipping fever	Zoontoic
Mycobacterium tuberculosis	162	10,624,349	Altering microbial populations and modifying microbiota	Tuberculosis	Zoonotic
		8,361,725	Detection and typing of bacterial strains		
		10,920,222	Treating and preventing microbial infections		

Table 5.2 continued…

Bacterial species	Number of Patents	Patent number	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Mycobacterium paratuberculosis	2	10,610,104	Gastrointestinal tract detection methods, devices and systems	Johne's disease	
		10,266,887	CRISPR effector system based diagnostics		
Sphaerophorus necrophorus	None	-	-	Calf necrotic diphtheria, Necrotic abscesses, Necrosis of rumen, nephritis, foot rot	
Campylobacter coli	14	10,953,090	Selectively altering microbiota for immune modulation	Gastrointestinal campylobacteriosis	Zoonotic, Food-borne
		10,920,222	Treating and preventing microbial infections		
Campyobacter jejuni	131	10,920,222	Treating and preventing microbial infections	Gastrointestinal campylobacteriosis	Zoonotic, Food-borne
Campylobacter fetus	17	10,920,222	Treating and preventing microbial infections	Genital campylobacteriosis	Zoonotic
Salmonella Typhimurium	74	10,953,090	Selectively altering microbiota for immune modulation	Salmonellosis, Enteritis	Zoonotic, Food-borne
Salmonella Dublin	None	-	-	Salmonellosis, Enteritis	Zoonotic, Food-borne
Salmonella Newport	None	-	-	Salmonellosis, Enteritis	Zoonotic, Food-borne
Salmonella Anatum	None	-	-	Salmonellosis, Enteritis	Zoonotic
Salmonella Arizona	None	-	-	Salmonellosis, Enteritis	Zoonotic

Table 5.2 continued…

Bacterial species	Number of Patents	Patent numbers of patents for antimicrobial use or bacterial detection	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Bacillus anthracis	101	10,260,111	Method of detecting sepsis-related microorganisms and detecting antibiotic-resistant sepsis-related microorganisms in a fluid sample	Anthrax	Zoonotic
		10,570,465	Method of improved identification of and antibiotic resistance of sepsis-related microorganisms		
		10,760,065	Tuning microbial populations with programmable nucleases		
Brucella abortus	28	-	-	Brucellosis, Abortion	Zoonotic
Dermatophilus congolensis	None	-	-	Dermatophilosis	Zoonotic
Leptospira pomona	None	-	-	Leptospirosis, cystitis	Zoonotic
Leptospira grippotyphosa	None	-	-	Leptospirosis, cystitis	Zoonotic
Leptospira hyos	None	-	-	Leptospirosis, cystitis	Zoonotic
Leptospira icterohaemorrhagica	None	-	-	Leptospirosis, cystitis	Zoonotic
Leptospira canicola	3	10,610,104	Gastrointestinal tract detection methods, devices and systems	Leptospirosis, cystitis	Zoonotic
Coxiella burneti	None	-	-	Q fever	Zoonotic
Pasteurella multocida	56			Shipping fever, Neonatal mortality	Zoonotic
Pasteurella haemolytica	4	-	-	Shipping fever	Zoonotic

Table 5.2 continued…

Bacterial species	Number of Patents	Patent numbers of patents for antimicrobial use or bacterial detection	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Mycoplasma mycoides	9	10,760,065	Tuning microbial populations with programmable nucleases	Contagious bovine pleuropneumonia	
Nocardia asteroides	8	10,610,104	Gastrointestinal tract detection methods, devices and systems	Nocardiosis	
		10,266,887	CRISPR effector system based diagnostics		
Mycobacterium bovis	26	10,933,102	Bacteria engineered to treat a disease or disorder	BRD[b]	
		10,266,887	CRISPR effector system based diagnostics		
		11,021,740	Devices for CRISPR effector system based diagnostics		
Diplococcus pneumoniae	4	10,920,222	Treating and preventing microbial infections	Pneumonia	
		10,752,906	Precise microbiota engineering at the cellular level		
		10,610,104	Gastrointestinal tract detection methods, devices and systems		
		10,596,255	Selectively altering microbiota for immune modulation		
Achromobacter anitratum	None	-	-	Septicemia	
Corynebacterium pyogenes	None	-	-	Abortion, Mastitis, Pyobacillosis, Neonatal mortality	Zoonotic

Table 5.2 continued…

Bacterial species	Number of Patents	Patent numbers of patents for antimicrobial use or bacterial detection	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Corynebacterium renale	None	-	-	Pyelonephritis	
Proteus mirabilis	33	9,248,175	Live-attenuated compositions for bacterial infections	Mastitis	Zoonotic
		10,260,111	Method of detecting sepsis-related microorganisms and detecting antibiotic-resistant sepsis-related microorganisms in a fluid sample		
		10,570,465	Method of improved identification of and antibiotic resistance of sepsis-related microorganisms		
		10,920,222	Treating and preventing microbial infections		
Enterobacter aerogenes	25	-	-	Mastitis	
Actinobacillus lignieresi	None	-	-	Big head, wooden tongue	Zoonotic
Alcaligenes faecalis	19	10,195,273	Selectively altering microbiota for immune modulation	Mastitis	Zoonotic
		10,920,222	Treating and preventing microbial infections		
Mannheimia haemolytica	None	-	-	BRD[b]	
Actinomyces bovis	3	10,953,090	Selectively altering microbiota for immune modulation	Lumpy jaw	Zoonotic
		10,920,222	Treating and preventing microbial infections		
		10,610,104	Gastrointestinal tract detection methods, devices and systems		

Table 5.2 continued…

Bacterial species	Number of Patents	Patent numbers of patents for antimicrobial use or bacterial detection	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Erysipelothrix insidiosa	None	-	-	Arthritis	Zoonotic
Moraxella bovis	2	10,953,090	Selectively altering microbiota for immune modulation	Infectious keratitis	
		10,920,222	Treating and preventing microbial infections		
Streptococcus pyogenes	417	10,624,349	Altering microbial populations and modifying microbiota	Mastitis, Neonatal mortality	Zoonotic
		8,361,725	Detection and typing of bacterial strains		
		10,260,111	Method of detecting sepsis-related microorganisms and detecting antibiotic-resistant sepsis-related microorganisms in a fluid sample		
		10,570,465	Method of improved identification of and antibiotic resistance of sepsis-related microorganisms		
		10,953,090	Selectively altering microbiota for immune modulation		
		10,660,943	Sequence specific antimicrobials		
Streptococcus agalactiae	85	10,624,349	Altering microbial populations and modifying microbiota	Mastitis	Zoonotic
		10,260,111	Method of detecting sepsis-related microorganisms and detecting antibiotic-resistant sepsis-related microorganisms in a fluid sample		

Table 5.2 continued…

Bacterial species	Number of Patents	Patent numbers of patents for antimicrobial use or bacterial detection	Patent Title	Common Cattle Diseases	Zoonotic / Food-borne
Streptococcus agalactiae	85	10,570,465	Method of improved identification of and antibiotic resistance of sepsis-related microorganisms	Mastitis	Zoonotic
		10,953,090	Selectively altering microbiota for immune modulation		
		10,920,222	Treating and preventing microbial infections		
		10,760,065	Tuning microbial populations with programmable nucleases		
Streptococcus uberus	None	-	-	Mastitis	
Streptococcus dysgalactiae	17	10,366,793	Method and system for characterizing microorganism-related conditions	Mastitis	
		10,752,906	Precise microbiota engineering at the cellular level		
		10,953,090	Selectively altering microbiota for immune modulation		
		10,920,222	Treating and preventing microbial infections		

[a] United States Patent Office Search, 2021
[b] Bovine respiratory disease

Delivery of CRISPR-Cas systems to bacterial cells in mammalian systems has been a challenge, but bacteriophages and nanoparticles have become promising delivery methods. Nanoparticles are microscopic particles with varying properties that make them beneficial for therapeutic use in the health industry (Anu Mary Ealia and Saravanakumar, 2017). Lipid and polymeric based nanoparticles have been researched with CRISPR-Cas9 for therapeutic use in

treating cancer and genetic disorders (Chen et al., 2017; Lee et al., 2018). Likewise, Kang et al. (2017) used nanocomplexes to deliver CRISPR-Cas to bacterial cells. The second delivery method is the use of bacteriophages, which are bacterial viruses that insert DNA into a bacterial host. Bacteriophages have two possible life cycles: lytic or lysogenic (Snyder, 2013). Lytic bacteriophages lyse bacterial cells, while lysogenic bacteriophages insert their DNA into the genome of bacterial cells (Snyder, 2013). Lysogenic bacteriophages have been used to insert CRISPR-Cas9 into *Escherichia coli* and *Staphylococcus aureus* in vitro (Bikard et al., 2014; Citorik et al., 2014; Yosef et al., 2015). Some differences between the two methods of delivery are that nanoparticles do not have target specificity unless a modality is added to make them specific and they can be easily modified, while most bacteriophages have species-specific specificity and bacterial resistance can quickly form (Merril et al., 2003; Gómez et al., 2015; Lino et al., 2018). Due to the specificity of CRISPR-Cas systems, delivery to more than the target bacteria should not cause other, possibly beneficial bacteria to be affected (Jinek et al., 2012a; Cong et al., 2013).

CRISPR-Cas Systems to Diagnose Cattle Diseases

Field deployable, quick, and reliable diagnostic tools are necessary for fast disease treatment in cattle production systems. Many CRISPR-Cas system diagnostic tools have been developed that fit these needs. For example, finding low abundance sequences by hybridization next generation sequencing (FLASH- NGS) is a CRISPR-Cas9 diagnostic tool that cleaves target DNA sequences into fragments properly sized for Illumina sequencing (Quan et al., 2019). Quan et al. (Quan et al., 2019) demonstrated that FLASH-NGS detected antimicrobial resistance genes in respiratory fluid and blood. Additionally, DETECTR, DNA endonuclease-targeted CRISPR trans reporter, has been demonstrated to have attomolar sensitivity for DNA detection of human papilloma virus and uses Cas12a ssDNase activation with isothermal amplification (Chen et al., 2018). Specific high-

sensitivity enzymatic reporter unlocking (SHERLOCK) uses CRISPR-Cas13's promiscuous RNA cleavage activity to detect target DNA sequences (Abudayyeh et al., 2016; East-Seletsky et al., 2016; Gootenberg et al., 2017). SHERLOCK has been used in conjunction with HUDSON, heating unextracted diagnostic nucleases to obliterate nucleases, to detect attomolar levels of Zika and Dengue viruses in saliva, urine, and blood serum samples in less than 2 hours (Myhrvold et al., 2018). Furthermore, STOP, SHERLOCK One-Pot Testing, was designed to detect SARS-CoV-2 (Segel et al., 2020). In 2018, Gootenberg et al. (2018) designed SHERLOCKv2, which uses Cas13 and Csm6, an auxillary CRISPR-Cas associated enzyme, to improve SHERLOCK so that it could detect multiple target DNA sequences in inputs as low as two attomolar and increase signal sensitivity. SHERLOCKv2 can detect more than one target DNA sequence though multiplexing and been demonstrated to have high sensitivity to Zika and Dengue viruses (Gootenberg et al., 2018). Moreover, Lu et al. (2019) designed a LwCas13a nanomachine to detect avain influenza A H7N1. In conjunction with reverse-transcription recombinase polymerase amplification assay (RT-RPA) and a T7 transcription system, this LwCas13a nanomachine was able to detect 1 fM of avian influenza A H7N1 within 50 minutes on a fluorescence plate reader (Liu et al., 2019). Moreover, Cas13a has been used to design a mobile phone-based fluorescence microscope that has no pre-amplification steps and can detect around 100 copies/ µL of target sequences from patient nasal swabs within 30 minutes of measurement time (Fozouni et al., 2021). Likewise, magnetic bead-quantum dots (MB-Qdots) have been used in conjunction with CRISPR-Cas12a to design a diagnostic tool that can be used with the human eye (Bao et al., 2020). Bao et al. (Bao et al., 2020) demonstrated the use of this MB-Qdot CRISPR assay on African swine fever and were able to detect limits of around 0.5 and 1.25 nM from buffer and porcine plasma, respectively.

These different illustrations show how many CRISPR-Cas systems could be applied as rapid and reliable diagnostic tools for use in in field diagnosis of cattle diseases.

Conclusions and Future Directions

CRISPR-Cas systems have the potential to diagnose bacterial pathogens and replace antibiotics to prevent, control, and treat bacterial infections in cattle production. CRISPR-Cas proteins occur naturally in bacteria to precisely cut DNA of invading viruses and disable the virus so it does not harm the bacteria similar to a human immune system. The purpose of this literature review was to find literature that used CRISPR-Cas systems to find and alter or detect DNA of cattle bacterial pathogens. The CRISPR-Cas complex scans bacterial DNA looking for a match to the gRNA within the CRISPR-Cas complex. Once the DNA match is found, a PAM sequence acts as a key that unlocks a door which allows the CRISPR-Cas complex editing access to the bacterial DNA within the pathogenic bacteria. Once the door is unlocked, the CRISPR-Cas acts as a molecular scalpel that cuts the bacterial DNA, which disables the bacteria from normal function, causes death of the bacteria, or can lead to detection of a bacterial species. The gRNA identifies the bacterial DNA, the PAM unlocks the door and the CRISPR-Cas surgically alters the bacterial DNA. The simplicity of CRISPR-Cas has made it easy to modify and cut DNA of many bacterial species. Due to the simplicity of modifying CRISPR-Cas systems it is capable of becoming a fast and reliable diagnostic tool or an antimicrobial alternative in cattle production. Studies need to be conducted on many cattle pathogens to determine if they have an endogenous CRISPR-Cas system, how well CRISPR-Cas systems work on the bacterium of interest, and how to effectively and safely deliver the CRISPR-Cas systems in cattle. If a bacterium has an endogenous CRISPR-Cas system, further studies should be conducted to determine the PAM(s) of that system and how the endogenous system can be used to alter, kill, or identify the bacterium.

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Chapter 6 - Preliminary data on CRISPR-Cas9 use as an antimicrobial alternative: A novel strategy to inhibit ruminal *Fusobacterium necrophorum* to prevent liver abscesses in feedlot cattle

Abstract

Liver abscesses are a major economic concern for cattle producers. The primary causative agent of liver abscesses is *Fusobacterium necrophorum* subsp. *necrophorum*, an anaerobic ruminal bacterium. Currently, tylosin, a macrolide antimicrobial, is regularly used to reduce the incidence of liver abscesses in feedlot cattle. Because of the public health concerns associated with the use of medically important antimicrobials in cattle used for food production, there is interest in finding an antimicrobial alternative to control liver abscesses. Clustered regularly interspaced short palindromic repeats (CRISPR) in conjunction with CRISPR-associated proteins are bacterial immune mechanisms that can be programmed to inhibit bacteria. Our main objectives were to determine if Cas9 with gRNAs could target the genes *rpsP* and *fomA* in *E. coli* and *F. necrophorum*, respectively.

A 1.3- to 3.3-$\log_{10}$ reduction was observed when the *rpsP* gene was targeted from a plasmid in *E. coli*. Likewise, when the *fomA* gene was targeted in *F. necrophorum*, there was a 1.0- to 3.6-$\log_{10}$ reduction in bacterial cell counts. However, the Cas9 control reduced bacterial cell counts by a 2.0-$\log_{10}$ in *F. necrophorum*, but not in *E. coli*. A possible reason for this is that *F. necrophorum*'s endogenous CRISPR-Cas type I system could be producing a gRNA compatible with Cas9. More studies should be conducted to determine whether CRISPR-Cas system-based bacterial inhibition

could be used to inhibit *F. necrophorum* in the rumen or liver as a strategy to reduce the incidence of liver abscesses in cattle.

Introduction

Fusobacterium necrophorum is an anaerobic, Gram-negative, rod-shaped bacterial opportunistic pathogen that causes liver abscesses in cattle (Langworth, 1977). The bacterium is a member of the ruminal bacterial flora, which travels from the rumen to the liver through portal circulation to set up abscesses. The pathogenesis includes rumenitis induced by chronic ruminal acidity that permits *F. necrophorum* to penetrate the ruminal wall to cause microabscesses. From these abscesses, the organisms enter portal blood to reach the liver. The organism is also a causative agent of foot rot and abscesses, and calf diphtheria in cattle (Tan et al., 1996). The species of *F. necrophorum* is classified into two subspecies, *necrophorum* and *funduliforme*, which differ in cell and colony morphologies, growth pattern in broth, and more importantly, in virulence. The subsp. *necrophorum* is more virulent, hence, more frequently involved in liver abscesses (Tadepalli et al., 2009). The major virulence factor that allows *F. necrophorum* to evade the defense mechanisms of the ruminal wall and liver is leukotoxin, a secreted protein that is cytotoxic to leukocytes and macrophages (Nagaraja et al., 2005). The leukotoxin is encoded by a three-gene operon, *lktABC*, of which the second gene, *lktA*, encodes the leukotoxin protein of 3,241 amino acids, with a molecular weight of 335,956 Da (Narayanan et al., 2002). The importance of leukotoxin as a virulence factor in *F. necrophorum* is indicated by a correlation between toxin production and the ability to induce abscesses in laboratory animals, an inability of non-leukotoxin-producing strains to induce foot abscesses in cattle following intradermal inoculation, and a relationship between antileukotoxin antibody titers and protection against infection in experimental challenge studies (Nagaraja et al., 2005). Furthermore, an outer membrane protein,

FomA, and a 30S ribosomal protein S16, RpsP, of *F. necrophorum* subsp. *necrophorum* are encoded by a likely nonessential and an essential gene, respectively (Menon, 2014; Kumar et al., 2015). FomA has been shown to be a porin protein in *F. nucleatum* that also aids in bacterial adhesion (Liu et al., 2010). In *F. necrophorum,* Kumar et al. (2015) demonstrated high binding affinity to cattle endothelial cells. The DNA sequence of the *F. necrophorum*'s *rpsP* gene has been shown to be specific to *F. necrophorum* with > 98 % and > 99 % identity between subsp. *fundiliforme* and *necrophorum*, respectively. The next closest species is *Leptotrichia*, with around 80 % identity.

The majority of liver condemnations in beef processing plants are due to liver abscesses (Reinhardt and Hubbert, 2015; Amachawadi and Nagaraja, 2016). The National Beef Quality Audit in 2016 reported 30.8 % liver condemnation compared to the 20.9 % liver condemnation rate in 2011. Around 60 % of the condemned livers were due to liver abscesses (Eastwood et al., 2017). In addition to the loss of liver, the economic impact of liver abscesses to the cattle industry includes decreased animal performance and lower carcass yield, as well as time and labor costs at cattle processing plants (Nagaraja and Lechtenberg, 2007). Currently, the most common antibiotic used to reduce the incidence of liver abscesses is tylosin (Nagaraja and Lechtenberg, 2007; Alicia et al., 2015; Müller et al., 2018). The mode of action of tylosin is to inhibit *F. necrophorum* and reduce ruminal concentration, which reduces the opportunity of the bacterium to enter the ruminal wall and reach the liver. There is concern with the tylosin use because the antibiotic belongs to the macrolide class, which is a medically important antibiotic class (Alicia et al., 2015; FDA, 2015; Müller et al., 2018). Antimicrobial resistance to medically important antibiotics has caused a shift in antibiotic stewardship, leading to the use of fewer antibiotics in food animal production (Sáenz et al., 2004; Hong et al., 2016; Müller et al., 2018; Oloso et al., 2018; Tyson et al., 2018; Salaheen

et al., 2019). Finding alternatives to these antibiotics are essential for the health, wellness, and productivity of food animals.

A possible alternative to antimicrobials is clustered regularly interspaced short palindromic repeats (CRISPR), along with CRISPR-associated (Cas) proteins. CRISPR-Cas systems are bacterial immunity mechanisms against foreign nucleic acids, including bacteriophages (Makarova et al., 2006; Brouns et al., 2008; Josiane et al., 2010). There are many different CRISPR-Cas proteins, but CRISPR-Cas9 is the most well studied. CRISPR-Cas9 is a class II CRISPR-Cas endonuclease that is guided by RNA and cleaves double stranded DNA (Deltcheva et al., 2011; Jinek et al., 2012). Cleavage is caused by complementary base pairing of the target DNA with a programmable single guide RNA (sgRNA) followed by a necessary protospacer adjacent motif (PAM) sequence in the target DNA (Jinek et al., 2012; Shah et al., 2013; Doudna and Charpentier, 2014). The binding of the PAM sequence after complementary base pairing of the target DNA is necessary for the double stranded cleavage of the target DNA (Gasiunas et al., 2012; Jinek et al., 2012; Sternberg et al., 2014). Furthermore, CRISPR-Cas9 has been used to edit bacterial genomes; therefore, CRISPR-Cas9 paired with an efficient delivery method will potentially be able to replace antibiotics (Jiang et al., 2013; Citorik et al., 2014; Gomaa et al., 2014; Moreb et al., 2017). For example, bacteriophages have been used to deliver CRISPR-Cas9 to *Escherichia coli*, where the nuclease was able to reduce the presence of antimicrobial resistance genes and inhibit bacterial growth (Citorik et al., 2014). Furthermore, CRISPR-Cas9 has also been delivered into methicillin- resistant *Staphylococcus aureus* through nanocomplexes and was demonstrated to be efficient at editing and inhibiting the growth of the bacterium (Kang et al., 2017). Bikard et al. (2014) demonstrated the inhibition of *S. aureus* with the use of CRISPR-Cas9 in a mouse skin colonization model. Moreover, CRISPR-Cas9 has been reported to cleave

plasmids in *E. coli* and *Klebsiella pneumonia* (Yosef et al., 2015; Hao et al., 2020). Because of CRISPR- Cas9's ability to efficiently cleave targeted DNA strands, the protein complex has the potential to effectively cleave and disrupt a gene in targeted bacteria.

Our objective was to demonstrate the use of a CRISPR-Cas9 system to inhibit *F. necrophorum*. This is a novel concept to potentially eliminate or reduce *F. necrophorum* in the rumen and a hypothetical strategy to reduce liver abscesses in cattle.

Materials and Methods

Plasmid construction

A plasmid with the *F. necrophorum rpsP* gene was designed with the *kanR* and *lacZ* genes (GenScript, Nanjing, China). The sequence was chosen from the complete genome of the *F. necrophorum* subsp. *necrophorum* ATCC 25286 (Genebank accession number CP034842.1). The inserted sequence size was 286 base pairs.

Insertion of *F. necrophorum rpsP* gene

New England Biolabs (NEB) 10-beta electrocompetent *E. coli* (New England Biolabs, Ipswich, MA) was placed on ice to thaw. After thawing, 25 µl of the electrocompetent *E. coli* was transferred to a chilled 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany) and 100 ng (0.1 µL) of the plasmid with the *F. necrophorum rpsP* gene or the pUC19 plasmid (control; New England Biolabs, Ipswich, MA) was gently mixed with the electrocompetent *E. coli* on ice. The mixture was then transferred to a 0.1 cm gap cuvette (Bio-Rad, Hercules, CA) and electroporated with a Gene Pulser Xcell Microbial System (Bio-Rad, Hercules, CA) set to 2 kV, 25 µF, and 200 Ω. Nine hundred and seventy-five microliters of pre-warmed NEB 10-beta outgrowth medium (New England Biolabs, Ipswich, MA) were immediately added to the cuvette and pipetted quickly and gently to mix. The electroporated cells were then transferred to a round-bottom culture tube

and placed in a shaking incubator at 37 °C. After 1 hr of incubation, 100 µL of the electroporated cells were serially diluted and each dilution was spread plated onto pre-warmed selective Lennox L. (LL) agar (Invitrogen, Waltham, MA) plates with x-gal (Promega, Madison, WI) and either kanamycin (MilliporeSigma, Burlington, VT) or ampicillin (MilliporeSigma, Burlington, VT). The cells were also streaked for purity on the previously described selection plates. All plates were placed in a 37° C incubator overnight. The following day, the plates were examined and PCR was used to confirm the insertion of the plasmid with the *F. necrophorum rpsP* gene from blue colonies on kanamycin plates. After confirmation, colonies were saved at -80 °C in Cyrobank freezer beads (COPAN Diagnostics, Murrieta, CA). PCR was also used to confirm pUC19 insertion from blue colonies on ampicillin plates.

PCR assays for *rpsP* and *fomA* genes

rpsP gene

*Escherichia coli rpsP*FI colonies were suspended in 50 µL sterile water, boiled for 10 min, and centrifuged for 2 min at 2,200 x g. On ice, 5 µL of 10x buffer, dNTP mix, forward and reverse primers (Table 6.1), 3 µL of Mg^{2+}, and 25 µL water were gently combined. Then, 1 µL of the boiled and centrifuged *E. coli rpsP*FI cells were added and, lastly, 1 µL KOD DNA polymerase (New England Biolabs, Ipswich, MA) was added. The mixture was centrifuged for 5 seconds at 5,000 x g and then a Mastercycler nexus GSX1 (Eppendorf, Hamburg, Germany) was used to run the PCR reaction in the sequence of 95 °C for 2 minutes 15 seconds, 54 °C for 30 seconds, 72 °C for 40 seconds for a total of 30 times, then the sequence went to 72 °C for 10 minutes before finishing. The samples were then placed in a QiAxel Advanced (Qiagen, Hilden, Germany) and outputs were analyzed.

fomA gene

F. necrophorum subsp. *necrophorum* colonies were suspended in 50 µL sterile water, boiled for 10 min, and centrifuged for 2 min at 2,200 x g. On ice, 25 µL GoTaq green master mix (Promega, Madison, WI), 2.5 µL forward and reverse primers (Table 6.1), 2.5 µL DNA template, and 17.5 µL nuclease free water were gently combined. Then, 1 µL of the boiled and centrifuged *F. necrophorum* subsp. *necrophorum* cells were added and, then 1 µL KOD DNA polymerase. The mixture was centrifuged for 5 seconds at 5,000 x g and then Mastercycler nexus GSX1 (Eppendorf, Hamburg, Germany) was used to run the PCR reaction in the sequence of 96° C for 3 minutes 15 seconds, 60 °C for 15 seconds, 54 °C for 15 seconds, 72 °C for 1 minute, repeated for a total of 34 times, then the sequence went to 72 °C for 5 minutes. The samples were then placed in a QiAxel Advanced (Qiagen) and outputs were analyzed.

Bacterial strains

*E. coli rpsP*FI

*Escherichia coli rpsP*FI was streaked onto LL agar with kanamycin and X-gal and incubated overnight at 37° C. Single blue colonies were inoculated into 10 mL LL broth and placed in a 37° C incubator overnight. The following day, 1 mL of overnight culture was pipetted into 10 mL LL broth and incubated until an OD_{600} of 0.4- 0.6 (around 2 hours).

F. necrophorum subsp. *necrophorum*

Fusobacterium necrophorum subsp. *necrophorum* strain 8L1, stored at -80° C, was streaked onto sheep blood agar plates (SBA; Remel Inc., Lenexa, KS) and incubated at 37 °C for 48 h in an anaerobic glove box (Forma Scientific Inc., Marietta, OH). Single colonies were inoculated into 10 ml pre-reduced (with 0.05 % cysteine HCl; Sigma-Aldrich, St. Louis, MO), anaerobically sterilized brain heart infusion broth (PRAS-BHI; Becton Dickinson, Sparks, MD)

and incubated overnight at 37 °C. The following day, 1 mL of overnight culture was pipetted into 10 mL PRAS-BHI broth and incubated until an OD_{600} of 0.4- 0.6 (around 4 hours).

Insertion of Cas9 protein and gRNA complex

*Escherichia coli rpsP*FI

One milliliter of *E. coli rpsP*FI culture, at an OD_{600} of 0.4-0.6, was pipetted into a pre-chilled 1.5 microcentrifuge tube kept on ice and centrifuged at 4 °C for five minutes at 7,000 x g. The supernatant was discarded, and the pellet was resuspended in 1 mL, sterile, ice-cold water and centrifuged at 4° C for five minutes at 7,000 x g. This was repeated for a total of three washes. Then, the pellet was resuspended with 20 µL ice water. Four microliters of the Cas9 protein (MilliporeSigma, Burlington, VT) at a concentration of 10 pmol/ µL was gently mixed with 12 µL gRNA at a concentration of 30 pmol/ µL and given fifteen minutes to combine at room temperature before being placed on ice. Two gRNAs that were designed to target the *F. necrophorum rpsP* gene insert were used independently and mixed (Table 6.2). When the gRNAs were mixed, each was allowed to combine separately, with 2 µL Cas9 and 6 µL gRNA, before being mixed together. Then, 12 µL reconstitution buffer (MilliporeSigma, Burlington, VT) and 20 µL electrocompetent *E.coli rpsP*FI was added to the Cas9-gRNA combination and gently mixed. The final volume was 48 µL and this was gently pipetted into a 0.1 cm gap cuvette and electroporated with a Gene Pulser Xcell Microbial System set to 2 kV, 25 µF, and 200 Ω. Immediately following electroporation, 952 µL of stable outgrowth medium (New England Biolabs, Ipswich, MA) was added to a cuvette and pipetted quickly and gently. The mixture was then serially diluted in stable outgrowth medium and 100 µL of each dilution was spread plated onto LL agar plates with x-gal only and LL agar plates with x-gal and kanamycin. The plates were incubated overnight at 37 °C, then colony counts were recorded, and colonies were picked for PCR. The controls used were *E. coli rpsP*FI only, *E.*

*coli rpsP*FI with individual gRNAs only, and *E. coli rpsP*FI with Cas9 only. Reconstitution buffer was used to bring the controls to a final volume of 48 µL.

***F. necrophorum* subsp. *necrophorum* 8L1**

One mL of *F. necrophorum* subsp. *necrophorum* culture at an OD_{600} of 0.4-0.6 was pipetted into a pre-chilled 1.5 microcentrifuge tube on ice and made electrocompetent as described previously. Two gRNAs that were designed to target the *fomA* gene insert were used independently and combined (Table 6.2). When the gRNAs were combined, each was allowed to bind separately, with 2 µL Cas9 and 6 uL gRNA, before being mixed together. Then, 12 µL reconstitution buffer and 20 µL electrocompetent *F. necrophorum* subsp. *necrophorum* was added to the Cas9-gRNA combination and gently mixed. The final volume was 48 µL and this was gently pipetted into a 0.1 cm gap cuvette and electroporated with a Gene Pulser Xcell Microbial System set to 2 kV, 25 µF, and 200 Ω. Immediately following electroporation, 952 µL of PRAS-BHI broth was added to a cuvette and pipetted quickly and gently. The mixture was then pipetted to a 1.5 mL centrifuge tube and placed in an anaerobic glove box, where it was serially diluted in PRAS-BHI broth. One hundred microliters of each dilution were spread plated onto SBA plates and incubated overnight at 37 °C. Colony counts were recorded, and colonies were picked for PCR. The controls used were *F. necrophorum* subsp. *necrophorum* only, *F. necrophorum* subsp. *necrophorum* with individual gRNAs only, and *F. necrophorum* subsp. *necrophorum* with Cas9 only. Reconstitution buffer was used to bring the controls to a final volume of 48 µL.

Statistical analysis

The cleavage and inhibitory activity of Cas9/gRNA complexes were evaluated using a PROC GLM procedure in SAS (Version Studio, Cary, NC). The statistical model included the treatments, replications (repeated measures as a random effect) and $\log_{10}$ of treatments. The mean comparisons

within and between treatment groups were tested using Student-Newman-Keuls Test with an alpha of ≤ 0.05.

Results

F. necrophorum rpsP gene targeting in *E. coli rpsP*FI with CRISPR-Cas9

On LL agar with x-gal and kanamycin a 1.3- to 3.3-$\log_{10}$ reduction was seen in bacterial cell counts depending on gRNA(s) used, compared to controls (*p*-value < 0.0001; Figure 6.1; Table 6.3). Very few white colonies were observed on LL agar with x-gal and these colonies were presumed to have lost the plasmid containing the *F. necroophorum rpsP* gene which was confirmed by PCR (Figure 6.2). Fewer white colonies with a blue center were observed on LL agar with x-gal when Cas9 and gRNA2 were tested and some were observed when the gRNAs were combined. A slight band for the *F. necrophorum rpsP* gene was observed in the PCR assay of these colonies.

CRISPR-Cas9 inhibition of *F. necrophorum* subsp. *necrophorum* 8L1

On SBA agar a 1.0- to 3.6-$\log_{10}$ reduction was seen in bacterial cell counts after 48 h incubation in an anaerobic glove box, compared to controls (*p*-value < 0.0001; Figure 6.3; Table 6.4). When gRNAs were used with Cas9 independently, there was a 1- to 3.6- $\log_{10}$ decrease; however, when used together there was a 1.5- to 3.5-$\log_{10}$ decrease. When comparing only *F. necrophorum* with *F. necrophorum* + Cas9 + gRNA 1, *F. necrophorum* + Cas9 + gRNA 2, and *F. necrophorum* + Cas9 + gRNA 1 and 2 combined the $\log_{10}$ differences were 3.6 (*p*-value = 0.0003), 3.0 (*p*-value = 0.0034), and 3.5 (*p*-value = 0.0005), respectively (Figure 6.4). Interestingly, a 2-$\log_{10}$ decrease in bacterial cell counts was observed when Cas9 was tested without a gRNA and a 0.9- to 1.1-$\log_{10}$ decrease was observed when individual gRNAs 1 and 2, respectively, were tested without Cas9, compared to when *F. necrophorum* was electroporated unaccompanied (*p*-value =

0.0229, 0.5987, and 0.2677, respectively). Nonetheless, no statistical differences were seen between *F. necrophorum* + Cas9 control and *F. necrophorum* + Cas9 + gRNA 1, 2, and combined (*p*-value = 0.3637, 0.2193, 0.2495, respectively; Figure 6.5).

Discussion

Currently, liver abscesses are controlled with antimicrobial feed additives. To control liver abscesses the FDA has approved several antimicrobial compounds for use in cattle feed (Feed Additive Compendium, 2021). Tylosin, which belongs to the macrolide antimicrobial class, is the most commonly used to prevent liver abscesses (Nagaraja and Chengappa, 1998). Tylosin is a mainly effective against Gram-positive bacteria, but is also affective against *F. necrophorum*, a Gram-negative bacteria (Gingerich et al., 1977; Tan et al., 1994; Lechtenberg et al., 1998). Although tylosin is only used in animals, it is a macrolide similar to erythromycin, and macrolide antimicrobials are considered medically important because they are commonly used in human medicine (Scott et al., 2019). Therefore, tylosin was placed under veterinary oversight due to a growing public health concern of the use of the antimicrobial in animals (FDA, 2021). There is some concern with the use of tylosin related to antimicrobial resistance within bacteria found in the digestive tract (Zaheer et al., 2013; Alicia et al., 2015; Müller et al., 2018). However, in tylosin-fed cattle there is no indication of macrolide resistance in *F. necrophorum* isolated from liver abscesses and studies have found minimal AMR impact of tylosin use on gut bacteria (Nagaraja et al., 1999; Amachawadi et al., 2017; Schmidt et al., 2020). The public health concern associated with the use of medically important antimicrobials in cattle diets has caused an interest in discovering alternatives to replace tylosin. Antimicrobial alternatives that have been evaluated include oil seeds (Mir et al., 2008), vaccines (Saginala et al., 1997; Jones et al., 2004), egg yolk immunoglobulins (Stotz et al., 2021), essential oils (Meyer et al., 2009; Elwakeel et al., 2013), and

direct-fed microbials (Huebner et al., 2019). For example, Samii et al. (2016) demonstrated the use of limonene to decrease ruminal *F. necrophorum* concentrations, with 40 mg limonene/ kg body weight reducing ruminal *F. necrophorum* concentrations the most. Moreover, vaccine development has been a major area of study because *F. necrophorum* virulence factors have been identified and a few have been well-characterized. (Amachawadi and Nagaraja, 2016). For example, Fox et al. (2009) compared two commercially available vaccines, Fusogard® (Novartis Animal Health US, Inc, Greensboro, NC) and Centurion™ (Intervet/Schering-Plough Animal Health, DeSoto, KS), and demonstrated no differences in liver abscess incidence or severity compared to cattle not given a vaccine. Because commercially available vaccines have had low efficacy at preventing liver abscesses, they have not replaced the use of tylosin in the cattle industry (Checkley et al., 2005).

CRISPR-Cas systems have been shown to exert inhibition of *E. coli, S. aureus, K. pneumoniae, C. difficile, S. enterica* and *M. tuberculosis* (Bikard et al., 2014; Gomaa et al., 2014; Kiga et al., 2019; Li et al., 2019; Hao et al., 2020; Selle et al., 2020a). For example, CRISPR-Cas9 has been shown to reduce the prevalence of antimicrobial resistance genes in *E. coli and S. aureus*, as well as inhibit bacterial growth *in vivo* and *in vitro* (Bikard et al., 2014; Citorik et al., 2014; Kang et al., 2017). CRISPR-Cas9 has also been shown to have the ability to remove plasmids containing antimicrobial resistance and virulence genes. For example, CRISPR-Cas9 has been reported to cleave plasmids in *E. coli* and *K. pneumonia* (Yosef et al., 2015; Hao et al., 2020). Furthermore, *E. coli* and *C. difficile* have been selectively inhibited with type I CRISPR-Cas systems (Gomaa et al., 2014; Selle et al., 2020b, a). CRISPR-Cas13a has also demonstrated inhibition of *E. coli* and *S. aureus* (Kiga et al., 2019).

The current study demonstrated a 1.3- to 3.3-log reduction in growth of *E. coli* with a plasmid containing the *F. necrophorum rpsP* gene. This gene encodes the 30S ribosomal subunit S16 protein and the DNA sequence is specific to *F. necrophorum* subsp (Genebank accession #CP034842, National Center for Biotechnology Information, Bethesda, MD). Furthermore, few white colonies were observed on LL agar with x-gal, these colonies were presumed to have lost the plasmid containing the *rpsP* gene, which was confirmed by PCR. Some of the white colonies had a blue center, indicating that the whole plasmid was not lost after *rpsP* cleavage. However, DNA sequencing should be done to determine if the whole plasmid was lost. There were few white colonies observed on LL agar with x-gal compared to the 1.3- to 3.3-log reduction on LL agar with x-gal and kanamycin. This could be because the bacterial cells were either dying when the *rpsP* gene was cleaved or because the plasmid was not totally removed and the *lac* operon was still functional, while the *kanR* gene was not functional.

Furthermore a 1.0- to 3.6-log reduction was seen when the *fomA* gene was targeted in *F. necrophorum*. The differences in reduction were due to controls with gRNAs 1 and 2 having a 0.9- and 1.1-log reduction, respectively, and the Cas9 control having a 2.0-log reduction compared the control with *F. necrophorum* only. This is interesting because when tested in *E. coli* the controls were all within 0.4 logs of each other, with the Cas9 control being 0.2 logs lower than the control with *E. coli* only. One possibility for this finding is that *F. necrophorum* is an anaerobic bacterium, thus some cells could have died when made electrocompetent and electroporated (Langworth, 1977). However, this possibility is doubtful because it occurred over multiple repetitions. Another possibility is that *F. necrophorum* has a CRISPR system, and therefore, could have produced gRNAs that worked with the Cas9 nuclease. This is unlikely because *F. necrophorum*'s endogenous CRISPR system is a type I with a Cas3 protein (Genebank accession #CP034842,

National Center for Biotechnology Information, Bethesda, MD). Still, some common PAM sequences of type I CRISPR systems are CCN and CG, which could be used with the Cas9 endonuclease if by random chance the CCN was a PAM of the *F. necrophorum* CRISPR-Cas system or if CG was a PAM and the target sequence was followed by CGG (Westra et al., 2013; Koskela et al., 2015; Guo et al., 2019; Musharova et al., 2019; Liu and Doudna, 2020). Even so, Cas9 requires a tracrRNA, while Cas3 only requires a crRNA making this possibility doubtful, nonetheless *F. necrophorum*'s CRISPR-Cas system has not been well characterized and could be why there is a reduction in bacterial cell counts when Cas9 in used alone (Deltcheva et al., 2011; Sinkunas et al., 2011; Gasiunas et al., 2014; Workman et al., 2021). Furthermore, when the Cas9 control was tested in NEB 10-beta *E. coli,* which also has a CRISPR-Cas type I system, bacterial concentrations were not reduced, indicating that *F. necrophorum'*s endogenous CRISPR-Cas system is likely not causing the reduction.

Moreover, the current study was preliminary and therefore there are many future studies that should be done before determining CRISPR-Cas9 and other CRISPR-Cas systems as potential antimicrobial alternatives to tylosin. For example, the Cas9/gRNA ratio should be optimized for maximum efficiency as well as the electroporation settings. Furthermore, western blot should be performed to confirm the presence of Cas9 in the bacterial cells, while rt-PCR should be performed to determine the presence of gRNAs (Campbell et al., 2019; Sullivan et al., 2019). Fluorescent markers could also be used to determine the presence of Cas9 (Chen et al., 2019; Liu et al., 2020). Moreover, testing several gRNAs should be done to determine the most efficient gRNAs to disrupt the *fomA* gene.

Fusobacterium necrophorum subsp. *necrophorum* has an endogenous type I system (Genebank accession #CP034842, National Center for Biotechnology Information, Bethesda,

MD). Therefore, this system should be evaluated to determine how it works and how it could be used for gene editing or bacterial self-destruction. One key experiment that should be performed would be to identify the PAMS of the endogenous system. This could be done by using bioinformatics, high-throughput experimental screenings to disrupt target DNA, or a PAM screen achieved by NOT-gate repression (PAM-SCANR) (Horvath et al., 2008; Mojica et al., 2009; Jiang et al., 2013; Pattanayak et al., 2013; Karvelis et al., 2015). Using the endogenous system to disrupt genes would be beneficial to cattle production systems because only gRNAs and not Cas proteins would need to be delivered to the bacteria.

Studies should also be conducted to evaluate CRISPR-Cas systems ability to disrupt the gene that is responsible for leukotoxin production, *lktA*. *Fusobacterium necrophorum* without the ability to produce leukotoxin will likely be unable to survive in the liver to cause abscesses. Experiments should determine if gene cleavage allows for the survival of *F. necrophorum* and, if so, whether gene disruption reduces or eliminates leukotoxin production.

A major obstacle will be delivering CRISPR-Cas complexes into *F. necrophorum* into the rumens of cattle. Two possible delivery methods are via nanoparticles and bacteriophages. Nanoparticles are microscopic particles with varying properties that have been used to deliver CRISPR-Cas systems to inhibit bacterial cells as well as for use in treating genetic disorders and cancer (Anu Mary Ealia and Saravanakumar, 2017; Chen et al., 2017; Lee et al., 2018). The second possible delivery method is the use of bacteriophages, which are bacterial viruses that insert DNA into a bacterial host. Bacteriophages can either be lytic or lysogenic and lysogenic bacteriophages have been used to insert CRISPR-Cas9 into *Escherichia coli* and *Staphylococcus aureus in vitro* (Snyder, 2013; Bikard et al., 2014; Citorik et al., 2014; Yosef et al., 2015). The delivery methods differ in multiple ways, with one being target specificity. Nanoparticles do not have target

specificity unless a modality is added, while most bacteriophages are very target specific (Merril et al., 2003; Lino et al., 2018). Furthermore, nanoparticles are able to directly deliver the protein-gRNA, while bacteriophages can be used to deliver the Cas genes and gRNA DNA sequence, which must be translated and transcribed in the bacterial cell (Citorik et al., 2014; Kang et al., 2017; Kiga et al., 2019). Therefore, delivery methods should be evaluated with different Cas systems, including *F. necrophorum*'s endogenous system, to determine the most efficient delivery method for the system to cleave target genes.

Lastly, safety and product stability studies need to be conducted. Safety studies should include determination that there are no cattle or human health risks when using the CRISPR-Cas system paired with the chosen delivery method and that viable residues are not in the meat or the environment. Product stability studies should determine that the delivery and CRISPR-Cas complexes could tolerate production, handling, and administration to cattle.

Conclusions

In conclusion, the *rpsP* and *fomA* genes were targeted with Cas9-gRNA complexes in *E. coli* and *F. necrophorum* to determine if CRISPR-Cas9 could possibly be used as an antimicrobial alternative. When the *rpsP* gene was targeted from a plasmid in *E. coli*, there was a 1.3- to 3.3-$\log_{10}$ reduction in bacterial cell counts observed. Likewise, when the *fomA* gene was targeted in *F. necrophorum*, a 1.0- to 3.6-$\log_{10}$ reduction was observed. However, in *F. necrophorum* the Cas9 control reduced bacterial cell counts by a 2.0-$\log_{10}$. This was not seen for the Cas9 control when tested in *E. coli*. This could be because *F. necrophorum* has an endogenous CRISPR-Cas type I system. More studies should be conducted to determine how this endogenous system works and to further demonstrate the potential use of CRISPR-Cas systems as antimicrobial alternatives to tylosin in cattle production.

Table 6.1. PCR assay primers for *rpsP* and *fomA* genes.

Primer	**Sequence**
rpsP forward primer	5’ -TCCCGGAAAGCTTTTAAAGTTAAGATTA- 3’
rpsP reverse primer	5’ -TCCGGGAAAGCTTTTTCTTTTTTGTTT- 3’
fomA forward primer	5’ - GCCGGATCCAAAGAAGTGATGCCTGCTCCT- 3’
fomA reverse primer	5’ - ACCTGCAGGAAAGTAACTTTCATACCAGC- 3’

Table 6.2. Sequences of guided RNAs (gRNA) targeting *rpsP* and *fomA* genes.

gRNA	**Sequence**
rpsP gRNA 1	5’ -AAAGTTAAGATTAACTCGATT- 3’
rpsP gRNA 2	5’ -CAAGAACTGTAAAATCTATTC- 3’
fomA gRNA 1	5’ -ATTAGGTTCTTTATTAGTCAT- 3’
fomA gRNA 2	5’ -GCCTTTACTTATACTTCTAC- 3’

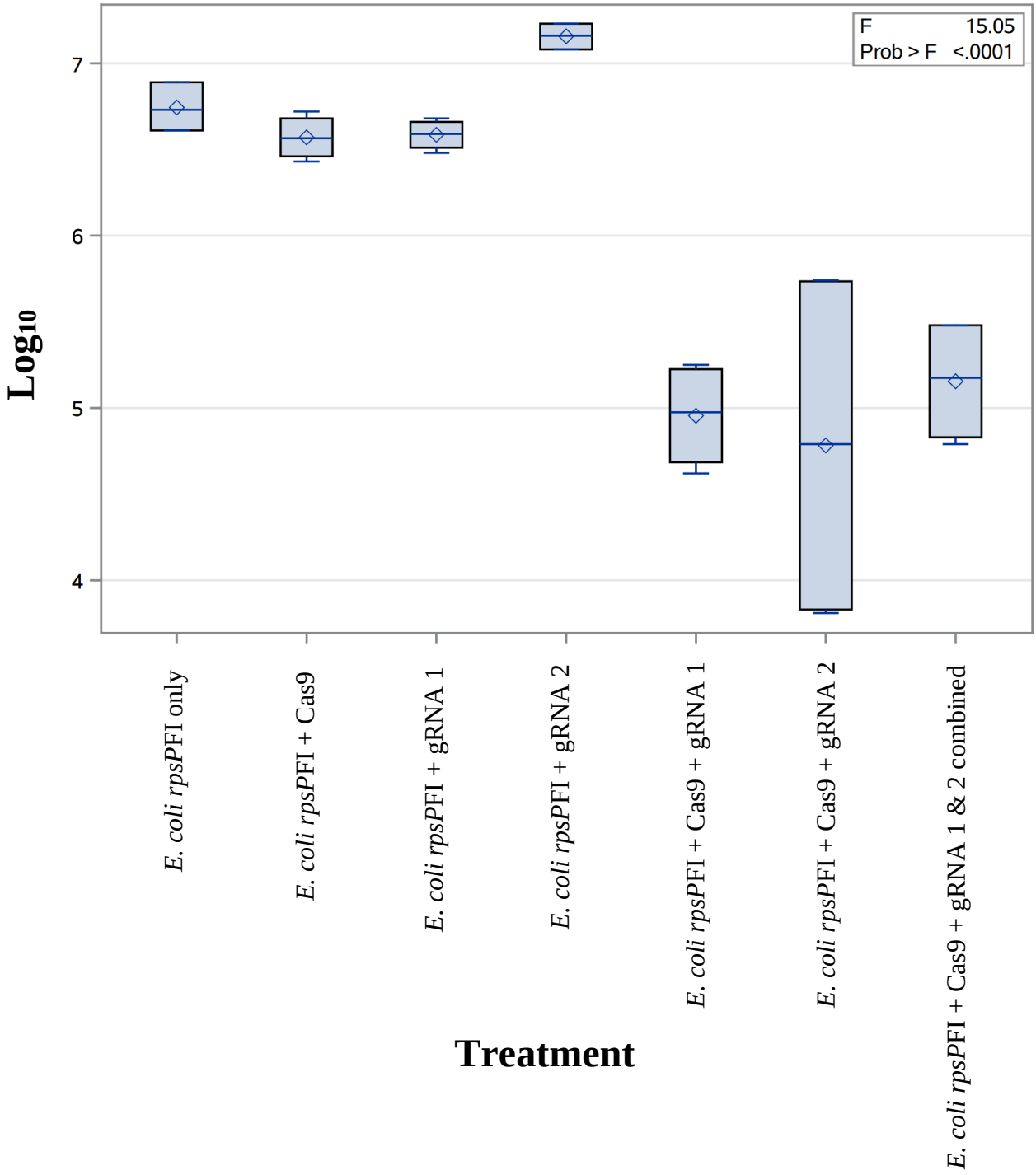

Figure 6.1. Distribution of log10 for *Fusobacterium necrophorum rpsP* gene deletion in *Escherichia. coli rpsP*FI with CRISPR-Cas9.

Table 6.3. *Fusobacterium necrophorum rpsP* gene targeting in *Escherichia coli rpsP*FI with CRISPR-Cas9.

Sample	Transformed CFU/ mL[a]	Total CFU/ mL[a]	Transformation Efficiency	CFU/ mL[b]	Log_{10}[b]	TM (ms)	Volts (V)
*Escherichia coli rpsP*FI	-	-	-	7.7E+06	6.9	5.2	1979
*Escherichia coli rpsP*FI + gRNA 1	-	-	-	3.3E+06	6.5	4.7	1977
*Escherichia coli rpsP*FI + gRNA 2	-	-	-	1.2E+07	7.1	4.7	1976
*Escherichia coli rpsP*FI + Cas9	-	-	-	4.9E+06	6.7	4.5	1975
*Escherichia coli rpsP*FI + Cas9 + gRNA 1	4.75E+03	6.20E+05	0.77 %	1.7E+05	5.2	3.7	1973
*Escherichia coli rpsP*FI + Cas9 + gRNA 2	2.40E+02	1.10E+04	2.18 %	6.7E+03	3.8	3.3	1973
*Escherichia coli rpsP*FI + Cas9 + gRNA 1 & 2	1.39E+03	1.26E+05	1.10 %	6.8E+04	4.8	3.7	1973

[a] Taken from LL agar with x-gal

[b] Taken from LL agar with x-gal and kanamycin

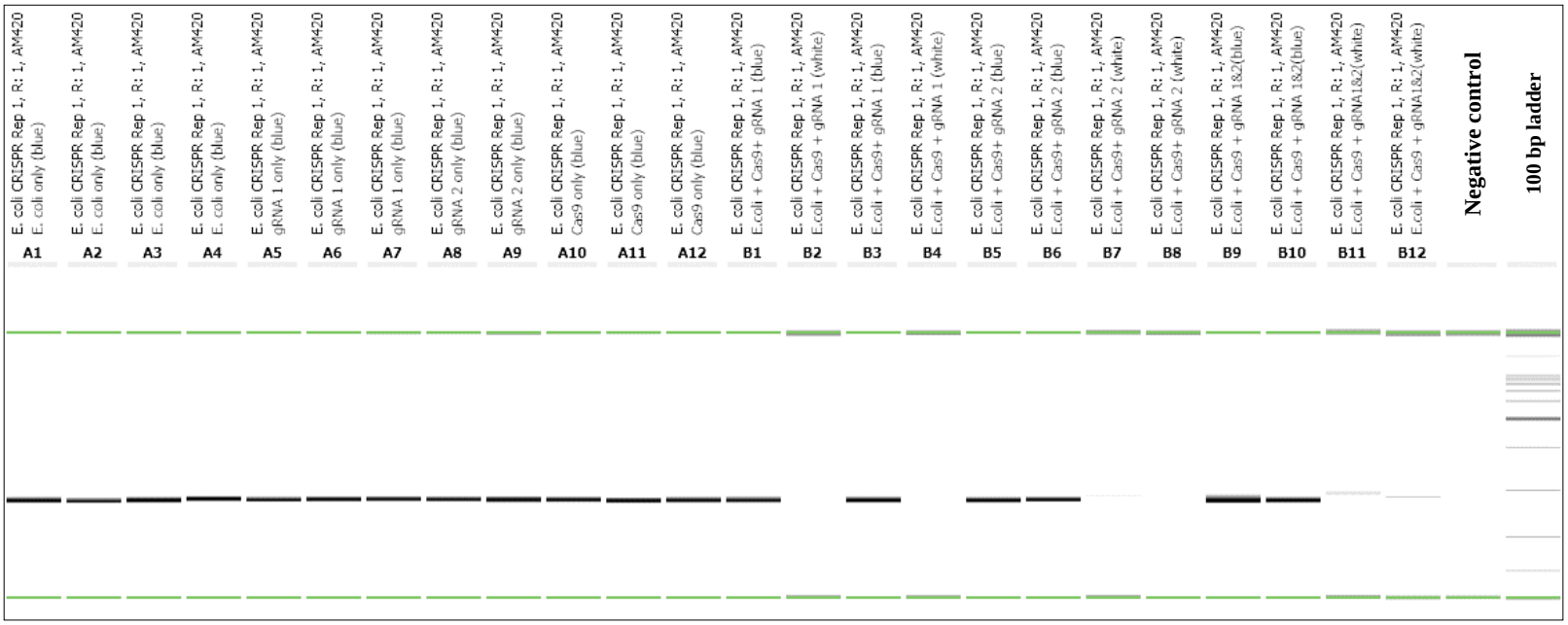

Figure 6.2. Polymerase chain reaction assay of *Escherichia coli* after insertion of Cas9/gRNA complex.

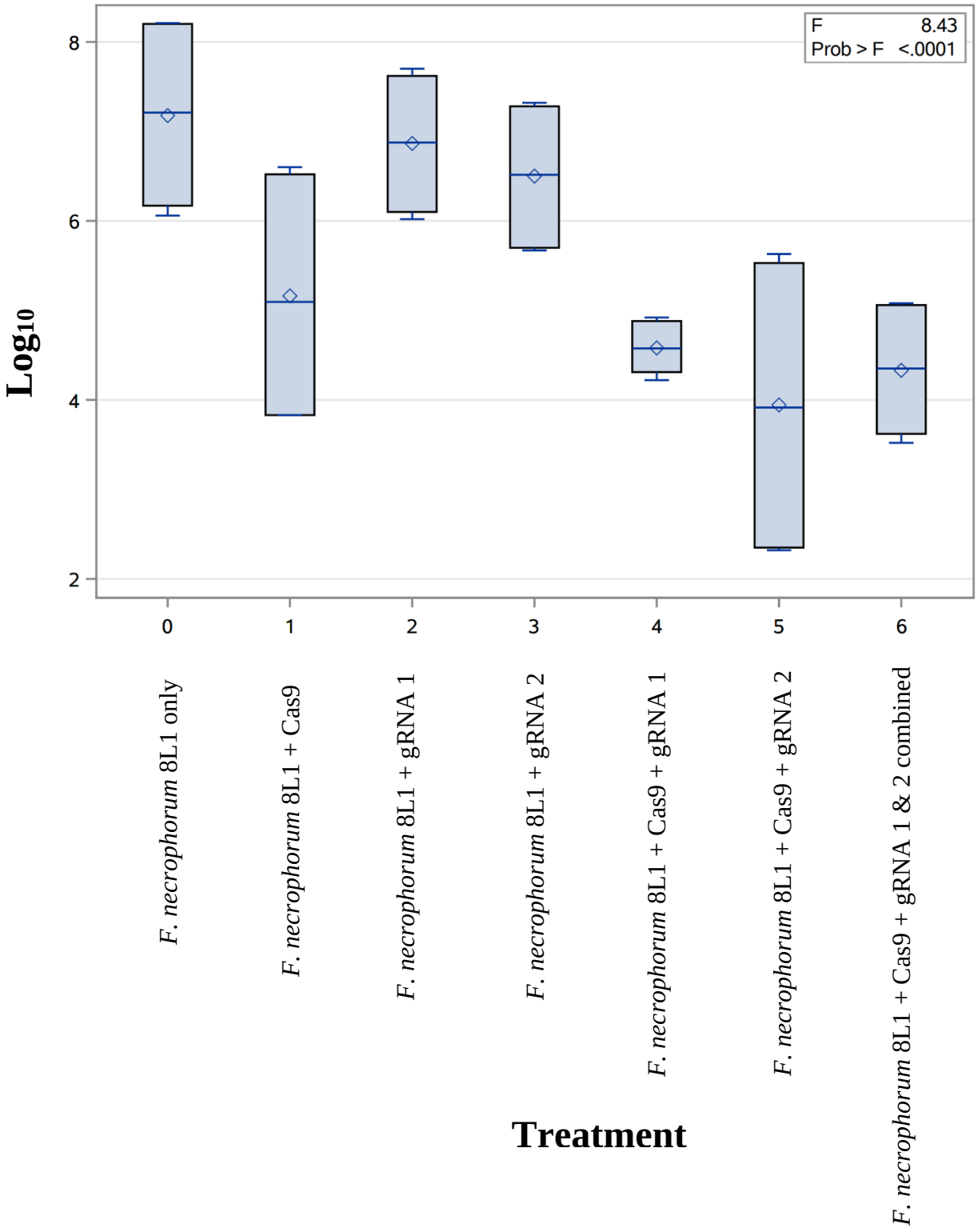

Figure 6.3. Distribution of $\log_{10}$ CRISPR-Cas9 inhibition of *Fusoacterium necrophorum* subsp. *necrophorum* 8L1.

Table 6.4. CRISPR-Cas9 inhibition of *Fusobacterium necrophorum* subsp. *necrophorum* 8L1.

Sample	CFU/ mL	Log_{10}	TM (ms)	Volts (V)
Fusobacterium necrophorum subsp. *necrophorum* 8L1	1.9E+08	8.3	5.1	1978
Fusobacterium necrophorum subsp. *necrophorum* 8L1 + gRNA 1	2.3E+07	7.4	5.0	1978
Fusobacterium necrophorum subsp. *necrophorum* 8L1 + gRNA 2	1.4E+07	7.2	5.0	1977
Fusobacterium necrophorum subsp. *necrophorum* 8L1 + Cas9	1.8E+06	6.3	3.7	1973
Fusobacterium necrophorum subsp. *necrophorum* 8L1 + Cas9 + gRNA 1	4.7E+04	4.7	3.5	1972
Fusobacterium necrophorum subsp. *necrophorum* 8L1 + Cas9 + gRNA 2	1.8E+05	5.3	3.6	1973
Fusobacterium necrophorum subsp. *necrophorum* 8L1 + Cas9 + gRNA 1 & 2	5.9E+04	4.8	3.7	1974

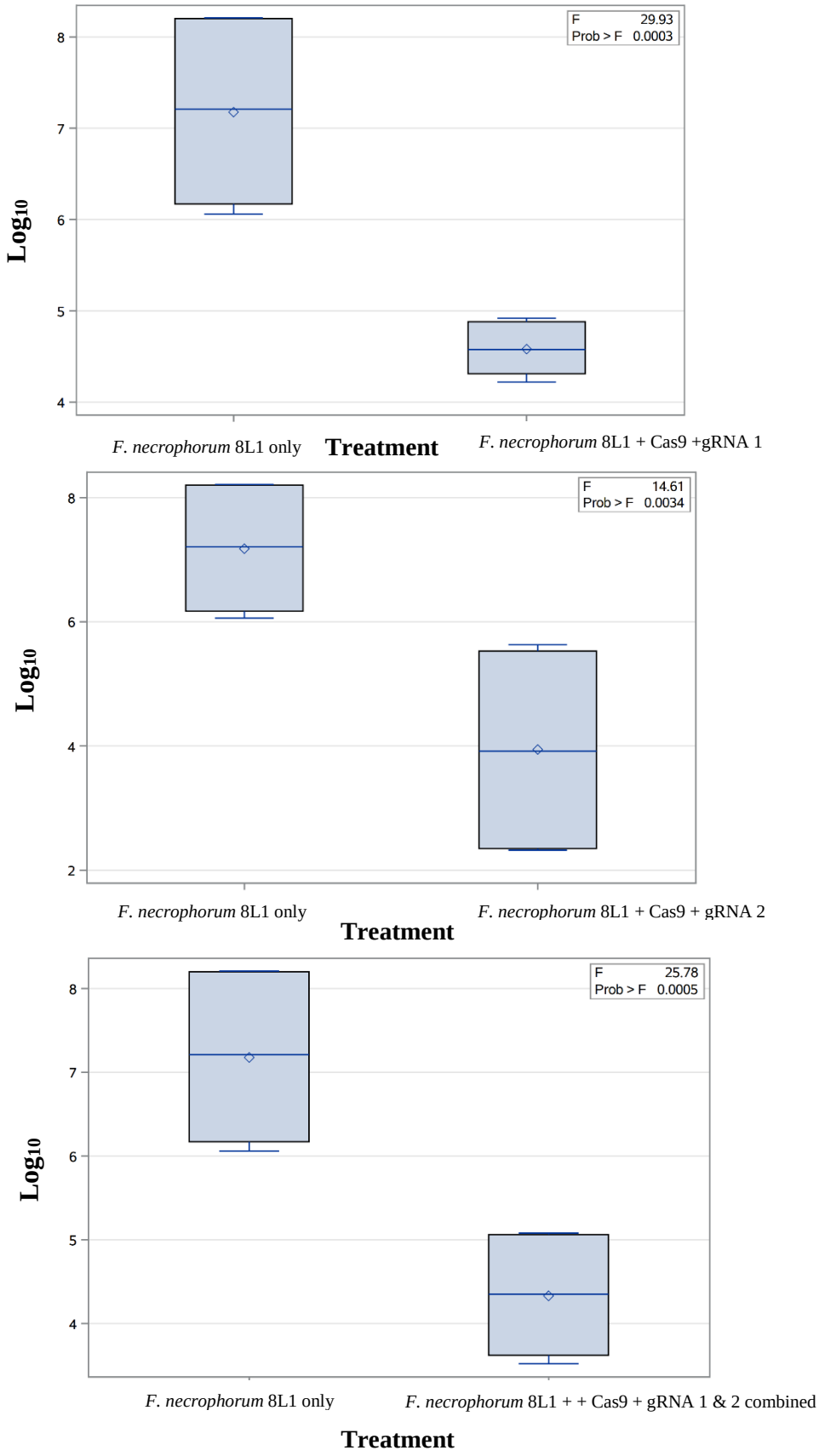

Figure 6.4. Distribution of log_{10} CRISPR-Cas9 inhibition of *Fusobacterium necrophorum* subsp. *necrophorum* 8L1 only and *F. necrophorum* subsp. *necrophorum* 8L1 + Cas9 + gRNA 1, 2, and combined.

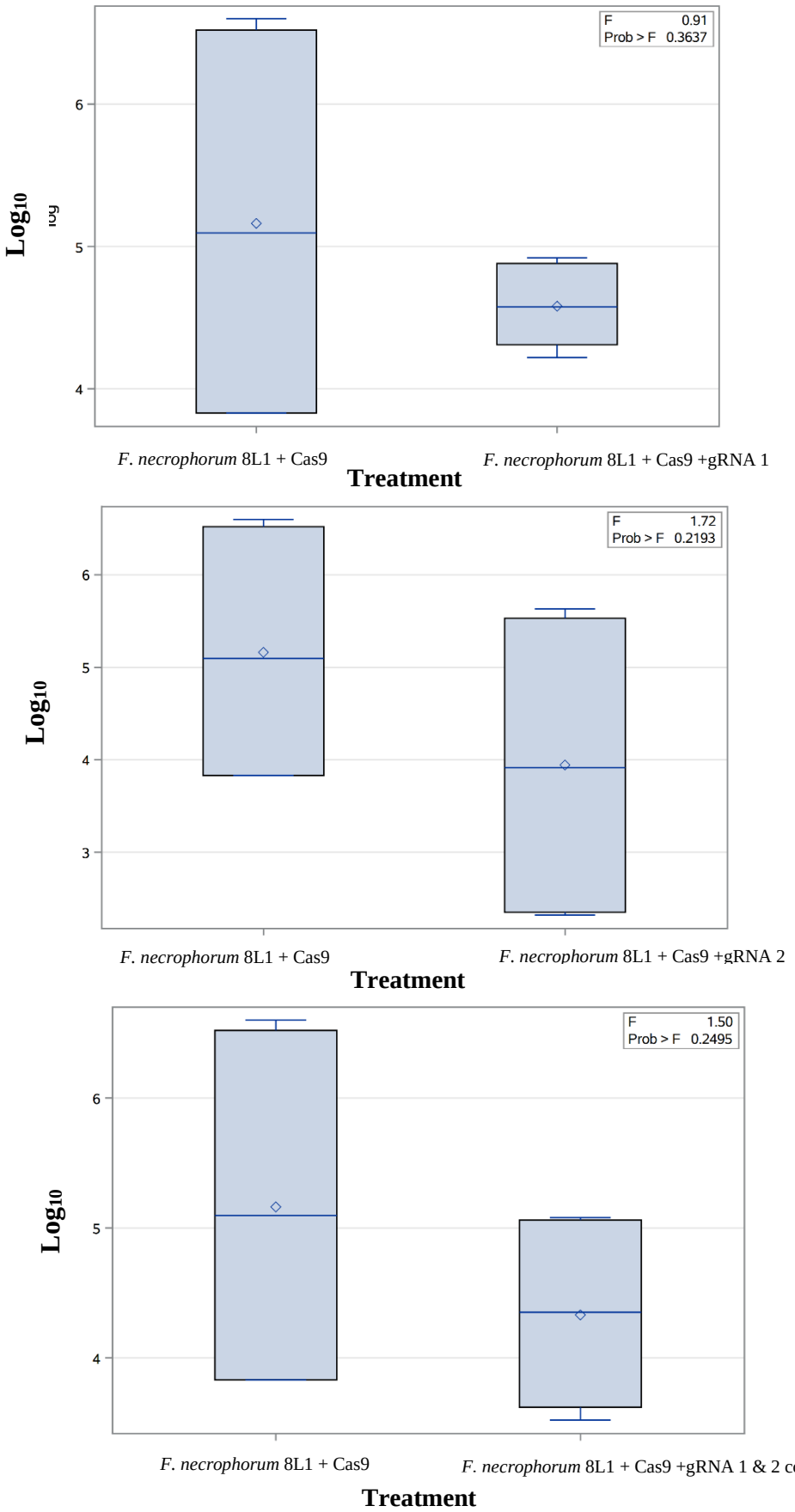

Figure 6.5. Distribution of $\log_{10}$ CRISPR-Cas9 inhibition of *F. necrophorum* subsp. *necrophorum* 8L1 + Cas9 and *F. necrophorum* subsp. *necrophorum* 8L1 + Cas9 + gRNA 1, 2, and combined.

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Chapter 7 - Antimicrobial activity of Sorghum Phenolic Extract on Bovine Foodborne and Mastitis Causing Pathogens

Abstract

Antimicrobial resistance in bacterial pathogens associated with bovine mastitis and human foodborne illnesses from contaminated food and water have an impact on animal and human health. Phenolic compounds have antimicrobial properties, and some specialty sorghum grains are high in phenolic compounds and the grain extract may have the potential as a natural antimicrobial alternative. The study's objective was to determine antimicrobial effects of sorghum phenolic extract on bacterial pathogens that cause bovine mastitis and human foodborne illnesses. Bacterial pathogens tested included *Escherichia coli*, *Salmonella* Typhimurium, *Campylobacter jejuni*, *Campylobacter coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Antibacterial activities of sorghum phenolic extracts were determined by agar well diffusion assay. Sorghum phenolic extract was added to the wells in concentrations of 0, 100, 200, 500, 1,000, or 4,000 µg/mL. The control wells did not receive phenolic extract. Plates were incubated for 18-24 hours and the diameter of each zone of inhibition was measured. The results indicated that sorghum phenolic extract had inhibitory effects on *Staphylococcus aureus, Enterococcus faecalis, Campylobacter jejuni,* and *Campylobacter coli*.

Introduction

Increased antimicrobial resistance due to agricultural use has become a major issue around the world (Aidara-Kane et al., 2018). An increase in resistance to medically important antimicrobials, such as macrolides, third generation and higher cephalosporins, fluoroquinolones,

tetracyclines, and glycopeptides, led to increased scrutiny in the use of antibiotics in food animals with emphasis on antibiotic stewardship (FDA, 2015; Torio and Padilla, 2018; WHO, 2019).

Zoonotic foodborne illnesses are a major public health concern and food-producing animals are a major reservoir for many of the foodborne pathogens. The three major foodborne bacterial pathogens that food animals harbor are Shiga toxin-producing *Escherichia coli*, *Salmonella enterica*, and *Campylobacter* species, particularly *jejuni* and *coli*. The Center for Disease Controls recognizes the threat of these pathogens to One Health, spanning both human, animals, and the environment (CDC, 2021). Brown et. al. reported that 21% of 176 *Salmonella* foodborne illness outbreaks in the United Stated between 2003 and 2012 were resistant to at least one antibiotic (Brown et al., 2017). Furthermore, Europe had 319 foodborne outbreaks linked to *Campylobacter* in 2019, which was 23 % of the total number of outbreaks caused by bacteria in Europe *(European Food Safety Authority and European Centre for Disease Prevention and Control, 2021)*. Likewise, *Campylobacter* has been related to foodborne illness outbreaks in the United States and China (Roels et al., 1998; Yu et al., 2020). In 2017, the CDC reported 23 Campylobacter outbreaks, 4 % of the total number of outbreaks caused by bacteria in the United States (CDC, 2017). Furthermore, illnesses involving *E. coli* O157 can lead to hemolytic uremic syndrome (Launders et al., 2016). In 2019, 42 Shiga toxin-producing *E. coli* outbreaks were reported in Europe, which was 3 % of the total number of outbreaks caused by bacteria in Europe (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021). Likewise, the United States reported that Shiga toxin-producing *E. coli* caused 3 % of the total number of bacterial outbreaks in 2017 (CDC, 2017). Hemolytic uremic syndrome (HUS) is a severe illness involving the kidneys and central nervous system that can lead to acute renal failure and is more often seen in children and immunocompromised adults (Haus-Cheymol et al., 2006;

Alpers et al., 2008). Furthermore, 87,923 human salmonellosis cases were reported in Europe in 2019 (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021). Antimicrobial resistance coupled with foodborne illness outbreaks among *Campylobacter*, *Escherichia coli* O157, and *Salmonella enterica* are a growing public health threat (Chigor et al., 2010; McCrackin et al., 2016; Brown et al., 2017).

Furthermore, mastitis is an important production disease of dairy cattle. Mastitis is typically caused by various bacterial pathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella*, and causes reduced milk yield and economic losses to producers (DeGraves and Fetrow, 1993; Hortet and Seegers, 1998; Gröhn et al., 2004; Park et al., 2014). Ohnishi et al. (2011) demonstrated that mastitis cases associated with *P. aeruginosa* can lead to acute systemic infections that are moderate to severe. Additionally, *P. aeruginosa* is known to be multidrug-resistant and, therefore, difficult to treat (Lister et al., 2009; Ohnishi et al., 2011; Saleh et al., 2016; Tomlinson et al., 2016). Tenhagen et al. (2014) reported that 631 isolates of *S. aureus* from various cattle production areas were resistant to at least one antimicrobial. Isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* have also shown resistance to antimicrobials (Liu et al., 2013). These findings demonstrate the need to find antimicrobial alternatives for food animal medicine, either for treatment or for prevention of diseases.

Antimicrobial alternatives are substitutions to existing antibiotics where bacterial resistance has emerged (Hoelzer et al., 2018). There are three major categories of antimicrobial alternatives: natural, synthetic, and biotechnology-based (Ghosh et al., 2019). Plant-based antimicrobial compounds are part of the naturally occurring antimicrobial alternative category (Baskaran et al., 2013; Ghosh et al., 2019). These compounds are typically secondary metabolites

derived from plants with antioxidant and antimicrobial activity (Srivastava et al., 2014; Anastasaki et al., 2017). The mode of actions for antimicrobial activity to combat these pathogens are believed to be the reduction of pH, disruption of the permeability of the bacterial membrane, or through manipulation of bacterial efflux pumps (Srivastava et al., 2014).

Sorghum phenolic extract is a collection of plant-based compounds and a potent natural antimicrobial alternative (Kil et al., 2009; Villalobos et al., 2016). Likewise, it has been shown to have many health and pharmaceutical benefits (Dykes and Rooney, 2006). Genetic diversity offers some sorghum specialty lines with high tannin content and/ or high phenolic compounds that demonstrate high antioxidant activity (Herald et al., 2012). Also, the increased concentrations of phenolic compounds have been associated with higher antioxidant activities, anticarcinogenic effects, and antimicrobial activities (Dykes et al., 2005; Guajardo Flores, 2008; Kil et al., 2009). Kil et al. (2009) reported that a cultivar of sorghum phenolic extract had minimum inhibitory concentrations (MIC) for *S. aureus*, *Bacillus subtilis*, *Salmonella* Typhimurium, *K. pneumoniae*, and *E. coli* of 250, 250, 500, 500, and 250 µg/mL, respectively. Furthermore, Villalobos et al. (2016) demonstrated antimicrobial activity of soy flour phenolic extract against *Listeria innocua*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Enterococcus faecalis*. Additionally, a sorghum extract with phenol present in high amount has been shown to attenuate replication of *Legionella pneumophila* in mouse macrophage cells (Gilchrist et al., 2020).

The current study's objective was to investigate antimicrobial activity of phenolic compounds extracted from sorghum bran against foodborne and mastitis-causing pathogens associated with cattle. We hypothesized that extracts from high phenolic sorghum grain would significantly inhibit pathogens in a dose-dependent manner.

Materials and Methods

Sorghum Plant Material

The diversified sorghum association panel (SAP) was evaluated at Manhattan, KS and at Puerto Vallarta, Mexico in 2014 along with other photosensitive lines. All lines were tested for total phenolic compound concentrations and oxygen radical absorption capacity (ORAC) values. One photosensitive line PI570481 was chosen for the study as this line had ORAC values greater than 150 µM Trolex equivalent. The line PI570481 is a black-grained sorghum selected due to its efficacy and high phenolic content as previously published (Smolensky et al., 2018; Gilchrist et al., 2020).

Total Phenolic Compounds Extraction and Quantification

A tangential abrasive dehulling device (Venables Machine Works, Saskatoon, Canada) was used to remove the bran. Twenty-five ml of acidified methanol (1 % HCl/methanol v/v) was used to suspend 0.5 g of the bran sample, which was then shaken for two hours. The mixture was centrifuged for 15 minutes, and the supernatant was transferred into 50 ml tubes. Total phenols in the sorghum bran extract were determined using the method described by Herald et al. (2012). Seventy- five µl of water was added to each well in a 96-well plate. Twenty-five µl of sample extract, Trolox standard (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), or blank, along with 25 µl Folin-Ciocalteu reagent, were then added to each well and allowed to equilibrate at room temperature for 6 minutes. After equilibrating, 100 µl of 7.5 % Na_2CO_3 was added to each well. The plate was placed at room temperature and left in the dark for 90 minutes. The absorbance was measured with a Synergy 2 microplate reader (BioTek, Winooski, VT) at 765 nm and reported as mg gallic acid equivalents (GAE)/g of sorghum bran. Four replicates were used to measure each sample and the extraction resulted in phenolic extract with 4.0-4.4 mg/ml concentration of phenols.

Antimicrobial Susceptibility Testing of Sorghum Phenolic Extract

Preparation of bacterial inocula

Staphylococcus aureus VDL3-SA-2017, *Pseudomonas aeruginosa* VDL4-PA-2017, *Klebsiella pneumoniae* VDL1-KP-2017, and *Klebsiella oxytoca* VDL2-KO-2017 isolated from bovine milk samples at the Kansas State Veterinary Diagnostic Laboratory, *Escherichia coli* O157:H7 (2017-5-590), *Escherichia coli* O157:H7 (2017-5-493), and *Escherichia coli* O157:H7 (380-94) isolated from bovine feces, and *Enterococcus faecalis* ATCC 29212 were streaked onto blood agar plates (Remel Inc., Lenexa, KS) and incubated at 37 °C for 24 hours. *Campylobacter jejuni* and *Campylobacter coli* were streaked onto Mueller-Hinton (MH) agar (Becton Dickinson, Sparks, MD) and incubated at 37 °C for 24 hours in a tri-gas incubator under a microaerophilic condition. A single colony of each species from the plates was then suspended into 10 ml of phosphate buffer saline (PBS; Becton Dickinson, Sparks, MD) until a turbidity equivalent to 0.5 McFarland standard was achieved per CLSI guidelines (CLSI, 2018).

Agar well diffusion assay

The antimicrobial activities of the sorghum phenolic extract were determined by a well diffusion assay (Rammelsberg and Radler, 1990). Sixteen mm diameter wells were punched into MH agar (Becton Dickinson and a small amount (approx. 30 µL) of melted MH agar was used to seal the bottom of the wells. A cotton swab was used to inoculate bacterial inoculum of the bacterial species to be tested onto MH agar with wells to obtain a lawn of growth. The wells were then filled with 100 µL of sorghum phenolic extract at concentrations of 0 (control), 100, 200, 500, 1,000, or 4,000 µg/ml with LB broth. Seventy percent ethanol was used to dissolve the sorghum phenolic extract and was added to wells as negative controls. The plates were incubated at 37 °C

for 24 hours aerobically or under microaerophilic conditions (for *Campylobacter* spp.). The diameter of the inhibitory zone was recorded.

Antibiotic Susceptibility Determinations

Minimum inhibitory concentrations of antibiotics were determined by micro-broth dilution method [48]. Antibiotics tested were erythromycin and tetracycline (Sigma-Aldrich, St. Louis, MO). Antibiotic stock solutions were prepared as per manufacturer's guidelines to obtain a concentration of 1,000 µg/ml based on potency of antibiotics. Antibiotics were tested at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 and 0.098 µg/ml. The bacterial inocula were prepared by preparing 1:100 dilutions of cultures grown in 10 ml Mueller Hinton broth for 6 to 8 h. The antimicrobial susceptibility was performed in 96-well micro titer plates (Becton and Dickinson, Franklin Lakes, NJ). Plates were incubated at 37 °C for 20-24 h under aerobic (*Enterococcus faecalis* and *Staphylococcus aureus*) and or microaerophilic conditions (*Campylobacter coli and C. jejuni*) and results were recorded as growth or no growth. The procedure was repeated again with different bacterial inoculum. As a reference standard, sorghum phenolic compound was also subjected for testing at the same concentration like antibiotics.

Statistical Analysis

The inhibitory activity of sorghum phenolic extract against foodborne illness and mastitis causing bacterial pathogens was evaluated using a PROC MIXED procedure in SAS (Version 9.4, Cary, NC). The statistical model included the concentrations of phenolic extract, replications (repeated measures as a random effect) and zone diameter of well diffusion assay. For the MIC data, values with >100 µg/ml were not analyzed. The mean comparisons within and between

bacterial isolates against various concentration of phenolic extract were tested using Tukey's Honest Significant Difference with an alpha of ≤ 0.05.

Results

Food borne pathogens

The sorghum phenolic extract did not inhibit *E. coli* O157:H7 and *S.* Typhimurium but inhibited *C. jejuni* and *C. coli* ($P \leq 0.0001$; Table 7.1). The average zones of inhibition demonstrated for *C. jejuni* at concentrations of 0 µg/ml (control), 100 µg/ml, 200 µg/ml, 500 µg/ml, 1,000 µg/ml, and 4,000 µg/ml were 0.7, 1.9, 4.6, 6.8, and 13.2 mm, respectively. The zone of inhibition with different concentrations did differ significantly ($P \leq 0.0001$; Tables 7.1 and 7.2). The average zones of inhibition against *C. coli* at concentrations of 0 µg/ml (control), 100 µg/ml, 200 µg/ml, 500 µg/ml, 1,000 µg/ml, and 4,000 µg/ml were 0.0, 1.2, 2.4, 4.1, 6.8, and 13.0 mm, respectively. The concentration effect on zone of inhibition was significant ($P < 0.05$; Tables 7.1 and 7.2).

Mastitic pathogens

Overall, sorghum phenolic extract did not have inhibitory effects on *P. aeruginosa*, *K. pneumoniae*, and *K. oxytoca* (Table 7.1). However, significant inhibitory effects were observed between different concentrations of sorghum phenolic extract against *P. aeruginosa*, *K. pneumoniae*, and *K. oxytoca* (Tables 7.1 and 7.2). There were statistical differences seen for the following comparisons of within and between sorghum phenolic extract concentrations when tested against *P. aeruginosa* and *K. pneumoniae* ($P < 0.0001$). The concentrations of sorghum phenolic extract with the largest zones of inhibition when used against *K. pneumoniae* were 100 µg/ml and 200 µg/ml, both with zones of inhibition of 1.3 mm. The zones of inhibition from sorghum phenolic extract tested against *K. oxytoca* demonstrated statistical differences for the

following concentration comparisons: 0 µg/ml (control) vs. 1,000 µg/ml, 100 µg/ml vs. 4,000 µg/ml, 200 µg/ml vs. 4,000 µg/ml, and 500 µg/ml vs. 4,000 ($P = 0.05$). The concentration that sorghum phenolic extract had the greatest zone of inhibition for *K. oxytoca* was 4,000 µg/ml with a zone of inhibition of 2.4 mm.

The overall inhibitory effect of sorghum phenolic extract was significant when tested against *Staphylococcus aureus* ($P \leq 0.0001$; Table 7.1). The average zones of inhibition recorded for *S. aureus* at various concentrations of 0 µg/ml (control), 100 µg/ml, 200 µg/ml, 500 µg/ml, 1,000 µg/ml, and 4,000 µg/ml were 0.0, 2.8, 3.4, 4.1, 5.2, and 7.7 mm, respectively, which was significant when tested between concentrations ($P < 0.05$). When compared between sorghum phenolic extract concentrations all comparisons had *P-values* less than 0.05 (Tables 7.2 and 7.3).

Opportunistic commensal bacteria

The overall inhibitory effect of sorghum phenolic extract was significant when tested against *Enterococcus faecalis* ($P \leq 0.0001$; Table 7.1). The average zones of inhibition demonstrated when sorghum phenolic extract was tested against *E. faecalis* at concentrations of 0 µg/ml (control), 100 µg/ml, 200 µg/ml, 500 µg/ml, 1,000 µg/ml, and 4,000 µg/ml were 0.0, 1.7, 2.3, 3.0, 4.3, and 8.2 mm, respectively. The zone of inhibition in comparison with the various tested concentrations did differ significantly ($P \leq 0.05$; Tables 7.2 and 7.3).

Minimum inhibitory concentrations of antibiotics and sorghum phenolic compounds

MIC testing was performed for the two most commonly used antibiotics in cattle production systems for prophylaxis and therapeutic purposes. Antimicrobial susceptibility testing was done for 4 bacterial isolates (*Enterococcus faecalis, Staphylococcus aureus, Campylobacter coli* and *C. jejuni*) which showed weak to moderate effects with sorghum phenolic compound.

Campylobacter coli and *C. jejuni* isolates were susceptible to erythromycin and tetracycline antibiotics. The *C. coli* isolate had an MIC value of 6.25 µg/ml and 1.56 µg/ml for erythromycin and tetracycline, respectively. The *C. jejuni* isolate had an MIC value of 3.12 µg/ml and 1.56 µg/ml for erythromycin and tetracycline, respectively. Both, *E. faecalis* and *S. aureus* isolates were resistant to erythromycin and tetracycline antibiotics (>100 µg/ml). Consistent with the well diffusion assay, the sorghum phenolic compound was found inhibitory against all four bacteria by the microbroth dilution procedure with an MIC value of 12.5 µg/ml, 12.5 µg/ml, 12.5 µg/ml, and 6.25 µg/ml for *Campylobacter coli, C. jejuni, E. faecalis* and *S. aureus* isolates, respectively.

Discussion

Antimicrobial alternatives to control foodborne illness and bovine mastitis causing pathogens are crucial for researchers to explore. Foodborne illness outbreaks caused by *Escherichia coli* O157, *Salmonella* Typhimurium, and *Campylobacter* cause economic impacts on communities due to decreased worker production and in some cases, they can cause serious disease capable of resulting in death (Launders et al., 2016; Vygen-Bonnet et al., 2017; Salaheen et al., 2019; Smith et al., 2019). Furthermore, mastitis causes economic losses to milk producers and is a major health concern for lactating cows (Halasa et al., 2007; Ohnishi et al., 2011). Our findings suggest that sorghum phenolic extract has antimicrobial effects against *Campylobacter*, but not *E. coli* O157 or *S.* Typhimurium. We also observed antimicrobial effects against *Staphylococcus aureus*, a Gram-positive mastitis causing bacterium, and *Enterococcus faecalis*, a Gram-positive bacterium, a major nosocomial pathogen, particularly in severely immunocompromised individuals. Sorghum phenolic extract had no observed antimicrobial effect on Gram-negative bacteria studied, except for *Campylobacter*, while it had antimicrobial effects on both Gram-positive bacteria tested. *Campylobacter* is a microaerophilic organism, and this could explain why

it was inhibited by the sorghum phenolic extract. Other high antioxidant compounds have been demonstrated to inhibit *Campylobacter* as well (Xiaonan et al., 2011; Duarte et al., 2015). For example, Duarte et. al. (2015) reported coriander essential oil and linalool to have zones of inhibition that were about 85 mm. The rest of the Gram-negative bacteria tested showed no inhibition and this could be due to their lipopolysaccharide outer member protecting them from penetration of sorghum phenolic extract (Villalobos et al., 2016). A limitation to this study was that the screening of sorghum was not exhaustive and instead represents a screening of sorghums with known high phenolics. Questions of which specific compound(s) in sorghum has efficacy, and the representation of those compound(s) in the diversity of sorghum was not in the scope of this project. Furthermore, a second limitation was that only two species of Gram-positive bacteria were tested, but the findings are similar to other researchers (Villalobos et al., 2016; Akogou et al., 2018). Akogou et. al. (2018) demonstrated no antimicrobial activity of red, phytoalexin- rich sorghum extract against *E. coli* O157. Likewise, Villalobos et al. (2016) demonstrated that Gram-positive bacteria were more likely to be inhibited by natural phenolic extract from soybeans. However, the previous authors did show inhibitory effects on *E. coli* and *Salmonella* at 1,250 and 1,500 µg/mL (Villalobos et al., 2016). Furthermore, Kil et al. (2009) demonstrated sorghum phenolic extract to have minimum inhibitory concentrations of less than 1,000 µg/ mL for *S. aureus*, *Salmonella* Typhimurium, *Klebsiella pneumonia*, and *Escherichia coli* for four different sorghum lines. These results are different than our findings but could be due to different methods used to determine antimicrobial activity or, more likely, differences in the sorghum cultivars used. Furthermore, an upward dose dependent trend of antimicrobial activity was seen for *C. jejuni, C. coli*, *E. faecalis*, and *S. aureus* with 100 ug/ mL having the lowest zone of inhibition and 4,000 ug/mL having the highest zone of inhibition (Figure 7.1).

Apart from *Campylobacter*, Gram-negative foodborne illness causing bacteria used in this study were not inhibited by sorghum phenolic extract. However, sorghum grain from different sorghum cultivars could still be used as an antimicrobial alternative to these bacteria. For example, Kumar et al. (2012) engineered silver glyconanoparticles using sweet sorghum syrup that were shown to have antimicrobial effects on *E. coli*, *P. aeruginosa*, and *Klebsiella planticola*, as well as some Gram-positive bacteria. Furthermore, Halder et al. (2019) identified a glycine and proline rich protein in sorghum that has demonstrated antimicrobial activity against *Bacillus subtilis*, *Rhodococcus fascians*, and *Escherichia coli* with minimum inhibitory concentrations of 7.5, 22.4, and 75 µg/mL, respectively. Together these findings demonstrate how sorghum grain can be used in different ways to develop antimicrobial alternatives and how further research should be conducted on sorghum antimicrobial effects.

Conclusions

Our data demonstrated that phenolic extract of a sorghum cultivar exhibited antibacterial activity. Of the mastitis-causing pathogens and foodborne pathogens prevalent in cattle tested, only *S. aureus*, *E. faecalis*, *C. jejuni*, and *C. coli* were inhibited by the phenolic extracts in a dose dependent manner. The selective inhibition of certain bacterial species, both Gram-positive and Gram-negative bacteria, and not against other bacterial species is an interesting observation that needs to be investigated. Our study provides impetus to test additional cultivars of phenolic acid extracts of sorghum cultivars and explore value-added benefits of sorghum grains as natural antimicrobial compounds against bacterial pathogens.

Table 7.1. Overall antimicrobial effects of sorghum phenolic extract.

	Sorghum phenolic extract concentration (µg/mL)						
	0	100	200	500	1000	4000	
Bacterial species and strain number	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	*P-value*
***Escherichia coli* O157 2017-5-590**	0	0	0	0	0	0	-
***Escherichia coli* O157 2017-5-493**	0	0	0	0	0	0	-
***Escherichia coli* O157 380-94**	0	0	0	0	0	0	-
***Campylobacter jejuni* 2016-12-17A**	0	0	0	4.1	5.8	11.1	0.0002
***Campylobacter coli* 2016-12-82A**	0	1.4	3.2	3.8	7.0	12.7	0.0016
***Campylobacter coli* 2016-12-80A**	0	0	0.8	3.8	5.8	11.7	<0.0001
***Campylobacter coli* 2016-12-181A**	0	2.3	3.3	4.8	7.7	14.7	0.0004
***Salmonella* Typhimurium ATCC 14028**	0	0	0	0	0	0	-
***Staphylococcus aureus* VDL3-SA-2017**	0	2.8	3.4	4.1	5.2	7.7	0.0001
***Pseudomonas aeruginosa* VDL4-PA-2017**	0	0	0	0	0	0.3	-
***Klebsiella pneumoniae* VDL1-KP-2017**	0	1.3	1.3	0.9	0	0.8	0.8887
***Klebsiella oxytoca* VDL2-KO-2017**	0	0	0	0	0.8	2.4	-
***Enterococcus faecalis* ATCC 29212**	0	1.7	2.3	3.0	4.3	8.2	0.0003

[a] ZOI: average zone of inhibition (diameter; mm)

Table 7.2. Antimicrobial effects of different sorghum phenolic extract concentrations.

	Sorghum Phenolic Extract Concentration (µg/mL)					**Mean differences with the *P-values***																			
	100	200	500	1000	4000	100 v. 200		100 v. 500		100 v. 1000		100 v. 4000		200 v. 500		200 v. 1000		200 v. 4000		500 v. 1000		500 v. 4000		1000 v. 4000	
Bacteria	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*
E. coli O157 2017-5-590	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli O157 2017-5-493	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli O157 380-94	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. jejuni 2016-12-17A	0	0	4.1	5.8	11.1	1.55E-15	1.00	-4.08	<.0001	-5.75	<.0001	-11.08	<.0001	-4.08	<.0001	-5.75	<.0001	-11.08	<.0001	-1.67	<.0001	-7.00	<.0001	-5.33	<.0001
C. coli 2016-12-82A[c]	1.4	3.2	3.8	7.0	12.7	-1.75	0.0001	-2.33	<.0001	-5.58	<.0001	-11.25	<.0001	-0.58	0.1226	-3.83	<.0001	-9.50	<.0001	-3.25	<.0001	-8.92	<.0001	-5.67	<.0001
C. coli 2016-12-80A[c]	0	0.8	3.8	5.8	11.7	-0.75	0.0255	-3.75	<.0001	-5.75	<.0001	-11.67	<.0001	-3.00	<.0001	-5.00	<.0001	-10.92	<.0001	-2.00	<.0001	-7.92	<.0001	-5.92	<.0001
C. coli 2016-12-181A	2.3	3.3	4.8	7.7	14.7	-1.083	0.1650	-2.50	0.0036	-5.42	<.0001	-12.42	<.0001	-1.42	0.0746	-4.33	<.0001	-11.33	<.0001	-2.92	0.0011	-9.92	<.0001	-7.00	<.0001
S. Typhimurium ATCC 14028	0	0	0	0	0	7.89E-31	1.00	-789E-33	1.00	0.00	1.00	2.31E-15	1.00	-158E-32	1.00	-789E-33	1.00	2.31E-15	1.00	7.89E-31	1.00	2.31E-15	1.00	2.31E-15	1.00
S. aureus VDL3-SA-2017[c]	2.8	3.4	4.1	5.2	7.7	-0.63	0.0007	-1.29	<.0001	-2.38	<.0001	-4.88	<.0001	-0.67	0.0004	-1.75	<.0001	-4.25	<.0001	-1.08	<.0001	-3.58	<.0001	-2.50	<.0001
P. aeruginosa VDL4-PA-2017	0	0	0	0	0.3	1.11E-16	1.00	2.22E-16	1.00	1.67E-16	1.00	-0.33	<.0001	1.11E-16	1.00	1.11E-16	1.00	-0.33	<.0001	5.55E-17	1.00	-0.33	<.0001	-0.33	<.0001
K. pneumoniae VDL1-KP-2017	1.3	1.3	0.9	0	0.8	-0.083	0.6079	0.33	0.0512	1.25	<.0001	0.42	0.0177	0.42	0.0177	1.33	<.0001	0.50	0.0057	0.92	<.0001	0.083	0.6079	-0.83	<.0001
K. oxytoca VDL2-KO-2017	0	0	0	0.8	2.4	-444E-18	1.00	4.44E-16	1.00	-0.83	0.4575	-2.38	0.0441	8.88E-16	1.00	-0.83	0.4575	-2.38	0.0441	-0.83	0.4575	-2.38	0.0441	-1.54	0.1771
E. faecalis ATCC 29212	1.7	2.3	3.0	3.4	6.5	-0.67	0.5496	-1.29	0.2527	-1.75	0.1268	-4.83	0.0003	-0.63	0.5746	-1.08	0.3348	-4.17	0.0013	-0.46	0.6800	-3.54	0.0045	-3.08	0.0113

[a] ZOI: average zone of inhibition (diameter; mm)
[b] SEM: standard error of the mean

Table 7.3. Comparison of antimicrobial effects of different concentrations of sorghum phenolic extract vs. control.

	Sorghum Phenolic Extract Concentration (µg/mL)						**Mean differences with the *P-values***									
	0	100	200	500	1000	4000	0 v. 100		0 v. 200		0 v. 500		0 v. 1000		0 v. 4000	
Bacteria	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	ZOI[a]	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*	SEM	*P*
E. coli O157 2017-5-590	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-
E. coli O157 2017-5-493	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-
E. coli O157 380-94	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-
C. jejuni 2016-12-17A	0[efg]	0	0	4.1[e]	5.8[f]	11.1[g]	0	1.0000	6.66E-16	1.0000	-4.08	<.0001	-5.75	<.0001	-11.08	<.0001
C. coli 2016-12-82A	0[h]	1.4[c]	3.2[d]	3.8[e]	7.0[f]	12.7[g]	-1.42	0.0010	-3.17	<.0001	-3.75	<.0001	-7.00	<.0001	-12.67	<.0001
C. coli 2016-12-80A	0[defg]	0	0.8[d]	3.8[e]	5.8[f]	11.7[g]	-178E-17	1.0000	-0.75	0.0255	-3.75	<.0001	-5.75	<.0001	-11.67	<.0001
C. coli 2016-12-181A	0[h]	2.3[c]	3.3[d]	4.8[e]	7.7[f]	14.7[g]	-2.25	0.0076	-3.33	0.0003	-4.75	<.0001	-7.67	<.0001	-14.67	<.0001
S. Typhimurium ATCC 14028	0.5	0	0	0	0	0	1.73	0.1004	1.73	0.1004	1.73	0.1004	1.73	0.1004	1.73	0.1004
S. aureus VDL3-SA-2017	0[h]	2.8[c]	3.4[d]	4.1[e]	5.2[f]	7.7[g]	-0.63	0.0007	-1.29	<.0001	-2.38	<.0001	-4.88	<.0001	-0.67	0.0004
P. aeruginosa VDL4-PA-2017	0[h]	0[c]	0[d]	0[e]	0[f]	0.3[g]	1.083	<.0001	1.083	<.0001	1.083	<.0001	1.083	<.0001	0.75	<.0001
K. pneumoniae VDL1-KP-2017	0[f]	1.3	1.3	0.9	0[f]	0.8	-0.1667	0.3101	-0.25	0.1346	0.17	0.3101	1.083	<.0001	0.25	0.1346
K. oxytoca VDL2-KO-2017	0[g]	0	0	0	0.8	2.4[g]	1.78E-15	1.0000	1.33E-15	1.000	2.22E-15	1.0000	-0.83	0.4575	-2.38	0.0441
E. faecalis ATCC 29212	0[defg]	1.7	2.3[d]	3.0[e]	3.4[f]	6.5[g]	-1.52	0.1447	-2.13	0.0468	-2.71	0.0145	-3.13	0.0058	-5.95	<.0001

[a] ZOI: average zone of inhibition (diameter; mm)
[b] SEM: standard error of the mean
[c] evidence of statistical difference between 0 v. 100 µg/mL at α = 0.05
[d] evidence of statistical difference between 0 v. 200 µg/mL at α = 0.05
[e] evidence of statistical difference between 0 v. 500 µg/mL at α = 0.05
[f] evidence of statistical difference between 0 v. 1000 µg/mL at α = 0.05
[g] evidence of statistical difference between 0 v. 4000 µg/mL at α = 0.05
[h] evidence of statistical difference between all sorghum phenolic extract concentrations at α = 0.05

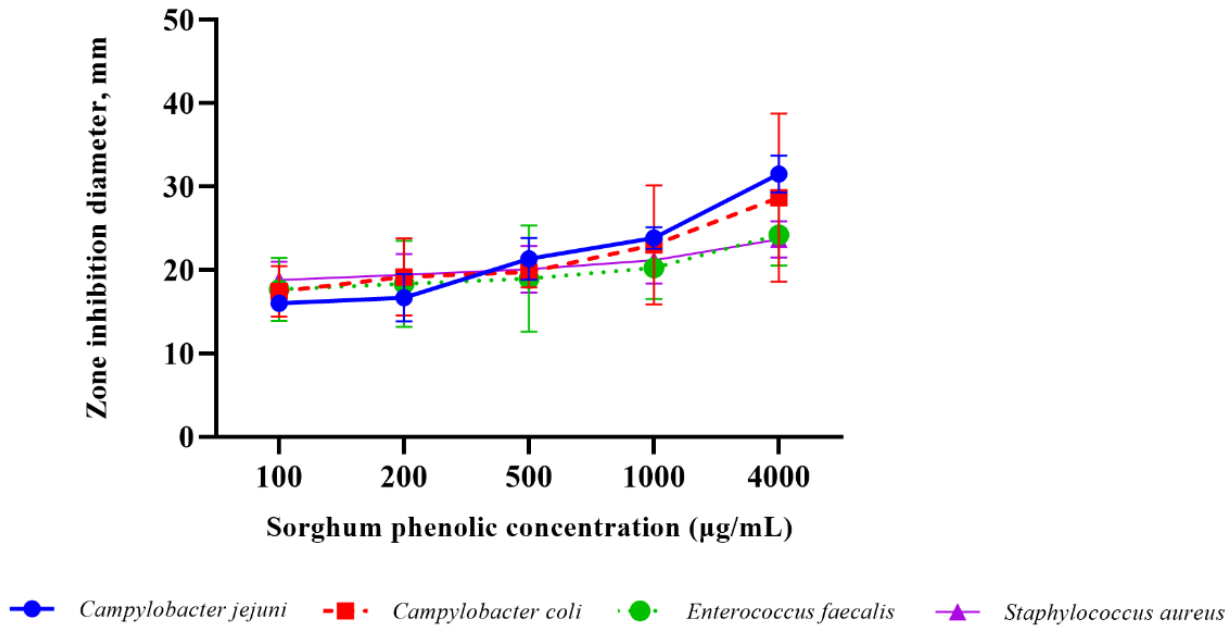

Figure 7.1. Antibacterial effects of sorghum phenolic extract concentrations for *Campylobacter jejuni*, *Campylobacter coli*, Enterococcus faecalis, and *Staphylococcus aureus*. Each line represents an average.

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Appendix A - Miscellaneous Photos