Search for antibiotic alternatives for prevention of liver abscesses in feedlot cattle

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Abstract

Liver abscesses are a significant animal health, economic loss, and animal welfare concern for the beef cattle feedlot industry. Liver abscesses occur in feedlot cattle because of a high grain feed diet. The primary causative agent is *Fusobacterium necrophorum*, a ruminal bacterium that enters portal circulation to reach the liver and causes abscesses. *Trueperella pyogenes* is the second most common pathogen identified, often in combination with *F. necrophorum*. *Salmonella enterica* has also been cultured from liver abscesses of cattle. The most common antibiotic used to prevent liver abscesses is tylosin, a macrolide antibiotic. This antibiotic class is a medically important antimicrobial class to human medicine; therefore, it is currently being regulated by the Veterinary Feed Directive. Because of the potential for emergence and dissemination of antimicrobial resistance associated with continuous administration of tylosin in the feed, there is considerable interest in finding an alternative. The research aimed to evaluate different antibiotic alternatives that are inhibitory to pathogens involved in liver abscesses. The following alternatives were evaluated: Sorghum phenolic compounds, probiotic cultures, medium-chain fatty acids, and essential oils. Antimicrobial activities of the sorghum phenolic compound tested were assessed 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. Plates were incubated for 24 hours, and the diameter of each zone of inhibition was measured. None of the sorghum phenolic compounds evaluated had inhibitory effects on *F. necrophorum* subsp. *necrophorum, F. necrophorum* subsp. *fundiliforme, T. pyogenes,* and *S.* Lubbock.

Probiotics are beneficial microbes that have the capability to improve intestinal health by promoting the development of a healthy microbiota, hindering enteric pathogens from colonizing the intestine, increasing digestive capacity, and improving mucosal immunity. The study evaluated the antimicrobial activities of *Bacillus pumilus, Bacillus subtilis, Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus acidophilus* 020526, *Lactobacillus buchneri* 110917*, Pediococcus acidilactici* 060117, and *Pediococcus pentosaceus* 032118 against liver abscess-causing pathogens. Antibacterial activities of the culture supernatant of probiotic cultures were determined by agar well diffusion assay. Probiotics culture supernatant with pH adjusted to around 7.0 or unadjusted were added to the wells to determine inhibitions. Plates were incubated for 24 hours, and the diameter of each zone of inhibition was measured. The results indicated that the probiotic cultures had inhibitory effects on *S.* Lubbock and *T. pyogenes.* None of the culture supernatants had inhibition against both subspecies of *F. necrophorum.*

The antimicrobial activities of medium-chain fatty acids, caproic acid (C6), caprylic acid (C8), capric acid (C10), alone or in combinations, were evaluated by the microbroth dilution method. Bacterial growth was monitored by measuring absorbance at 600 nm at 6, 12, 24, and 48 hours. From the data reviewed, it can be concluded that the efficacy of organic acids was more effective when used in combination.

Essential oils, which are plant-based compounds, contain a wide variety of secondary metabolites that can inhibit or slow the growth of bacteria. Antimicrobial activities of the selected essential oils were assessed by the microbroth dilution method. Bacterial growth was monitored by measuring absorbance at 600 nm at 6, 12, 24, and 48 hours. The results indicated that the tested essential oils had no effect against the primary liver abscess-causing pathogens.

Antimicrobial resistance has become a major issue in human and animal health around the world. This study provides momentum for the search of MCFA combinations to be potentially used to prevent liver abscesses in feedlot cattle. However, future studies are warranted.

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Dedication

I want to devote this accomplishment to my family who have molded me into the person I am today. Mom and Dad, you taught me determination, persistence, hard work, encouraged my passion for learning and gave me endless support and love that I am forever grateful for. I am truly blessed to have you as my parents. My sisters, nieces, and nephews, for providing love and words of encouragement during this time and always. My friends who are more like family and talked me through some of the toughest times and been some of my biggest cheerleaders. My husband, thank you for being my rock, sounding board, and biggest supporter. This dissertation is for you all.

Chapter 1 - Control of Liver Abscesses in Feedlot Cattle: A Review of Literature

Liver Abscesses are pus-filled capsulated lesions found on the surface or deep in the liver. They may occur at all ages and in all types of cattle; however, liver abscesses are most common in feedlot cattle, therefore, are of most economic concern. In addition to liver condemnation, liver abscesses have a negative impact on animal performance because of reduced feed intake, reduced weight gain, and decreased feed efficiency. The National Beef Quality Audit (NBQA) is performed approximately every five years to evaluate and characterize quality regarding producer-related beef quality. In the most recent audit, total abscess incidence was 17.8%, is higher compared to all previous audits (Eastwood et al., 2017). However, it is speculated that the increase may be due to the rise in the numbers of Holsteins raised for beef production. The incidence rate can range from as little as 2% to as high as 95%; however, the average is typically around 12-32% (Brink et al., 1990).

Economic Importance

The leading cause for liver condemnation of beef cattle at slaughter is liver abscesses. Liver abscesses are of economic importance to the producer, packer, and eventually to the consumers. The loss of the liver itself is not the most significant economic impact, but the reduction of animal performance and carcass yield (Nagaraja et al., 1996b). The performance impact of liver abscesses includes: reduced feed intake, reduced weight gain, decreased feed efficiency, and decreased carcass weight. Although, decrease in animal performance is not always exhibited (Smith, 1994; Harman et al., 1989), an 11% decline of daily gain, and a decrease of feed efficiency of almost 10% have been reported (Brink et al., 1990.)

The condemnation of the liver is not a major economic concern; however, the reduced animal performance and carcass yield can become a significant financial loss (Nagaraja and Lechtenburg, 2007). This is more evident in cattle with the most severe abscesses, particularly those with the adhesions (Brown and Lawrence, 2010). Liver abscesses are categorized, based on severity, using the Elanco Liver Check System, as follow: normal = healthy liver; A- = 1 to 2 small abscesses or scars; A = 1 to 2 large abscesses or multiple small abscesses; A+ = multiple large abscesses; A+AD = liver attached to the diaphragm or any other surrounding organs; A+OP = open liver abscess (Brown and Lawrence, 2010). Cattle with abscesses in the A and A- categories do not have a measurable loss in performance. A+ liver abscesses may require more carcass trimming due to adhesion of the abscesses to the diaphragm or any other surrounding organs. Consequently, A+ abscesses, being large, could get ruptured causing the entire carcass to be contaminated. The interruption in the flow of carcasses on the slaughter floor costs time and labor (Nagaraja and Lechtenberg, 2007).

The severity of the abscesses will make the effects more evident. Cattle with abscesses scoring an A- (one or two small abscesses) or A (two to four abscesses) have little to no effect on performance or feed efficiency, whereas a score of A+ (one or more large, or a numerous of small abscesses) may significantly decline feed intake (up to almost 14%), overall weight gain (11.4%), feed efficiency (29.5%), and final carcass weight (4.6%) (Brink et al., 1990). Data from twelve research trials were evaluated to compare the association of abscess severity to growth characteristics (Brink et al., 1990). Brink et al. (1990) concluded a 5.1% reduction in intake, 9.4% decrease in average daily gain, a 9.7% increase in feed-to-gain, and a 1.5% cutback in dressed carcass yield. Severe abscesses may be ruptured or be adhered to the diaphragm and

abdominal cavity. Additional trimming may have to occur to guarantee that adhered abscesses are fully extracted from the carcass.

A study involving 1,447 commercially fed cattle from seven feedlots in the Texas Panhandle was examined to assess the effect of liver abscess severity on carcass grading and trimming (Montgomery, 1985). According to the observations, when documenting feeding performance, cattle with severe abscesses (A+ score) had negative impact on carcass outcome regarding trim and overall performance. Subsequently, cattle with liver abscesses required more carcass trimming than cattle with normal liver (0.46% vs. 0.02% of carcass weight (Montgomery, 1985). When more surrounding lining or organs are needed to be excised, this may significantly drop carcass weight resulting in a less valuable carcass.

Etiology

Liver abscesses are polymicrobial infections. The predominant bacteria are Gram-negative anaerobes (Amachawadi and Nagaraja, 2016). Many studies have concluded that the primary causative agent is *Fusobacterium necrophorum*, which has been isolated as a single pathogen from an abscess or in association with other bacteria (Lechtenberg et al,. 1988). Other bacteria that have been isolated include *Truperella pyogenes*, *Bacteroides* spp., *Clostridium* spp., *Pasteurella* spp., *Peptostreptococcus* spp., *Staphylococcus* spp. (Reinhardt and Hubbert, 2015), and *Salmonella enterica* (Amachawadi and Nagaraja, 2015). *Trueperella pyogenes* (previously known as *Arcanobacterium pyogenes*) is the second most prevalent organism and there is evidence to suggest a pathogenic synergy between *F. necrophorum* and *T. pyogenes* (Takeuchi et al., 1983).

Fusobacterium necrophorum

Fusobacterium necrophorum is an aerotolerant, non-spore forming Gram negative, rod-shaped, or pleomorphic bacterium (Langworth, 1977). The pathogen is also involved in other infections such as foot rot, foot abscesses in cattle, and necrotic laryngitis (Tan et al., 1996). This organism is part of the normal gastrointestinal, respiratory, and urogenital flora of animals and humans, and is also a soil bacterium (Amachawadi and Nagaraja, 2015). *Fusobacterium necrophorum* is a normal inhabitant of the rumen (Berg and Scanlan, 1982; Tan et al., 1994a), but when the cattle are switched from a roughage diet to a grain-based diet, the concentration increases about 10- to 100-fold (Tan et al., 1994b). *Fusobacterium necrophorum* has a fermentative role in the rumen, utilizing lactic acid to produce volatile fatty acids and breakdown feed and rumen epithelial proteins and amino acids. The change to a high-grain diet places the cattle at a higher risk for liver abscesses due to increased lactate availability from starch fermentation.

Fusobacterium necrophorum has been classified into four biovars/biotypes; A, B, AB, and C. Biotype AB is rarely seen in liver abscesses but is commonly isolated from foot lesions of cattle and sheep. The two biotypes, given a subspecies status (Shinjo et al., 1990), differ in cell morphology, colony morphology, growth patterns in broth, and biochemical and biological characteristics (Chengappa and Nagaraja, 1998). Virulence factor production is critical in the development of liver abscesses. The difference in the production of virulence factors reflects the difference in the prevalence of the two subspecies. Subspecies *necrophorum* is more virulent compared to subsp. *fundiliforme*, thus, is more frequently isolated from liver abscesses. The difference in virulence would explain the occurrence of subsp. *necrophorum* in 71 to 95% of

liver abscesses compared to subsp. *fundiliforme* in 5 to 29% of liver abscesses (Lechtenberg et al., 1988).

Several virulence factors are linked to the pathogenesis of *F. necrophorum* infections and are crucial for the bacterial survival and evasion of host defenses. The virulence factors include leukotoxin, endotoxic lipopolysaccharide (LPS), hemolysin, hemagglutinin, capsule, adhesins, platelet aggregation factor, dermonecrotic toxin, and several extracellular enzymes (Nagaraja and Chengappa, 1998). However, leukotoxin and endotoxic lipopolysaccharide are believed to be the major virulence factors involved in fusobacterial infections (Tan et al., 1996). Several studies have reported that leukotoxin production is correlated with the ability to induce abscesses in laboratory animals (Nagaraja et al., 1996).

Trueperella pyogenes

Trueperella pyogenes is a Gram-positive, rod-shaped, and capnophilic organism, and is the second most common bacterium isolated from liver abscesses. The organism is a normal inhabitant of the mucus membranes of the upper respiratory and digestive tracts of animals (Biberstein, 1990). The source of *T. pyogenes* in liver abscesses is believed to be the ruminal wall rather than the ruminal contents (Narayanan et al., 1998), which clarifies the relevance of *T. pyogenes* in liver abscesses. *Trueperella pyogenes* are often co-isolated with *F. necrophorum* leading to the pathogenic synergy between the two species. *Trueperella pyogenes* may remain dormant and until conditions are favorable for growth. In order for *F. necrophorum* to overcome the oxygen-rich state of the liver, *T. pyogenes* creates anaerobic conditions using the oxygen that is available from the blood circulation. Consequently, the end product of *T. pyogenes* is lactic acid, which is a primary substrate of *F. necrophorum*. Therefore, the occurrence of *T. pyogenes* creates a favorable environment for *F. necrophorum* (Tadepalli et al., 2009).

Trueperella pyogenes is more prevalent when there is more ruminal damage and lesions. Consequently, if there is more damage in the ruminal wall due to the acidosis, then the probability of *T. pyogenes* entering the portal blood increases. On rare occasions, *T. pyogenes* was the only organism isolated from a liver abscess (Narayanan et al., 1998), which raises the question of whether *T. pyogenes* alone can cause liver abscesses (Nagaraja and Lechtenburg, 2007). Steers inoculated intraportal with *T. pyogenes* in a pure culture did not develop liver abscesses (Lechtenberg et al., 1993). However, steers inoculated with pure cultures of *T. pyogenes* and *F. necrophorum,* or *T. pyogenes* mixed with leukotoxin developed liver abscesses, suggesting that the leukotoxin provided by *F. necrophorum* allows *T. pyogenes* to colonize in the liver and assist in abscess formation (Lechtenberg et al., 1993).

Virulence factors associated with *T. pyogenes* include: hemolysin, proteases, DNases, and extracellular matrix binding protein (Narayanan et al., 1998). The most important virulence factor is the hemolysin, also known as pyolisin, is cytotoxic to polymorphonuclear leukocytes (Billington et al., 1997). The virulence factors are involved in adherence, colonization, and pathogenicity (Billington et al., 1997).

Salmonella enterica

Salmonella enterica has been isolated from liver abscess samples under anaerobic conditions. The Lubbock serotype was the predominant serotype (Amachawadi and Nagaraja, 2015). It is still unclear if *S. enterica* is an etiologic agent of liver abscesses or entry occurred after *F. necrophorum* began the development of an abscess. *Salmonella* in the gut can cross the intestinal lumen, enter portal circulation, and become localized in the liver to initiate infection. Entry through the gut epithelium could be facilitated through inflammation or gut acidosis.

Salmonella are facultative intracellular pathogens causing them to adapt quickly to adverse environmental conditions, including anaerobic conditions. In fact, in anaerobic conditions, *Salmonella* is more invasive, more virulent, and adheres better to mammalian cells than in aerobic conditions (Lee, 1990). However, more research is needed to understand the role of *Salmonella* in the liver-abscess complex.

Pathogenesis

The pathogenesis of liver abscesses is believed to begin with ruminal acidosis resulting from fermentation when cattle are fed a high-grain diet. Therefore, the term rumenitis-liver abscess complex was proposed for this pathway of infection. The correlation between ulcerative lesions of the rumen and liver abscesses in feedlot cattle was first observed by Smith (1944). Jensen et al. (1954) later confirmed this observation to be accurate. Although, Weiser et al. (1966) found no interrelation between the incidence of liver abscesses and ruminal lesions. Therefore, there is no precise pathogenic mechanism (Nagaraja and Chengappa, 1998). However, the literature generally agrees that rumenitis, resulting from acidosis, is a predisposing factor for liver abscesses (Jensen et al.,1954; Nagaraja and Chengappa, 1998).

Cattle fed highly fermentable carbohydrates have increased organic acid production that accumulates in the rumen, resulting in acidosis. Rumenitis, as a result of acidosis, usually occurs with a sudden dietary change, change in feeding patterns, not feeding cattle in a timely manner, and even weather and seasonal factors aid in the process (Elam, 1976). Further ruminal damage can occur from sharp feed particles, foreign objects in the feed, or ingestion of hair (Fell, 1972). Damaged ruminal wall caused by acidosis or penetration of foreign objects becomes susceptible to invasion and colonization of *F. necrophorum* and *T. pyogenes.* Once colonization has occurred, *F. necrophorum* enters the blood or causes ruminal wall abscesses and can eventually

shed bacterial emboli to the portal circulation (Nagaraja and Chengappa, 1998). Bacteria is later filtered by the liver from the portal circulation, leading to possible infection and abscess formation.

The virulence factors of *F. necrophorum* play a critical role from the penetration of the ruminal epithelium to the eventual colonization and infection of the liver. The liver is a highly vascular and highly defended organ due to its rich number of phagocytic cells. Therefore, *F. necrophorum* must overcome a hostile environment to survive and proliferate preceding abscess formation (Reinhardt and Hubbert, 2015). The cytotoxic effect of leukotoxin, secreted by *F. necrophorum,* degrades ruminal cells aiding in penetration of the ruminal wall. The primary virulence factors that aid the survival of *F. necrophorum* in the liver are leukotoxin and endotoxic lipopolysaccharide, which protect it from phagocytosis (Emery et al., 1986; Tan et al., 1996). The destruction of phagocytes leads to a release of oxygen metabolites and cytolytic enzymes, resulting in adverse effects on the surrounding liver tissue. *Fusobacterium necrophorum* also has the ability to lyse the erythrocytes (hemolysin production), provoking the deterioration of oxygen transport. Synergism with facultative bacteria such as *T. pyogenes* occurs by utilizing oxygen; thus, creating further anaerobic conditions and a more favorable environment for *F. necrophorum*. These factors all contribute to forming an anaerobic microenvironment within the liver, essential assets for the eventual development of a liver abscess. Liver abscesses may differ in size and severity; however, pathogenesis for all are the same.

Diagnosis

Liver abscesses are difficult to diagnose cattle exhibit no clinical signs; they are usually detected at the time of slaughter. Liver function tests are not reliable in detecting liver abscesses

(Nagaraja and Lechtenburg, 2007). Ultrasonography is an imaging technique using echoes of ultrasound pulses to depict objects or areas of different densities in the body. Ultrasonographic imagery is an excellent approach for the liver due to the location and tissue density of this particular organ. The technique has helped detect the onset and development of experimentally induced liver abscesses (Nakajima et al., 1986). However, the equipment is not an inexpensive one making it burdensome for feedlots to use this application. Not to mention the major limitations which include: not being able to visualize the entire liver, lack of rumen fill, lung tissue that may overshadow liver, position, and excessive internal fat (Nagaraja and Lechtenburg, 2007). These limitations are too substantial and the reason why they are typically not used in feedlots and only used in research settings.

Prevention and Control

Thus far, the prevention and control of liver abscesses have included antimicrobial feed additives, vaccines, and nutritional management.

Antimicrobial feed Additives

Liver abscesses are caused by bacteria and the rumen provides an adequate environment where the bacteria that causes liver abscesses can reside and thrive. Therefore, cattle are continuously being exposed to bacteria that cause liver abscesses due to their favorable environment being dependent on a symbiotic relationship with other microorganisms. There are a few approaches to take when preventing and controlling this infection, but the cattle industry has primarily focused on using antimicrobial feed additives to decrease the incidence of liver abscesses in feedlot cattle.

The US *Feed Additive Compendium* (Feed Additive Compendium, 2018) states that five antibiotics include: bacitracin methylene disalicylate, chlortetracycline, oxytetracycline, tylosin,

and virginiamycin have approval for control of liver abscesses. Their inhibitory effects vary, with bacitracin being the less effective and tylosin being the most effective. Although, the minimum inhibitory concentration assays do not correlate with the effectiveness of each of the antibiotics, with the exception of bacitracin (Brown et al., 1973; Haskins et al., 1967; Rogers et al., 1995). Tylosin, being the most effective, is the most widely used in combination with monensin. Many studies have confirmed the efficacy of tylosin; inclusion in the feed has resulted in a 40-70% decrease of liver abscesses (Brink et al., 1990; Brown et al., 1975; Heinemann et al., 1978; Pendlum et al., 1978). The addition of the antimicrobial compounds, resulting in a decrease in liver abscesses and the improvement of weight gain and feed efficiency (Rogers et al., 1995; Potter et al., 1985).

Tylosin is a macrolide antibiotic that is generally effective against Gram-positive bacteria but has shown to affect Gram-negative *F. necrophorum* (Berg and Scanlan, 1982; Tan et al., 1994; Lechtenburg et al., 1998). Macrolide antibiotics are bacteriostatic compounds that are identified by a large macrocyclic lactone ring. They are protein synthesis inhibitors that reversibly bind to the 23S rRNA in the 50S ribosome subunit and inhibit mRNA-directed protein synthesis. Furthermore, stimulation occurs by preventing peptidyltransferase from adding the growing peptide attached to tRNA to the next amino acid as well as inhibiting ribosomal translation (Kaneko et al., 2007). It has been observed that tylosin has antimicrobial benefits outside of the rumen, but it is effective primarily in the rumen (Gingerich et al., 1977).

Tylosin's antimicrobial activity on ruminal bacteria has a moderating effect on fermentation rates, reducing the prevalence of ruminal acidosis and hepatic abscesses (Nagaraja et al., 1999); however, cattle fed tylosin still have 12 to 18% liver abscess incidence (Reinhardt and Hubbert, 2015). The use of tylosin in feedlot cattle periodically raises concerns for the

consumers. Bacterial resistance has resulted from low-level feeding (Linton 1977 a,b; Smith 1977a; Braude 1978; Richmond and Linton 1980). Antibiotic resistance is transmissible. In most situations, resistance is presented by R-plasmids. R-plasmids can mediate their conjugational transfer in addition to specifying resistance to certain antibiotics (National Research Council, 1980).

Zoonotic infections, sewage, food animals, and humans all can transfer selected resistance due to antibiotic use. Antibiotics used in human medicine belong to the same class as those used in animals; even if the antibiotic is not the same, they still may have the same mode of action. As a result, the Food and Drug Administration has restricted the use of tylosin in the feedlot under veterinary oversight. Consequently, there is increased interest in developing alternatives for the control of liver abscesses in feedlot cattle.

Vaccines

Antimicrobial agents are the principal method for prevention and control, but do not eradicate the problem. For that reason, the development of an effective vaccine has become of high importance. An effective vaccine would ease concerns of antibiotic resistance created by the antibiotics in the feed. Two vaccines have been developed thus far, to prevent liver abscesses: Fusoguard® (Elanco Animal Health, Greenfield, IN) and Centurion® (Merck Animal Health, Madison, NJ). Fusoguard is a *F. necrophorum* bacterin, a suspension of killed or attenuated bacteria, approved for the control of liver abscesses and foot rot in cattle. Centurion was a combination of the leukotoxoid from *F. necrophorum* and a *T. pyogenes* bacterin; however, it is no longer commercially available (Jones et al., 2004).

In a randomized and blinded field trial, Fusoguard decreased the prevalence of A and A+ liver abscesses from 9.5% to 2.8% in cattle with a low incidence (10%) but was not seen to be

effective in cattle with a high incidence (30%) of liver abscesses (Checkley et al., 2005). Fox et al., (2009) conducted a study to assess the efficacy of these two commercial vaccines in natural-fed feedlot cattle. Feedlot cattle were randomly assigned to 1 of 3 treatments: no vaccination (control), high-antigen dose vaccine, low-antigen dose vaccine; all animals were fed a diet consisting of 73% steam-flaked corn and 13% roughage. Cattle were gathered after 238 days on feed, the total prevalence of liver abscesses (56%) and severe abscesses, grade of A+, (39%) were relatively high. Unfortunately, the vaccination approach did not affect the prevalence of either total liver abscesses or severe abscesses.

The effectiveness of recombinant leukotoxin of *F. necrophorum* was evaluated in a mouse model for liver abscesses (Narayanan et al., 2003). The gene that encodes leukotoxin (*lktA*) was cloned, sequenced, and expressed in *E. coli* (Narayanan et al., 2001). Expression levels were significantly low; consequently, problems arose with purifying the full-length recombinant protein, and the physical instability of the protein. Five recombinant truncated polypeptides (designated as BSBSE, SX, GAS, SH, and FINAL) were expressed in *E. coli* (Narayanan et al., 2001). Even though all polypeptides were immunogenic, two polypeptides (BSBSE and SH) showed more promising results, inducing significant protection in mice with *F. necrophorum* infections. These two polypeptides showed surpassing efficacy compared to the full-length native leukotoxoid or inactive culture supernatant containing leukotoxoid (Narayanan et al., 2003).

Another antigen of *F. necrophorum* that has been the target for vaccine development is the outer membrane proteins, responsible for mediating *F. necrophorum's* adhesion to bovine cells (Kumar et al., 2013). Evidence that must be considered is the fact that the outer membrane protein of subsp. *necrophorum* works differently than the outer membrane protein in subsp.

fundiliforme (Kumar et al., 2014). Thus, implicating that the vaccine is subspecies specific, which would decline its efficacy. Bacterial adhesion to bovine cells is crucial in establishing infection and disease pathogenesis of many Gram-negative bacteria (Bavington and Page, 2005). This particular pathogenesis is the key component for the liver abscess complex concept. Without the adhesion of *F. necrophorum* to the ruminal wall, the development of a rumen wall abscess and the consequential leakage of bacteria into the portal blood would be impossible.

FomA, an outer membrane protein similar to a protein of *F. nucleatum,* a human oral pathogen (Han et al., 2005) binds *F. necrophorum* with strong affinity. This bond will reduce the binding between subsp. *necrophorum* to bovine adrenal gland capillary endothelial cells (EJG cells); therefore, making it a potential candidate for the development of a vaccine against fusobacterial infections in cattle (Kumar et al., 2015). A vaccine containing the FomA protein of *F. nucleatum* has been tested in a mouse model and was shown to prevent oral infection (Liu et al., 2010). Further research is needed to conclude if targeting *F. necrophorum* outer membrane protein effectively prevents and controls liver abscesses in feedlot cattle.

Nutritional Management

Proper nutrition and bunk management are critical component to minimize rumen imbalance and the consequential evolvement of liver abscesses. There are two types of acidosis, acute and subacute, also known as clinical and subclinical acidosis (Gonzalez et al., 2012). In acute acidosis, cattle exhibit symptoms of acute acidosis following consumption of readily fermentable carbohydrates in high amounts and may result in death (Nagaraja and Titgemeyer, 2007); subacute acidosis is commonly asymptomatic, but feed consumption and performance may dwindle (Owens et al., 1998). Subacute acidosis has ruminal fluid pH is below 5.6; however, once pH goes below 5.0 it is considered acute acidosis (Owens et al., 1998; Nagaraja

and Titgemeyer, 2007). In order to decrease liver abscess prevalence, the incidence and longevity of acidosis in feedlot cattle must be reduced due to liver abscess being sequential to acidosis.

Feedlot nutritionists have given a few recommendations to diminish the risk of acidosis. Some of which include: adapting cattle to high-grain diets gradually, avoiding either under- or overfeeding, providing feed several times per day to spread the intake, increasing the roughage content of the feed, imposing quality control in mixing feeds, and providing adequate bunk space (Bartle and Preston, 1991; Hale 1985; Elam, 1976; Jensen et al., 1954). Feedlot diets often contain high concentrations of readily fermentable carbohydrates (Gonzalez et al., 2012). Starch ferments rapidly to lactate by lactic-acid producing bacteria in the rumen, which will result in the death of lactate-fermenting bacteria and an ultimate pH reduction due to the unbalanced rumen microbiome that is not able to stabilize the environment due to increasing lactate concentrations (Nagaraja and Titgemeyer, 2007). When starch concentrations are gradually increased in the diet it allows lactate-fermenting bacteria to adapt to the wavering pH changes; thus, ultimately resulting in a reduction of acidosis.

Roughage is high in fiber, meaning that it will take ruminal microbes much longer to ferment, resulting in a slower rate of pH decline (Allen, 1997). Increased roughage in the diet results in less grain and other concentrates. The consequence is a diet that is less energy-dense. When cattle consume a diet low in starch, the rate of ruminal fermentation is slow (Gonzalez et al., 2012), resulting in lower animal productivity. Hence, producers need to balance the animal's diet. Doing so will make fermentation more efficient and present a less acidic environment in the rumen.

Antibiotic Alternatives

Research has been executed to observe the effects of alternative feed additives regarding liver abscess incidence. The native gut microbiome is responsible for synthesizing vitamins, bioconversion of toxic compounds to non-toxic residues, triggering an immune response, gut peristalsis, and intestinal mucosal maintenance, and plays a role against the colonization of pathogens (Chaucheyras-Durand and Durand, 2010). The leading antibiotic alternative is probiotics or direct-fed microbials (DFM). Probiotics have the ability to alter rumen fermentation in order to decline the incidence of ruminal acidosis.

Probiotics

Probiotics are live microorganisms (bacteria and fungi, most commonly yeasts) that add to the beneficial bacteria normally present in the gastrointestinal tract. If these microorganisms become abundant in the rumen, which is the main site of feed digestion, this will result in a beneficial microbial balance and consequently affect feed efficiency, performance, and decreased acidosis. In order for the probiotics to be effective, they must have the ability to amplify and establish in the ruminal population (Ouwerkerk et al., 2002). This would be the desired outcome for using this methodology of applying probiotics into the feed. However, probiotics have been observed to be the most effective when they have been implemented during a stressful stage for the animal's gut microbiome. Ruminal acidosis varies in severity depending on the lactic acid production present in the rumen (Nagaraja and Titgemeyer, 2007).

Megasphaera elsdenii is a Gram-negative, large coccus bacterium that is the leading ruminal microorganism involved in the fermentation of lactic acid; therefore, it has a pivotal role in preventing ruminal lactic acid accumulation (Counotte et al., 1981; Nagaraja and Titgemeyer, 2007). *Megasphaera. elsdenii* is the only rumen microorganism known to ferment DL-lactate

(60-95%) to propionic acid via the acrylate pathway (Counotte et al., 1981). The capability of *M. elsdenii* to ferment most of the lactate in the rumen is crucial for pH regulation. Lactate accumulating in the rumen will decline the pH to a more acidic environment; subsequently, the eventual development of acidosis. There have been *in vitro* and *in vivo* studies in which cattle are inoculated with *M. elsdenii* and shown to modify ruminal fermentation and prevent lactic acid build up in between diet changes, which is not seen in newly received feedlot cattle (Greening et al., 1991; Kung and Hession, 1995).

Kung and Hession (1995) performed an *in vitro* study to determine if *M. elsdenii* would prevent the accumulation of lactic acid during the change in diet from low to high concentrate. The experiment was performed in three flasks with an uninoculated flask, a low dose flask of *M. elsdenii* (8.7×10^5 cfu/mL), or a high dose flask (8.7×10^6 cfu/mL). The pH would drop once the flask was inoculated with *M. elsdenii* due to the accumulation of lactic acid. Therefore, inoculating *M. elsdenii* into the cattle orally can prevent lactic acid build up (Kung and Hession, 1995). However, further research needs to be done to establish the mode of action by which probiotics improve performance in cattle fed a high-grain diet (Ghorbani et al., 2002). With this knowledge, feedlots have applied the administration of *M. elsdenii*; however, *M elsdenii* does not inhibit *F. necrophorum*. Its action is to regulate pH, which may decrease the incidence of acidosis and eventually reduce the incidence of liver abscesses.

The most common probiotic for ruminants is the live yeast, *Saccharomyces cerevisiae*. There is evidence that live yeast stabilizes ruminal pH and decreases the risk of acidosis (Chaucheyras-Durand et al., 2008). *In vitro* studies have shown beneficial effects of yeast culture due to their ability to provide soluble growth factors (i.e., amino acids, B vitamins, and organic acids) to promote the growth of rumen bacteria that use lactate and digest cellulose (Callaway

and Martin, 1997; Yang et al., 2016; Yoon and Stern, 1996). For that reason, supplementing with yeast cultures has the potential to stabilize rumen conditions for cattle fed a high-grain diet. One study evaluated the effectiveness of *Saccharomyces cerevisiae* fermentation products (SCFP) on liver abscess prevalence, performance, and carcass characteristics compared to cattle fed monensin, tylosin and direct-fed microbial feed additives. However, the study showed no change in final body weight, gain to feed ratio, carcass characteristics, morbidity and mortality, or liver abscess prevalence (Scott et al., 2017). Further research regarding SCFP is needed to conclude the effectiveness towards the rumen and liver abscess microbiome.

Essential Oils

Essential oils are volatile liquid and aromatic plant-based compounds, which have antimicrobial properties due to their unique structures and a wide variety of sidechains (Nazzaro et al., 2013). Essential oils contain a wide range of secondary metabolites that can inhibit or slow the growth of bacteria, yeasts and molds (Nazzaro et al., 2013). Their effectiveness depends on the amount of compound present; at low concentrations, they disrupt enzyme activity involved in energy production, and at high concentrations, they can denature proteins (Tiwari et al., 2009). Generally, Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria (Nazzaro et al., 2013). This is due to the cell wall of Gram-positive bacteria, which will allow hydrophobic compounds to permeate the cell (Nazzaro et al., 2013). The complexity of the cell membrane of Gram-negative bacteria allows for more effective resistance to essential oils. It has a peptidoglycan layer covered by an outer membrane, linked to the lipopolysaccharide matrix consisting of lipid A, the core polysaccharide, and the O-side chain, which provide the “quid” that greatly inhibits the ability of hydrophobic compounds to cross this membrane (Nazzaro et

al., 2013). Gram-negative bacteria can still be affected by essential oils due to the wide array of reactive sidechains attached to these molecules.

The antibacterial properties of essential oils have been evaluated in their efficacy against *F. necrophorum in vitro* and *in vivo*. Five essential oils (eugenol, vanillin, thymol, guaiacol, and limonene) were observed on their effectiveness against the growth of *F. necrophorum in vitro* and concluded that limonene (20 or 100 μg/mL), and thymol (100 μg/mL) inhibited *F. necrophorum* growth (Elwaakeel et al., 2013). Although, when these two essential oils were evaluated in combination with the other five essential oils, the growth of *F. necrophorum* was not inhibited (Elwakeel et al., 2013). The authors believed this loss of affect was caused by the lowered concentrations of limonene and thymol in the mixture as compared to when they were applied individually. Essential oils that had no effect on *F. necrophorum* is primarily due to their inability to cross the outer membrane and disrupt the proteins inside the cell. A minimum inhibitory concentration assay is needed to conclude the effectiveness of the concentration at which the essential oils obtain complete inhibition. Due to some essential oil exhibiting inhibition towards *F. necrophorum* growth *in vitro*, the efficacy of these compounds was observed *in vitro*.

Feedlot steers were fed an essential oil mixture containing thymol, eugenol, vanillin, guaiacol, and limonene targeted at 1.0 g/steer daily. The incidence of liver abscess was generally reduced when compared to the control (Control = 27.2%; Essential Oil Mixture = 16.6%; Meyer et al., 2009). Although essential oils showed inhibition, liver abscess incidence was still higher than cattle fed monensin and tylosin (21.6% vs. 6.5% liver abscess incidence). Therefore, essential oils may have antibiotic capabilities, but their efficacy has not yet exhibited a match for a true antibiotic compound. Essential oils appear to have bacterial inhibition in a petri dish, but

once they are applied to the ruminal environment; their efficacy diminishes possibly due to the volume and bacterial load of the rumen. Continued research for other antibiotic alternatives has heightened interest regarding sorghum compounds.

Sorghum compounds

Sorghum is a widely cultivated cereal native to warm regions and is a major source of grain and feed for livestock. Interests in sorghum have heightened due to agronomic advantages such as high drought tolerance, high yields, low cost, potential health benefits that include slow starch digestibility, cardiovascular disease reduction, antioxidant activity, anti-inflammatory and anticarcinogenic properties (Awika et al., 2009; Awika and Rooney, 2004). Sorghum compounds that are intriguing for researchers are phenolic compounds such as condensed tannins (proanthocyanidins), 3-deoxyanthocyanins, and other flavonoids concentrated in the sorghum bran (Awika et al., 2005).

Tannins are naturally occurring plant polyphenols and their main characteristic is that they bind and precipitate proteins. They act as a defense mechanism in plants against pathogens, herbivores and hostile environmental conditions. High molecular weight condensed tannins appear to have more powerful antioxidant activity *in vitro* and *in vivo* than any other simple phenols (Hagerman et al., 1998; Tian et al., 2012). Sorghum anthocyanidins are unique since they do not display a hydroxyl group in the C-ring 3-position; thus, are called 3-deoxyanthocyanidins (3-DA) (Carbonneau et al., 2014). Therapeutic properties claimed for anthocyanins are highly associated to their antioxidant properties (Awika and Rooney; 2004). Few pigmented sorghums are also potentially a rich source of unique anthocyanins (Nip and Burns, 1969; Gous, F., 1989).

An *in vitro* study was conducted to observe the effectiveness of crude sorghum extract. Five bacterial species and one fungal strain were evaluated: *Bacillus subtilis, Staphylococcus aureus, Salmonella Typhimurium, Klebsiella pneumoniae,* and *Candida albicans*. Sorghum cultivars used were Gumeunchalsusu, Bulkeunchalsusu, Jangsususu, and Neulsusu. Two methods using the sorghum extracts were assessed: the two-fold dilution assay and the paper disc diffusion assay. Antimicrobial activity in the sorghum extract varies, depending not only on the presence of phenolic compounds but also on the various secondary metabolites present (Gordana et al., 2007). Of the four cultivars used, Neulsusu showed the highest level of antimicrobial activity in the two-fold dilution assay method (Kil et al., 2009). Cultivar Bulkeunchalsusu exhibited the highest level of antimicrobial activity using the paper disc diffusion assay method (Kil et al., 2009). This study suggests that sorghum may be used as a natural additive with biological function for its antioxidant and antimicrobial characteristics; however, further studies on sorghum are required to understand its effectiveness of sorghum against bacterial species involved in liver abscesses.

Medium-Chain Fatty Acids

Medium-chain fatty acids (MCFAs) have an aliphatic tail of 6-12 carbon atoms. They occur naturally as medium-chain triglycerides (MCTs) in milk lipids of many animal species and in plants, especially in coconut oil. The MCFAs have a larger scale of carbon and hydrogen in their molecule; therefore, MCFA have a lower energy density than long-chain fatty acids (LCFA). Medium-chain fatty acids are identified as saturated and unbranched monocarboxylic acids. They include caproic acid (C6:0, hexanoic acid), caprylic acid (C8:0, octanoic acid) and capric acid (C10:0, decanoic acid). Lauric acid (12:0, dodecanoic acid) is often classified with the group of MCFAs (Bach and Babayan, 1982). Medium-chain fatty acids have a low melting

point and a comparatively high solubility in water due to a shorter hydrocarbon chain length compared to long-chain fatty acids. When in neutral pH, MCFAs are mostly dissociated (ionized) (Bach and Babayan, 1982).

An autolysin appears to be involved with the bacterial death and cellular lysis provoked by MCFA (Tsuchido *et al*., 1985). Some scientists concluded that Gram-negative bacteria could metabolize MCFA (Cherrington et al.,1991), while others do not quite agree (Fay and Farias, 1975). Researchers interpret this theory by illustrating that the MCFA enter the membrane via porins and once, inside the cell, degradation occurs via the β-oxidation cycle. There is evidence that pathogenic bacteria may be inhibited by MCFA (Kabara *et al.* 1972; Kabara, 1984; Isaacs *et al*. 1990, 1992; Boddie & Nickerson, 1992; Wang & Johnson, 1992; Guthery, 1993; Oh & Marshall, 1993; Kinderlerer *et al*. 1996; Petrone *et al.* 1998; Petschow *et al*. 1998; Sprong *et al*. 2002). However, the role and potential of MCFA in preventing the growth and colonization of food-borne pathogens or for the prevention of liver abscesses need further research regarding its acid tolerance, mechanisms of resistance and associated virulence.

Animals with a high content of MCFAs in their milk include mice, rats, rabbits, goats, horses, and elephants (Zentek *et al*., 2011). Whereas cow, sheep, and human breast milk contain small amounts of MCFAs (Zentek *et al*., 2011). The difference of MCFAs concentrations between species is not fully understood. Dierick (2003) performed an *in vivo* study using *Cuphea* seeds. *Cuphea* seed oil is an exemplary source of MCFAs, exhibiting significant diversity between species. *Cuphea lanceolate* and *Cuphea ignea* oils contain over 80% capric acid and slight amounts of caprylic acid which were used as the source of MCTs in the Dierick (2003) study applied on piglets. Many authors contemplate that the improved performance in the piglets is associated with the antibacterial effects of the MCFAs in the intestinal lumen, particularly

against pathogenic strains (Decuypere and Dierick, 2003). MCFA and MCTs have nutritional and metabolic effects specific to rapid digestion, passive absorption and obligatory oxidation. Thus, raising interest in the feedlot cattle industry for the prevention of liver abscesses.

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Chapter 2 – Sorghum phenolic compound: Evaluation of antimicrobial activities and potential to alter ruminal fermentative activities

Abstract

The escalation of antimicrobial resistance in human health has led to the urgency of discovering antimicrobial alternatives in the agriculture industry. Sorghum grain contain phenolic compounds with antimicrobial properties that have the potential as a natural antimicrobial alternative. The study's objective was to determine the antimicrobial effects of sorghum phenolic extract on bacterial pathogens responsible for liver abscess in feedlot cattle. Bacterial pathogens tested included *Fusobacterium necrophorum* subsp. *necrophorum, Fusobacterium necrophorum* subsp. *fundiliforme, Trueperella pyogenes,* and *Salmonella* Lubbock. Antibacterial activities of sorghum phenolic compounds 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. Plates were incubated for 24 hours and the diameter of each zone of inhibition was measured. The data exhibited that sorghum phenolic compounds had no inhibitory effects on *Fusobacterium necrophorum* subsp. *necrophorum, Fusobacterium necrophorum* subsp. *fundiliforme, Trueperella pyogenes,* and *Salmonella* Lubbock.

Introduction

Sorghum is a staple cereal food crop in many parts of the world. The ability to adapt to semi-dry and dry conditions and high temperatures make this a highly cultivatable crop. Sorghum is growing in popularity due to agricultural advantages, such as high yields, low cost,

and potential health benefits including slow starch digestibility, cardiovascular disease reduction, antioxidant activity, anti-inflammatory and anticarcinogenic properties (Barros et al., 2012). Thousands of strains (germplasm) of sorghum contain high concentrations of grain polyphenols (Harrison, 2017). Free radicals, chemical reactions, and several redox reactions of many compounds may cause protein oxidation, DNA damage, and lipid peroxidation in living cells (Morrissey and O'Brien, 1998). Sorghum has an abundance of phytochemicals, including tannins, phenolic acids, anthocyanins, phytosterols, and policosanols (Awika and Rooney, 2004).

Tannins are phenolic compounds that precipitate proteins. Tannins exist primarily as condensed (CT) or hydrolyzable forms (HT). Condensed tannins are complexes of oligomers and polymers of flavonoid units linked by carbon-carbon bonds (Hagerman and Butler, 1991). Condensed tannins are of interest in ruminant nutrition because of their reactivity with forage proteins after the plant has been masticated. After CT-containing forage is chewed, insoluble CT proteins complexes are subsequently formed (Jones and Mangan, 1977), and CT in the rumen binds to cell coat polymers of bacterial cells (Jones et al., 1994). Condensed tannins in the diet have been observed to induce changes in the morphology of several species of rumen bacteria (Chiquette et al., 1988; Jones et al., 1994). Condensed tannins interact with proteolytic rumen bacterial cell surface, such as cell-bound extracellular enzymes, inhibiting their activity. Inhibition rates may vary depending on the different types of CT (Min. et al., 2003). Further studies are required on CT-bacterial interactions to determine the antimicrobial activity of tannins from sorghum grains.

Anthocyanins constitute a major flavonoid class in sorghum. Sorghum anthocyanidins are unique due to not exhibiting a hydroxyl group in the C-ring 3-position. Therefore, they are named 3-deoxyanthocyanidins (Carbonneau et al., 2014). These polyphenols are speculated to

exert health benefits to consumers; for example, the incidence of esophageal and gastrointestinal cancers was reduced based on epidemiological studies (Isaacson, 2005). Antimicrobial activity in plant extracts, such as sorghum, rely on not only the presence of phenolic compounds but as well as the presence of various secondary metabolites (Gordana et al., 2007). Studies show that the antimicrobial activity of sorghum is due to the presence of tannic acid; however, other phenolic acid-like phenols are thought to provide plant defenses against pests and pathogens (Awika and Rooney, 2004). An *in vitro* study was performed to test for antimicrobial activity using n-butanol purified saponin extract of sorghum bicolor against three pathogenic microbes, which included: *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. *Staphylococcus aureus* was inhibited by the sorghum extract and concluded that the saponins were effective against Gram-positive organisms, but not on the Gram-negative organisms and fungi (Soetan et al., 2006). Not much information is available concerning the antimicrobial effects of sorghum phenolic compounds. Therefore, our objective was to evaluate the antimicrobial effectiveness of sorghum compounds on major bacterial species involved in liver abscesses.

Material and Methods

Plant Material

The sorghum varieties chosen for this study were selected from two populations: 1) the sorghum association panel (SAP), consisting of ~300 genetically and phenotypically diverse varieties, which are grown in Manhattan, KS, and 2) a panel consisting of 72 varieties selected based primarily on the presence of a black colored grain, which are grown in Puerto Vallarta, Mexico. The concentration of total phenolic compounds and oxygen radical absorption capacity (ORAC) values were measured in the flour of all of the varieties. Four varieties with ORAC values greater than 150 μM Trolex equivalent were chosen for the study. The ORAC value

threshold selected is based on the reported literature for blueberries that are often cited as the gold standard for antioxidant health promoting compound potential. The selected varieties are 1) a high tannin/ high ORAC (PI 570481) from the black sorghum panel, 2) a high tannin/high 3-deoxyanthocyanidin (PI 576391) from the SAP; 3) a high tannin/ high ORAC (PI 534117) from the SAP; and 4) a non-tannin/low ORAC (Macia; PI 656121) from the SAP.

Total Phenols Extraction and Quantification Procedure

Using a tangential abrasive dehulling device, Bran was removed from the grain by decortication (Venable Machine Works, Saskatoon, Canada). Extractions were conducted in duplicate for each sorghum variety, resulting in a 50 ml of a concentrated extract. Bran samples (0.5 g) were suspended in 25 ml acidified methanol (1% HCL/methanol v/v), shaken for 2 h, and centrifuged for 15 minutes. The supernatant was then decanted into 50 ml tubes. The method developed by Herald et al. (2012) was used to determine total phenols in the sorghum bran extracts. To each of 96 wells, 75 µl of water were added, followed by 25 µl of either sample, Trolex standard (6-hydroxy-2578-tetramethylchromane-2-carboxylic acid), or blank and 25 µl Folin-Ciocalteu reagent. The solutions were allowed to equilibrate at room temperature for 6 min and then 100 µl of 7.5% Na_2CO_3 were added to each well. The plate was then left in the dark for 90 min at room temperature. The absorbance was measured at 765 nm with a Synergy 2 microplate reader (BioTEK, Winooski, VT) and reported as mg gallic acid equivalents (GAE)/g of sorghum bran. Each sample was measured with four replicates.

Preparation of *Fusobacterium nechrophorum* cultures

Fusobacterium necrophorum subsp. *necrophorum* 2016-13/115B, 2013-16/104A, 2013-16/113A and subsp. *fundiliforme* 2016-13/101, 2016-13/126A, 2016-13/138 previously isolated from bovine liver abscesses were used. The bacterial cultures, stored at -70 C, were streaked onto

blood agar plates (Remel Inc., Lenexa, KS) and incubated for 48 h at 39° C in an anaerobic glove box (80% N_2, 10% H_2, 10% CO_2; Forma Scientific Inc., Marietta, OH). Both strains were reconfirmed by biochemical tests with a commercial identification kit, RapID ANAII System (Innovative Diagnostic Systems Inc., Atlanta, GA). Single colonies from the blood agar plates were then inoculated into 10 mL of prereduced (with 0.05% cysteine-HCl) anaerobically sterilized brain heart infusion broth (PRAS-BHI; Becton Dickinson, Sparks, MD) until the absorbance reached turbidity matching 0.5 McFarland standard.

Preparation of *Trueperella pyogenes* cultures

Trueperella pyogenes 2016-13/102, 2016-13/107, 2016-13/109, previously isolated from liver abscesses and stored at -70 C, were streaked onto blood agar plates and incubated for 48 h at 37° C in a 5% CO_2 incubator. Single colonies from the blood agar plates were then inoculated into 10 ml of Mueller-Hinton broth (MH; Becton Dickinson, Sparks, MD) until the absorbance reached 0.5 McFarland turbidity standard.

Preparation of *Salmonella* enterica serotype Lubbock cultures

Salmonella Lubbock 2016-13/26, 2016-13/34, 2016-13/38 isolated from liver abscesses were streaked onto blood agar plates and incubated for 24 h at 37° C. A single colony from the blood agar plate was inoculated into 10 mL of MH broth until the absorbance reached 0.5 McFarland turbidity standard.

Antibacterial activity using agar well diffusion assay

Antibacterial activities of sorghum phenolic compounds on liver abscess pathogens were determined. A well with a 12 mm diameter was punched into the agar aseptically using a sterile cork borer. The bottom of each well was then coated with 20 µl of agar medium to provide a seal and prevent the extract from exuding the well. A sterile cotton swab was used to inoculate the

agar surface with the bacterial culture to obtain a lawn. A concentration (100 µl, 200 µl, 500 µl, 1,000 µl, and 4,000 µl) of the antimicrobial extract was pipetted into the well. Next, agar plates were incubated under suitable conditions depending on the bacterial species of interest. The diameters of the inhibition zones were measured with a ruler (in mm) starting from the end of the well to the end of the inhibition zone.

Results

The well diffusion assay analysis indicated that the sorghum compound tested did not have antibacterial activity against any of the liver abscess causing pathogens. (Table 2.1)

Discussion

There is a rising interest in natural plant compounds for the development of antibacterial drugs because of the concern associated with antimicrobial resistance in bacteria. Antimicrobial alternatives that are accessible to increase animal productivity and performance include, organic acids, probiotics, phytogenics, prebiotics, synbiotics, enzymes, antimicrobial peptides, hyperimmune egg yolk antibodies, bacteriophages, clay and metals (Gadde, 2017). Phytogenetics, also known as phytochemicals, are natural bioactive compounds that have been extracted from plants and integrated into animal feed. Phytochemicals can come in different forms: solid, dried, ground, or extract form. Sorghum is rich in phytochemicals such as tannins, phenolic acids, anthocyanins, phytosterols, and policosanols (Awika & Rooney, 2004). In ruminants, phytochemicals such as tannins have been used to improve health and productivity. Tannins bind and precipitate proteins and other organic compounds. Specific blends of tannins were developed to increase the range of benefits from each tannin in livestock (Lillehoj et al., 2018). These compounds are used in many countries to increase the quality and production of

milk, eggs, and meat. However, the general agreement is that they all lack consistency and their efficacies fluctuate among regions.

It was hypothesized that the sorghum phenolic compound would inhibit the growth of liver abscess pathogens. Based on the well diffusion assay, sorghum extract did not inhibit *Salmonella Lubbock*, *Trueperella pyogenes*, or *Fusobacterium necrophorum*. On the contrary, Abdel *et al.* (2007) reported that commercial tannins have high antibacterial activity against *Salmonella typhimurium.* Abdel *et al.* (2007), tested three cultivars of sorghum, *Tabat, Dabar,* and *Feterita*. The objective of the study was to test the tannin concentration levels as well as their efficacy to inhibit. The three sorghum cultivars had different concentrations of tannins; however, all three exhibited inhibition toward *Salmonella typhimurium*. *Dabar* cultivar had the highest levels of tannins compared to the other two. These findings complied with Harris and Burns (1970), who observed that the deep colors of sorghum grain correlated with the tannin content. However, Bullard et al., (1980) did not have the same observations between the tannin contents and color of the grain. The variation of sorghum grain tannin concentrations across these studies could be due to many factors such as weather, geographic location, or the class of sorghum extract.

Yuru et al. (2020) attempted to elucidate the antibacterial activity and mechanism of action of Luteolin against *Trueperella pyogenes.* Luteolin has exhibited antibacterial properties against *Bacillus subtilis, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli,* and *Pseudomonas fluoresces (*LV et al., 2009 and Qian et al., 2020*).* Luteolin, a natural polyphenolic compound, expressed significant antibacterial activity towards *Trueperella pyogenes* (Yuru, 2020). The author suggested the mode of action of luteolin against *T. pyogenes* in four different ways: destroying the integrity of the cell wall and cell membrane, affecting protein expression,

inhibiting nucleic acid synthesis, and interfering with energy metabolism. This preliminary research exhibited broad prospects for a new drug against *T. pyogenes.*

Durmic *et al.* (2000) demonstrated that in pigs fed sorghum-based diets, there was no effect on the total number of anaerobes in feces. These pigs were fed a variety of cereal grains with different resistant starch (RS) and soluble non-starch polysaccharides (sNSP). *Fusobacterium necrophorum, Bacteroides vulgatus, F. nucleatum, Clostridium perfringens, Listeria dentrificans* and some other *Bacteroides* species work synergistically with *Brachyspira hydosenteriae* and are found in low numbers in the gut, however, become abundant when the pigs are diseased (Durmic, 2000; Meyer *et al.,* 1975; Alexander *et al.,* 1976; Barnum and Fullerton 1976; Whipp *et al.,* 1979; Hayashi *et al.,*1990; Williams Smith and Jones 1963). The goal of this study was to reduce the number of bacteria in order to regain healthy measures. However, there was no significant reduction in anaerobes, sorghum fed pigs actually had an increase in numbers. Which, in fact, validates the results of the present study observing *Fusobacterium necophorum*.

Plant byproducts play an important role in the development of new antibacterial drugs. Secondary metabolites of normal metabolic pathways of plants have promising antibacterial activities, some of which include: terpenes, alkaloids, flavonoids, and phenols (Yuru, 2020). Polyphenols are a large class of compounds that vary in composition, from simple structures to polymeric compounds, such as tannins. Tannin's ability to hinder many different pathogens has been of high interest. Mechanisms that impede bacterial growth are due to the non-specific capacity of tannins to bind bacterial enzymes, inhibition of oxidative phosphorylation, or the ability to aggregate transitional metal ions, which are critical for bacterial growth (Koleckar, 2008). The lack of inhibition of sorghum extracts on the pathogens responsible for liver

abscesses, may have been due to the low concentration of antimicrobial activity for the sorghum phenolic compound tested. The increase concern of drug-resistant pathogens and the lack of potential antibiotics for the treatment of human and animal diseases has resulted in an urgent need for the development of antibiotic alternatives. Therefore, investigations like we did in this study are so critical.

Conclusion

The results of this study demonstrated that sorghum phenolic compound did not have any inhibitory effect against these pathogens. Future studies are needed to test the antibacterial activities of phenolic compounds extracted from different varieties of sorghum.

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Tables

Table 2.1. Overall antimicrobial effects of sorghum phenolic extract.

	Sorghum phenolic extract concentration (ug/mL)					
Bacterial species and strain number	0	100	200	500	1000	4000
Fusobacterium necrophorum subsp. *necrophorum* 2016-13/115B	-	-	-	-	-	-
Fusobacterium necrophorum subsp. *necrophorum* 2016-13/104A	-	-	-	-	-	-
Fusobacterium necrophorum subsp. *necrophorum* 2016-13/113A	-	-	-	-	-	-
Fusobacterium necrophorum subsp. *fundiliforme* 2016-13/101	-	-	-	-	-	-
Fusobacterium necrophorum subsp. *fundiliforme* 2016-13/126A	-	-	-	-	-	-
Fusobacterium necrophorum subsp. *fundiliforme* 2016-13/138	-	-	-	-	-	-
Trueperella pyogenes 2016-13/102	-	-	-	-	-	-
Trueperella pyogenes 2016-13/107	-	-	-	-	-	-
Trueperella pyogenes 2016-13/109	-	-	-	-	-	-
Salmonella Lubbock 2016-13/26	-	-	-	-	-	-
Salmonella Lubbock 2016-13/34	-	-	-	-	-	-
Salmonella Lubbock 2016-13/38	-	-	-	-	-	-

Chapter 3 – Determination of Antimicrobial Activities of Probiotic Bacterial Cultures

Abstract

Probiotics are beneficial microbes that have the capability to improve intestinal health by promoting the development of a healthy microbiota, hindering enteric pathogens from colonizing the intestine, increasing digestive capacity, and improving mucosal immunity. The study's objective was to determine the antimicrobial activities of *Bacillus pumilus, Bacillus subtilis, Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus acidophilus* 020526, *Lactobacillus buchneri* 110917*, Pediococcus acidilactici* 060117, and *Pediococcus pentosaceus* 032118 against liver abscess-causing pathogens. Bacterial pathogens tested included *Fusobacterium necrophorum* subsp. *necrophorum, Fusobacterium necrophorum* subsp. *fundiliforme, Trueperella pyogenes,* and *Salmonella enterica,* serotype Lubbock. Antibacterial activities of the culture supernatant of probiotics cultures were determined by agar well diffusion assay. Probiotics culture supernatant with pH adjusted to around 7.0 or unadjusted were added to the wells to determine inhibitions. Plates were incubated for 24 hours and the diameter of each zone of inhibition was measured. The results indicated that the probiotic cultures had inhibitory effects on *Salmonella* Lubbock and *Trueperella pyogenes.* None of the culture supernatants had inhibition against both subspecies of *Fusobacterium necrophorum.*

Introduction

Probiotics used as feed additives to promote animal health and performance have increased considerably due to public health concerns of antimicrobial resistance associated with antibiotics. Currently, the antibiotic tylosin is used in the feed to reduce the incidence of liver abscesses (Nagaraja and Lechtenburg, 2007). Even though tylosin is only used on animals, the

antibiotic belongs to the macrolide class considered medically important to public use (FDA, 2019). Due to public concern about using a medically important antibiotic in food animals, it is of major importance to find an alternative to replace the usage of tylosin. The major liver abscess-causing pathogens are *Fusobacterium necrophorum* subsp. *necrophorum*, *Fusobacterium necrophorum* subsp. *funduliforme*, *Trueperella pyogenes*, and *Salmonella* Lubbock.

Probiotics, also called direct-fed microbials, are described as live strains of selected microorganisms, either bacteria or fungi, which, when applied in adequate amounts, could have beneficial effects on the host (Markowiak and Śliżewska, 2018). After consumption, the probiotic microorganisms may balance the gastrointestinal microbiota, whose role is essential to maintain gut equilibrium (Chaucheyras-Durand and Durand, 2008). Large numbers of bacteria, archaea, ciliate protozoa, flagellate protozoa, anaerobic fungi and bacteriophage particles inhabit the rumen (Fonty and Chaucheyras-Durand, 2006).

Liver abscesses are a persistent concern for the cattle feeding industry in the United States, with the latest National Beef quality Audit recording 17.8% of the fed cattle seen in slaughterhouses to have had liver abscesses (Eastwood, et. al., 2017). Brink, et al. (1990) evaluated data from 12 different experiments where each animal was fed individually. Components altered between normal livers and those with A+ liver scores included end weights, HCW (hot carcass weight), dressing percentage and feed intake. Consequently, there can be a loss of HCW, ribeye area, and marbling scores for cattle (Brown and Lawrence, 2010). Liver abscesses with an A+ scoring can have a detrimental effect, specifically when attached to the diaphragm, abdominal organs, or body cavity (Brown and Lawrence, 2010). An estimation for severe liver abscesses can reduce the value of an affected carcass by $38 (Brown and Lawrence,

2010) to more than $52 (Reinhardt and Hubbert, 2015), however, this value could be even higher today due to the cattle market fluctuations. Under these circumstances, probiotics can be a useful tool to stabilize the rumen microflora, possibly inhibiting pathogens involved in liver abscesses, consequently, limiting these problems.

Selecting probiotic strains that adhere to certain criteria and are safe to use is crucial for optimizing their use (Anadón et al., 2014). Their mechanism of action as direct-fed microbials is not fully understood. By adhering to the alimentary tract, probiotic organisms may endure harsh conditions and benefit the stability and protection of the intestinal microflora (Markowiak and Śliżewska, 2018). A microbe to be considered a 'probiotic' must possess certain criteria, which include, but are not limited to, an effective count of viable cells, a beneficial effect on a host's health, and a beneficial effect on the function of the alimentary tract (Markowiak and Śliżewska, 2018). Moreover, probiotic cultures supplemented in feed should endure arduous temperatures and stress used in the processing of feeds, such as pelleting. Probiotics may consist of one or more selected microbial strains. Most microorganisms used as feed additives are bacteria. Most probiotics are Gram-positive bacteria, such as *Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus.* The safety of all these organisms mentioned has been analyzed for the host; however, some *Enterococcus* species may aid in the transmission of antibiotic resistance and *Bacillus cereus* strains can produce exotoxins and emetic toxins (Anadón et al., 2006).

Stressful periods for the gut microbiota are the best stage to implement probiotics to the animal. The main site of feed digestion in adult ruminants is the rumen compartment; therefore, probiotics that target this compartment need to be selected (Chaucheyras-Durand and Durand, 2010). Brossard et al. (2006) reported that one strain of *Saccharomyces cerevisiae* could prohibit

pH decrease by triggering specific populations of ciliate protozoa, which rapidly ingest starch; therefore, effectively competing with lactate-producing amylolytic bacteria (*Enterococcus, Lactobacillus*), which would maintain a consistent level of lactic acid, resulting in the lactate-utilizing species to multiply (Nocek et al., 2022; Nocek and Kautz, 2006). This may signify a possible route to limit acidosis in high concentrate fed animals. According to past studies, the response to probiotics has had a positive potential; however, specific mechanisms are not clearly understood.

The current study's objective was to determine antimicrobial activities of the selected probiotic bacterial cultures on the major bacterial species involved in liver abscesses in feedlot cattle.

Material and Methods

Preparation of Culture Supernatants

The following probiotic bacterial cultures were used in the study: *Bacillus pumilus, Bacillus subtilis, Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus acidophilus* 020526, *Lactobacillus buchneri* 110917*, Pediococcus acidilactici* 060117, and *Pediococcus pentosaceus* 032118. Each bacterial species was cultured in Mueller Hinton broth (Becton Dickinson, Sparks, MD) until an absorbance of 0.60 to 0.65 at 600 nm, early log phase, was achieved. Incubation conditions were dependent for each bacterial species: 37° C in a 5% CO_2 incubator for *Trueperella pyogenes,* 37° C for *Salmonella* Lubbock, and 39° C in an anaerobic glove box (80% N_2, 10% H_2, 10% CO_2; Forma Scientific Inc., Marietta, OH) for Fusobacterium spp. Each culture was then centrifuged at 4,000 x g for 10 min to sediment the cells. The pH of each culture supernatant was recorded. An aliquot of the culture supernatant was adjusted with 0.1 N sodium hydroxide to a pH of 7.0. The original culture supernatant (pH-unadjusted) and

pH-adjusted culture supernatant were then filter sterilized using a 0.22 µm pore size membrane filter. The filtrate was stored at -20°C until further use to detect antimicrobial activity.

Preparation of *Salmonella enterica*

Salmonella enterica serotype Lubbock (strains 2016-13/23, 2016-13/34, and 2016-13/38) previously isolated from liver abscesses, were used. Cultures were streaked onto blood agar plates (Remel Inc., Lenexa, KS) and incubated for 24 h at 37°C. The purity of the isolates was checked, microscopic morphology was determined. A single colony was taken from the pure culture and inoculated into a 10 mL Mueller-Hinton broth until it reached 0.5 McFarland Standard.

Preparation of *Trueperella pyogenes* cultures

Trueperella pyogenes (strains 2016-13/102, 2016-13/107, and 2016-13/109) isolated from liver abscesses were streaked onto blood agar plates (Remel Inc.) and incubated for 48 h at 37°C in a 5% CO_2 incubator. The purity of the isolates was checked, microscopic morphology was determined. The pure culture was then inoculated into 10 mL of Mueller-Hinton broth until it reached 0.5 McFarland Standard.

Preparation of *Fusobacterium necrophorum* cultures

Fusobacterium necrophorum subsp. *necrophorum* (strains 2016-13/107, 2016-13/110, 2016-13/111) and subsp. *funduliforme* (strains 2016-13/126, 2016-13/132, 2016-13/134) were used. The bacterial cultures were streaked onto blood agar plates (Remel Inc.) and incubated for 48 h at 39°C in an anaerobic glove box (80% N_2, 10% H_2, 10% CO_2; Forma Scientific Inc., Marietta, OH). The purity of the isolates was checked, microscopic morphology determined. Both the species and subspecies were reconfirmed by biochemical tests with a commercial identification kit, RapID ANAII System (Innovative Diagnostic Systems Inc., Atlanta, GA). The

pure culture was then inoculated into a 10 mL anaerobic BHI broth until it reached 0.5 McFarland Standard.

Agar well diffusion assay

Pure cultures of the strains of liver abscess-causing pathogens were prepared as described above. Ten mm diameter wells were punched into MH agar or MH with 5% sheep blood agar (Becton Dickinson) with a sterile cork borer and a small amount (approx. 10 µL) of melted MH agar was used to seal the bottom of the wells. Each bacterial inoculum was inoculated onto the plate using a sterile cotton swab to obtain a lawn. The wells were then filled with 100 µL of probiotic culture supernatants, both pH unadjusted and pH adjusted (pH 7.0). The inoculated plates were incubated at 37°C or 39°C for 24 hours. After 24 hours, the plates were examined for zones of inhibition and the diameter of the zones was measured. Each probiotic culture was tested on two more different occasions with different bacterial inocula preparations.

Results

Salmonella enterica serotype Lubbock was the only bacterial culture that showed inhibition with each pH unadjusted probiotic culture (Table 3.1). *Trueperella pyogenes* only showed inhibition toward both pH adjusted and unadjusted *Bacillus pumi*lus (Table 3.2). Both subspecies of *Fusobacterium* showed no inhibition with either pH adjusted or unadjusted culture supernatants (Tables 3.3 and 3.4).

Statistical analysis

The inhibitory activity of probiotic supernatants against liver abscess causing bacterial pathogens was evaluated using a PROC GLM procedure in SAS (Version 9.4, Cary, NC). The statistical model included the various concentrations of probiotic supernatants, replications (repeated measures as a random effect) and zone diameter of disc diffusion assay. The mean

comparisons within and between bacterial isolates against the various concentration of phenolic extracts were tested using Tukey's Honest Significant Difference with an alpha of ≤ 0.05.

Discussion

Antimicrobial alternatives to control liver abscess-causing pathogens are crucial due to the public health concern associated with the use of medically important antimicrobials in food animal production (Torio and Padilla, 2018; WHO, 2019). Various components, such as dietary and management restrictions, have been shown to affect the composition and actions of gut microbial communities in cattle (Dehority, 2003). Factors such as diet composition, feeding practices, and farm management have a major effect on the composition and functions of the microbiota in livestock animals (Zoetendal et al., 2004). Liver abscesses have a significant economic impact, depending on the severity. The Elanco Liver Check System, commonly used in the feedlot industry, is as follows: normal = healthy liver; A- = 1 to 2 small abscesses or scars; A = 1 to 2 large abscesses or multiple small abscesses; A+ = multiple large abscesses; A+AD = liver attached to the diaphragm or any other surrounding organs; A+OP = open liver abscess (Brown and Lawrence, 2010). Cattle with abscesses in the A and A- categories do not have a measurable loss in performance. However, A+ liver abscesses may require more carcass trimming due to adhesion of the abscesses to the diaphragm or any other surrounding organs. Consequently, A+ abscesses, being large, could get ruptured causing the entire carcass to be contaminated. The interruption in the flow of carcasses on the slaughter floor costs time and labor (Nagaraja and Lechtenberg, 2007).

Our findings suggest that one of the probiotics tested (*Bacillus pumilis*) exhibited antimicrobial effects against *Trueperella pyogenes*, a few (list here) against *Salmonella* Lubbock

and none against *Fusobacterium* spp. Probiotics tested had no antimicrobial effect on anaerobic Gram-negative bacteria, but did on the aerobic Gram-negative, *Salmonella* Lubbock and the Gram-positive, *Trueperella pyogenes.* Chateau et al. (1993) analyzed the antimicrobial properties of *Lactobacillus* sp. strains isolated from commercial probiotic products. The most distinct inhibition was detected in *Listeria monocytogenes*, a Gram-positive rod, similar results to the current study's observations of *Trueperella pyogenes*.

Apart from *Trueperella pyogenes*, anaerobic Gram-negative liver abscess causing pathogens were not inhibited by the probiotics, but the aerobic Gram-negative pathogen expressed inhibition. Different probiotics or probiotic combinations could be used as an antimicrobial alternative for these bacteria. For example, Guzel-Seydim et al. (2016) reported the anti-*Fusobacterium nucleatum* promising efficacy of kefir, commercial Kefir, and yogurt against *F. nucleatum* by disc diffusion assays. Natural kefir exhibited the largest inhibitory effect against *F. nucleatum.* This data demonstrates how probiotics can be used in different ways to develop antimicrobial alternatives and how further research should be conducted on direct-fed microbial effects. A limitation to this study was no probiotic combinations was tested to determine whether it would be more effective.

Conclusion

Our data demonstrated that the probiotics tested exhibited antibacterial activity on some bacterial strains. *Trueperella pyogenes* were inhibited by *Bacillus pumilus* and *Salmonella* Lubbock was inhibited by all probiotic cultures, except the two *Bacillus* species tested. The selective inhibitions are interesting observations that need to be further investigated. Our study provides momentum to test additional probiotics along with probiotic combinations as antimicrobial compounds against bacterial pathogens involved in liver abscesses.

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Tables

Table 3.1 Antimicrobial effects of different probiotics against Salmonella Lubbock. Average of 3 replications.

***Salmonella* Lubbock**								
	pH unadjusted				**pH adjusted**			
Probiotic cultures	**2014-3/26**	**2014-3/34**	**2014-3/38**	**pH value**	**2014-3/26**	**2014-3/34**	**2014-3/38**	**pH value**
Bacillus pumilis	-	-	-	6.4	-	-	-	7
Bacillus subtilis	-	-	-	5.7	-	-	-	7
Pediococcus acidilactici	+ (2 mm)	+ (1.67 mm)	+ (1.8 mm)	4.4	-	-	-	7
Pediococcus pentosaceous	+ (2.3 mm)	+ (2.2 mm)	+ (2.4 mm)	4.1	-	-	-	7
Lactobacillus acidophilus	+ (1.7mm)	+ (1.9 mm)	+ (1.7 mm)	4.8	-	-	-	7
Lactobacillus buchneri	+ (1.7 mm)	+ (1.5 mm)	+ (1.7 mm)	4.6	-	-	-	7
Lactobacillus helviticus	+ (3.25 mm)	+ (3.4 mm)	+ (1.6 mm)	3.9	-	-	-	7
Lactobacillus rhamnosus	+ (3 mm)	+ (2.7 mm)	+ (2.7 mm)	4.1	-	-	-	7

Table 3.2 Antimicrobial effects of different probiotics against *Trueperella pyogenes.* Average of 3 replications.

Trueperella pyogenes						
	pH unadjusted			**pH adjusted**		
Probiotic cultures	**2016-13/109**	**2016-13/102**	**2016-13/107**	**2016-13/109**	**2016-13/102**	**2016-13/107**
Bacillus pumilis	+ (3.5 mm)	+ (4 mm)	+ (4.3 mm)	+ (3.3 mm)	+ (4.2 mm)	+ (4.2 mm)
Bacillus subtilis	-	-	-	-	-	-
Pediococcus acidilactici	-	-	-	-	-	-
Pediococcus pentosaceous	-	-	-	-	-	-
Lactobacillus acidophilus	-	-	-	-	-	-
Lactobacillus buchneri	-	-	-	-	-	-
Lactobacillus helviticus	-	-	-	-	-	-
Lactobacillus rhamnosus	-	-	-	-	-	-

Table 3.3 Antimicrobial effects of different probiotics against *Fusobacterium necrophorum* subsp. *necrophorum.* Average of 3 replications.

Fusobacterium necrophorum* subsp. *necrophorum						
	pH unadjusted			**pH adjusted**		
Probiotic cultures	**2016-13/110**	**2016-13/111**	**2016-13/107**	**2016-13/110**	**2016-13/111**	**2016-13/107**
Bacillus pumilis	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-
Pediococcus acidilactici	-	-	-	-	-	-
Pediococcus pentosaceous	-	-	-	-	-	-
Lactobacillus acidophilus	-	-	-	-	-	-
Lactobacillus buchneri	-	-	-	-	-	-
Lactobacillus helviticus	-	-	-	-	-	-
Lactobacillus rhamnosus	-	-	-	-	-	-

Table 3.4 Antimicrobial effects of different probiotics against *Fusobacterium necrophorum* subsp. *fundiliforme.* Average of 3 replications.

Fusobacterium necrophorum* subsp. *fundiliforme						
	pH unadjusted			**pH adjusted**		
Probiotic cultures	**2016-13/132**	**2016-13/134**	**2016-13/126**	**2016-13/132**	**2016-13/134**	**2016-13/126**
Bacillus pumilis	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-
Pediococcus acidilactici	-	-	-	-	-	-
Pediococcus pentosaceous	-	-	-	-	-	-
Lactobacillus acidophilus	-	-	-	-	-	-
Lactobacillus buchneri	-	-	-	-	-	-
Lactobacillus helviticus	-	-	-	-	-	-
Lactobacillus rhamnosus	-	-	-	-	-	-

Chapter 4 – Evaluation of Antimicrobial Activities of Medium Chain Fatty Acids on Major Bacterial Species Involved in Liver Abscesses of Cattle

Abstract

A structured diet is a major factor affecting the fatty acid composition of meat and milk from ruminants because the fatty acids (FA) that reach the duodenum are, at least in part, a dietary element and a product of rumen microbial biohydrogenation (BH) of dietary lipids. Currently, numerous feed supplements are being investigated as effective antibiotic alternatives in animal agriculture such as prebiotics, probiotics, acidic compounds, competitive exclusion products, herbs, essential oils, and bacteriophages. However, acidic compounds consisting of organic acids show promising features as antibiotic alternatives. Organic acids, by altering the composition of the microbiome, protect ruminants from pH-sensitive pathogens. Thus, the objective of this study was to evaluate '*in vitro'* antimicrobial activities of caproic acid, caprylic acid, capric acid, alone or in combinations on major bacterial species implicated in liver abscesses. Bacterial pathogens tested included *Fusobacterium necrophorum* subsp. *necrophorum, Fusobacterium necrophorum* subsp. *fundiliforme, Trueperella pyogenes,* and *Salmonella* Lubbock. Antimicrobial activities of the organic acids were demonstrated by the microbroth dilution method. Bacterial growth was monitored by measuring absorbance at 600 nm at 6, 12, 24, and 48 hours. From the data reviewed, it can be concluded that the efficacy of organic acids was more effective when used in combination.

Introduction

The food animal industry is scrutinized for determining a legitimate antimicrobial alternative with similar capabilities as antibiotics (Cervantes, 2015; Cheng et al., 2014). Feed efficiency and cost of time and labor are typically improved with the use of antibiotics. However, there are raised concerns about increasing antimicrobial resistance and possible transmission from livestock to human consumption. The rumen environment is characterized by a consortium of bacteria, protozoa, fungi and yeasts, a temperature of 38-39°C, pH range of 6.0 and 6.7, and a redox potential of -150-250mV. Any alternation of these conditions may disrupt the microbial population and their fermentation products. Carboxylic acids are considered to inhibit bacteria by altering rumen pH (Sheoran and Tewatia, 2017). Organic acids have been studied in ruminants and have 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). Lipid characteristics that determine their antimicrobial effects in the rumen consists of the type of functional group, number of carbons, saturated or unsaturated, degree of unsaturation, formation of carboxylate salts, and physical cooperation of lipids with surfaces of feed particles and microbes.

Organic acids are defined as organic compounds with acidic properties (Papatisiros et al., 2013). Organic acids primarily consist of carboxylic acids (-COOH) and generally embody SCFAs (≤ C6), also known as volatile fatty acids (VFA), such as formic (C1), acetic (C2), propionic (C3), butyric (C4), valeric (C50, and caproic (C6), either straight or branched chain. The other fatty acids include medium-chain fatty acids (MCFA; C7 to C10) and long-chain fatty acids (LCFA; ≥ C11). Furthermore, there are two groups of organic acids. The first group (lactic,

fumaric, citric) have the capability of reducing the pH of the stomach, resulting in a decline of acid sensitive bacteria present indirectly. The second group (butyric, formic, acetic, propionic and sorbic acids) lower the pH in the GIT by directly acting upon the cell wall of Gram-negative bacteria (Papatisiros et al., 2013; Diener et al., 1993). Organic acids improve the environment of the GIT in consequence of the reduction of GIT pH, promoting proteolytic enzyme activity and nutrient digestibility, promoting pancreatic secretions, stimulating digestive enzyme activity, developing balance of the microbial population, promoting the growth of beneficial bacteria, and by implementing bacteriostatic and bactericidal to pathogenic bacteria (Papatisiros et al., 2013).It has been observed that organic acids like fumaric, propionic, lactic, and sorbic acid acquire the capability to decrease colonization of pathogenic bacteria and the production of toxic metabolites by means of acidification of the diet (Kirchgessner and Roth, 1988).

In ruminants, organic acids have been shown to improve rumen fermentation, maintain rumen pH, have buffering effect in the rumen, decrease methane production, and reduce harmful bacteria on the intestinal wall (Callaway and Martin, 1996; Khampa et al., 2006). Overall, organic acids have exhibited traits to improve cattle health and production (Kung et al., 1982; Khampa et al., 2006). In addition, these naturally occurring antimicrobial agents have little or no human or animal toxicity and induce no problems of residues and cross-resistance induction. They are prospective alternatives to in-feed antibiotics to reduce the risk of liver abscesses in feedlot cattle.

Here, we report on the evaluation of '*in vitro'* antimicrobial activity of caproic, caprylic, and capric at different concentrations and combinations on the major bacterial species (*Fusobacterium necrophorum*, *Trueperella pyogenes*, *Salmonella enterica*) implicated in liver abscesses.

Material and Methods

Preparation of *Fusobacterium necrophorum* cultures

Fusobacterium necrophorum subsp. *necrophorum* and subsp. *funduliforme* (liver abscess strains) were streaked onto blood agar plates (Remel Inc., Lenexa, KS) and incubated for 48 h at 39°C in an anaerobic glove box (80% N_2, 10% H_2, and 10% CO_2; Forma Scientific Inc., Marietta, OH). The purity, species and subspecies of the isolates were confirmed by microscopic morphology and with the commercial identification kit, RapID ANAII System (Innovative Diagnostic Systems Inc., Atlanta, GA). A single colony from the blood agar plate were then inoculated into 10 ml of pre-reduced (with 0.05% cysteine-HCl) anaerobically sterilized brain heart infusion broth (PRAS-BHI; Becton Dickinson, Sparks, MD) and incubated at 39°C overnight. A hundred microliter of the overnight culture were then inoculated into 10 mL of PRAS-BHI broth and incubated at 39° C for 4 to 6 h or until the absorbance reached 0.6 to 0.65 at 600 nm and the bacterial concentration were adjusted to 0.5 McFarland Standard (per CLSI guidelines).

Preparation of *Trueperella pyogenes* cultures

Trueperella pyogenes strains were streaked onto blood agar plates (Remel Inc.,) and incubated for 48 h at 39° C in a 5% CO_2 incubator. A single colony from the blood agar plates was inoculated into 10 ml of Mueller-Hinton broth (MH; Becton Dickinson, Sparks, MD) and incubated at 39° C overnight. One hundred microliter quantity of the overnight culture will then be inoculated into 10 mL of MH broth and incubated at 39° C for 5 to 6 h or until the absorbance reached 0.6 to 0.65 at 600 nm and the bacterial concentration was adjusted to 0.5 McFarland Standard (per CLSI guidelines).

Preparation of *Salmonella enterica* cultures

Salmonella enterica serotype Lubbock, isolated from liver abscesses was used. Cultures were streaked onto blood agar plates (Remel Inc.) and incubated for 24 h at 37°C. The purity of the isolates was confirmed by microscopic morphology. A single colony from the blood agar plates was then inoculated into 10 ml of MH broth (Becton Dickinson) and incubated at 39° C overnight. One hundred microliter quantity of the overnight culture was then inoculated into 10 mL of MH broth and incubated at 39° C for 3.5 to 5 h or until the absorbance reached 0.6 to 0.65 at 600 nm and the bacterial concentration was adjusted to 0.5 McFarland Standard (per CLSI guidelines).

***In vitro* efficacy of MCFA on bacterial cultures**

The compounds, at different concentrations, were tested by the microbroth dilution method. As far as possible, we followed Clinical Laboratory Standard Institute (CLSI) guidelines. Each concentration was inoculated, in duplicates, into 10 ml of anaerobic BHI (for both subspecies of *Fusobacterium*) and MH broth (for *Trueperella* and *Salmonella*). Each tube was then inoculated with 100 µl of bacterial inoculum prepared as above and incubated at 39°C. The growth was monitored by measuring absorbance at 600 nm at 6, 12, 24, and 48 hours.

Results

Medium-chain fatty acids all demonstrated strong evidence of their antimicrobial properties against *Fusobacterium necrophorum* subsp. *necrophorum* (strains 2013-16/104A, 2013-16/113A, 2013-16/146B) (Table4.1), *Fusobacterium necrophorum* subsp. *funduliforme* (strains 2013-16/101, 2013-16/126A, 2016-13/138) (Table 4.2), *Trueperella pyogenes* (strains 2018-12/102, 2018-12/107, 2018-12/109) (Table 4.3), and *Salmonella* Lubbock (strains 2016-13/26, 2016-13/34, 2016-13/38) (Table 4.4). Most effective results were obtained when organic

acids were tested in combinations, most likely due to the fatty acid chain length determining the antimicrobial mode of action and its synergistic effects.

Discussion

Antimicrobial feed additives are currently used to control liver abscesses. The antibiotic that is commonly used is a macrolide antibiotic, tylosin (Nagaraja and Chengappa, 1998). Tylosin is considered a narrow-spectrum antibiotic, predominantly affecting Gram- positive bacteria (Gingerich et al., 1977). However, some Gram-negative bacteria are inhibited, such as *F. necrophorum* (Tan et al., 1994; Lechtenburg et al., 1998). Tylosin is exclusively used on animals but has similar characteristics to erythromycin and antibiotics in the macrolide class that are considered medically essential to human medicine (Scott et al., 2019). Thus, raising concerns in the development of antimicrobial resistance. Global interests have raised in finding alternatives to replace tylosin.

Medium-chain fatty acids are organic acids that have been tested as alternatives to antibiotics and potentially have the characteristic to withhold the expansion of antibiotic-resistant genes in bacteria (Decuypere and Dierick, 2003; Desbois and Smith, 2009; Petschow et al., 1996; Ruzin and Novick, 2000). Medium-chain fatty acids are identified as saturated fatty acids with 6 (caproic acid, C6:0), 8 (caprylic acid, C8:0), 10 (capric acid, C10:0), or 12 (lauric acid, C12:0) carbon atoms. The mode of action has been interpreted as the ability of the fatty acids to invade bacterial cell membrane, acidifying cell cytoplasm, thus inhibiting bacterial growth (Kashket, 1987; Hirshfield et al., 2003; Salsali et al., 2008). Additionally, it has also been proposed that organic acids may reduce ATP production by uncoupling electron transport or interrupt nutrient uptake by invading bacterial cell membrane (Russell, 1992; Alakomi et al., 2000; Mani-Lopez et al., 2012).

Medium-chain fatty acids are demonstrated as a class of organic acid that may be considered as a replacement of antibiotics (Decuypere and Dierick, 2003). Milk fat and numerous feed ingredients, such as coconut palm oils, are sources of medium-chain triglycerides (MCT) (Rossi et al., 2010). Research has shown improvements with a MCFA blend (1:1:1 ration of C6:0, C8:0, and C10:0) at 1.0% and 2.0% of a diet may reduce *Salmonella Typhimurium* ATCC 14028 when present as well as the enhancement of RNA degradation of PEDV in swine feed and ingredients (Chochrane et al., 2016a, 2016b). Similar observations were concluded when individual fatty acids were tested (C6:0, C8:0, and C10:0) at 0.66% of the diet (Cochrane et al., 2017). Many MCFA including C12:0 and its monoglyceride ester monolaurin, target gram-positive bacteria (Ruzin and Novick, 2000, Dansen, 2016). Similarly, the MCFA examined in the current study also conferred inhibition against liver abscess causing pathogens, observing even more efficacy when used in combinations.

One current limitation in using MCFA is the effect they would have on feed intake. Free MCFA may radiate a strong goat-like odor, causing reduced feed intake (Cera et al., 1989; Timmermann, 1993; Molimard et al., 1997). Creating blends of MCFA, other organic acids, and MCFA monoglycerides, means targeting different populations of bacteria within the gut. Unfortunately, this may result in a negative effect on rumen fermentation. Future studies will have to evaluate the best combinations of MCFA and probiotics for an improved effect on pathogenic bacteria. Additionally, MCFA may activate the release of cholecystokinin, resulting in satiety thus, lowering feed intake (Mabayo et al., 1992; Dierick et al., 2002). Field studies are not always successful even though they have demonstrated evident antimicrobial properties (Lynch et al., 2017; Walia et al., 2017; Koyuncu et al., 2013). In summary, our results show

promising in vitro antimicrobial effects of MCFA against tested major bacterial species involved in feedlot cattle liver abscesses.

Conclusion

Our results indicated MCFA to have in vitro bactericidal activity against the four-liver abscess causing pathogens. Several combinations of these organic acids impel interest in using various combinations of these antimicrobials in preventative strategies in feedlots to reduce the risk of liver abscesses in cattle.

Tables

Table 4.1. Antimicrobial effects of different MCFA concentrations against *Fusobacterium necrophorum* subsp. *necrophorum.* Average of 3 replications

Fusobacterium necrophorum subsp. *necrophorum*															
strains	Concentrations (mg/mL)	**Caproic acid (C6)**		**Caprylic acid (C8)**		**Capric acid (C10)**		**Caproic (C6) + Caprylic acids (C8)**		**Caproic acid (C6) + Capric acid (C10)**		**Caprylic acid (C8) + Capric acid (C10)**		**Caproic acid (C6) + Caprylic acid (C8) + Capric acid (C10)**	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
2013-16/104A	0 (Control)	0.4	0.4	0.4	0.47	0.5	0.43	0.4	0.43	0.58	0.59	0.55	0.59	0.61	0.63
	0.125	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.1	0.09	0.06	0.04	0	0
	0.063	0.04	0.04	0.1	0.09	0.05	0.05	0.03	0.02	0.1	0.06	0.1	0.1	0.05	0.05
	.031	0.14	0.2	0.2	0.19	0.13	0.12	0.04	0.03	0.17	0.11	0.23	0.22	0.12	0.12
	.015	0.23	0.23	0.21	0.24	0.26	0.26	0.15	0.17	0.3	0.3	0.37	0.25	0.27	0.24
2013-16/113A	0 (Control)	0.51	0.51	0.51	0.51	0.5	0.5	0.51	0.5	0.59	0.6	0.6	0.58	0.64	0.62
	0.125	0.01	0.02	0.02	0.03	0.02	0.02	0.01	0.01	0.04	0.05	0.05	0.06	0.01	0
	0.063	0.01	0.02	0.02	0.03	0.05	0.06	0.05	0.04	0.07	0.1	0.2	0.2	0.08	0.06
	.031	0.08	0.17	0.1	0.12	0.17	0.14	0.13	0.12	0.17	0.21	0.28	0.26	0.15	0.15
	.015	0.47	0.41	0.36	0.33	0.27	0.28	0.25	0.24	0.27	0.39	0.41	0.36	0.27	0.3
2013-16/146B	0 (Control)	0.54	0.54	0.51	0.51	0.47	0.5	0.5	0.6	0.5	0.54	0.51	0.57	0.5	0.6
	0.125	0.02	0.03	0.01	0.02	0.17	0.18	0.01	0.01	0.03	0.03	0.05	0.03	0.01	0
	0.063	0.07	0.08	0.13	0.14	0.06	0.06	0.05	0.02	0.06	0.06	0.12	0.11	0.04	0.02
	.031	0.2	0.2	0.25	0.25	0.13	0.13	0.07	0.06	0.13	0.13	0.19	0.21	0.13	0.16
	.015	0.35	0.3	0.3	0.32	0.3	0.4	0.22	0.26	0.34	0.4	0.3	0.27	0.28	0.28

Fusobacterium necrophorum subsp. *funduliforme*								
strains	Concentrations	**Caproic acid**	**Caprylic acid**	**Capric acid**	**Caproic (C6)**	**Caproic acid**	**Caprylic acid**	**Caproic acid**

	(mg/mL)	**(C6)**		**(C8)**		**(C10)**		**+ Caprylic acids (C8)**		**(C6) + Capric acid (C10)**		**(C8) + Capric acid (C10)**		**(C6) + Caprylic acid (C8) + Capric acid (C10)**	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
2016-13/101	0 (Control)	0.56	0.51	0.56	0.5	0.5	0.4	0.54	0.56	0.49	0.5	0.51	0.6	0.5	0.55
	0.125	0.02	0.01	0.02	0.01	0.06	0.04								
	0.063	0.02	0.01	0.03	0.02	0.12	0.11								
	.031	0.35	0.4	0.05	0.05	0.16	0.15	0.03	0.03	0.03	0.03	0.03	0.04	0.02	0
	.015	0.57	0.52	0.19	0.3	0.33	0.3	0.06	0.07	0.09	0.08	0.1	0.07	0.06	0.04
	.008	0.58	0.53	0.55	0.5	0.49	0.47	0.2	0.24	0.15	0.17	0.15	0.2	0.14	0.1
	.004							0.35	0.36	0.34	0.35	0.35	0.35	0.24	0.26
	.002							0.33	0.48	0.43	0.56	0.45	0.6	0.51	0.4
2013-16/126A	0 (Control)	0.51	0.5	0.5	0.5	0.52	0.49	0.56	0.58	0.59	0.6	0.6	0.58	0.64	0.62
	0.125	0.02	0.04	0.02	0.01	0.04	0.02								
	0.063	0.03	0.04	0.02	0.03	0.05	0.05								
	.031	0.49	0.46	0.04	0.04	0.2	0.13	0.05	0.06	0.61	0.07	0.04	0.04	0.03	0.02
	.015	0.5	0.5	0.33	0.15	0.25	0.23	0.09	0.09	0.19	0.21	0.12	0.12	0.08	0.07
	.008	0.58	0.6	0.57	*0.6*	0.58	0.47	0.15	0.14	0.26	0.24	0.18	0.23	0.23	0.3
	.004							0.37	0.35	0.4	0.42	0.34	0.4	0.38	0.49
	.002							0.49	0.57	52	0.55	0.57	0.53	0.5	0.63
2016-13/138	0 (Control)	0.55	0.57	0.57	0.57	0.49	0.5	0.54	0.54	0.45	0.47	0.45	0.5	0.5	0.6
	0.125	0.02	0.02	0.02	0.03	0.01	0.03								
	0.063	0.04	0.04	0.03	0.03	0.02	0.05								
	.031	0.1	0.19	0.06	0.09	0.08	0.14	0.03	0.03	0.03	0.04	0.04	0.02	0.01	0.01
	.015	0.57	0.57	0.16	0.51	0.42	0.36	0.06	0.06	0.06	0.08	0.04	0.06	0.04	0.03
	.008	0.6	0.59	0.54	0.6	0.5	0.5	0.1	0.1	0.18	0.19	0.1	0.15	0.1	0.09
	.004							0.4	0.39	0.27	0.32	0.26	0.26	0.23	0.24
	.002							0.56	0.47	0.44	0.49	0.47	0.44	0.43	0.55

Table 4.2. Antimicrobial effects of different MCFA concentrations against *Fusobacterium necrophorum* subsp. *fundiliforme.* Average of 3 replications.

Table 4.3. Antimicrobial effects of different MCFA concentrations against *Trueperella pyogenes.* Average of 3 replications.

Trueperella pyogenes															
strains	Concentrations (mg/mL)	**Caproic acid (C6)**		**Caprylic acid (C8)**		**Capric acid (C10)**		**Caproic (C6) + Caprylic acids (C8)**		**Caproic acid (C6) + Capric acid (C10)**		**Caprylic acid (C8) + Capric acid (C10)**		**Caproic acid (C6) + Caprylic acid (C8) + Capric acid (C10)**	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
2018-12/102	0 (Control)	0.4	0.5	0.41	0.5	0.4	0.49	0.38	0.45	0.42	0.49	0.42	0.49	0.43	0.5
	0.063	0.04	0.06	0.04	0.04	0.05	0.05								
	.031	0.12	0.12	0.11	0.09	0.12	0.1								
	.015	0.24	0.19	0.2	0.18	0.2	0.22	0.07	0.07	0.07	0.08	0.04	0.06	0	0.01
	.008	0.43	0.36	0.35	0.38	0.4	0.4	0.16	0.12	0.18	0.17	0.16	0.15	0.05	0.06
	.004							0.27	0.24	0.26	0.23	0.26	0.25	0.14	0.13
	.002							0.41	0.43	0.41	0.48	0.43	0.41	0.43	0.41
2018-12/107	0 (Control)	0.4	0.5	0.41	0.46	0.42	0.5	0.42	0.5	0.41	0.49	0.43	0.51	0.44	0.51
	0.063	0.08	0.09	0.08	0.07	0.07	0.05								
	.031	0.12	0.13	0.13	0.13	0.17	0.12								
	.015	0.23	0.22	0.23	0.25	0.25	0.25	0.08	0.07	0.06	0.07	0.05	0.06	0.02	0.02
	.008	0.44	0.4	0.4	0.4	0.41	0.41	0.15	0.11	0.15	0.16	0.11	0.14	0.1	0.06
	.004							0.27	0.23	0.26	0.26	0.17	0.23	0.16	0.15
	.002							0.4	0.45	0.45	0.39	0.45	0.41	0.4	0.45
2018-12/109	0 (Control)	0.49	0.54	0.47	0.51	0.48	0.53	0.45	0.5	0.43	0.52	0.42	0.5	0.49	0.55
	0.063	0.08	0.08	0.07	0.08	0.07	0.06								
	.031	0.15	0.16	0.16	0.15	0.16	0.13								
	.015	0.25	0.23	0.27	0.25	0.28	0.28	0.07	0.06	0.08	0.05	0.05	0.05	0.01	0.02
	.008	0.43	0.43	0.41	0.4	0.43	0.45	0.16	0.14	0.13	0.18	0.11	0.13	0.07	0.05
	.004							0.25	0.25	0.23	0.25	0.19	0.24	0.17	0.15
	.002							0.44	0.44	0.43	0.41	0.43	0.44	0.41	0.43

Table 4.4. Antimicrobial effects of different MCFA concentrations against *Salmonella* Lubbock. Average of 3 replications.

Salmonella Lubbock															
strains	Concentrations (mg/mL)	**Caproic acid (C6)**		**Caprylic acid (C8)**		**Capric acid (C10)**		**Caproic (C6) + Caprylic acids (C8)**		**Caproic acid (C6) + Capric acid (C10)**		**Caprylic acid (C8) + Capric acid (C10)**		**Caproic acid (C6) + Caprylic acid (C8) + Capric acid (C10)**	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
2016-13/26	0 (Control)	0.8	0.9	0.8	0.86	0.71	0.81	0.74	0.8	0.78	0.83	0.78	0.8	0.72	0.82
	0.125	0.06	0.14	0.12	0.2	0.11	0.2	0.02	0.05	0.05	0.07	0.06	0.06	0.02	0.02
	0.063	0.39	0.48	0.42	0.44	0.22	0.25	0.09	0.06	0.21	0.22	0.09	0.13	0.13	0.15
	.031	0.5	0.53	0.52	0.56	0.4	0.4	0.3	0.25	0.3	0.37	0.32	0.24	0.2	0.26
	.015	0.5	0.57	0.57	0.61	0.51	0.5	0.47	0.41	0.5	0.5	0.5	0.45	0.4	0.3
	.008	0.5	0.6	0.58	0.62	0.57	0.7	0.71	0.71	0.67	0.61	0.7	0.71	0.62	0.7
2016-13/34	0 (Control)	0.75	0.86	0.79	0.86	0.7	0.8	0.8	0.91	0.72	0.83	0.75	0.84	0.7	0.8
	0.125	0.03	0.07	0.11	0.11	0.09	0.21	0.03	0.04	0.04	0.04	0.06	0.06	0.02	0.01
	0.063	0.3	0.39	0.38	0.41	0.29	0.29	0.05	0.05	0.21	0.23	0.12	0.13	0.09	0.08
	.031	0.5	0.6	0.51	0.52	0.44	0.47	0.33	0.25	0.47	0.47	0.41	0.35	0.16	0.17
	.015	0.58	0.68	0.54	0.64	0.55	0.6	0.5	0.44	0.58	0.63	0.47	0.5	0.43	0.4
	.008	0.6	0.7	0.54	0.65	0.6	0.75	0.62	0.74	0.86	0.86	0.7	0.72	0.65	0.72
2016-13/38	0 (Control)	0.79	0.86	0.79	0.87	0.71	0.81	0.73	0.76	0.78	0.85	0.78	0.8	0.73	0.8
	0.125	0.12	0.13	0.15	0.21	0.18	0.2	0.02	0.03	0.05	0.08	0.08	0.07	0.02	0.02
	0.063	0.4	0.52	0.42	0.48	0.42	0.54	0.08	0.06	0.32	0.34	0.18	0.11	0.08	0.08
	.031	0.5	0.6	0.53	0.58	0.5	0.6	0.29	0.23	0.43	0.48	0.37	0.32	0.23	0.17
	.015	0.54	0.61	0.56	0.64	0.51	0.61	0.45	0.43	0.58	0.58	0.48	0.47	0.45	0.45
	.008	0.52	0.63	0.51	0.55	0.61	0.69	0.72	0.71	0.77	0.78	0.64	0.69	0.65	0.63

Chapter 5 – Effects of Essential Oils on the Growth of bacterial Pathogens Involved in Liver Abscesses

Abstract

Screening of alternative antimicrobial compounds is essential for their use to control pathogens in feedlot cattle production due to raising concerns of antibiotic resistance. In nature, essential oils play an important role in the protection of plants. Essential oils contain a wide variety of secondary metabolites that can inhibit or slow the growth of bacteria. Bacterial pathogens evaluated included *Fusobacterium necrophorum* subsp. *necrophorum, Fusobacterium necrophorum* subsp. *fundiliforme, Trueperella pyogenes,* and *Salmonella enterica,* serotype Lubbock. Antimicrobial activities of the selected essential oils were assessed by the microbroth dilution method. Bacterial growth was monitored by measuring absorbance at 600 nm at 6, 12, 24, and 48 hours. The results indicated that the tested essential oils had no effect against the major liver abscess causing pathogens.

Introduction

The increase of antimicrobial resistance, consequence of food animal use, has raised concerns (Aidara-Kane et al., 2018). Antimicrobials such as macrolides, third generation and higher cephalosporins, fluoroquinolones, tetracyclines, and glycoproteins are medically important. Therefore, resulting in veterinary oversight for the control of these antibiotics in food animals (FDA,2015; Torio and Padilla, 2018; WHO, 2019). Thus, increasing urgency to discover antimicrobial alternatives for agricultural use against disease-causing bacterial pathogens (Pukrop, 2017).

Essential oils are volatile and aromatic compounds extracted from plants (Castillejos et al., 2007). They can be present in all plant organs, including buds, flowers, leaves, seeds, twigs,

stems, flowers, fruits, roots, wood, or bark but are generally stored by the plant in secretory cells, cavities, or epidemic cells (Nazzaro et al., 2013). Research has demonstrated that they enhance digestibility, immunity, and improve gut health in production animals (Brenes and Roura, 2010; Chitprasert and Sutaphanit, 2014; Omonijo et al., 2018). Essential oils are present as variable mixtures of terpenes, terpenoids, and phenylpropenes with various other molecules such as acids, alcohols, aldehydes, aliphatic hydrocarbons, acyclic esters or lactones; rare nitrogen- and sulfur-containing compounds (Nazzaro et al., 2013). Additionally, essential oils possess antimicrobial, antioxidant, and anti-inflammatory activities (Zhang et al., 2006). Their antimicrobial activity is caused by damaging the structure and function of bacterial cell membrane due to their hydrophobicity, leading to cell death (Burt, 2004; Xu et al., 2008; Yap et al., 2014).

Liver abscesses are polymicrobial bacterial infections (Nagaraja and Lechtenburg, 2007; Amachawadi and Nagaraja, 2016), and it is believed that the major route of entry is the rumen (Smith, 1944; Nagaraja and Lechtenburg, 2007). Prevalence and severity of abscesses are caused by the adverse effects relating to roughage levels in the diet (Bartle and Preston, 1991; Foster and Wood, 1970; Gill et al., 1979; Harvey et al., 1968; Zinn and Plascencia, 1996). Liver abscesses in feedlot cattle have a major economic impact on the feedlot industry because of liver condemnation and reduced animal performance and carcass yield. The prevalence averages from 12% to 32% in most feedlots and is altered by dietary and management factors (Brink et al., 1990).

Antibiotics have been used in food animal production to control, prevent, and treat disease, and some are used for growth promotion. A veterinarian must prescribe antimicrobials that are of human use for handling in food animal production (FDA, 2015). Currently, the antibiotic tylosin is used to reduce the prevalence of liver abscesses (Nagaraja and Lechtenburg,

2007). Supplemented tylosin has been shown to hinder the increase in *F. necrophorum* linked with feeding high-grain diets (Nagaraja et al., 1999). Tylosin is a macrolide antibiotic, this class of antibiotics are considered critically important in human medicine (FDA, 2003). Therefore, growing interests to discover antimicrobial alternatives are due to rising antimicrobial resistance in disease-causing bacterial pathogens and for growth promotion purposes in the cattle industry (Pukrop, 2017).

The mode of action of essential oils relies on their chemical structure, and their antimicrobial activity is not attributable to a unique mechanism but is instead a cascade of reaction involving the entire bacterial cell (Burt, 2004). Therefore, essential oils may inhibit the growth of bacterial cells and impede the production of toxic bacterial metabolites. Gram-positive bacteria appear to be more affected by essential oils compared to Gram-negative bacteria. This is presumably due to the differences in the cell membrane compositions (Chorianopoulos et al., 2008; Gutierrez et al., 2008; Marino et al., 2001). This may indicate a potential method to decrease the incidence of liver abscess in high-grain diets.

The current study's objective was to determine the antimicrobial activities of the selected essential oils on the pathogenic bacterial species involved in liver abscesses in feedlot cattle.

MATERIALS AND METHODS

Preparation of *Salmonella enterica*

Salmonella enterica serotype Lubbock (2016/13-23,2016/13- 34, and 2016/13-38), isolated from liver abscesses, were used. Cultures were streaked onto blood agar plates (Remel Inc.,) and incubated for 24 h at 37°C. The purity of the isolates was checked, microscopic morphology was determined. A single colony was taken from the pure culture and inoculated into a 10 mL Mueller-Hinton broth until it reached 0.5 McFarland Standard.

Preparation of *Trueperella pyogenes* cultures

Trueperella pyogenes (2018/12-102, 2018/12-107, and 2018/12-109) isolated from liver abscesses were streaked onto blood agar plates (Remel Inc.,) and incubated for 48 h at 37°C in a 5% CO_2 incubator. The purity of the isolates was checked, microscopic morphology was determined. The pure culture was then inoculated into 10 ml of Mueller-Hinton broth until it reached 0.5 McFarland Standard.

Preparation of *Fusobacterium necrophorum* cultures

Fusobacterium necrophorum subsp. *necrophorum* (strains 2013/16-104A, 2013/16-113A, 2016/13-146B) and subsp. *funduliforme* (strains 2016/13-101, 2016/13-126A, 2016/13-138) were used. The bacterial cultures were streaked onto blood agar plates (Remel Inc., Lenexa, KS) and incubated for 48 h at 39°C in an anaerobic glove box (80% N_2, 10% H_2, 10% CO_2; Forma Scientific Inc., Marietta, OH). The purity of the isolates was checked, microscopic morphology was determined. Both the species and subspecies were reconfirmed by biochemical tests with a commercial identification kit, RapID ANAII System (Innovative Diagnostic Systems Inc., Atlanta, GA). The pure culture was then inoculated into a 10 mL anaerobic BHI broth until it reached 0.5 McFarland Standard.

***In vitro* Efficacy of Essential oils on bacterial cultures via MIC**

The compounds, at different concentrations, will be tested by the microbroth dilution method. As far as possible, we followed Clinical Laboratory Standard Institute (CLSI) guidelines. Each concentration was inoculated, in duplicates, into 10 ml of anaerobic BHI (for both subspecies of *Fusobacterium*) and MH broth (for *Trueperella* and *Salmonella*). Each tube was then inoculated with 100 µl of bacterial inoculum prepared as above and incubated at 39°C. The growth was monitored by measuring absorbance at 600 nm at 6, 12, 24, and 48 hours.

Results

The tested essential oils indicated little to no evidence of its antimicrobial activity against *Trueperella pyogenes, Fusobacterium necrophorum* subsp. *necrophorum, Fusobacterium necrophorum* subsp. *fundiliforme,* and *Salmonella* Lubbock. (Tables 5.1a, 5.1b, 5.2, 5.3 and 5.4)

Discussion

Although tylosin is widely used to control liver abscesses, the use is under veterinary oversight (FDA, 2015). Consequently, there are ample interests in evaluating antibiotic alternatives, such as essential oils to control liver abscesses. Studies have reported the efficacy of synergistic approaches of plant-derived antimicrobials (Brenes and Roura, 2010; Maréchal *et al.,* 2011), combinations of active compounds improve efficiency, leading to a reduction of toxicity and higher yield. This is an important observation because this could lead to developing a potent, cost-effective combination that can reduce liver abscess causing pathogens.

Essential oils are available as variable mixtures of terpenes, terpenoids, and phenylpropenes with various other molecules such as acids, alcohols, aldehydes, aliphatic hydrocarbons, acyclic esters or lactones; rare nitrogen- and sulfur-containing compounds (Nazzaro et al., 2013). Terpenes are hydrocarbons that are assembled by combining several isoprene units. Most terpenes do not obtain high implicit antimicrobial activity. Terpenoids are terpenes with added oxygen molecules or that have had their methyl groups moved or removed by designated enzymes (Caballero, 2003). The antimicrobial activity of most terpenoids is correlated to their functional groups, and the hydroxyl group of the phenolic terpenoids and the presence of delocalized electrons are critical elements for their antimicrobial action. Phenylpropenes are termed as such due to their structure of a six-carbon aromatic phenol group and a three-carbon propene tail from cinnamic acid, which is formed during the first step of

phenylpropanoid biosynthesis. The antimicrobial activity of the phenylpropenes also relies on the type and number of substitutions on the aromatic ring and comparable to most other essential oils, on the microbial strain and condition in which the EO is tested (Pauli and Kubeczka, 2010).

Helander et al. (1997) reported the effect of the inherent properties of essential oils on the outer membrane permeability. Tea tree oil caused damage to the cell membrane complex that subsequently decreased viability for all three microorganisms included in their study. Later confirmed that the cause of cell death was due to membrane damage. Essential oils can penetrate the microbial cells due to their hydrophobic traits; therefore, causing changes in their structure and abilities. This elucidates why essential oils are typically more efficacious, with some exceptions (Kim et al., 1995), against Gram-positive microorganisms. Possible reason why there was efficacy against the only Gram-positive organism, *Trueperella pyogenes,* in the current study. The outer layer of some Gram-negative bacteria restricts or prevents the entry of essential oils into the microbial cell.

A limitation of this study is that it is difficult to foresee the susceptibility of particular species or even a certain strain within that species to the essential oils. De Martino et al. (2009) noticed that two strains of *Bacillus cereus* exhibited different outcomes when introduced to the same essential oils and their singular compounds. Determining the mode of action of essential oils requires further study of their chemical structures, and the mode of action should also be observed in multiple strains and species of microorganisms. The essential oils used in the current study were blind; therefore, we did not know what antimicrobial properties were tested. Essential oils with terpenoids or phenylpropenes could have possibly expressed better effectiveness against the Gram-negative liver abscess causing pathogens. Broadening our basic knowledge of

the molecules present in the essential oils will aid future studies into the modes of antimicrobial activity of essential oils.

Conclusion

The results obtained allow us to conclude that the essential oils tested were not effective against Gram-negative liver abscess pathogens. However, it did express inhibition against the Gram-positive organism, *Trueperella pyogenes*. The narrow-spectrum inhibition points interest to be further investigated. Our study provides momentum to test additional essential oils as antimicrobial compounds against bacterial pathogens involved in liver abscesses.

Tables

Table 5.1a. Antimicrobial effects of different essential oil concentrations against *Salmonella* Lubbock

Salmonella Lubbock																				
Strains	Concentrations	Essential Oils																DMSO			
		#001				#002				#003				#004							
		6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h
2016/13-26	0 (Control	0.64	0.68	0.71	0.82																
	0.02%	0.69	0.80	0.86	0.86	0.7	0.79	0.86	0.87	0.7	0.79	0.86	0.88	0.69	0.80	0.83	0.88	0.71	0.83	0.84	0.84
	0.2%	0.68	0.72	0.77	1.04	0.63	0.71	0.80	1.01	0.64	0.75	0.83	1.02	0.64	0.75	0.82	1.03	0.67	0.75	0.84	0.95
2016-13/34	0 (Control	0.62	0.68	0.73	0.87																
	0.02%	0.70	0.79	0.86	0.93	0.69	0.77	0.82	0.68	0.80	0.84	0.86	0.69	0.81	0.84	0.77	0.77	0.70	0.77	0.86	0.81
	0.2%	0.68	0.72	0.75	1.06	0.62	0.71	0.77	1.03	0.65	0.74	0.81	1.04	0.65	0.74	0.82	1.04	0.67	0.72	0.80	1.12
2016-13/38	0 (Control	0.62	0.68	0.73	0.87																
	0.02%	0.71	0.84	0.86	0.95	0.68	0.72	0.82	0.86	0.66	0.79	0.83	0.86	0.67	0.79	0.87	0.86	0.68	0.80	0.82	0.90
	0.2%	0.63	0.70	0.79	1.11	0.63	0.82	0.79	1.06	0.64	0.73	0.80	1.05	0.63	0.73	0.83	1.06	0.65	0.74	0.84	1.03

Table 5.1b. Antimicrobial effects of different essential oil concentrations against *Salmonella* Lubbock

Salmonella Lubbock																	
Strains	Concentrations	Essential Oils															
		#001				#002				#003				#004			
		6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h
2016/13-26	0 (Control	0.71	0.74	0.75	0.73												
	1%	0.63	0.12	0.11	0.12	0.07	0.07	0.09	0.1	0.09	0.10	0.14	0.12	0.08	0.09	0.09	0.11
	0.5%	0.49	0.65	0.59	0.69	0.53	0.60	0.59	0.60	0.55	0.72	0.71	0.74	0.56	0.74	0.72	0.77
2016-13/34	0 (Control	0.69	0.71	0.73	0.70												
	1%	0.03	0.07	0.11	0.10	0.08	0.10	0.12	0.10	0.11	0.19	0.23	0.23	0.09	0.09	0.10	0.10
	0.5%	0.51	0.52	0.49	0.61	0.52	0.60	0.58	0.61	0.55	0.64	0.70	0.73	0.54	0.63	0.71	0.78
2016-13/38	0 (Control	0.70	0.72	0.74	0.72												
	1%	0.01	0.06	0.12	0.18	0.13	0.16	0.18	0.15	0.12	0.15	0.19	0.16	0.10	0.08	0.14	0.13
	0.5%	0.51	0.52	0.52	0.61	0.54	0.58	0.61	0.63	0.56	0.63	0.71	0.74	0.55	0.66	0.76	0.81

Table 5.2 Antimicrobial effects of different essential oil concentrations against *Trueperella pyogenes*

Trueperella pyogenes																				
Strains	Concentrations	Essential Oil																DMSO			
		#001				#002				#003				#004							
		6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h
2018-12/102	0 (Control	0.23	0.34	0.41	0.49																
	0.02%	0.11	0.14	0.17	0.13	0.13	0.17	0.21	0.19	0.15	0.19	0.21	0.20	0.17	0.23	0.27	0.24	0.23	0.34	0.42	0.5
	0.2%	0	0	0	0	0	0	0	0	0	0	0	0	0.64	0.75	0.82	1.03	0.21	0.29	0.35	0.43
2018-12/107	0 (Control	0.27	0.38	0.49	0.46																
	0.02%					0.14	0.17	0.21	0.19	0.14	0.18	0.22	0.17	0.18	0.25	0.29	0.27	0.27	0.38	0.49	0.46
	0.2%					0	0	0	0	0	0	0	0	0	0	0	0	0.27	0.32	0.4	0.43
2018-12/109	0 (Control	0.26	0.47	0.48	0.54																
	0.02%					0.14	0.20	0.23	0.17	0.17	0.22	0.27	0.23	0.16	0.23	0.27	0.29	0.25	0.46	0.41	0.5
	0.2%					0	0	0	0	0	0	0	0	0	0	0	0	0.5	0.53	0.47	0.39

Table 5.3 Antimicrobial effects of different essential oil concentrations against *Fusobacterium necrophorum* subsp. *necrophorum*

Fusobacterium necrophorum subsp. *necrophorum*																				
Strains	Concentrations	Essential Oil																DMSO			
		#001				#002				#003				#004							
		6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h
2013-16/104A	0 (Control	0.48	0.65	0.84	0.85																
	0.02%	0.48	0.68	0.88	0.91	0.48	0.66	0.84	0.90	0.46	0.66	0.85	0.87	0.44	0.66	0.84	0.86	0.44	0.69	0.85	0.85
	0.2%	0.47	0.61	0.84	0.78	0.47	0.58	0.82	0.77	0.45	0.60	0.82	0.76	0.44	0.65	0.77	0.81	0.43	0.69	0.82	0.86
2013-16/113A	0 (Control	0.49	0.68	0.88	0.91																
	0.02%	0.47	0.69	1.01	0.99	0.46	0.66	1.02	0.96	0.47	0.66	0.99	0.98	0.49	0.65	1	0.96	0.50	0.69	0.98	0.10
	0.2%	0.46	0.67	0.96	0.93	0.45	0.67	0.95	0.88	0.48	0.66	0.96	0.93	0.9	0.64	0.92	0.96	0.51	0.68	0.10	0.94
2013-16/146B	0 (Control	0.48	0.66	0.92	0.82																
	0.02%	0.48	0.69	0.92	0.93	0.49	0.68	0.98	0.87	0.48	0.66	0.98	0.88	0.47	0.69	0.93	0.87	0.47	0.68	0.95	0.87
	0.2%	0.48	0.67	0.93	0.80	0.47	0.67	0.93	0.82	0.47	0.66	0.91	0.83	0.46	0.68	0.96	0.85	0.47	0.64	0.9	0.81

Table 5.4 Antimicrobial effects of different essential oil concentrations against *Fusobacterium necrophorum* subsp. *fundiliforme*

Fusobacterium necrophorum subsp. *fundiliforme*																				
Strains	Concentrations	Essential Oil																DMSO			
		#001				#002				#003				#004							
		6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h	6h	12h	24h	48h
2016-13/101	0 (Control	0.45	0.90	0.96	1.01																
	0.02%	0.42	0.87	1.04	0.96	0.42	0.83	0.92	0.98	0.43	0.87	1.07	0.92	0.45	0.91	1.13	0.94	0.48	0.90	0.90	1.01
	0.2%	0.41	0.83	0.99	0.96	0.45	0.82	0.93	0.98	0.41	0.83	1.01	0.94	0.42	0.87	1.07	0.99	0.44	0.90	0.87	0.10
2016-13/126A	0 (Control	0.47	0.88	1.11	1.06																
	0.02%	0.44	0.88	1.06	0.99	0.45	0.87	1.03	0.99	0.46	0.87	1.05	1.03	0.45	0.89	1.01	1.03	0430	0.90	1.06	1.06
	0.2%	0.43	0.84	1.09	1.11	0.42	0.86	1.02	1.00	0.42	0.86	1.06	1.04	0.45	0.87	1.06	1.09	0.41	0.91	1.05	1.11
2016-13/138	0 (Control	0.48	0.89	1.03	1.06																
	0.02%	0.47	0.89	1.01	1.02	0.48	0.91	1.10	1.02	0.47	0.93	1.05	1.05	0.46	0.91	1.01	1.11	0.48	0.91	1.06	1.09
	0.2%	0.44	0.88	1.09	1.15	0.46	0.89	1.02	1.12	0.46	0.90	1.06	1.12	0.44	0.89	1.06	1.16	0.46	0.90	1.05	1.16

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