# EFFECT OF LIMONENE ON RUMINAL *FUSOBACTERIUM NECROPHORUM*

by

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# Abstract

Seven ruminally cannulated heifers approximately 225 kg initial BW were used in a $7 \times 4$ Youden square design to determine the effects of different levels of limonene on ruminal *Fusobacterium necrophorum* populations. Treatments included: 1) control, 2) limonene at 10 mg/kg diet DM, 3) limonene at 20 mg/kg diet DM, 4) limonene at 40 mg/kg diet DM, 5) limonene at 80 mg/kg diet DM, 6) CRINA-L (a blend of essential oil components) at 180 mg/kg diet DM, 7) tylosin at 12 mg/kg diet DM. Each period included 11 d with 10 d washouts between periods. Samples were collected on d 0 (before treatment initiation), 4, 7, and 10 for measuring *F. necrophorum* by most probable number (MPN) method using selective culture medium. Results indicate that CRINA-L ($P = 0.52$) and tylosin ($P = 0.19$) did not affect ruminal *F. necrophorum* populations. Limonene linearly decreased ($P = 0.03$) *F. necrophorum* populations, and the optimal dietary concentration for limonene was 40 mg/kg DM. Limonene did not affect ruminal degradation rate of lysine, $NH_3$ concentration, or VFA profiles in ruminal fluid. Limonene was useful for reducing ruminal concentrations of *F. necrophorum*. It may have potential to control liver abscesses, although further research will be needed to assess the effect of limonene under feedlot conditions.

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# Dedication

To Nahid Balazadeh (my Mother).You are the meaning of life for me.

# Chapter 1 - A Review of Literature

Liver abscesses are one of the most challenging problems for the feedlot industry (Nagaraja and Lechtenberg, 2007). Cattle that are exposed to high-concentrate diets or managed poorly may experience acidosis and rumenitis, which consequently leads to liver abscesses (Elam, 1976). *Fusobacterium necrophorum* is known to be the most common bacterial cause of liver abscesses. *Fusobacterium necrophorum* has two sub species, *necrophorum* and *funduliforme*, which are frequently isolated from abscessed livers along with other pathogenic bacteria (e.g., *Arcanobacterium pyogenes*). These two invaders attach to the ruminal wall and then penetrate from the area of lesion in the rumen epithelium and subsequently reach the portal blood and access the liver (Jensen et al., 1954; Scanlan and Hathcock, 1983; Tadepalli et al., 2009). Tylosin is an antibiotic that is commonly added to feedlot diets to prevent liver abscesses. Tylosin can reduce liver abscesses by reducing *F. necrophorum* in the rumen (Brown et al., 1973; Meyer et al., 2009). Tylosin is considered a useful management tool to control liver abscesses; however, antibiotics are not well accepted in the public. Therefore, it is valuable to search for alternatives that can control liver abscesses, while minimizing public concern. Researchers have investigated effects of different essential oils along with their major components, such as thymol and eugenol, as feed additives on animal performance, ruminal fermentation profile, and liver abscesses (Gustafson and Bowen, 1997; Dorman and Deans, 2000; McIntosh et al., 2003). Essential oils and their components are active against bacteria, particularly gram-negative bacteria (Helander et al., 1998; Calsamiglia et al., 2007). Limonene is found in lemons, oranges, and grapefruit (Castillejos et al., 2006). Few researchers have studied

the effects of limonene in vitro on *F. necrophorum* (Elwakeel et al., 2013). Our study was designed to investigate the effect of limonene in vivo on ruminal *F. necrophorum* populations.

## *Incidence and factors affecting liver abscesses*

Brink et al. (1990) studied the relationship between liver abscesses and feed intake, gain, and feed efficiency in 12 experiments which involved 566 individually fed cattle. They scored all livers based on size and number of abscesses on liver. They tested pooled data for homogeneity of variance and formed 2 groups. In one group (4 experiments), incidence of liver abscesses was 32%, and in a second group (5 experiments) the prevalence of liver abscesses was 78%. They reported that cattle with severe liver abscesses had significantly lower weight gain, hot carcass weight, and feed intake. Bartle and Preston (1991) studied the effect of dietary roughage on feedlot performance. They tested 2 roughage regimes. One treatment was fed less than 2% roughage from d 22 until d 84 and 10% roughage from d 85 through to final day of finishing d 133 (2/10% group). A second treatment group was fed 10% roughage for the entire finishing period (10/10% group). They reported that the incidence of liver abscesses for the 2/10% group was 24% and for the 10/10% group was 15%. Meyer et al. (2013) studied the effects of monensin, tylosin, and grain processing on 3,632 crossbred steers that received dry-rolled corn and high-moisture corn in equal amounts in experiment 1 and steam-flaked corn for experiments 2 and 3. They observed ranges of 8.3% to 42.2% liver abscesses for experiment 1 and 15.7% to 46% in experiments 2 and 3. Practically, the incidence of liver abscesses can range from 1% to 90% (Nagaraja and Lechtenberg, 2007).

Prevalence of liver abscesses varies among feedlot cattle. The occurrence and variation rate for liver abscesses could be related to different factors. A study was conducted to evaluate the effect of different roughage levels and thiopeptin (antibiotic for controlling acidosis) on performance of feedlot steers (Gill et al., 1979). Yearling Hereford steers (n = 112) were fed high-concentrate rolled-corn diets with thiopeptin and 2 levels of cotton seed hulls (15 and 5%)

in a factorial experiment. Liver abscesses were more prevalent for steers receiving the low roughage level than for steers fed the high level of roughage (Gill et al., 1979). Zinn and Plascencia (1996) studied the feeding value of yellow grease (0 and 6%) with 2 levels of forage (10 and 30%) in 96 crossbred steers in a 135-d finishing trial. They reported that liver abscesses were inversely related to forage level. Steers that received the lower level of alfalfa had a greater rate of liver abscesses.

Harvey et al. (1968) studied the effects of different sources of roughage and antibiotic on animal performance and liver abscesses for finishing period. They used 128 Hereford steers in two $2 \times 4$ factorial experiments. Roughage sources were ground rice hulls, whole rice hulls, and long hay; chlortetracycline was used as an antibiotic. Steers fed chlortetracycline had a lower prevalence of liver abscesses than those not fed antibiotics. They also observed that steers fed no roughage or ground roughage had a greater incidence of liver abscesses.

Dietary roughage level did not always have a relationship with prevalence of liver abscesses. Effects of grain type, roughage level, monensin, and their interaction were tested on cattle performance in 4 trials (Stock et al., 1990). Four levels of roughage were used (0, 3, 6, and 9%). Surprisingly, roughage level did not have a negative relationship with occurrence of liver abscesses. The 9% roughage level led to more abscesses than 0% roughage. Observations of Kreikemeier et al. (1990) were similar to those of Stock et al. (1990). They studied dietary roughage and feed intake on animal performance. They used 126 steers which were fed steam-rolled wheat with different levels of roughage (0, 5, 10, and 15%). They observed no relationship between levels of roughage and incidence of liver abscesses.

Owens et al. (1997) reviewed several studies with regard to the effect of grain source and grain processing on feedlot cattle performance. They stated that extensive processing and rapidly

fermentable grains could lead to low ruminal pH and acidosis, which is a predisposing factor for liver abscesses. Feeding combinations of grain products could be another influential factor for incidence of liver abscesses. Research was conducted to evaluate the effect of combination of high-moisture corn and dry corn in the finishing period. Steers that were fed a mixture of high-moisture corn and dry corn were more efficient in terms of gaining weight and gain to feed ratio compared with those steers that received only 100% high-moisture corn or 100% dry corn. High-moisture corn is a rapidly fermentable grain, whereas dry corn is fermented more slowly; the combination of both can improve efficiency of steers due to reduction of acidosis (Stock et al., 1987a). Stock et al. (1987b) conducted similar research but with different source of grains. They investigated the effect of combination of high-moisture corn and dry-rolled grain sorghum. The conclusions were similar to that of Stock et al. (1987a), which suggests that a combination of highly digestible grain with a more slowly fermentable grain could reduce the risk of acidosis, which consequently could reduce prevalence of liver abscesses.

Fulton et al. (1979a,b) conducted 2 studies on beef cattle adaptation to high concentrate diets. The first study was designed to evaluate feed intake and ruminal pH, volatile fatty acids, and lactate in 4 ruminally cannulated steers fed corn and wheat diets in vivo. They reported that steers fed wheat had lower ruminal pH, less feed intake, and greater fluctuation in ruminal pH than steers fed the corn-based diet. This indicated the importance of grain type in inducing chronic acidosis, which ultimately leads to rumenitis and sequentially liver abscesses. The second study evaluated effect of ruminal pH alteration on feed intake. Steers were infused with mixture of sodium and potassium hydroxide to maintain a less acidic ruminal pH. Steers infused with hydroxide had higher feed intake than the control group. This finding was in agreement with their first study, in which low pH can lead to less feed intake.

Factors such as having a skillful crew to manage feeding so cattle are fed on a regular basis (to lower the risk of over-consuming high concentrate diet), having strong management plans to adapt cattle gradually to high concentrate diets, and weather and season (cattle can develop more acidosis in warmer season) are important elements which might be considered to reduce the prevalence of liver abscesses (Elam, 1979).

Hicks et al. (1990) studied the effect of breed and gender on feed intake of feedlot cattle. They analyzed the data from large feedlots and reported that beef heifers had lower feed intake (2%) than beef steers and that Holstein steers had a greater (12%) dry matter intake than beef steers with similar initial weight.

In conclusion, roughage level, grain type, grain processing method, gender and breed, and management methods are key factors which could affect acidosis, and consequently liver abscesses.

## *Economic impact of liver abscesses*

Liver abscesses contaminate livers and are associated with less feed intake, weight gain, and dressing percentage. Meanwhile they increase trimming and as a consequence damage the profitability of feedlot producers and slaughter plant owners (Nagaraja and Lechtenberg, 2007). Research was conducted to study the effect of tylosin and chlortetracycline on liver abscesses, weight gain, and feed efficiency in 4 feedlot trials (Brown et al., 1975). Cattle (n = 1,329) were used with or without continuous feeding of 75 mg/d tylosin and 70 mg/d chlortetracycline. The investigators used abscess scores to measure the impact of liver abscesses on animal performance: 0 (no abscess), A⁻ (1 or 2 small abscesses or small scars), A (small number of large abscesses), or A⁺ (multiple large organized abscesses). Cattle that received antibiotics had better feed efficiency, greater weight gain, and less frequent liver condemnation than cattle in the

control group. They also reported that moderately or lowly severe liver abscesses had no significant effect on animal performance; on the other hand, very severe liver abscesses ($A^+$) had a significant negative effect on performance of feedlot cattle. This illustrates that liver abscesses are a big liability for feedlot producers.

Several studies have revealed that liver abscesses in cattle reduce feed intake, weight gain, feed efficiency, and dressing percentage. Therefore, it is reasonable to assume that a relationship exists between severity of liver abscesses and animal performance (Brown et al., 1973; Meyer et al., 2009). However, this is not always a case. Harman et al. (1989) evaluated the effect of year, duration of feeding, and housing on finishing cattle performance and onset of liver abscesses. They recorded weight gain and incidence of liver abscesses for 3,570 steers for almost 5 yr. They did not observe any siginificant difference for daily gain between cattle with a healthy liver and cattle with an abscessed liver ($P > 0.19$). Their findings lead to the conclusion that daily gain is more affected by seasonal change and type of housing than incidence of liver abscesses. This conclusion was partially in agreement with the findings of Brink et al. (1989). As previously described, Brink et al. (1989) evaluated the relationships of severity of liver abscesses with daily gain, feed efficiency, and feed intake. They did not observe any significant relationship between severity of liver abscesses and animal performance in 4 of their experiments. However, they scored liver abscesses based on their size and number of abscesses to assess the severity of liver abscesses, which was not done in the Harman et al. (1989) study.

In summary, liver abscesses are a major economic problem for feedlot producers and packers; economic damage may be directly related to level of severity of liver abscesses.

## *Etiologic agents*

A study was conducted to evaluate the susceptibilities of bacterial flora of liver abscesses of cattle fed tylosin or no tylosin (Nagaraja et al., 1999). Liver abscesses were collected from 2 groups of feedlot cattle based on history of receiving tylosin. Bacterial flora of liver abscesses was cultured for both anaerobic and facultative bacteria. *Fusobacterium necrophorum* was isolated from all liver abscesses, and incidence of *F. necrophorum* in mixed culture was 81% in cattle fed no tylosin. *Arcanobacterium pyogenes*, a facultative bacteria, was isolated as the second most frequent bacteria in liver abscesses. The incidence of *A. pyogenes* in liver abscesses of cattle fed no tylosin was 10% (Nagaraja et al., 1999). Kanoe et al. (1977) studied the incidence of bovine ruminal lesions and relationships between rumenitis, rumen parakeratosis, traumatic reticulitis, and reticular abscess with hepatic abscesses in cattle. *F. necrophorum* was isolated from all liver abscesses. These results were in agreement with Scanlan and Hathcock (1983) and Berg and Scanlan (1981); both articles considered *F. necrophorum*, previously called *Sphaerophorus necrophorum*, as the main etiological agent for causing liver abscesses. *A. pyogenes* is the pathogenic bacteria which is second most frequently isolated from liver abscesses (Scanlan and Hathcock, 1983; Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007).

In addition to *F. necrophorum* and *A. pyogenes*, several other anaerobic and facultative bacteria have been isolated from bovine hepatic abscesses (Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007).

## *Fusobacterium necrophorum*

*F. necrophorum* is a gram-negative, anaerobic, nonmotile, nonsporeforming, rod-shaped (pleomorphic) bacterium, is a normal inhabitant of the rumen and respiratory tract, and is a

member of *Bacteroidaceae* (Langworth, 1977; Nagaraja and Lechtenberg, 2007; Tadepalli et al., 2009). *F. necrophorum* was considered as a pathogenic agent for liver abscesses in cattle, foot rot in domestic animals, and calf diphtheria (Langworth, 1977; Tan et al., 1996; Nagaraja and Lechtenberg, 2007; Tadepalli et al., 2009). Jang and Hirsh (1994) took isolates of specimens obtained from different animals such as canine, equine, porcine, and ruminants and then identified the characterization, distribution, and morphology of these isolates. They concluded that *F. necrophorum* was the most isolated species and the most frequent sites for isolation of *Fusobacterium* were abscesses, respiratory tract, and pleural and peritoneal cavities. *F. necrophorum* was previously named *Spherophorus necrophorus*, which was considered incorrect because it did not define *F. necrophorum* as a separate species. In order to differentiate *F. necrophorum* from other species, several researchers examined carbohydrate fermentation and indole production by species in the *Bacteroidaceae* family (Langworth, 1977). Böe and Thjötta (1943) studied the differences between *F. necrophorum* and *Leptotrichia* (another member of *Bacteroidaceae*), and they found that *Leptotrichia* could ferment carbohydrate but did not produce indole; in contrast, *F. necrophorum* acted as a weak carbohydrate fermenter, but had strong indole production. In another study to investigate the differences between *Fusobacterium* and *Leptotrichia*, 27 strains of fusiform non-sporing anaerobes were collected and examined (Werner et al., 1971). Gas chromatography was used to determine fermentation end-products. All strains had similar morphological characteristics, but they had pronounced biochemical differences. They determined 17 clinical isolates of fusiform were weak in carbohydrate digestion, strong in amino acid decomposition, high in indole production, and also high in ammonia and propionate production from threonine. In addition all 17 strains produced butyric acid in peptone-yeast extract medium. They found that these strains had characteristics very

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similar to *Fusobacterium* strains. On the other hand, the remaining 10 fusiforms had opposite biochemical characteristics with the above 17 strains, and had high similarities with strains considered *Leptotrichia* (Werner et al., 1971).

A study designed to examine factors that influence leukotoxin activity of *Fusobacterium necrophorum* revealed further characteristics of *F. necrophorum* (Tan et al., 1992). They used 24 *F. necrophorum* strains isolated from bovine liver abscesses, which were grown in brain-heart infusion broth, liver infusion, and Eugon broths; growth rate was measured spectrophotometrically, and leukotoxin activity was assessed by its effect on polymorphonuclear neutrophil leukocytes as target cells. Optimal growth conditions for *F. necrophorum* and leukotoxin activity was pH from 6.6 to 7.7, with the temperature of 39$^{o}$C. The optimal redox potential for this bacteria was within the range of -230 to -280 mV. Low iron concentration reduced *F. necrophorum* growth rate.

Bacteriology and histology of hepatic abscesses were studied by Lechtenberg et al. (1988). Abscessed livers were collected from feedlot cattle, and isolates were obtained from 49 abscesses for bacteriological and histological examination. They isolated and enumerated facultative and obligate anaerobic bacteria. *F. necrophorum* was isolated from all abscessed livers. It was shown that *F. necrophorum* generally did not ferment carbohydrate as a source of energy, but did weakly ferment glucose and fructose. End product fermentation analysis indicated that lactic acid was the major energy source for *F. necrophorum*, whereas acetate and butyrate were the major end products of fermentation with a small molar proportion of propionate.

Extracellular enzymes of *F. necrophorum* subspecies were studied by Amoako et al. (1993). Twenty strains were tested for extracellular enzyme production. They practiced different

laboratory techniques such as DNase agar and GAM agar to test DNase production and hemolysin production, respectively. They reported 13 extracellular enzymes activity from *F. necrophorum* subspecies.

## *Classification*

Langworth (1977) and Scanlan and Hathcock (1983) classified *F. necrophorum* in mice, based on its biological and biochemical properties, into biotype (subspecies or biovar) A, B, and C. Shinjo et al. (1991) determined the differences between biotype A and B based on biological and biochemical properties, DNA base composition, and DNA-DNA homology of these 2 biotypes. They used 10 strains which were supplied by different labs. They compared G + C contents of DNA from labeled biovar A strains with other biotype A strains and found a high rate of similarity between them. Also, they compared DNA composition of labeled biotype B strains with other biotype B strains and similarity between them was high. *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* were named respectively, for biotype A and B.

Biotype C is a nonpathogenic bacteria and has been named *F. pseudonecrophorum* (Shinjo et al., 1990). Biotype AB was studied by Emery et al. (1985) from 61 isolates of *F. necrophorum* obtained from lesions of foot abscesses in cattle and sheep, and some from lesions of skin of cattle. They identified the subspecies based on their colonial morphology, production of indole, and haemolytic and leucocidal activity. They reported that biotype AB had characteristics similar to biotype A and B. Research was conducted to study the relationship of biotype A, B, and AB based on their 16s rRNA sequence (Nicholson et al., 1994). They obtained biotype strains from ovine foot abscess lesions, isolates were indentified based on their

characteristics, genomic DNA was extracted from *F. necrophorum,* 16S rRNA genes were amplified, and DNA sequencing and phylogenetic analysis was performed. They concluded that biotype AB is closely related to biotypes A and B (Nicholson et al., 1994).

Molecular techniques such as 16S rRNA, DNA restriction fragment length polymorphism (RFLP), PCR, and ribotyping is used to differentiate biotypes A and B at the molecular level in addition to biological differences (leukotoxic, hemolytic, hemagglutination activity) between these 2 subspecies (Tadepalli et al., 2009). Okwumabua et al. (1996) used the ribotyping method to differentiate *F. necrophorum* subsp. *necrophorum* from *F. necrophorum* subsp. *funduliforme* isolated from ruminal contents and liver abscesses. They isolated 8 strains of *F. necrophorum* from ruminal contents and 2 strains of *F. necrophorum* from liver abscesses for each subspecies of *F. necrophorum*. Chromosomal DNA was isolated and digested by restriction endonuclease, and then restriction fragments were separated on electrophoresis apparatus. Labeled probe was prepared by a non-radioactive labeling system, and from there the hybridization process was completed. Isolates differing in their bands in the hybridization process were identified as different strains. *F. necrophorum* subsp. *necrophorum* had a 2.6-kb band, whereas a 4.3-kb band was identified in *F. necrophorum* subsp. *funduliforme* but not subsp. *necrophorum*, regardless of isolation source (ruminal contents and liver abscesses). Overall, the ribotyping method is a useful technique to differentiate subspecies of *F. necrophorum.*

A study was conducted to investigate the differences between biotype A and B based on their leukotoxin operon promoter regions (Zhang et al., 2006). Four strains for each subspecies of *F. necrophorum* were collected from rumen contents and liver abscesses, chromosomal DNA was isolated from *F. necrophorum* strains, and then the leukotoxin promoter region was amplified by polymerase chain reaction (PCR), and the PCR products were separated by gel

electrophoresis. The results indicated that *F. necrophorum* subsp. *necrophorum* was different

than *F. necrophorum* subsp. *funduliforme* in nucleotide sequence of the leukotoxin promoter

region (Zhang et al., 2006).

Bacteriologic and histologic studies of liver abscesses by Lechtenberg et al. (1988)

demonstrated that *F. necrophorum* biotype A is mostly isolated from liver abscesses in pure

culture, but *F. necrophorum* biotype B frequently is isolated from liver abscesses in mixed

culture with other pathogenic bacteria such as *Corynebacterium pyogenes*, *Staphylococcus* spp,

and *Streptococcus* spp. Other findings were that biotype A produces more leukotoxin and occurs

in more infections than biotype B.

## *F. necrophorum in rumen contents, rumen wall, and liver abscesses*

Ribotyping technique was used in the research study to compare *F. necrophorum* strains

isolated from liver abscesses, ruminal contents, and rumen walls (Narayanan et al., 1997). The

goal of the study was to examine the genetic differences between subspecies of *F. necrophorum*

from liver abscesses, rumen walls, and rumen contents to find the origin of particular subspecies

of *F. necrophorum* which cause liver abscesses. They collected samples of rumen content, rumen

walls, and liver abscesses from 11 cattle for comparison of *F. necrophorum* strains. *F.

necrophorum* strains were isolated, and biological characterization was based on their leukotoxic

and hemolytic activities. Chromosomal DNA was extracted and digested with restriction

endonucleases such as EcoRI, probe was prepared with the mixture of 16S and 23S rRNA from

*Escherichia coli* and labeled with digoxigenin and then a hybridization process was performed.

The ribotyping method identified the genetic similarity between isolates from liver abscesses and

rumen walls, but not those from rumen contents. They assumed that lack of genetic similarity

between isolates from liver abscesses and rumen contents might be related to existence of a great number of *F. necrophorum* strains of which only one or a few attach to the ruminal wall and cause infection. They also found out that strains from ruminal walls are the main causative agent for liver abscesses (Narayanan et al., 1997).

The concentration of *F. necrophorum* in ruminal contents of cattle was studied during rapid change to a high-grain diet with addition of tylosin (antimicrobial agent) to the diet (Nagaraja et al., 1999). Six ruminally cannulated steers were randomly assigned to 2 treatments (control vs. tylosin-fed), and the diet was changed rapidly from no concentrate to 85% concentrate to induce acidosis and consequently rumenitis. Ruminal content samples were collected before and after treatment (tylosin), and *F. necrophorum* concentration was measured with the most-probable-number technique. Results demonstrated that the concentration of *F. necrophorum* was increased by a sudden change to a high-concentrate diet in the control group, whereas the tylosin-fed group had a lower *F. necrophorum* concentration. Therefore, *F. necrophorum* concentration was affected by type of diet, and also by the presence of tylosin (Nagaraja et al., 1999).

### *Role of F. necrophorum on rumen community*

Russell (2005) conducted research to examine the degradation of lysine by ruminal bacteria. He collected rumen samples from lactating dairy cows fed a commercial ration and non-lactating cows fed timothy hay ad libitum. Mixed ruminal bacteria were transferred to serum bottles containg: 1) no addition, 2) 50 mM lysine, 3) 5 mg/mL trypticase, or 4) 5 mg/mL trypticase plus 50 mM lysine. Chromosomal DNA was isolated and compared with known DNA sequences in the GenBank database. Based on DNA sequence comparison, lysine degrading isolates were closely related to *F. necrophorum* D4 (strain isolated from the rumen of grazing

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cattle in New Zealand). His results indicated that lactate was converted to acetate and propionate; and lysine was utilized as an energy source resulting in the production of acetate, butyrate, and ammonia. According to the data, lysine degradation was dependent on lysine concentration and pH (optimum pH was 6.7). However, further research was required to assess factors affecting lysine degradation.

Russell (2006) studied factors affecting lysine degradation by ruminal fusobacteria. His objectives were to study the effect of addition of dietary lysine on lysine-degrading bacteria in vivo, and in vitro factors which might affect fusobacteria. Rumen samples were collected from 3 non-lactating cows initially fed timothy hay and then receiving commercial pelleted grain with protein mix and lysine for 21 d. *F. necrophorum* JB2 strain was grown on basal medium with addition of lysine and lactate as an energy source, and also *F. necrophorum* was grown in continuous culture with addition of HCl to reduce pH. They observed that factors such as a ratio of ruminal fluid to basal medium more than 25%, clarified and autoclaved ruminal fluid, low pH, fermentation products, and sodium acetate at pH 6.1 could inhibit growth of *F. necrophorum* JB2 in vitro, and also they observed that fermentation products could be inhibitory to *F. necrophorum* growth in vivo by adding more lysine to the diet without detecting any increase in ammonia production.

A research study in New Zealand monitored and characterized ammonia-hyperproducing bacteria (HAP) in pasture grazing dairy cattle, deer, and sheep (Attwood et al., 1998). Recognized HAP bacteria such as *Clostridium aminophilum, Clostridium sticklandii, Peptostreptococcus anaerobius,* and *Streptococcus bovis* were supplied by different laboratories, rumen samples from fistulated animals were collected and enumerated for HAP bacteria, and HAP isolates were grown in HAP liquid medium. DNA was extracted from rumen samples,

restriction fragment length polymorphisms (RFLP) were performed on 16S rRNA genes of isolates, and DNA of isolates were detected by 16S rDNA sequencing for phylogenetic analyses. The phylogenic analyses showed that isolate D4 had high similarity with *F. necrophorum*. 16S RFLP showed that all isolates were different from recognized HAP bacteria; however, biochemical tests indicated that carbon substrate utilization patterns of isolates were similar to HAP bacteria. Nitrogen source (tryptone and casamino acids) utilization patterns and growth rates demonstrated that isolate D4 grew fast in HAP liquid medium. In conclusion, Attwood et al. (1998) characterized *F. necrophorum* as HAP bacteria. However, it is not exactly clear whether *F. necrophorum* is a HAP bacteria or not.

Gharbia and Shah (1989) studied the capacity of *Fusobacterium* species for uptake of amino acids in chemically defined medium. Different sources of *Fusobacterium* strains were used (2 strains for *F. necrophorum*), defined medium contained 1 mM of each acidic, basic, nonpolar neutral, and polar neutral amino acids. Results indicated that *F. necrophorum* could utilize lysine as an energy source. Furthermore, *Fusobacterium* utilized significant amounts of arginine, histidine, and glutamate (non-neutral amino acids), as well as serine, threonine, and asparagine (polar neutral amino acids). However, *F. necrophorum* did not utilize extensively glycine, tyrosine, or alanine.

Elwakeel et al. (2013) studied in vitro lysine degradation by mixed ruminal bacteria and *F. necrophorum*. Seven experiments included: 1) addition of pure cultures of *F. necrophorum* to ruminal fluid sources to evaluate lysine degradation by mixed rumen bacteria, 2) evaluation of the effects of pH and fermentation acids (VFA) on lysine degradation, 3) study of the degradation of alanine and glutamate by mixed ruminal bacteria, 4 and 5) measurement of the growth of *F. necrophorum* strains on lysine, lactate, tryptophan, alanine, glutamate, methionine,

and histidine, and 6 and 7) study of the effect of monensin, tylosin, and essential oil components on *F. necrophorum* growth. Some of the experiments will be discussed later in detail, but the current discussion will involve experiments 1 through 5.  Rumen samples were collected from 2 cannulated cows, supernatants from rumen contents were used as rumen bacterial inoculum which was dispensed with McDougall's buffer into serum bottles. For experiment 1, a 2 × 3 factorial design was arranged for treatments and included 2 lysine concentrations, and 3 inocula of *F. necrophorum*. In experiment 2, a 2 × 3 factorial design was used for treatments and included 2 levels of pH, and fermentation acids (acetate and propionate) which were added to the rumen fluid-based fermentation culture. In experiment 3, cultures contained alanine, glutamate, and 2 levels of lysine (25 mM and 50 mM). For experiment 4 and 5, they tested lysine and amino acid (AA) degradation by pure cultures of *F. necrophorum*. For experiment 4, 7 strains of *F. necrophorum* including both biotype A and B were added to the basal medium which contained lysine, lactate, and lysine plus lactate as a substrate. In experiment 5, 2 strains of *F. necrophorum* including both biotype A and B were added to the basal medium which contained tryptophan, alanine, glutamate, methionine, and histidine as substrates. For the first experiment, they observed that lysine was degraded to ammonia in fermentation cultures, and addition of *F. necrophorum* did not increase lysine degradation; this was in disagreement with Russell (2005) which reported increased lysine degradation by addition of *F. necrophorum* strains to fermentation cultures. They concluded that fermentation cultures might have enough lysine-degrading bacteria including *F. necrophorum* to utilize lysine, which would explain the lack of increase in ammonia production by addition of *F. necrophorum*. In experiment 2, they reported higher ammonia production at pH 6 than pH 7 with the absence of VFA products in the media, whereas, addition of VFA significantly reduced ammonia production at pH 6, with no changes at

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pH 7. In experiment 3, they observed higher $NH_3$ production for 50 mM lysine at 48 h than 25 mM lysine, glutamate, and alanine in mixed rumen bacteria, whereas $NH_3$ production for glutamate at 36 h was higher than all concentrations of lysine and alanine. In experiment 4, they observed that both subspecies of *F. necrophorum* (*necrophorum* and *funduliforme*) could grow on both lysine and lactate as an energy source. Results indicated that lysine plus lactate provided more growth for *F. necrophorum* subspecies than lysine or lactate alone as a substrate. They reported that end products of lysine degradation were $NH_3$, acetate, and butyrate, whereas end products of lactate degradation were propionate and acetate. Data indicated that *F. necrophorum* subsp. *necrophorum* produced slightly more fermentation products than *F. necrophorum* subsp. *funduliforme*. For experiment 5, data illustrated that *F. necrophorum* had the greatest growth with lysine and glutamic acid, whereas subspecies *necrophorum* could grow with histidine and methionine; no observation of growth was detected for subspecies *funduliforme* with histidine and methionine.

In conclusion, *F. necrophorum* utilizes both lactate and lysine as C and energy sources, with $NH_3$, acetate, propionate, butyrate production in the rumen; little carbohydrate fermentation is observed for *F. necrophorum*.

## *Virulence factors of F. necrophorum*

In this section we will discuss about three major virulence factors of *F. necrophorum*: 1. Hemagglutinin (HA), 2. Lipopolysaccharide (LPS), and 3. Leukotoxin.

## *Hemagglutinins*

Nagai et al. (1984) conducted research to characterize *F. necrophorum* HA. *F. necrophorum* was cultured in modified heart infusion broth, and HA activity was determined. The HA was separated from bacterial cells with trypsinization followed by gel filtration. The molecular weight and amino acid content of *F. necrophorum* HA was determined. Results indicated that HA was a heat labile, low molecular weight protein (19,000 daltons), rich in alanine, glutamine, and histidine. They also assumed that HA is the cell surface component that helps bacteria attach to the rumen wall (Nagai et al., 1984).

Pathogenicity of human and animal *F. necrophorum* strains was studied by Smith and Thornton (1993). Mice were used as an animal model, 8 human and 3 animal strains of *F. necrophorum* were studied, and strains of *F. necrophorum* were injected subcutaneously and intravenously. They reported that 7 of 8 human strains and biovar B isolates produced mild lesions and were not considered  major virulent strains, whereas isolates of biovar A produced severe lesions in liver tissue and were considered major virulent strains. The reason for greater virulence by subspecies *necrophorum* than subspecies *funduliforme* might be the lack of HA protein in *funduliforme* subspecies (Tadepalli et al., 2009).

Kanoe and Iwaki (1987) studied the role of HA on adhesion of *F. necrophorum* to the rumen wall. Three strains were used, adherence of bacteria to rumen wall was assayed, and HA activity was measured. Scanning electronmicroscopy was used to detect adhesion of bacteria to the rumen wall. Results showed that HA plays an important role for adherence of *F. necrophorum* to the rumen epithelium. Furthermore, results using HA antiserum suggested that adhesion of *F. necrophroum* is mediated by HA.

### *Lipopolysaccharide (LPS)*

Inoue et al. (1985) studied the biological properties of LPS from *F. necrophorum*. Two strains of *F. necrophorum* biotype A and B were used and cultured in modified heart infusion broth. The LPS was extracted from cells, and the protein, hexose, aminosugar, and crude lipid A content was determined. Also, pyrogenicity in rabbits as well as hemorrhage and death of chick embryos were determined. Results showed a difference in chemical composition between LPS of subspecies *necrophorum* and *funduliforme*, with more glucose and aminosugar for *funduliforme* and more lipid A for *necrophorum* subspecies. They also reported that subspecies *necrophorum* was more virulent than subspecies *funduliforme* based on lethal effects on chick embryos (Inoue et al., 1985).

Nakajima et al. (1985) studied the process of infection by comparing liver abscess formation by intact *F. necrophorum* with extracted components of *F. necrophorum* such as LPS. Sixty female mice were inoculated intravenously with intact *F. necrophorum*, LPS was extracted from the cell wall and then administered intraperitoneally in 65 mice. The LPS was detected by the fluorescent microscope method. Data illustrated that both LPS and intact *F. necrophorum* induced liver necrosis and were detectable in sinusoid tissues.

They concluded that LPS is one of the components of *F. necrophorum* which could induce liver abscesses.

### *Leukotoxin*

### *Biological and biochemical characterization of leukotoxin*

Tan et al. (1992) studied factors affecting *F. necrophorum* leukotoxin activity in vitro. Twenty-seven strains of *F. necrophorum* including biotypes A and B were used, leukotoxin

activity was assayed by tetrazolium dye with bovine polymorphonuclear neutrophil (PMN) leukocytes as target cells, and leukotoxin production was measured. The effects of growth media, pH, incubation temperature, redox potential, and iron concentration on bacterial growth and leukotoxin production were determined. Biotype A had higher leukotoxin titer than biotype B, with the peak of toxin production at late-log and early stationary phase for both subspecies.

Tan et al. (1994) examined biological and biochemical properties of *F. necrophorum* leukotoxin. Six strains were grown in brain heart infusion broth at 39$^{\circ}$C, incubated for 7 h, and examined for leukotoxic, hemolytic, and dermonecrotic activities. Leukotoxin activity was assayed with PMN of cattle, horses, sheep, pigs, and rabbits as target cells, effects of pH, temperature, chemical treatment, enzymatic treatment and protease inhibitors on leukotoxin activity were tested, and gel filtration was used to estimate molecular weight of leukotoxin. The leukotoxin titer of *F. necrophorum* subspecies *necrophorum* was higher than subspecies *funduliforme*, and leukotoxin of *F. necrophorum* was toxic to PMN of cattle and sheep. The optimal pH for leukotoxin activity was in range of 6.6 to 7.8; furthermore, reducing agents such as sodium sulfide inactivated the toxin, but did not inhibit leukotoxin production. Findings from gel filtration demonstrated that leukotoxin is a high molecular weight protein. Results also indicated that leukotoxin is a water-soluble and heat-labile exotoxin.

Narayanan et al. (2001) studied leukotoxin at the molecular level. They isolated the leukotoxin gene, and the leukotoxin gene open reading frame (ORF; *lktA*) was cloned and expressed. Data from Southern blot hybridization analysis illustrated differences in *lktA* hybridization patterns of subspecies *necrophorum* and *funduliforme*.

### *Importance of leukotoxin*

Saginala et al. (1997) determined the protection against liver abscesses offered by different doses of *F. necrophorum* leukotoxoid vaccine for cattle. Twenty-five steers were used for five treatments (control, 1.0, 2.0, 5.0 culture supernatant, and 2.5 concentrated supernatant). *F. necrophorum* strain A25 was used for preparation of vaccine. They observed higher antileukotoxin antibody titers for steers receiving vaccine than for the control group; furthermore, those steers which received a higher dose of vaccine had higher rate of protection against liver abscesses. These findings illustrate the importance of leukotoxin as an important virulence factor for *F. necrophorum*.

## *Synergistic interaction of F. necrophorum and A. pyogenes*

Tadepalli at al. (2009) stated that *Actinomyces pyogenes* is a gram-positive, pleomophic, rod-shaped, and a facultative anaerobic bacterium which is the second most frequently isolated bacteria from liver abscesses.

Takeuchi et al. (1983) studied the synergistic effect of *F. necrophorum* and *A. pyogenes* in induction of hepatic abscesses in mice. The objective was to understand the pathway of formation of liver abscesses, and pathogenic synergism of *F. necrophorum* and *A. pyogenes* in cattle by using mice as a model. *F. necrophorum* biotype A was inoculated intravenously or intraperitoneally into the 6 mice. Liver abscess formation in mice injected with a mixture of *F. necrophorum* and *A. pyogenes* was enhanced compared to the injection *F. necrophorum* alone. They assumed that *F. necrophorum* could inhibit phagocytosis by the host through exotoxin, to provide a safe living environment for *A. pyogenes*. In return, *A. pyogenes* utilizes oxygen to help establish *F. necrophorum* in the liver (Tadepalli et al., 2009).

## Susceptibility of F. necrophorum

### Tylosin

Tylosin is a macrolide antibiotic which is effective against gram-positive bacteria and some gram negative bacteria (*F. necrophorum*), and it is widely used as a feed additive to control liver abscesses in feedlot cattle. Tylosin is a natural fermentation product of *Streptomyces fradiae* with a molecular weight of 915 daltons. The mode of action of tylosin is to inhibit bacterial protein synthesis (Berg and Scanlan, 1982; Lechtenberg et al., 1998; Nagaraja and Chengappa, 1998; Wikipedia, 2014b).

Nagaraja et al. (1999) studied the effect of tylosin on *F. necrophorum* in cattle during an abrupt shift to a high-grain diet. Six ruminally cannulated steers were randomly assigned to either control (no tylosin) or tylosin (90 mg/d). *F. necrophorum* was isolated from ruminal contents and quantified by most-probable-number. Steers receiving tylosin had lower concentrations of *F. necrophorum* than the control group during feeding of a high-concentrate diet, and also their results showed that for the 31-d trial no *F. necrophorum* was resistant to tylosin, suggesting that tylosin might be a beneficial tool to control liver abscesses in cattle.

Nagaraja et al. (1999) studied the susceptibilities of bacterial flora of liver abscesses to tylosin and determined the minimum inhibitory concentration (MIC) of tylosin and other antimicrobial compounds. Liver abscesses were collected from tylosin-fed and non-tylosin-fed groups, bacterial flora were isolated and characterized, and antimicrobial susceptibility was determined. Results indicated a lower incidence of *F. necrophorum* subspecies *necrophorum* in mixed infection in the tylosin-fed group than in the non-tylosin-fed group. The MIC for the tylosin-fed group was 9.2 μg/mL and for the non-tylosin-fed group was 10.1 μg/mL, which was

not significantly different, suggesting no tylosin resistance in *F. necrophorum* (Nagaraja et al., 1999).

Elwakeel et al. (2013; experiment 6) tested tylosin on both subspecies of *F. necrophroum* grown with lactate or lysine as substrates to determine the MIC. Strains A21 and B35, which represent biotype A and B respectively, were used; the MIC was determined by micro-broth dilution. They observed that MIC for both subspecies was 25 μg/mL, whether they were grown in lactate or lysine as an energy substrate.

## CRINA® Ruminants

CRINA Ruminants is a commercially available blend of essential oils which includes thymol, eugenol, vanillin, guaiacol, and limonene on an organic carrier intended to improve rumen metabolism and animal performance (DSM Nutritional Products, Parsippany, NJ). There is no research available on the direct effect of CRINA Ruminants on *F. necrophorum* populations in the rumen. The product used in our research was CRINA-L, which contains extra limonene added to the original CRINA Ruminants product. However, there is little research that has studied the effect of CRINA Ruminants on rumen fermentation or liver abscesses.

Meyer et al. (2009) studied the effect of essential oils on rumen fermentation, diet digestibility and liver abscesses in finishing steers. Steers (n = 1,468) were randomly assigned to 5 treatments including: 1) control, no feed additive; 2) CRINA Ruminant, 1.0g/d; 3) experimental essential oil mixture (EXP), containing guaiacol, linalool, and α-pinene, 1.0 g/d; 4) CRINA Ruminants, 1.0 g/d, plus tylosin, 90 mg/d; 5) monensin, 300 mg/d, plus tylosin, 90 mg/d. Steers were slaughtered and liver abscess occurrences were recorded. CRINA Ruminants plus tylosin tended to reduce severe liver abscesses, whereas CRINA Ruminants alone tended to reduce total liver abscesses.

McIntosh et al. (2003) studied the effect of CRINA Ruminants on rumen microorganisms in pure-culture. They observed that CRINA Ruminants reduced the rate of deamination of amino acids with the effect on monensin sensitive bacteria, particularly hyper-ammonia producing bacteria; however, the range of effectiveness was small for most ruminant species.

Elwakeel et al. (2013; experiment 7) tested CRINA Ruminants with the same essential oil components used by McIntosh et al. (2003) to determine its effect on *F. necrophorum* growth. Concentrations of 20 or 100 μg/mL were tested on *F. necrophorum* strains A21 and B 35. Results indicated that CRINA Ruminants at 20 μg/mL did not inhibit growth of *F. necrophorum*. However, CRINA Ruminants at 100 μg/mL moderately inhibited both strains at 24 h but only inhibited A21 at 48 h.

## *Limonene*

Limonene is a hydrocarbon cyclic terpene that is abundant in lemons, oranges, and grapefruit (Castillejos et al., 2006; Wikipedia, 2014a). There is little research available on the effect of limonene on rumen microorganisms in vitro, moreover no in vivo research has been published; therefore one of our objectives was to study the effect of limonene on *F. necrophorum* in vivo.

Dorman and Deans (2000) tested effects of volatile oils from different plants and their components as antibacterial agents against various bacterial pathogens in vitro. Nine gram-positive and 16 gram-negative bacterial isolates were assessed. Limonene was toxic to gram-negative bacteria. In contrast, in vitro research conducted by Oh et al. (1967) to study the effect of essential oils from Douglas fir tree needles on rumen microorganism of sheep demonstrated no effect of limonene on rumen microbes.

Elwakeel et al. (2013) studied the effect of limonene on *F. necrophorum* growth in vitro. They tested 20 or 100 μg/mL of limonene on *F. necrophorum* strains A21 and B35. Limonene at 20 or 100 μg/mL significantly reduced growth by both strains of *F. necrophorum* at 24 and 48 h. These results suggested that limonene can be a promising tool to naturally reduce *F. necrophorum* and subsequently control liver abscesses.

## *Conclusions*

Liver abscesses are a big economical problem in the feedlot industry. *F. necrophorum* is a gram-negative anaerobe, and it is the primary bacterial pathogen that induces liver abscesses in cattle. *F. necrophorum* has 4 biovars in which biotype A is considered the most virulent subspecies. This bacterium utilizes lactate and lysine as sources of energy. Hemagglutinins, LPS, and leukotoxin are virulence factors of *F. necrophorum* which help bacteria to attach to the rumen wall and penetrate to portal blood and cause liver abscesses. Many studies have been conducted to evaluate means of reducing *F. necrophorum* populations in the rumen. Tylosin is an antibiotic active against gram-negative bacteria, and it is commonly used in feedlot diets to prevent liver abscesses by reducing *F. necrophorum*. CRINA Ruminant is a blend of essential oils which was assumed to have antimicrobial effects against *F. necrophorum* due to its components. Limonene is a hydrocarbon cyclic terpene active against gram-negative bacteria, and it has been shown to have a growth inhibiting effect on *F. necrophorum*.

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# Chapter 2 - Effect of Limonene on ruminal *Fusobacterium necrophorum* populations

## *Abstract*

Seven ruminally cannulated heifers approximately 225 kg initial BW were used in a $7 \times 4$ Youden square design to determine the effect of different levels of limonene on ruminal *Fusobacterium necrophorum* populations. Treatments included: 1) control, 2) limonene at 10 mg/kg diet DM, 3) limonene at 20 mg/kg diet DM, 4) limonene at 40 mg/kg diet DM, 5) limonene at 80 mg/kg diet DM, 6) CRINA-L (a blend of essential oil components) at 180 mg/kg diet DM, and 7) tylosin at 12 mg/kg diet DM. Each period included 11 d with 10 d washouts between periods. Samples were collected on d 0 (before treatment initiation), 4, 7, and 10 for measuring *F. necrophorum* by most probable number (MPN) method using selective culture medium. Results indicate that CRINA-L ($P = 0.52$) and tylosin ($P = 0.19$) did not affect ruminal *F. necrophorum* populations. CRINA-L significantly decreased $NH_3$ and 3-methyl butyrate, whereas tylosin significantly decreased propionate and increased both acetate and butyrate. Limonene linearly decreased ($P = 0.03$) *F. necrophorum* populations, and the optimal dietary concentration for limonene was 40 mg/kg DM. Limonene did not affect ruminal degradation rate for lysine, $NH_3$ concentration, or VFA profiles in rumen fluid. Limonene demonstrated usefulness in reducing ruminal concentrations of *F. necrophorum* suggesting that it may have potential to control liver abscesses, although further research will be needed to assess the effect of limonene under feedlot conditions.

## *Introduction*

Liver abscesses are a major economic problem in the feedlot industry, and prevalence is high when grain-based diets are fed (Nagaraja et al., 1996, Nagaraja and Lechtenberg, 2007). *Fusobaterium necrophorum* is the main pathogenic bacterium that causes liver abscesses and is also a normal inhabitant of the rumen (Tadepalli et al., 2009). *F. necrophorum* can attach to the rumen wall and penetrate from lesions on the rumen wall to gain access to the portal blood. Subsequently, *F. necrophorum* can reach the liver where they may cause abscesses (Jensen et al., 1954; Scanlan and Hathcock, 1983; Tadepalli et al., 2009).

Tylosin is a macrolide anitibiotic used frequently in feedlots to reduce prevalence of liver abscesses (Nagaraja et al., 1996). Although tylosin effectively prevents liver abscesses, it raises public concern as an antibiotic; therefore, scientists are looking for alternatives to satisfy the public view while meeting the needs of the beef industry. Essential oils and their major components, such as thymol and eugenol, have been investigated as an alternative to antibiotics for many years (Dorman and Deans, 1999; McIntosh et al., 2003), and it has been shown that they are active against gram-negative bacteria (Helander et al., 1998; Calsamiglia et al., 2007). CRINA Ruminants is a blend of essential oil components that contains thymol, guaiacol, eugenol, vanillin, and limonene on an organic carrier (DSM Nutritional Products Inc., Parsippany, NJ), and it has been claimed to improve rumen metabolism and animal performance. Limonene is an organic compound which is found in lemons, oranges, and grapefruit (Castillejos et al., 2006) and has been shown active mainly against gram-negative bacteria (Dorman and Deans, 2000). Elwakeel et al. (2013) studied the effect of limonene on *F. necrophorum* growth in vitro, but we are unaware of any in vivo evaluations of the effect of limonene on *F. necrophorum*. Therefore, our objective was to determine the effect of limonene, tylosin, and a

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blend of essential oil components containing 11% limonene on in vivo ruminal *F. necrophorum* populations in cattle. We also evaluated effects of these treatments on ruminal fermentation and ruminal lysine degradation.

## *Materials and Methods*

Procedures involving animal use were approved by the Kansas State University Institutional Animal Care and Use Committee.

### *Animals and Treatments*

Seven ruminally cannulated Holstein heifers (210 ± 10 d of age, and approximately 225 kg initial BW) were used in a 7 × 4 Youden square design (7 treatments, 7 heifers, 4 periods) to evaluate the effect of different levels of limonene on ruminal *Fusobacterium necrophorum* populations. Treatments were allocated so, in periods 2 through 4, each treatments followed 3 different treatments.Diet composition is presented in Table 2.1. All heifers were fed 5 kg of diet (as fed) daily, with equal amounts provided at 12-h intervals. One heifer received a diet with a higher concentration of alfalfa because it would not adequately consume the other diet. Treatments were mixed thoroughly into diets before feeding and included: 1) control; 2) 10 mg limonene/kg diet DM; 3) 20 mg limonene/kg diet DM; 4) 40 mg limonene/kg diet DM); 5) 80 mg limonene/kg diet DM); 6) 12 mg tylosin/kg DM (Tylan 40; Elanco, Animal Health, Indianapolis, IN); 7) 180 mg CRINA-L Ruminant/kg DM (DSM Nutritional Products AG; Kaiseraugst, Switzerland). The CRINA-L treatment provided 20 mg limonene/kg diet DM. The limonene was provided as a product containing 20% D-limonene added to a carrier (DSM Nutritional Products). The CRINA-L was formulated from CRINA Ruminant by increasing D-limonene in the product to 11%.

Heifers were housed in tie-stalls in a controlled environment room (22°C) with 16 h of lighting daily. Each period lasted 11 d with 10 d of washout between periods. One observation for the CRINA-L treatment was missed from the final period due to issues not related to treatment.

*Sample Collection and Analysis*

On d 0 (prior to initiation of treatments), 4, 7, and 10 of each period, 100 mL of ruminal fluid was collected before the morning feeding. Ruminal fluid pH was measured by pH meter (ATI, Orion, Boston, MA), then samples were immediately strained through 4 layers of cheesecloth into a 50-mL conical bottom tube and then transferred to the lab for enumeration of *F. necrophorum* populations. The procedure of Tan et al. (1994) was used for selection and enumeration of *F. necrophorum* with minor modification to the medium; $(NH_4)_2SO_4$ concentration was 0.45 g/L and $MgSO_4·7H_2O$ was 0.045 g/L. Most-probable-number (MPN) was used to determine the *F. necrophorum* concentrations following growth in selective medium and positive identification of indole production with Kovac' reagent (Sigma, St Louis, MO). The MPN were conducted in 96-well plates and were calculated from 8 rows of serial dilutions for each sample. In the first period, we used 0.1 mL of ruminal fluid in the first column and conducted 10/1 serial dilutions. In the remaining 3 periods, we used 0.2 mL of ruminal fluid in the first column and conducted 5/1 serial dilutions. The change in protocol was made because there were no positive wells at the higher dilutions in the first period, and the lower dilutions allowed for better sensitivity.

On d 10, ruminal fluid samples were collected at 2, 4, 6, 8, and 10 h after the morning feeding. From the fluid collected prior to feeding as well as at all time points after feeding, pH was measured and then 1 mL of rumen fluid strained through 4 layers of cheesecloth was mixed

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with 0.25 mL of 25% (wt/vol) meta-phosphoric acid and stored frozen for later analysis of $NH_3$ and organic acids. In addition, 10 mL of strained rumen fluid was mixed with 0.5 mL of 70% (wt/wt) perchloric acid and stored frozen for later peptide analysis.

On d 11, a 1-L solution of Cr-EDTA (2.83 g Cr) containing 38.2 g L-lysine-HCl (30 g lysine) was dosed through the ruminal cannula just before the morning feeding. At 3, 6, 9, and 12 h after dosing, 100 mL of ruminal fluid was collected and strained through 4 layers of cheesecloth. Strained ruminal fluid was stored frozen for later Cr analysis. In addition, 1 mL of strained rumen fluid was mixed with 0.25 mL of 25% (wt/vol) meta-phosphoric acid and then stored frozen for $NH_3$ analysis. A sample of strained ruminal fluid (0.75 mL) was mixed with 0.75 mL SeraPrep (Pickering Laboratories, Mountain View, CA) containing 1 m*M* norleucine and stored frozen for later analysis of lysine. Data from d 11 was used to estimate ruminal lysine disappearance rate, liquid dilution rate, and lysine degradation rate (Binnerts et al., 1968; Merchen, 1988).

### *Laboratory Analyses*

Thawed ruminal samples were prepared for analysis by centrifuging at $17,000 \times g$ for 15 min at $4^oC$ and collecting the supernatant. Concentrations of VFA and lactate were measured using gas chromatography (Column: 2 m $\times$ 2mm i.d. Carbopak B-DA [Supelco, Bellefonte, PA], injection temperature $200^oC$, detector temperature $200^oC$, oven temperature $175^oC$, flow rate: 24 mL/min of $N_2$). The procedure of Broderick and Kang (1980) was used to measure $NH_3$ concentrations. Automated trinitrobenzene sulfonic acid analysis (Palmer and Peters, 1969) was used to determine concentrations of ruminal α-amino N (AAN) before and after acid hydrolysis; peptide N was calculated as the increase in AAN upon hydrolysis (Ives et al., 2002). For analysis of lysine, samples were thawed and centrifuged ($13,800 \times g$; 10 min; $4^oC$), and the supernatant

was frozen for later lysine separation by cation exchange chromatography and measurement by fluorimetry after postcolumn *o*-phthalaldehyde derivitization (Beckman System Gold, Beckman, Palo Alto, CA). Concentrations of Cr were measured by atomic absorption spectrophotometry.

## *Calculations and Statistical Analyses*

***Most Probable Numbers (MPN) of F. necrophorum.*** The MPN data were log10 transformed prior to analysis to normalize the data. The analysis of variance was conducted using the Mixed Procedure of SAS. The model included fixed effects of period, treatment, day, and treatment × day. The log-transformed MPN from day 0 was included as a covariate. Heifer was included as a random effect. Day was considered as a repeated variable within each cell, and the covariance structure was compound symmetry. The autoregressive and unstructured covariance structures were also considered, but compound symmetry led to lower AIC. The denominator degrees of freedom were based on the Kenwood-Rogers method. Means were separated with contrasts for 1) control vs. tylosin, 2) control vs. CRINA-L, and 3 through 5) linear, quadratic, and cubic effects of limonene based on unequally spaced treatments. Outliers were removed when |studentized residuals| > 3. For MPN, there were 2 outliers (d 7 and 10 for a heifer receiving CRINA-L).

The pH data collected at the time of sampling for *F. necrophorum* were analyzed the same as for the MPN data. No outliers were observed for these ruminal pH data.

***Ruminal Characteristics on d 10.*** Data were analyzed similar to that for MPN, except time after feeding, rather than day, was the repeated measure. For all ruminal characteristics, the covariance structure used was compound symmetry, because compound symmetry led to lower AIC for more variables than autoregressive, and the unstructured option did not allow the model to converge for all characteristics. With the exception of molar percentage of acetate, no

treatment × hour interactions were observed; for acetate, evaluation of the patterns over time suggested that this interaction was trivial. Thus, only main effects of treatment are presented. Means were separated using the contrasts described above. Outliers were removed as follows: pH (0), ammonia (2; 1 for CRINA-L at 4 h after feeding, and 1 for 10 ppm limonene at 4 h after feeding), α-amino-N (3; 1 for control at 2 h after feeding, 1 for 10 ppm limonene at 2 h after feeding, and 1 for 10 ppm limonene [different cell than previous outlier] at 2 h after feeding), peptide N (4; 3 were the same as for α -amino-N, and 1 for CRINA-L at 2 h after feeding), and total organic acids (0). Outliers were not removed from the data for VFA profiles because removal of data for one acid would then impact all of the others. Concentrations of lactate were included in the calculation of total organic acids, but statistical analysis was not conducted for lactate because 86% of observations were below detection limits, only 7% were $> 1$ m$M$, and none were $> 5$ m$M$.

   ***Lysine Degradation on d 11.*** With the use of the NLIN Procedure of SAS, ruminal Cr concentrations for each heifer in each period were fit to the model: Cr concentration = Cr concentration at dosing × $e^{-kp \cdot t}$, where kp is the liquid passage rate and t = time after dosing. The concentration at dosing was used to calculate ruminal liquid volume as: Cr dose (g)/Cr concentration at dosing. Ruminal lysine concentration at dosing (included in data for calculating lysine disappearance) was calculated as: lysine dose/ruminal liquid volume. With the NLIN Procedure of SAS, ruminal lysine concentrations were fit to the model: Lysine concentration = lysine concentration at dosing (model calculated) × $e^{-kdis \cdot t}$, where kdis is the lysine disappearance rate and t = time after dosing. Lysine degradation rate was calculated as lysine disappearance rate minus liquid passage rate. Analysis of variance with the Mixed Procedure of SAS was then used to analyze the parameter estimates. The model included period and treatment as fixed

effects, and heifer was included as a random effect. Means were separated using the contrasts described above.

## *Results*

There were no significant interactions between time and treatment on d 10 (data in Table 2.3), except for molar proportion of acetate ($P = 0.02$). There were no significant interactions between sampling day and treatment for MPN ($P = 0.64$)

### *Effect of limonene*

Limonene linearly decreased ($P = 0.03$) ruminal *F. necrophorum* concentrations (Table 2.2). However, limonene did not have an effect on pH at time of sampling for *F. necrophorum* measurements (Table 2.2). On d 10, limonene tended to linearly reduce ($P = 0.07$) molar proportions of propionate and 2-methyl butyrate (Table 2.3). Furthermore, limonene linearly decreased ($P = 0.05$) valerate proportions. However, limonene had no effect on pH ($P \geq 0.20$) or concentrations of $NH_3$ ($P \geq 0.13$), peptide N ($P \geq 0.15$), α-amino N ($P \geq 0.13$), or total organic acids ($P \geq 0.34$). Molar proportions of acetate ($P \geq 0.14$), isobutyrate ($P \geq 0.34$), butyrate ($P \geq 0.10$), and 3-methyl butyrate ($P \geq 0.14$) in ruminal fluid were not affected by limonene (Table 2.3). On d 11, limonene did not have any effect on $NH_3$ concentrations ($P \geq 0.62$), lysine degradation rate ($P \geq 0.39$), lysine disappearance rate ($P \geq 0.14$), liquid passage rate ($P \geq 0.26$), or rumen liquid volume ($P \geq 0.24$; Table 2.4).

### *Effect of tylosin*

Tylosin had no effect on concentrations of *F. necrophorum* (Table 2.2). On d 10, tylosin tended to increase ($P = 0.08$) acetate proportions. Tylosin had a significant effect on propionate

and butyrate proportions. Propionate proportions were reduced by tylosin ($P = 0.01$), and proportions of butyrate were increased by tylosin ($P < 0.01$). Tylosin tended to increase ($P = 0.08$) acetate proportions. Tylosin did not affect pH ($P = 0.20$), or concentrations of $NH_3$ ($P = 0.49$), peptide N ($P = 0.88$), $\alpha$-amino N ($P = 0.62$), or total organic acids ($P = 0.58$). Molar proportions of isobutyrate ($P = 0.51$), 2-methyl butyrate ($P = 0.35$), 3-methyl butyrate ($P = 0.14$), and valerate ($P = 0.12$) in ruminal fluid were not affected by tylosin (Table 2.3). On d 11, tylosin did not have any effect on ($P \geq 0.27$) $NH_3$ concentrations, lysine degradation rate, lysine disappearance rate, liquid passage rate, or rumen liquid volume (Table 2.4).

### *Effect of CRINA-L*

*F. necrophorum* concentrations and ruminal pH at time of sampling were not affected by CRINA-L ($P = 0.33$; Table 2.2). On d 10, CRINA-L reduced ($P = 0.03$) $NH_3$ concentrations (Table 2.3). CRINA-L also decreased both isobutyrate ($P = 0.05$) and 3-methyl butyrate ($P < 0.01$) proportions (Table 2.3). CRINA-L did not affect pH ($P = 0.27$) or concentrations of peptide N ($P = 0.78$), $\alpha$-amino N ($P = 0.48$), total organic acids ($P = 0.67$). Molar proportions of acetate ($P = 0.73$), propionate ($P = 0.80$), butyrate ($P = 0.23$), 2-methyl butyrate ($P = 0.48$), and valerate ($P = 0.23$) in the rumen were not affected by CRINA-L. On d 11, CRINA-L had no effect on $NH_3$ concentrations ($P = 0.63$), lysine degradation rate ($P = 0.70$), lysine disappearance rate ($P = 0.40$), liquid passage rate ($P = 0.35$), or rumen liquid volume ($P = 0.77$; Table 2.4).

## *Discussion*

### *Effect of limonene*

Limonene is a monoterpene compound which is prevalent in lemons, oranges, and grapefruit (Castillejos et al., 2006). The alkenyl substituent of limonene is important to increase its antimicrobial activity against gram-negative bacteria (Dorman and Deans, 1999).

In our study, 4 levels of limonene were tested for the ability to modulate *F. necrophorum* populations in the rumen. Limonene linearly decreased *F. necrophorum* concentrations (log 10) in ruminal contents, and 40 mg/kg of limonene in the diet could be interpreted as the best concentration to reduce populations of *F. necrophorum* in our in vivo study.

Assuming instantaneous mixing of limonene from the diet with ruminal liquid, no degradation or ruminal absorption of limonene, and first order passage of limonene from the rumen at a rate equal to our observed average liquid passage rate, the average ruminal concentration of limonene was 6 mg/L for the treatment providing 40 mg/kg of limonene. This was based on: concentration at feeding = (dose of limonene fed/ruminal volume) + residual amount prior to feeding, where residual amount prior to feeding = $0.542 \times$ concentration after feeding, where $0.542 = (e^{-12 \times 0.051})$. The average concentration was calculated as the average of the concentrations directly after feeding and at 12 h after feeding, following adaptation.

Elwakeel et al. (2013) investigated the effect of limonene on *F. necrophorum* growth in vitro. In that study, limonene at concentrations of 20 or 100 mg/L inhibited the growth of *F. necrophorum* strains A21 and B35 at 24 h and 48 h when grown in pure cultures in brain heart infusion broth. Unpublished data from in vitro cultures of *F. necrophorum* biotypes A and B grown in brain heart infusion broth with limonene added in concentrations ranging up to 100 mg/L also demonstrated that limonene could inhibit *F. necrophorum* growth, with different *F.*

*necrophorum* strains showing some differences in susceptibility to limonene. Further unpublished data demonstrated that limonene at concentrations as low as 6 mg/L inhibited growth of *F. necrophorum* biotypes A and B at 12 h, whereas concentrations as low as 12 mg/L were inhibitory at 24 h. In that work, limonene at 6 to 24 mg/L demonstrated growth inhibition of *F. necrophorum* similar to that observed for tylosin at 3 to 12 mg/L. In addition, limonene at 50 and 500 mg/L decreased $NH_3$ production (6 and 15 %, respectively), suggesting that limonene has a toxic effect on $NH_3$ producing bacteria such as *F. necrophorum* (Castillejos et al., 2006).

*F. necrophorum* is known to be a primary lysine degrading bacteria, which can utilize lysine as a sole energy source while producing $NH_3$, acetate, and butyrate (Gharbia et al., 1989; Russell, 2006; Nagaraja and Lechtenberg, 2007; Elwakeel et al., 2013). In our study, limonene reduced *F. necrophorum* concentrations in rumen, but it did not affect lysine degradation rate (d 11), $NH_3$ concentration (d 10), or proportions of branched-chain VFA in ruminal fluid (d 10). It was hypothesized that by inhibiting growth of *F. necrophorum*, lysine degradation rate would be reduced, which consequently might decrease $NH_3$ production and ruminal concentrations; however, this effect was not evident. In agreement, Elwakeel et al. (2013) studied the effect of adding *F. necrophorum* to mixed ruminal cultures supplemented with lysine, and they found no increase in lysine degradation as measured by $NH_3$ production. One explanation for this might be that *F. necrophorum* may not be the primary lysine-degrading bacteria or that other lysine-fermenting bacteria compensated when *F. necrophorum* was decreased by limonene. This is the first in vivo study of limonene effects on ruminal *F. necrophorum* populations, and further research on a larger scale will be needed to investigate the effect of limonene on *F. necrophorum* and subsequently on liver abscesses.

### *Effect of CRINA-L*

Essential oil components, especially those with terpenoid and phenolic structures, have shown antimicrobial activity (Dorman and Deans, 1999). Some essential oil components are active against a wide range of bacteria, particularly gram-negative bacteria (Calsamiglia et al., 2007). Essential oils can inhibit the growth of bacteria by disintegrating the cell membrane (Helander et al., 1998; Dorman and Deans, 1999). The mode of action of terpenoid compounds has been attributed to the effect of isoprene units on cell membranes to inhibit electron and protein transport, phosphorylation reactions, and other important chemical reaction; these effects are related to the lipid characteristics and solubility of terpenoids in the phospholipid bilayer of the cell membrane (Dorman and Deans, 1997).

The mode of action of essential oils with phenolic structures, such as thymol and carvacrol, is related to reduction of intracellular ATP of gram-negative bacteria, leading to destruction of cell membrane activity (Helander et al., 1998). Furthermore, essential oils can inhibit bacterial growth through inhibition of RNA and DNA synthesis (Calsamiglia et al., 2007).

CRINA Ruminants (DSM Nutritional Products, Basel, Switzerland) is a commercial blend of essential oils containing thymol, guaiacol, eugenol, vanillin, and limonene. CRINA-L is a new product of the same company containing extra limonene (11%). Meyer et al. (2009) reported that CRINA Ruminants tended ($P = 0.08$) to reduce total liver abscesses. McIntosh et al. (2003) studied the effect of CRINA Ruminants product on dairy cattle, and demonstrated that blend of essential oil compounds could decrease $NH_3$ production by hyperammonia producing (HAP) bacteria. Attwood et al. (1998) classified *F. necrophorum* as an HAP bacteria, and therefore we can hypothesize that CRINA products might be able to inhibit growth of *F. necrophorum*. However, CRINA failed to inhibit *F. necrophorum* in vitro, probably because

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concentrations of limonene in the mixture of essential oil components was too low (Elwakeel et al., 2013). Of the 5 essential oil components found in CRINA Ruminants, only limonene at 20 or 100 mg/L and thymol at 100 mg/L were effective in inhibiting growth of *F. necrophorum* in vitro (Elwakeel et al., 2013).

In our study, we tested CRINA-L product, which was similar to CRINA Ruminants with addition of more limonene (11%). CRINA-L provided 20 mg limonene/kg DM. However, responses were different between the CRINA-L and 20 mg/kg limonene treatments; CRINA-L did not reduce ruminal prevalence of *F. necrophorum*, whereas limonene by itself did decrease ruminal *F. necrophorum* populations. On d 10, CRINA-L reduced $NH_3$ concentration compared to control ($P = 0.03$), which might be related to inhibition of ammonia-producing bacteria. In addition, this mixture of essential oil components reduced isobutyrate and 3-methyl butyrate concentrations, suggesting decreased catabolism of AA within the rumen. Our data suggest that these responses to CRINA-L may have been mediated by components of the mixture besides limonene because similar results were not obtained by limonene alone. As for limonene alone, CRINA-L did not affect rate of lysine degradation on d 11. Overall, CRINA-L should be considered as a rumen modifier, but little research has studied the effect of CRINA in feedlot conditions, and no research is available on the effects of CRINA-L. Therefore, further research is required to study the effects of this blend on animal performance or liver abscess incidence.

## *Effect of Tylosin*

Numerous studies have demonstrated that tylosin reduces prevalence of liver abscesses, increases feed efficiency, and increases weight gain (Brown et al., 1975; Potter et al., 1985; Vogel and Laudert, 1994). The reduction of liver abscesses is related to the reduction of *F.*

*necrophorum* concentrations in the rumen and liver (Nagaraja et al., 1996), and the mode of action of tylosin is to inhibit protein synthesis within bacteria (Tan et al., 1994).

In our study, tylosin surprisingly had no effect on ruminal *F. necrophorum* concentrations, and in fact led to numerical increases in *F. necrophorum*. These findings are not in agreement with those of Nagaraja et al. (1999), who investigated the susceptibilities of bacterial flora of liver abscesses to tylosin. Their results did not show any resistance of *F. necrophorum* to tylosin. Furthermore, our results do not match with findings in Elwakeel et al. (2013), who investigated the effect of tylosin on both biotype A and B of *F. necrophorum* in vitro and observed that tylosin was effective in inhibiting growth of *F. necrophorum*. One explanation for these differences might be that *F. necrophorum* in our cattle were resistant to tylosin. Our results were not in agreement with Nagaraja et al. (1999), which they reported no significant effect of tylosin on VFA. However, our results were in agreement with Nagaraja et al. (1999), in which tylosin did not have any effect on ruminal pH, and $NH_3$ concentrations.

## *Conclusion*

Our results demonstrate that limonene was effective in reducing ruminal concentrations of *F. necrophorum* in vivo, suggesting that it might be effective in reducing liver abscesses in feedlot conditions. This research is the first in vivo experiment with limonene supplementation to cattle, and further research will be beneficial to determine the effect of limonene on a larger scale.

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**Table 2.1 Diet compositions (% DM)**

| | % DM | |
| --- | --- | --- |
| Ingredient | 6 heifers | 1 heifer |
| Alfalfa hay | 28.3 | 46.2 |
| Molasses, cane | 2.7 | 2.0 |
| Dry rolled corn | 59.4 | 44.5 |
| Solvent soybean meal | 8.2 | 6.1 |
| Limestone, 38% Ca | 1.4 | 1.0 |
| Vitamins/Minerals | 0.12[1] | 0.09[2] |

[1] Provided (per kg diet DM): 1,906 IU vitamin A, 190 IU vitamin D, 36 IU vitamin E, 49 mg Mn, 49 mg Zn, 8 mg Cu, 0.11 mg Co, 0.52 mg I, and 0.1 mg Se.

[2] Provided (per kg diet DM): 1429 IU vitamin A, 142 IU vitamin D, 27 IU vitamin E, 37 mg Mn, 37 mg Zn, 6 mg Cu, 0.08 mg Co, 0.39 mg I, 0.08 mg Se.

**Table 2.2 Effect of dietary supplementation with limonene, CRINA-L, or tylosin on ruminal _F. necrophorum_ concentrations and pH in heifers before feeding on d 4, 7, and 10**

| | Treatment | | | | | | | | Contrast[1] _P_-value | | | | |
| | Limonene, mg/kg of diet DM | | | | | | | | | | | | |
| Item | Control | 10 | 20 | 40 | 80 | CRINA-L[2] | Tylosin[3] | SEM[4] | Lim-L | Lim-Q | Lim-C | CRINA-L | Tylosin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | 4 | 4 | 4 | 4 | 4 | 3 | 4 | | | | | | |
| Fn[5], log MPN | 2.65 | 2.29 | 2.51 | 2.15 | 2.18 | 2.49 | 2.90 | 0.15 | 0.03 | 0.22 | 0.95 | 0.52 | 0.19 |
| Ruminal pH | 6.43 | 6.22 | 6.30 | 6.28 | 6.34 | 6.26 | 6.56 | 0.13 | 0.98 | 0.36 | 0.46 | 0.33 | 0.37 |

[1] Lim-L = Linear effect of limonene. Lim-Q = Quadratic effect of limonene. Lim-C = Cubic effect of limonene. CRINA-L = Control vs. CRINA-L. Tylosin = Control vs. Tylosin.

[2] CRINA-L at 180 mg/kg diet DM.

[3] Tylosin at 12 mg/kg diet DM.

[4] Average of SEM among treatments.

[5] Ruminal concentration of _Fusobacterium necrophorum_, $\log_{10}$ of MPN/mL.

**Table 2.3 Effect of dietary supplementation with limonene, CRINA-L, or tylosin on ruminal pH, NH$_3$, α-amino N, peptide N, and organic acids on d 10 in heifers[1]**

| | | Treatment | | | | | | | Contrast[2] $P$-value | | | | |
| | | Limonene, mg/kg of diet DM | | | | | | | | | | | |
| Item | Control | 10 | 20 | 40 | 80 | CRINA-L[3] | Tylosin[4] | SEM[5] | Lim-L | Lim-Q | Lim-C | CRINA-L | Tylosin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | 4 | 4 | 4 | 4 | 4 | 3 | 4 | | | | | | |
| pH | 6.06 | 6.06 | 6.34 | 6.22 | 6.27 | 5.88 | 6.26 | 0.14 | 0.21 | 0.29 | 0.49 | 0.27 | 0.20 |
| | | | mM | | | | | | | | | | |
| NH$_3$ | 11.6 | 9.5 | 8.2 | 9.2 | 9.7 | 7.2 | 10.5 | 1.3 | 0.56 | 0.13 | 0.23 | 0.03 | 0.49 |
| α-amino N | 4.27 | 3.51 | 3.40 | 3.39 | 3.67 | 4.69 | 4.00 | 0.38 | 0.54 | 0.13 | 0.39 | 0.48 | 0.62 |
| Peptide N | 6.41 | 5.45 | 5.41 | 5.53 | 5.87 | 6.60 | 6.51 | 0.49 | 0.81 | 0.15 | 0.29 | 0.78 | 0.88 |
| Organic acids | 124 | 114 | 103 | 114 | 111 | 131 | 116 | 13 | 0.63 | 0.44 | 0.34 | 0.67 | 0.58 |
| | | | mol/100 mol | | | | | | | | | | |
| Acetate[6] | 54.3 | 58.8 | 58.9 | 57.7 | 59.1 | 53.3 | 59.2 | 2.0 | 0.25 | 0.38 | 0.14 | 0.73 | 0.08 |
| Propionate | 25.9 | 21.6 | 16.9 | 20.2 | 17.7 | 26.8 | 16.8 | 2.5 | 0.07 | 0.18 | 0.10 | 0.80 | 0.02 |
| Isobutyrate | 1.01 | 0.98 | 1.05 | 0.96 | 0.97 | 0.88 | 0.98 | 0.057 | 0.34 | 0.98 | 0.47 | 0.05 | 0.51 |
| Butyrate | 11.6 | 11.3 | 14.0 | 13.0 | 13.4 | 13.1 | 15.1 | 0.84 | 0.10 | 0.19 | 0.57 | 0.23 | <0.01 |
| 2-methyl butyrate | 4.36 | 4.73 | 6.51 | 5.95 | 6.41 | 3.54 | 5.36 | 0.76 | 0.07 | 0.23 | 0.45 | 0.48 | 0.35 |
| 3-methyl butyrate | 1.07 | 1.05 | 1.02 | 0.98 | 0.97 | 0.79 | 0.96 | 0.062 | 0.14 | 0.43 | 0.84 | ≤0.01 | 0.14 |
| Valerate | 1.64 | 1.37 | 1.26 | 1.36 | 1.19 | 1.41 | 1.36 | 0.13 | 0.05 | 0.33 | 0.12 | 0.23 | 0.10 |

[1] Values represent average of concentrations at 0, 2, 4, 6, 8, and 10 h after feeding. Except for acetate, no treatment $\times$ hour interactions were observed.

[2] Lim-L = Linear effect of limonene. Lim-Q = Quadratic effect of limonene. Lim-C = Cubic effect of limonene. CRINA-L = control vs. CRINA-L. Tylosin = Control vs. Tylosin.

[3] CRINA-L at 180 mg/kg diet DM.

[4] Tylosin at 12 mg/kg diet DM.

[5] Average of SEM among treatments

[6] Treatment $\times$ hour interaction ($P = 0.02$)

**Table 2.4 Effect of dietary supplementation with limonene, CRINA-L, or tylosin on $NH_3$, lysine degradation and disappearance rates, liquid passage rate, and rumen liquid volume on d 11 in heifers following ruminal dosing of 30 g L-lysine[5]**

| Item | Treatment | | | | | | | | Contrast[1] $P$-value | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Limonene, mg/kg of diet DM | | | | | | | | | | | |
| | Control | 10 | 20 | 40 | 80 | CRINA-L[2] | Tylosin[3] | SEM[4] | Lim-L | Lim-Q | Lim-C | CRINA-L | Tylosin |
| N | 4 | 4 | 4 | 4 | 4 | 3 | 4 | | | | | | |
| $NH_3$, mM | 9.8 | 9.7 | 9.7 | 10.0 | 9.3 | 9.3 | 11.2 | 1.1 | 0.62 | 0.79 | 0.71 | 0.63 | 0.36 |
| Lysine disappearance rate, $h^{-1}$ | 0.264 | 0.266 | 0.232 | 0.244 | 0.218 | 0.234 | 0.285 | 0.023 | 0.14 | 0.82 | 0.68 | 0.40 | 0.53 |
| Liquid passage rate, $h^{-1}$ | 0.055 | 0.052 | 0.045 | 0.057 | 0.034 | 0.038 | 0.073 | 0.012 | 0.26 | 0.58 | 0.40 | 0.35 | 0.27 |
| Lysine degradation rate, $h^{-1}$ | 0.210 | 0.215 | 0.187 | 0.186 | 0.184 | 0.195 | 0.211 | 0.025 | 0.39 | 0.62 | 0.96 | 0.70 | 0.97 |
| Rumen volume, L | 23.0 | 26.4 | 25.7 | 23.4 | 27.0 | 24.2 | 20.8 | 2.83 | 0.50 | 0.82 | 0.24 | 0.77 | 0.54 |

[1] Lim-L = Linear effect of limonene.  Lim-Q =  Quadratic effect of limonene. Lim-C = Cubic effect of limonene. CRINA-L = Control vs. CRINA-L. Tylosin = Control vs. Tylosin.

[2] CRINA-L at 180 mg/kg diet DM.

[3] Tylosin at 12 mg/kg diet DM.

[4] Average of SEM among treatments.

[5] Samples were collected at 3, 6, 9, and 12 h after dosing