In vitro degradation of lysine by ruminal fluid-based fermentations and by *Fusobacterium necrophorum*¹

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**ABSTRACT**

The objective of these studies was to characterize some factors affecting lysine degradation by mixed ruminal bacteria and by ruminal *Fusobacterium necrophorum*. Mixed ruminal bacteria degraded lysine, and addition of pure cultures of *F. necrophorum* did not increase lysine degradation. Addition of acetic or propionic acid strikingly reduced NH₃ production from lysine by mixed ruminal bacteria at pH 6, but not at pH 7. Although typical ruminal environments with acidic pH and normal concentrations of volatile fatty acids might inhibit lysine degradation by *F. necrophorum*, ruminal fluid contained enough bacteria with a lysine-degrading capacity to ferment 50 mM lysine in vitro. Of 7 strains of ruminal *F. necrophorum* tested, all grew on both lactate and lysine as the primary energy source. Both subspecies of ruminal *F. necrophorum* (necrophorum and funduliforme) used lysine as a primary C and energy source. Lysine and glutamic acid were effectively fermented by *F. necrophorum*, but alanine and tryptophan were not, and histidine and methionine were fermented only to a minor extent. The end products of lactate fermentation by *F. necrophorum* were propionate and acetate, and those of lysine degradation were butyrate and acetate. Fermentation of glutamic acid by *F. necrophorum* yielded acetate and butyrate in a ratio near to 2:1. The minimum inhibitory concentration of tylosin for *F. necrophorum* was not dependent on whether bacteria were grown with lactate or lysine, but *F. necrophorum* was more susceptible to monensin when grown on lysine than on lactate. Although *F. necrophorum* is generally resistant to monensin, the ionophore may reduce lysine degradation by *F. necrophorum* in the rumen. The essential oil components limonene, at 20 or 100 μg/mL, and thymol, at 100 μg/mL, inhibited *F. necrophorum* growth, whereas eugenol, guaiacol, and vanillin had no effect. Our findings may lead to ways to minimize ruminal lysine degradation and thus increase its availability to the animal.

**Key words:** *Fusobacterium necrophorum*, lysine, rumen

**INTRODUCTION**

Ruminal bacteria responsible for AA degradation are broadly grouped into bacteria that are numerically abundant but have low activity and bacteria that have high activity (hyper-NH₃-producing bacteria) but are relatively low in number, and only a few species of ruminal bacteria have been identified as hyper-NH₃-producing bacteria (Russell et al., 1991; Wallace et al., 1997). In addition to deamination abilities, hyper-NH₃-producing bacteria can utilize peptides, but they are considered specialists in the rumen because they are not proteolytic and do not ferment carbohydrates.

*Fusobacterium necrophorum*, a gram-negative and rod-shaped organism, was initially identified as a lactate-utilizing bacterium in the rumen. Two subspecies of *F. necrophorum* are recognized, namely, ssp. *necrophorum* (biotype A) and ssp. *funduliforme* (biotype B), which are distinguishable by morphological, biochemical, and molecular characteristics (Tadepalli et al., 2009). *Fusobacterium necrophorum* is present in high numbers (up to 10⁷/mL) in the rumen, generally about 10- to 100-fold higher in grain-fed than forage-fed cattle (Tan et al., 1994c), and it is a proteolytic AA fermenter with low NH₃-producing activity. Gharbia and Shah (1989) reported that *F. necrophorum* extensively utilized lysine, arginine, histidine, glutamate, serine, threonine, and asparagine; partially used aspartate, methionine, and ornithine; but did not extensively use glycine, cysteine, tyrosine, or alanine. However, Attwood et al. (1998) reported that a strain isolated from mixed rumen fluid enriched with tryptone and casamino acids was a hyper-NH₃-producing bacterium. Russell (2005) isolated *F. necrophorum* from ruminal fluid enriched with lysine as the sole energy source and suggested that *F. necrophorum* plays an important role in ruminal lysine degradation.
*Fusobacterium necrophorum* is susceptible to tylosin, a macrolide antimicrobial agent that is widely used to prevent liver abscesses in feedlot cattle (Nagaraja and Chengappa, 1998). The organism is generally resistant to the ionophore antibiotic monensin (Tan et al., 1994a), but monensin can reduce lysine degradation by *F. necrophorum* by inhibiting lysine uptake (Russell, 2006). The effect of essential oils on growth and activities of *F. necrophorum* has not been evaluated directly.

A better understanding of the ruminal bacterial degradation of lysine, particularly with regard to the role of *F. necrophorum*, could lead to methods to control lysine degradation in the rumen. The objectives of this study were to identify some factors (pH, and acetate and propionate) affecting lysine degradation by mixed ruminal bacteria and by *F. necrophorum*, as well as to evaluate the effects of essential oil components (limonene, thymol, eugenol, guaiacol, and vanillin) and antibiotics (monensin and tylosin) on lysine degradation by *F. necrophorum*.

**MATERIALS AND METHODS**

**Lysine and AA Degradation by Mixed Ruminal Bacteria**

Three experiments were conducted to evaluate lysine degradation by ruminal fluid-based fermentations in vitro. Experiments evaluated additions of pure cultures of *F. necrophorum* (experiment 1) and alterations in pH and VFA concentrations (experiment 2) on lysine degradation, as well as degradation of glutamate and alanine (experiment 3).

**General Description of In Vitro Fermentations in Experiments 1 Through 3.** Ruminal contents were collected before the morning feeding from 2 ruminally cannulated Holstein dairy cows fed a typical dairy diet (Table 1). The use of cattle was approved by the Kansas State University Institutional Animal Care and Use Committee. Ruminal contents were filtered through 4 layers of cheesecloth and transported in thermally insulated containers to the laboratory. Rumen fluids were centrifuged at 150 × g for 5 min to precipitate protozoa and feed particles, and the supernatants were used as the ruminal bacterial inocula.

Ruminal bacterial inoculum (10 mL) and McDougall’s buffer (40 mL; McDougall, 1948) were dispensed into 60-mL serum bottles or 100-mL centrifuge tubes. For each treatment, duplicate fermentations for each of the 2 rumen fluid sources were inoculated under anaerobic conditions using O2-free CO2. Containers were capped with butyl rubber stoppers fitted with Bunsen valves, vortexed, and incubated at 39°C for 48 h.

Samples were collected over 48 h (at 12-h intervals for experiments 1 and 3, and at 24-h intervals for experiment 2) by removing 1 mL of fermentation fluid and transferring it to a microcentrifuge tube, acidifying it with 200 μL of 1 M HCl, and then freezing it at −20°C. After thawing, acidified samples were centrifuged at 30,000 × g for 15 min at 4°C and analyzed for NH3 (Broderick and Kang, 1980).

**Preparation of *F. necrophorum* Cultures.** *Fusobacterium necrophorum* ssp. *necrophorum* (strains A21, A27, and A29) and *F. necrophorum* ssp. *funduliforme* (strains B33, B34, B35, and B36), all of ruminal origin (Tan et al., 1994c), were used. The cultures were streaked on blood agar plates (Remel Inc., Lenexa, KS) and incubated for 48 h at 39°C in an anaerobic glove box (10% H2, 10% CO2, 80% N2; Forma Scientific Inc., Marietta, OH). The species and subspecies of strains were reconfirmed with a commercial identification kit (RapID ANA II System; Innovative Diagnostic Systems Inc., Atlanta, GA). A single colony from each plate was inoculated into 10 mL of prereduced (with 0.05% cysteine-HCl) and anaerobically sterilized brain heart infusion broth (PRAS-BHI; Becton Dickinson, Sparks, MD) and incubated at 39°C for 16 h. A 100-μL quantity of the culture was then inoculated into 10 mL of PRAS-BHI broth and incubated at 39°C for 4 to 7 h (absorbance at 600 nm near 0.6 to 0.65) and used as inoculum. The inoculum generally contained 1 to 5 × 109 cfu/mL. The purity of the inocula was verified by microscopic examination of Gram-stained smears.

**Experiment 1: Effect of Addition of *F. necrophorum* on In Vitro Lysine Degradation by Mixed Ruminal Bacteria.** This study was conducted to determine if addition of pure cultures of

| Table 1. Composition of the diet fed to cows serving as rumen fluid donors (experiments 1, 2, and 3) |
|---------------------------------|----------------|
| Ingredient                      | Amount, % of DM |
| Alfalfa hay                     | 16.5            |
| Corn silage                     | 20.8            |
| Wet corn gluten feed           | 31.9            |
| Cottonseed                     | 5.3             |
| Ground corn                    | 17.0            |
| Mechanically extracted soybean meal | 4.5          |
| Fish meal                      | 0.3             |
| Minerals-vitamins              | 2.5             |
| Rumen bypass fat               | 0.8             |
| Molasses                       | 0.3             |
| Yeast1  | 0.2          |
| Rumensin2                       | +               |

1Culture of *Saccharomyces cerevisiae* (Diamond V XP; Diamond V, Cedar Rapids, IA).
2Provided 10 mg of monensin/kg of diet DM (Rumensin; Elanco Animal Health, Greenfield, IN).
F. necrophorum to in vitro fermentations with mixed ruminal bacteria increased the degradation of lysine. Treatments were arranged as a 2 × 3 factorial and included 2 lysine concentrations (0 or 50 mM added as free l-lysine, >97%; Sigma-Aldrich, St. Louis, MO) and 3 inocula of Fusobacterium strains (none, 1 to 5 × 10^9 cfu of F. necrophorum sps. necrophorum strain A21, and 1 to 5 × 10^9 cfu of F. necrophorum sps. funduliforme strain B35).

Experiment 2: Effects of pH and Addition of Acetate or Propionate on Lysine Degradation. In this experiment, all fermentations contained 50 mM lysine. Treatments were arranged as a 2 × 3 factorial and included 2 initial pH (6 or 7) and 3 VFA additions (none, 50 mM acetate, or 50 mM propionate). Stock solutions of acetate and propionic acids were adjusted to pH 7 with NaOH before addition. To study the effect of pH, McDougall’s buffer was prepared with pH 7.0 or with pH adjusted to 6.0 with HCl.

Experiment 3: Fermentation of Alanine and Glutamate by Mixed Ruminal Bacteria. For this experiment, fermentations with mixed ruminal bacteria contained no supplemental AA or they contained 50 mM L-alanine, 25 mM L-lysine, or 50 mM L-lysine.

Lysine and AA Degradation by F. necrophorum Strains

Two experiments were conducted to evaluate lysine and AA degradation by pure cultures of F. necrophorum in vitro. Experiments measured the growth of 7 ruminal strains of F. necrophorum (sps. necrophorum and sps. funduliforme) on lysine with or without lactate (experiment 4) and fermentation of various AA by 2 strains of F. necrophorum (experiment 5).

Methods for Experiments 4 and 5. Basal medium for growing Fusobacterium was made according to Russell (2005) and contained (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of (NH_4)_2SO_4, 480 mg of NaCl, 100 mg of MgSO_4·7 H_2O, 64 mg of CaCl_2·2 H_2O, 1 g of Trypticase (Becton Dickinson), 0.5 g of yeast extract, and 4 g of Na_2CO_3. The medium (10 mL) was dispensed into Hungate tubes, was prereduced with cysteine-HCl (0.06%) and anaerobically sterilized. Experiments were run twice, and treatments within each run were conducted in duplicate tubes.

Tubes were inoculated anaerobically and incubated for 48 h at 39°C. Absorbance at 600 nm was measured every 12 h to assess growth. After 48 h, 1-mL samples for NH_3 and VFA analysis were acidicified with 250 μL of 25% (wt/vol) m-phosphoric acid and stored in a freezer at −20°C. Upon thawing, samples were centrifuged at 30,000 × g for 15 min at 4°C, and supernatants were analyzed for NH_3 (Broderick and Kang, 1980). Supernatants were also analyzed for VFA and lactate concentration by GLC (model 5890A; Hewlett-Packard, Palo Alto, CA) after 0.4 mL of supernatant was neutralized with 0.02 mL of 6 M NaOH and then mixed with 1.2 mL of a solution containing 4 mM pivalic acid (internal standard), 0.04 M oxalic acid, and 0.23 M formic acid. For GLC, the injection temperature was 200°C and the packed column (2 m × 2 mm; 4% Carbowax 20M on 80/120 Carbopack B-DA; Supelco, Bellefonte, PA) was maintained at 175°C. The carrier gas was He at a flow rate of 24 mL/min, and total run time was 20 min.

Experiment 4: Growth of F. necrophorum Strains on Lysine or Lactate. Seven strains of F. necrophorum (sps. necrophorum and sps. funduliforme) were grown in basal medium with lysine (50 mM), lactate (50 mM), or lysine plus lactate (50 mM each) as the growth substrate. Substrates were dissolved in the basal medium and adjusted to pH 7.0 before prereducing and autoclaving the medium. Seven strains (0.1 mL of 6-h cultures as described before) of ruminal F. necrophorum (sps. necrophorum strains A21, A27, and A29 and sps. funduliforme strains B33, B34, B35, and B36) were inoculated into the media containing lysine, lactate, or lysine plus lactate.

Experiment 5: Growth of F. necrophorum Strains on Various AA. Basal medium containing 50 mM of L-tryptophan, L-alanine, L-glutamic acid, L-methionine, L-histidine (all cell cultures tested; Sigma-Aldrich), or L-lysine (>97%; Sigma-Aldrich) were prepared as before. Tubes were inoculated with 0.1 mL of 6-h cultures of ruminal F. necrophorum strain A21 or B35.

Experiment 6: MIC of Monensin and Tylosin Against F. necrophorum Grown with Lactate or Lysine as the Energy Source

Fusobacterium necrophorum strains A21 and B35 were used to determine MIC of tylosin and monensin. The MIC was determined by micro-broth dilution (CLSI, 2008) in 96-well microtiter plates (Falcon Microtest U-Bottom with a low evaporation lid; Becton Dickinson). Stock solutions (1 mg/mL) of tylosin and monensin were prepared in water and methanol, respectively. The concentrations of each antimicrobial agent for MIC determinations ranged from 50 to 0.097 μg/mL (Lechtenberg et al., 1998).

Dilutions, inoculations, and incubations (39°C for 48 h) were conducted in an anaerobic glove box. Data were recorded as growth (turbidity or formation of bacterial pellet) or no growth [no red color formation after addition of Kovac’s reagent (Becton, Cockeysville, MD), indicating the absence of indole production; Tan et al.,
1994b]. The MIC was determined as the lowest concentration of the antimicrobial agent at which no visible bacterial growth was observed. Within a run, duplicate rows for each strain (A21 and B35) and antimicrobial agent (tylosin or monensin) combination were used on each plate, and separate plates were used for each substrate (lysine or lactate). The MIC determinations were performed 4 times.

Experiment 7: Effect of Essential Oil Components on the Growth of F. necrophorum

Effects of 5 different essential oil components (eugenol, vanillin, thymol, guaiacol, limonene) tested individually and of CRINA (DSM Nutritional Products, Basel, Switzerland), a commercially available mixture of the 5 essential oil components, on the growth of F. necrophorum were determined. Stock solutions of essential oil components were prepared to contain 2 or 10 mg/mL in methanol. Just before the inoculation of 10 mL of PRAS-BHI broth in Hungate tubes with F. necrophorum, 100 μL of stock solutions of essential oil components were pipetted to provide 20 or 100 μg/mL. The tubes were then inoculated with 0.1 mL of 4-h cultures of F. necrophorum strains A21 and B35 prepared as before. The inoculations were performed in an anaerobic glove box. Duplicate tubes were incubated for 48 h at 39°C, and absorbance at 600 nm was measured at 24 and 48 h.

Statistical Analyses

Data were analyzed by ANOVA for complete block designs, with rumen fluid donor or replicate as the blocking factor, by using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Model effects included block and treatments (plus all treatment interactions, when appropriate). For experiment 4, the effects of strain were separated into effects of subspecies and of strain within subspecies. For observations collected over time, analyses were for repeated measures, with time and all interactions of time with treatments included in the model.

RESULTS AND DISCUSSION

Lysine and AA Degradation by Mixed Ruminal Bacteria

Experiment 1: Effect of Addition of F. necrophorum on In Vitro Lysine Degradation by Mixed Ruminal Bacteria. Ammonia concentrations increased (P < 0.01) over time when lysine was included (Figure 1), indicating lysine degradation.

Ammonia concentration increased between 12 and 24 h and peaked at 36 h. At 36 h, NH₃ concentrations were 78 mM greater for tubes containing lysine than for those without lysine. No lysine degradation occurred in the first 12 h of incubation, and this might reflect catabolite repression. A total of 100 mM of NH₃ is expected from complete degradation of 50 mM lysine. The less than complete recovery of lysine-N as NH₃ likely reflects incorporation of N into the microbial cell mass in response to the energy provided by the lysine, although incomplete degradation of lysine cannot be excluded. Addition of F. necrophorum strains A21 or B35 did not increase NH₃ concentration, indicating no effect on lysine degradability (Figure 1).

In our study, an almost complete degradation of lysine was observed by 36 h without addition of F. necrophorum to the fermentation, in contrast to the poor degradation of 50 mM lysine by mixed ruminal microbes reported by Russell (2005). However, when Russell (2005) inoculated F. necrophorum in incremental concentrations to a rumen fluid-based fermentation enriched with lysine (50 mM), a linear increase in NH₃ concentration was obtained, and almost complete degradation of lysine was observed at 24 h when F. necrophorum was added. Further work by Russell (2006) demonstrated that ruminal fluid at more than 20% of the total fermentation volume inhibited lysine degradation; this effect apparently was reversed by the addition of F. necrophorum to the culture. Thus, the
poor degradation of lysine by mixed ruminal bacteria in the study by Russell (2005) might reflect inhibition by the ruminal fluid (possibly related to pH and VFA concentrations; Russell, 2006).

On the basis of the procedure by Russell (2006), our fermentations contained only 20% of the total fermentation volume as ruminal fluid, which should have prevented the inhibition of lysine degradation by ruminal fluid. Under our conditions, adequate amounts of \( F. \) necrophorum (or other lysine-degrading bacteria) appear to have been present in the ruminal fluid to support extensive degradation of lysine; thus, increases in lysine degradation were not observed by addition of \( F. \) necrophorum cultures.

**Experiment 2: Effects of pH and Addition of Acetate or Propionate on Lysine Degradation.** Ammonia produced from lysine fermentation was greater \((P < 0.01)\) at pH 6 compared with pH 7 in the absence of added acetate or propionate (Table 2). Adding acetate or propionate reduced \( \text{NH}_3 \) concentration strikingly at pH 6 \((P < 0.01)\), but not at pH 7 \((P \geq 0.17)\). Increasing initial pH from 6 to 7 increased \((P < 0.05)\) pH at 48 h only from 7.01 to 7.22. Clearly, our buffer system did not maintain the targeted pH throughout the fermentation, although the pH treatments did affect fermentation. The pH of the rumen fluids that served as sources of bacterial inocula were 5.66 and 6.89.

Because the response to low pH and organic acids was similar to that of Russell (2006) with cultures of \( F. \) necrophorum, much of the lysine degradation in our fermentations appeared to be due to the activity of \( F. \) necrophorum. However, other lysine-degrading organisms could be similarly affected by \( \text{pH} \) and organic acids; fermentation acids can inhibit many bacteria at low \( \text{pH} \) (Russell and Diez-Gonzalez, 1998).

Russell (2006) reported that the addition of sodium acetate in concentrations of 20 to 100 m\( M \) reduced lysine degradation by \( F. \) necrophorum more at pH 6.1 than at pH 6.6, and that reducing the pH of continuous cultures of \( F. \) necrophorum from 6.7 to 5.5 by using HCl reduced the degradation of lysine, which was the energy substrate. Although \( F. \) necrophorum is sensitive to acidic conditions (Russell, 2006), this bacterium is a lactate fermenter that increases in the rumen of animals fed grain-based diets (Tan et al., 1994c).

Our fermentations were conducted with mixed ruminal bacteria, so our responses to changes in \( \text{pH} \) and VFA could reflect their effects on myriad organisms or the indirect effect of other organisms or their end products on \( F. \) necrophorum. Russell (2005) reported that the rate of lysine transport by \( F. \) necrophorum decreased when \( \text{pH} \) was reduced with HCl and that transport of lysine was negligible at pH 5; however, in later research, Russell (2006) suggested that the negative effect of low \( \text{pH} \) in the presence of VFA was a general effect on growth of \( F. \) necrophorum and was not specifically related to lysine transport.

**Experiment 3: Fermentation of Alanine and Glutamate by Mixed Ruminal Microbes.** Ammonia production from alanine, glutamate, and lysine increased \((P < 0.01)\) over time (12 through 48 h; Figure 2). The greater concentration of \( \text{NH}_3 \) at 48 h for 50 m\( M \) lysine compared with 50 m\( M \) alanine or 50 m\( M \) glutamate likely reflects the greater amount of N present in lysine than in glutamate or alanine. The \( \text{NH}_3 \) concentration produced from glutamate (50 m\( M \)) fermentation was greater at 12, 24, and 36 h of fermentation than that from alanine (50 m\( M \)) or lysine (25 m\( M \)).

Through 36 h, \( \text{NH}_3 \) production from fermentations containing 25 m\( M \) lysine and from those containing 50 m\( M \) lysine were generally similar (Figure 2). Thus, zero-order kinetics were observed with regard to lysine concentration, suggesting that the lysine-degrading capabilities of the ruminal bacteria were saturated by as little as 25 m\( M \) lysine. The greater \( \text{NH}_3 \) production at 48 h from 50 m\( M \) lysine relative to 25 m\( M \) lysine reflects that, by 36 h, the lysine concentration in fermentations

| Table 2. Ammonia concentrations at 24 or 48 h and \( \text{pH} \) at 48 h from in vitro fermentation of 50 m\( M \) lysine by ruminal bacteria with initial culture \( \text{pH} \) of 6 or 7 and without or with addition of 50 m\( M \) acetate or 50 m\( M \) propionate (experiment 2) \( ^1 \) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Substrate**   | **Initial \( \text{pH} \)** | **24-h fermentation** | **48-h fermentation** | **Final \( \text{pH} \)** |
| Lysine          | 6               | 20.6            | 67.1            | 6.81            |
| Lysine          | 7               | 6.0             | 54.5            | 7.21            |
| Lysine + acetate| 6               | 3.7             | 13.1            | 7.09            |
| Lysine + acetate| 7               | 10.3            | 47.4            | 7.23            |
| Lysine + propionate | 6          | 2.9             | 9.6             | 7.13            |
| Lysine + propionate | 7          | 12.5            | 40.0            | 7.24            |
| **SEM**         |                 | 2.8             | 0.96            |                 |

\(^1\) For \( \text{NH}_3 \), effects were observed for substrate, initial \( \text{pH} \), time, and all interactions \((P < 0.05)\). For \( \text{pH} \), effects were observed for substrate and initial \( \text{pH} \) \((P < 0.05)\).
that began with only 25 mM lysine was reduced below saturating concentrations.

The temporal pattern of NH₃ production from lysine in this experiment was generally similar to that in experiment 1. In both experiments, little NH₃ was produced from the lysine-containing fermentations by 12 h, but linear increases in NH₃ production were observed between 12 and 36 h. In experiment 1, however, most of the lysine degradation was completed by 36 h, whereas in experiment 3, for fermentations initially containing 50 mM lysine, the NH₃ production by 36 h accounted for less than half of the initial lysine-N. Possible explanations for the slower fermentation in experiment 3 are that concentrations of lysine-deaminating organisms may have differed between inocula or some minor differences in fermentation conditions may have caused lysine degradation to occur at a slower rate in experiment 3.

**Conclusions from Fermentations with Mixed Ruminal Bacteria.** Our observations regarding the fermentation of large concentrations of lysine and other AA (alanine or glutamate) by mixed ruminal bacteria were consistent with the observation that *F. necrophorum* is the primary ruminal bacterium responsible for lysine degradation. The lack of response to addition of *F. necrophorum* into mixed ruminal bacteria suggests that *F. necrophorum* were already present in the rumen fluid in adequate concentrations to support the observed lysine degradation. Ruminal degradation of lysine was inhibited by acetate or propionate, particularly at acidic initial pH, which is consistent with the observations of Russell (2006) with *F. necrophorum*. Moreover, the ruminal fermentations were capable of degrading lysine and glutamic acid more rapidly than alanine. Gharbia and Shah (1989) did not observe the utilization of alanine by *F. necrophorum* of human origin (Gharbia and Shah, 1989). Degradation of lysine and glutamate by *F. necrophorum* but alanine degradation by bacteria other than *F. necrophorum* might explain the lag time associated with alanine degradation in experiment 3 (Figure 2). Given the similarities between our observations of lysine degradation by ruminal bacteria and previous research with *F. necrophorum*, studying *F. necrophorum* in pure culture with more defined media has potential to provide more specific information about ruminal lysine degradation.

**Lysine and AA Degradation by F. necrophorum**

**Experiment 4: Growth of F. necrophorum Strains on Lysine or Lactate.** Both *F. necrophorum* ssp. *necrophorum* and *F. necrophorum* ssp. *funduliforme* had the ability to grow on both lactate and lysine. For both subspecies, the greatest accumulation of cell mass was observed for lactate plus lysine (Figure 3), which likely reflects the provision of more total substrate. In most cases, growth on lactate was complete by 12 h, whereas the growth curve was more extended when lysine was the substrate, with maximal optical density typically near 36 h. No difference was detected among *F. necrophorum* strains for their growth on the 3 substrates (strain within subspecies, *P* = 0.27; strain within subspecies × substrate, *P* = 0.99). Some differences between subspecies were observed for growth rate as indicated by *F. necrophorum* ssp. *funduliforme* having greater optical density at 12 h, whereas *F. necrophorum* ssp. *necrophorum* had greater optical density at 24, 36, and 48 h (subspecies × time, *P* < 0.01).

The growth data clearly show the ability of both subspecies of *F. necrophorum* (*necrophorum* and *funduliforme*) to utilize lysine as the sole energy source. The greater accumulation of cell mass when grown on lactate compared with lysine could be related to the difference in ATP yield as a result of their fermentation, differences in transport rate, or other unknown factors. The ability of other *Fusobacterium* spp. to utilize AA as an energy source has been reported previously (Gharbia and Shah, 1989). In addition, our results agree with the complete utilization of lysine by *F. necrophorum* observed by Russell (2005), who isolated 2 strains of *F. necrophorum* (JB2 and JB7) from rumen fluid by enrichment with lysine and Trypticase, with JB2 having characteristics similar to *F. necrophorum* ssp. *funduliforme*.

Clear differences among substrates were observed for end products. The greatest NH₃ concentration was observed for lysine, followed by lactate plus lysine (Table 3). The lower concentration of NH₃ with lactate as the growth substrate was expected because of the absence of N in lactate. The lower concentration of NH₃ for lac-
tate plus lysine than for lysine alone is likely reflective of a greater uptake of NH₃ as a consequence of greater bacterial growth that increased utilization of NH₃; this conclusion is supported by measures of growth (Figure 3). Acetate produced by *F. necrophorum* (Table 3) was greatest (*P* < 0.01) for lysine, followed by lactate plus lysine. The mean propionate concentration across all strains of *F. necrophorum* was greatest for lactate, followed by lactate plus lysine, with virtually no propionate produced from lysine. Butyrate concentrations were greatest (*P* < 0.01) for lysine, followed by lysine plus lactate, with much less butyrate recorded for lactate. The presence of lysine in the medium increased the uptake of lactate by *F. necrophorum*, as shown by lower concentrations of residual lactate from fermentations receiving lactate plus lysine (4.2 mM) compared with lactate alone (7.7 mM).

Our data demonstrate that the end products of lactate fermentation by *F. necrophorum* are primarily propionate and acetate, which is in agreement with other research (Nagaraja and Chengappa, 1998; Russell, 2005). All strains produced NH₃, butyrate, and acetate as end products of lysine degradation, which agrees with the observations of Russell (2005) for ruminal *F. necrophorum* and those of Barker et al. (1982) for other *Fusobacterium* spp.

Although differences existed in the concentrations of end products produced for different substrates, the similar patterns for the production of NH₃, acetate, propionate, and butyrate by each subspecies suggest that similar fermentation pathways for lysine and lactate were used by *F. necrophorum* ssp. *necrophorum* and *F. necrophorum* ssp. *funduliforme*. The *F. necrophorum* ssp. *necrophorum* strains produced more (*P* < 0.05) acetate, propionate, butyrate, and NH₃ than the *F. necrophorum* ssp. *funduliforme* strains. The slightly lower production of end products by *F. necrophorum* ssp. *funduliforme* was reflected by more (*P* < 0.05) lactate remaining at 48 h for *F. necrophorum* ssp. *funduliforme* than for *F. necrophorum* ssp. *necrophorum*.

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**Figure 3.** Growth (absorbance at 600 nm) of *Fusobacterium necrophorum* subspecies grown with 50 mM lactate (×), 50 mM lysine (□), or 50 mM lactate plus 50 mM lysine (○; experiment 4). *Fusobacterium necrophorum* ssp. *necrophorum* included strains A21, A27, and A29, whereas *F. necrophorum* ssp. *funduliforme* included strains B33, B34, B35, and B36. Absorbance was affected by time, substrate, time × substrate, and time × subspecies (*P* < 0.001), but no effects of subspecies or of time × subspecies × substrate were observed.

**Table 3.** End product concentrations after 48 h of fermentation of 50 mM lactate, 50 mM lysine, or 50 mM lactate plus 50 mM lysine by *Fusobacterium necrophorum* ssp. *necrophorum* or ssp. *funduliforme* (experiment 4)

<table>
<thead>
<tr>
<th>Subspecies and substrate</th>
<th>NH₃</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssp. <em>necrophorum</em>²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>10.5</td>
<td>18.7</td>
<td>24.0</td>
<td>6.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>96.1</td>
<td>45.4</td>
<td>0.6</td>
<td>44.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Lactate + lysine</td>
<td>58.5</td>
<td>26.4</td>
<td>19.9</td>
<td>41.5</td>
<td>1.5</td>
</tr>
<tr>
<td>SEM</td>
<td>5.5</td>
<td>2.5</td>
<td>0.60</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>ssp. <em>funduliforme</em>²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>11.3</td>
<td>16.2</td>
<td>21.0</td>
<td>5.9</td>
<td>10.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>80.4</td>
<td>37.3</td>
<td>0.5</td>
<td>35.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Lactate + lysine</td>
<td>42.6</td>
<td>20.9</td>
<td>18.9</td>
<td>31.8</td>
<td>6.9</td>
</tr>
<tr>
<td>SEM</td>
<td>5.1</td>
<td>2.3</td>
<td>0.52</td>
<td>2.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

¹Substrate and subspecies affected (*P* < 0.05) NH₃, acetate, propionate, butyrate, and lactate concentrations, but their interaction was significant only for propionate, butyrate, and lactate.

²Subspecies *necrophorum* was represented by 3 strains (A21, A27, and A29), and subspecies *funduliforme* was represented by 4 strains (B33, B34, B35, and B36).
Experiment 5: Growth of *F. necrophorum* Strains on Various AA. The greatest growth was observed for lysine and glutamic acid (Table 4). Some growth was evident with histidine and methionine for strain A21, but not for strain B35. The optical density for strain A21 on glutamic acid or lysine was maximal at 24 h. When grown on lysine or glutamic acid, strain B35 reached the maximal optical density at 36 h.

Ammonia concentrations at 48 h produced by *F. necrophorum* strains A21 and B35 were greatest (Table 5) for lysine and glutamic acid. The data demonstrated that ruminal *F. necrophorum* strains A21 and B35 degraded glutamic acid and lysine almost completely by 48 h of incubation.

Slight differences between strains were observed (P < 0.01) for acetate and butyrate concentrations, but not for propionate (Table 5); strain A21 yielded greater concentrations of acetate and butyrate than strain B35. The greatest acetate concentrations were produced from glutamic acid and lysine, with significant but relatively small amounts of acetate produced from histidine by strain A21. Propionate concentrations were elevated only for methionine, and the amount produced by either A21 or B35 was fairly small. Butyrate concentration was greatest for lysine, followed by glutamic acid, with no butyrate production from other AA. No lactate production was observed in this experiment (data not shown). As we observed in experiment 4, acetate and butyrate were produced in a ratio near 1:1 from the fermentation of lysine. In contrast, fermentation of glutamic acid yielded acetate and butyrate in a ratio near 2:1.

The utilization of AA by *Fusobacterium* spp. as C and energy sources has been reported (Barker et al., 1982). Our results on the utilization of AA by ruminal strains of *F. necrophorum* are in general agreement with observations on *F. necrophorum* of human origin (Gharbia and Shah, 1989). Gharbia and Shah (1989) investigated the utilization of different AA by *F. necrophorum* in chemically defined media that contained 1 mM of each AA (20 AA were used). They found that *F. necrophorum* utilized almost 100% of the basic AA (lysine, arginine, and histidine) and utilized 100% of glutamic acid, with no utilization of alanine. In contrast, degradation of histidine in our study was not extensive. Unlike the study of Gharbia and Shah (1989), which used all AA at concentrations of 1 mM each, we used single AA at a large concentration (50 mM), which reduced competition among AA for uptake. This key difference between our study and that of Gharbia and Shah (1989) likely explains the different range of AA that are extensively degraded, although differences possibly exist among the different strains of *F. necrophorum*. Conclusions about the inability of our ruminal *F. necrophorum* to actively ferment a broad range of AA should be confined to situations in which large concentrations are involved as the predominant energy source.

Experiment 6: MIC of Monensin and Tylosin Against *F. necrophorum* Grown with Lactate or Lysine

The MIC for tylosin was 25 μg/mL for both *F. necrophorum* A21 and B35 whether they were grown with lactate or lysine (Table 6); thus, the susceptibility of *F.
necrophorum to tylosin appears to be independent of the growth substrate.

When \textit{F. necrophorum} was grown on lactate, the MIC for monensin was more than 50 μg/mL for strain A21 and 10.9 μg/mL for strain B35; however, when grown on lysine, the MIC for monensin was 6.25 μg/mL for strain A21 and 3.9 μg/mL for strain B35, demonstrating that both strains were more susceptible to the antimicrobial effects of monensin when grown on lysine rather than lactate (Table 6).

Ruminal \textit{F. necrophorum} is a causative agent for liver abscesses in feedlot cattle, and ruminal acidosis is a predisposing factor. The increased acidity of the ruminal digesta during bouts of acidosis causes inflammation of the rumen wall, which enables \textit{F. necrophorum} to penetrate the rumen wall and then travel through the portal vein to the liver, where it causes abscesses (Scanlan and Hathcock, 1983). Many studies have shown that tylosin is effective in controlling liver abscesses and improving animal performance (Vogel and Laudert, 1994). The mode of action of monensin is to bind to bacterial ribosomes and prevent protein synthesis. Tan et al. (1994b) reported that the susceptibility of \textit{F. necrophorum} to tylosin was not different between the 2 subspecies, which is in accordance with our results, but they obtained slightly lower MIC than ours (16 and 12.9 μg/mL for \textit{F. necrophorum} and \textit{funduliforme}, respectively, compared with 25 μg/mL for both subspecies in our experiment). Differences could be related to the growth media; they used BHI, whereas we used lactate or lysine as the sole energy source, although MIC for tylosin were similar between the substrates.

In our study, both subspecies of \textit{F. necrophorum} (A21, B35) were much more susceptible to monensin when grown on lysine than when grown on lactate. Ruminal \textit{F. necrophorum} is a gram-negative bacterium and is generally resistant to monensin (MIC >50 μg/mL; Tan et al., 1994b), and monensin had no effect on the incidence of liver abscesses when fed to feedlot cattle (Potter et al., 1985). The difference in the MIC of monensin to \textit{F. necrophorum} could be related to the different media that have been used; however, monensin does reduce NH₃ concentration in the rumen (Yang and Russell, 1993). Russell (2005) reported that monensin (7 μg/mL) reduced the uptake of lysine by ruminal \textit{F. necrophorum}, which could explain the increased susceptibility of \textit{F. necrophorum} to monensin when grown on lysine. However, we did not measure lysine uptake and can therefore not verify this hypothesis. Attwood et al. (1998) reported that a ruminal isolate of \textit{F. necrophorum} was inhibited by monensin at a concentration

### Table 5. Concentrations of NH₃, acetate, propionate, and butyrate after 48 h of fermentation of various AA by \textit{Fusobacterium necrophorum} ssp. necrophorum strain A21 or ssp. \textit{funduliforme} strain B35 (experiment 5)

<table>
<thead>
<tr>
<th>Strain and substrate</th>
<th>NH₃</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssp. necrophorum strain A21</td>
<td>12.2</td>
<td>4.0</td>
<td>0.36</td>
<td>1.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>51.0a</td>
<td>48.5a</td>
<td>0.48</td>
<td>23.4a</td>
</tr>
<tr>
<td>Alanine</td>
<td>10.9</td>
<td>4.3</td>
<td>0.28</td>
<td>1.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6.6</td>
<td>3.9</td>
<td>0.38</td>
<td>1.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.4</td>
<td>3.0</td>
<td>2.85a</td>
<td>1.4</td>
</tr>
<tr>
<td>Histidine</td>
<td>17.0</td>
<td>8.8a</td>
<td>0.63</td>
<td>3.8a</td>
</tr>
<tr>
<td>Lysine</td>
<td>91.5a</td>
<td>45.3a</td>
<td>0.50</td>
<td>45.1a</td>
</tr>
<tr>
<td>ssp. funduliforme strain B35</td>
<td>10.7</td>
<td>2.9</td>
<td>0.36</td>
<td>1.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>49.7a</td>
<td>43.8a</td>
<td>0.40</td>
<td>20.5a</td>
</tr>
<tr>
<td>Alanine</td>
<td>10.7</td>
<td>3.2</td>
<td>0.28</td>
<td>1.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6.0</td>
<td>3.1</td>
<td>0.25</td>
<td>1.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.3</td>
<td>2.9</td>
<td>2.13a</td>
<td>1.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>11.4</td>
<td>4.3</td>
<td>0.65</td>
<td>2.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>81.4a</td>
<td>40.7a</td>
<td>0.43</td>
<td>38.9a</td>
</tr>
</tbody>
</table>

aWithin strain, value exceeds \((P < 0.05)\) that of the control (none).

### Table 6. Minimum inhibitory concentration of tylosin and monensin on \textit{Fusobacterium necrophorum} ssp. necrophorum strain A21 and ssp. \textit{funduliforme} strain B35 grown with lysine or lactate as substrate (experiment 6)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Tylosin</th>
<th>Monensin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssp. necrophorum strain A21</td>
<td>Lysine</td>
<td>25</td>
<td>6.25</td>
</tr>
<tr>
<td>ssp. necrophorum strain A21</td>
<td>Lactate</td>
<td>25</td>
<td>&gt;50</td>
</tr>
<tr>
<td>ssp. funduliforme strain B35</td>
<td>Lysine</td>
<td>25</td>
<td>3.9</td>
</tr>
<tr>
<td>ssp. funduliforme strain B35</td>
<td>Lactate</td>
<td>25</td>
<td>10.9</td>
</tr>
</tbody>
</table>
of 3.5 μg/mL, which is comparable with our result for strain B35 grown on lysine.

**Experiment 7: Effect of Essential Oil Components on the Growth of F. necrophorum**

Limonene, at 20 or 100 μg/mL, and thymol, at 100 μg/mL, inhibited \((P < 0.01)\) *F. necrophorum* growth, whereas eugenol, guaiacol, and vanillin had no effect (Table 7). These effects were generally consistent across strains A21 and B35 and across time, although limonene, at 100 μg/mL, was more inhibitory for strain A21 than for strain B35. Overall, optical densities were greater \((P < 0.01)\) for strain A21 than for strain B35.

The failure of the mixture of the 5 essential oil components to inhibit *F. necrophorum* could be related to the low concentrations of limonene and thymol; the specific proportions of each essential oil component in the mixture are unknown to us, although the data suggest that the mixture contained less than 20% limonene because that proportion would have provided an inhibitory concentration of 20 μg/mL of limonene in 100 μg/mL of the mixture. It is interesting that the mixture at 100 μg/mL caused a modest inhibition of *F. necrophorum* growth at 24 h for both A21 and B35, but it led to greater optical density in cultures of strain A21 at 48 h; we have no explanation for this observation. Taken as a whole, these results indicate that thymol and limonene could be useful in inhibiting *F. necrophorum* in the rumen. Most research has attributed the antimicrobial activity of essential oil components to disintegration of the bacterial membrane (Helander et al., 1998; Lee et al., 2004). Helander et al. (1998) reported that thymol reduced the intracellular ATP and increased extracellular ATP, which indicated the destruction of the cell membrane, and they attributed this action to the phenolic structure of thymol. Other research showed that feeding essential oil components tended \((P = 0.08)\) to reduce the incidence of liver abscesses compared with the control (Meyer et al., 2009), which could be related to the inhibition of ruminal *F. necrophorum*, the main cause of the disease (Nagaraja and Chengappa, 1998). No research is available on the direct effect of essential oils on the inhibition of *F. necrophorum*.

A blend of essential oil components (containing the same components that we tested) reduced NH₃ production by ruminal bacteria (McIntosh et al., 2003), but the effect was abolished by monensin; this suggests that the reduction in NH₃ production was mediated by the effects of essential oil components on monensin-sensitive bacteria. In experiment 6, we demonstrated that *Fusobacterium necrophorum* can be sensitive to monensin, particularly when grown on lysine as the primary substrate (Table 6). Thus, in the study by McIntosh et al. (2003), the inhibitory effects of essential oil components on NH₃ production by ruminal bacteria could be reflective of the inhibition of *F. necrophorum*. The inhibition of *F. necrophorum* by limonene could be a useful tool to reduce lysine degradability in the rumen. More research is needed to study the effect of essential oil components on lysine degradability.

**CONCLUSIONS**

Our results demonstrate that ruminal *F. necrophorum* has the ability to use lysine as a sole energy source, which agrees with the general characteristics of other

<table>
<thead>
<tr>
<th>Essential oil component</th>
<th>Concentration, μg/mL</th>
<th>ssp. necrophorum A21</th>
<th>ssp. funduliforme B35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>1.22</td>
<td>1.13</td>
</tr>
<tr>
<td>Eugenol</td>
<td>20</td>
<td>1.16</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.07</td>
<td>1.03</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>20</td>
<td>1.08</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.19</td>
<td>1.10</td>
</tr>
<tr>
<td>Limonene</td>
<td>20</td>
<td>0.01/*</td>
<td>0.24/*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.05/*</td>
<td>0.03/*</td>
</tr>
<tr>
<td>Thymol</td>
<td>20</td>
<td>1.11</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.01/*</td>
<td>0.03/*</td>
</tr>
<tr>
<td>Vanillin</td>
<td>20</td>
<td>1.09</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.20</td>
<td>1.07</td>
</tr>
<tr>
<td>Mixture</td>
<td>20</td>
<td>1.10</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.86/*</td>
<td>1.40/*</td>
</tr>
</tbody>
</table>

\(^*\)Within strain and hour, absorbance differs from the control \((P < 0.01)\).

\(^1\)Treatment, strain, time, and all interactions affected absorbance \((P < 0.05)\).
Fusobacterium spp. This finding is particularly notable because all our strains of ruminal F. necrophorum were isolated by using a selective medium with lactate as the sole energy source. Fusobacterium necrophorum effectively fermented lysine and glutamic acid, but alanine and tryptophan were not used, and histidine and methionine were fermented only to a minor extent.

Although typical ruminal environments with acidic pH and normal concentrations of VFA might inhibit lysine degradation by F. necrophorum, ruminal fluid from cows fed a typical dairy diet contained enough bacteria with lysine-deaminating capacity to ferment 50 mM lysine in vitro, although this required a significant period of time (36 h) and conditions in which the ruminal VFA were diluted 5-fold before the in vitro fermentation. Fusobacterium necrophorum likely was the key bacterial degrader of lysine in our in vitro system, but the greater concentrations of VFA may limit the role of F. necrophorum in lysine catabolism in vivo. Future work to assess the importance of F. necrophorum to in vivo lysine degradation would be valuable.

It is interesting that although F. necrophorum is generally considered resistant to monensin, this ionophore may be able to reduce lysine degradation by F. necrophorum. The essential oil components limonene and thymol were effective in inhibiting F. necrophorum, suggesting that they may be effective in reducing ruminal lysine degradation as well as liver abscesses. Our findings may lead to methods of reducing ruminal lysine degradation.

REFERENCES


