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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 NH_3 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 m*M* 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-NH_3-producing bacteria) but are relatively low in number, and only a few species of ruminal bacteria have been identified as hyper-NH_3-producing bacteria (Russell et al., 1991; Wallace et al., 1997). In addition to deamination abilities, hyper-NH_3-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 10^7/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 NH_3-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-NH_3-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.

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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 these studies 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 O_2-free CO_2. 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 NH_3 (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% H_2, 10% CO_2, 80% N_2; 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 × 10^9 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
Yeast[1]	0.2
Rumensin[2]	+

[1]Culture of *Saccharomyces cerevisiae* (Diamond V XP; Diamond V, Cedar Rapids, IA).

[2]Provided 10 mg of monensin/kg of diet DM (Rumensin; Elanco Animal Health, Greenfield, IN).