Optimizing Ruminal Fermentation with Organic Acids

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Introduction

Much interest has been generated over the past few years aimed at evaluating alternative means to manipulate the gastrointestinal microflora in production livestock. Motivation for examining these alternatives comes from increasing public scrutiny about the use of antibiotics in the animal feed industry. However, compared to the amount of information available detailing the effects of antimicrobial compounds on ruminal fermentation, little research has been conducted to evaluate alternatives to antimicrobial compounds. In the past 10 yr, interest has increased in direct-fed microbial (**DFM**) products and research has been conducted to examine the effects of DFM on ruminant performance. While some of these products have shown promise in favorably altering the ruminal fermentation and improving animal performance, the effects are variable and inconsistent (Martin and Nisbet, 1992). Recent research has shown that organic acids stimulate growth of the prominent ruminal bacterium *Selenomonas ruminantium*, favorably alter the mixed ruminal microorganism fermentation, and improve the performance of feedlot steers (Nisbet and Martin, 1990, 1991, 1993, 1994; Callaway and Martin, 1996; Martin et al., 1999). Therefore, the objective of this paper is to provide an overview of this research with organic acids and discuss the potential applications in beef and dairy cattle.

Pure Culture Studies

Batch Culture Experiments

S. ruminantium is a common gram-negative ruminal bacterium that can account for up to 51% of the total viable bacterial counts in the rumen (Caldwell and Bryant, 1966). This bacterium can grow under a variety of dietary conditions and ferment many different soluble carbohydrates (Hungate, 1966). When *S. ruminantium* is grown in batch culture with glucose, a homolactic fermentation occurs (Hobson, 1965). However, after the glucose is depleted from the medium, *S. ruminantium* then utilizes the lactate as a carbon and energy source (Scheifinger et al., 1975). Only some strains of *S. ruminantium* (subspecies *lactilytica*) are able to ferment lactate (Stewart and Bryant, 1988).

Early research showed that *S. ruminantium* HD4 requires L-aspartate, CO_2, *p*-aminobenzoic acid, and biotin for growth in a lactate-salts medium (Linehan et al., 1978). In addition, the requirement for L-aspartate can be replaced by L-malate or fumarate. Because little work had been done in this area since the report by Linehan et al. (1978), my laboratory initiated studies to evaluate the effects of aspartate, fumarate, and malate on growth and lactate uptake by *S. ruminantium* HD4 (Nisbet and Martin, 1990). Growth of *S. ruminantium* HD4 in medium that contained DL-lactate was stimulated approximately two-fold by 10 m*M* L-aspartate, fumarate, or L-malate after 24 h of incubation (Nisbet and Martin, 1990). Both L-aspartate and fumarate increased L-lactate uptake over 4-fold, while L-malate stimulated uptake over 10-fold (Figure 1). In addition, different concentrations (0.03 to 10 m*M*) of L-malate stimulated L-lactate uptake by *S. ruminantium* HD4 in a dose response fashion (Table 1). D-Lactate uptake by *S. ruminantium* HD4 was also stimulated by 10 m*M* L-malate (Nisbet and Martin, 1993).

Sodium concentrations between 25 and 100 m*M* stimulated L-lactate uptake by *S. ruminantium* HD4 in the presence of 10 m*M* L-malate, whereas uptake in the absence of L-malate was low regardless of the Na^+ concentration (Nisbet and Martin, 1994; Figure 2). These results suggest that both L-malate and Na^+ play a role in stimulating L-lactate utilization by *S. ruminantium* HD4. Sodium is the predominant cation in the rumen and concentrations range between 90 to 150 m*M* (Durand and Kawashima, 1980).

A more recent isolate, *Selenomonas ruminantium* H18, also requires organic acids to grow on lactate (Strobel and Russell, 1991). However, strain H18 differs from strain HD4 in that lactate could be used to support growth only when Na^+ and aspartate were added to the medium. Malate or fumarate could replace aspartate, but Na^+ was not required (Strobel and Russell, 1991).

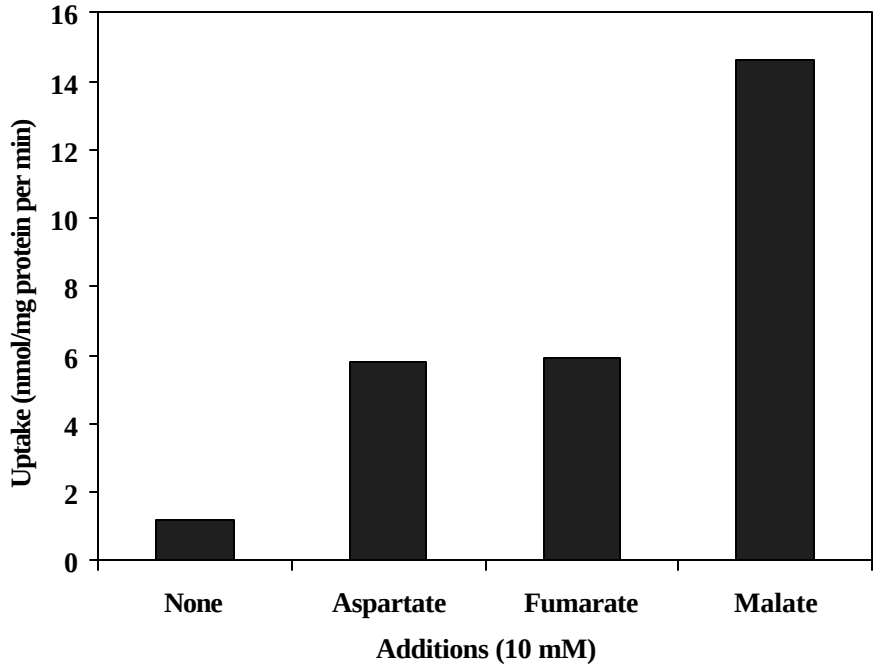

Figure 1. Effects of aspartate, fumarate, and malate on lactate uptake by whole cells of *Selenomonas ruminantium* HD4 (Nisbet and Martin, 1990).

Fumarate and malate are four-carbon dicarboxylic acids that are commonly found in biological tissues because they are intermediates of the citric acid cycle (Lehninger, 1975). Some strictly anaerobic bacteria use a reductive or reverse citric acid cycle known as the succinate-propionate pathway to synthesize succinate and(or) propionate (Gottschalk, 1986). Both malate and fumarate are key intermediates in the succinate-propionate pathway, and *S. ruminantium* uses this pathway (Gottschalk, 1986). The fact that dicarboxylic acids, especially malate and fumarate, stimulate lactate utilization is consistent with the presence of this pathway in this ruminal anaerobe.

Continuous Culture Experiments

In general, the dilution rate within the rumen is between 0.05 and 0.10 h^{-1} (Hungate, 1966). Most studies aimed at evaluating lactate utilization by *S. ruminantium* have been conducted in batch culture, and few experiments have been performed in continuous culture. Therefore, experiments were conducted to examine the effects of extracellular pH, lactate concentration, and malate addition on growth of *S. ruminantium* HD4 in continuous culture (Evans and Martin, 1997). When *S. ruminantium* HD4 was grown on lactate at pH 6.8, the primary end products were acetate and propionate with all concentrations of lactate. Little succinate or malate was produced. Even though not all lactate was utilized, as the lactate concentration was increased there was a corresponding increase in optical density at 600 nm ($\mathbf{OD}_{600}$), protein, and carbohydrate. These results suggested that lactate was limiting growth. Lactate utilization ranged between 35 and 50%.

S. ruminantium HD4 was unable to grow (culture washout) on 6 m*M* lactate at an extracellular pH of 5.5 (Evans and Martin, 1997). Growth did occur on 30 and 54 m*M* lactate at this pH and acetate and propionate were the main end products that were produced. Little malate or succinate accumulated. Bacterial protein and OD_{600} increased as lactate concentration increased and there was a decrease in cellular carbohydrate. When 8 m*M* malate was added to the growth medium, strain HD4 was able to grow on 6 m*M* lactate at pH 5.5 and 80% of the lactate was utilized. Acetate, propionate, and succinate were the primary fermentation products produced with all three

Table 1. Effect of increasing concentrations of L-malate on L-lactate uptake by *Selenomonas ruminantium* (Nisbet and Martin, 1991).

L-Malate, m*M*	Specific activity[a]
0	0.8 ± 0.04
0.03	1.4 ± 0.03*
0.06	2.7 ± 0.13*
0.12	3.2 ± 0.13*
0.48	5.6 ± 0.13*
2.5	8.5 ± 0.22*
5.0	10.4 ± 1.40*
10.0	12.2 ± 0.09*

[a]Nanomoles per milligram of protein per minute, mean ± SD.

*$P < 0.05$.

lactate concentrations in the presence of malate. Malate addition increased the amount of lactate utilized and OD_{600}, as well as the concentrations of protein and cellular carbohydrate synthesized by strain HD4. Lactate utilization ranged between 77 and 80% in the presence of malate compared to 40 and 70% in the absence of malate. Malate utilization ranged between 51 and 64%. Similar effects were seen when strain HD4 was grown at a dilution rate of 0.10 h^{-1} (Evans and Martin, 1997).

When domestic ruminants (beef and dairy cattle) are fed diets high in rapidly fermentable carbohydrates (i.e., cereal grains), lactate can accumulate and decrease ruminal pH (Slyter, 1976; Russell and Hino, 1985; Russell and Strobel, 1989). Lactate concentrations as high as 29 m*M* have been observed with these types of diets (Counotte et al., 1981). If lactate concentrations remain elevated, ruminal pH will drop below 6.0 and this leads to a variety of microbial and physiological problems (decreased fiber digestion, decreased digesta turnover, decreased salivation, rumen ulceration, founder, death) (Slyter, 1976; Russell and Hino, 1985).

Based on our continuous culture results, it appears that malate enhances the ability of strain HD4 to grow on all three lactate concentrations at an extracellular pH of 5.5 (Evans and Martin, 1997). These results are consistent with the observation that malate treatment increased final pH and decreased lactate concentrations in mixed ruminal microorganism fermentations of cracked corn and soluble starch (Martin and Streeter, 1995; Callaway and Martin, 1996). Therefore, by adding malate to the diets of ruminants fed high levels of rapidly fermentable carbohydrates, it may be possible to improve the ability of *S. ruminantium* HD4 to utilize lactate at pH 6.0.

Mixed Culture Studies

Based on the stimulation of lactate utilization by dicarboxylic acids in *S. ruminantium* and because information was limited detailing the effects of organic acids on ruminal fermentation, experiments were conducted to evaluate the effects of L-aspartate, fumarate, and DL-malate on the *in vitro* mixed ruminal microorganism fermentation (Martin and Streeter, 1995; Callaway and Martin, 1996). Fermentation of cracked corn in the presence of 8 or 12 m*M* DL-malate resulted in an increase in final pH and propionate concentration (Martin and Streeter, 1995). Total VFA tended to increase, while final concentrations of L-lactate numerically decreased. In the case of soluble starch, 8 and 12 m*M* DL-malate caused a decrease in CH_4 concentration. When only ruminal fluid (no added anaerobic medium) was used as the inoculum rather than 20% ruminal fluid medium, similar results for final pH, propionate, L-lactate, and total VFA were observed for soluble starch and corn incubations treated with DL-malate. These changes in fermentation products are analogous to ionophore effects (Russell and Strobel, 1989).

To determine whether organic acids plus monensin have an additive effect on ruminal fermentation, the effects of organic acid (L-aspartate, fumarate, or DL-malate) and monensin treatment on the *in vitro* mixed ruminal microorganism fermentation were evaluated (Callaway and Martin,

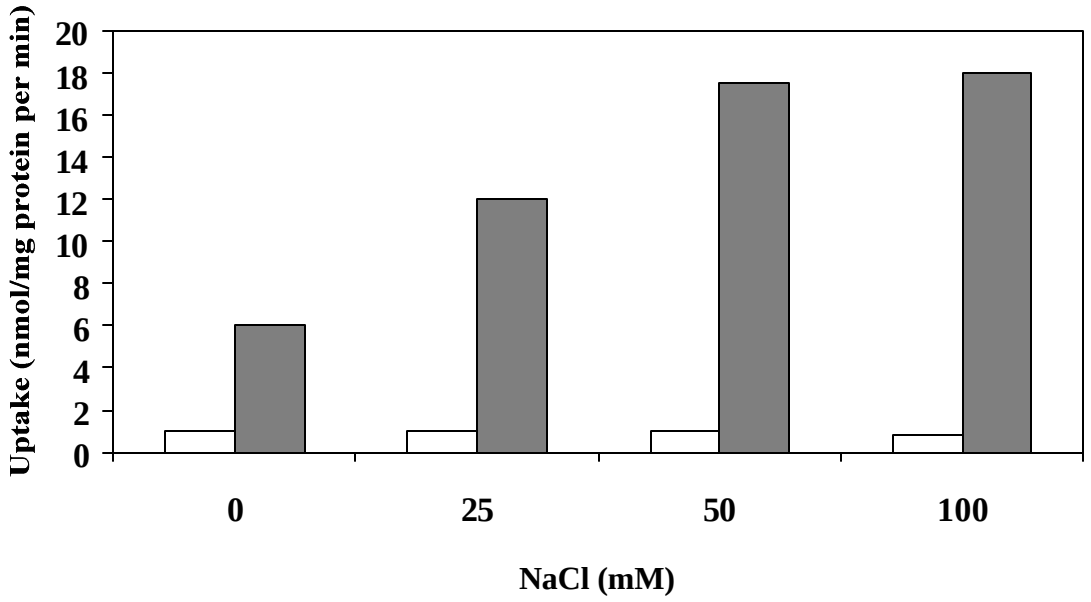

Figure 2. Effect of different concentrations of NaCl on L-lactate uptake by whole cells of *Selenomonas ruminantium* HD4 in the absence (open bars) and presence (shaded bars) of 10 m*M* L-malate (Nisbet and Martin, 1994).

1996). The addition of 8 and 12 m*M* organic acids to cracked corn fermentations increased final pH, tended to increase total gas production and CO_2 concentration, and decreased the acetate:propionate ratio. Organic acids tended to decrease CH_4 concentrations and H_2 concentration was not altered. DL-Malate addition at all levels reduced L-lactate accumulation.

Monensin is thought to alter ruminal fermentation primarily by inhibiting gram-positive bacteria, specifically *Streptococcus bovis*, that are responsible for much of the ruminal lactate, ammonia, and H_2 production (Dennis et al., 1981; Russell and Strobel, 1989). Reduction of ruminal lactate production is thought to help increase ruminal pH due to the low pKa of lactate compared with the VFA normally associated with ruminal fermentation (pKa 3.9 vs. 4.8 for acetate) (Russell and Hino, 1985). Therefore, due to inhibition of lactate production by *S. bovis* and increased lactate utilization by monensin-resistant *S. ruminantium*, it was hypothesized that an additive effect of organic acid and monensin treatment on the mixed ruminal microorganism fermentation might occur. An additive effect was observed with incubations containing DL-malate and monensin as well as fumarate and monensin (Callaway and Martin, 1996).

Lactate levels were lowest in incubations containing DL-malate and monensin; however, final pH was highest in incubations containing only DL-malate (Callaway and Martin, 1996). Therefore, despite the decrease in lactate accumulation in organic acid treated fermentations, not all of the increase in final pH can be attributed to a reduction of lactate levels. Sodium bicarbonate has been incorporated into dairy cattle diets to buffer ruminal pH for over 20 yr. It is generally believed by some that bicarbonate increases the buffering capacity of ruminal fluid, in part, by increasing the amount of dissolved CO_2. In addition to stimulating lactate utilization by *S. ruminantium*, organic acid addition to mixed ruminal microorganism fermentations increased CO_2 concentration (Callaway and Martin, 1996). Carbon dioxide is an end product of lactate fermentation to propionate via the succinate-propionate pathway that is utilized by *S. ruminantium* (Gottschalk, 1986). Therefore, organic acid treatment may act to buffer ruminal contents by a dual mechanism of increased lactate utilization and CO_2 production by *S. ruminantium.* Although the gas analysis results did not always show an increase in CO_2 concentration, the total gas measured was found to be composed of 95% CO_2 and 5% CH_4 (Callaway and

Martin, 1996). Therefore, it was suggested that the method used to quantify CO_2 (thermal conductivity gas chromatography) did not possess the sensitivity needed to detect changes in the amount of CO_2 produced, and that the increase in total gas production was primarily composed of CO_2. When *S. ruminantium* is grown in medium that contains lactate plus organic acids, we routinely observe *ruminantium* is grown in medium that contains lactate plus organic acids, we routinely observe more gas pressure in these cultures compared with lactate only cultures. Therefore, it seems that stimulation of *S. ruminantium* in mixed culture by organic acid addition helps to increase ruminal pH by increasing lactate utilization as well as the concentration of CO_2.

Organic acids have been suggested to act as an electron sink for *S. ruminantium* (Nisbet and Martin, 1991; Martin and Park, 1996). Treatment of mixed ruminal microorganism fermentations with DL-malate yielded responses similar to those of ionophores (i.e., increased propionate, decreased CH_4, decreased lactate), suggesting that organic acids have an effect on electron flow (Martin and Streeter, 1995). Ionophore effects closely associated with electron redistribution (decreased lactate, increased propionate) were enhanced by organic acid treatment (Callaway and Martin, 1996). Therefore, by providing an electron sink in the form of organic acids, the effects of monensin are enhanced in some cases.

All of the studies reviewed up to now have dealt with examining the effects of organic acids on ruminal microorganism fermentation. Experiments have also been conducted to determine the effects of cellobiose and monensin on the *in vitro* fermentation of all three organic acids by mixed ruminal bacteria (Callaway and Martin, 1997). Cellobiose addition to organic acid fermentations increased the rate of organic acid utilization by the mixed bacterial population. Total VFA concentrations were increased by cellobiose addition to all fermentations. A lag period ($\leq$ 4 h) occurred in monensin treated fermentations before organic acids were utilized; however, total VFA were increased and the acetate:propionate ratio was decreased by monensin treatment. When cellobiose and monensin were added together, propionate production and organic acid utilization were increased. Disappearance rates of organic acids and concentrations of total VFA were found to be highest, and the acetate:propionate ratio was the lowest, in incubations treated with cellobiose plus monensin. Therefore, the beneficial effects (i.e., increased total VFA and propionate concentrations) of organic acids on fermentation by mixed ruminal bacteria are apparently enhanced by the addition of cellobiose and monensin.

In Vivo Studies

Limited *in vivo* research has been conducted to evaluate the effects of organic acids on ruminant performance. Kung et al. (1982) reported that feeding 140 g of malate per d resulted in an increased milk persistency in lactating cows and increased total VFA during early lactation. Other parameters, including ruminal pH, were not altered by malate treatment; however, ruminal fluid samples were collected by stomach tube and lactate concentrations were not reported (Kung et al., 1982). Feeding malate to Holstein bull calves improved ADG and feed efficiency, but had little effect on blood serum constituents (Sanson and Stallcup, 1984). Even though *in vitro* studies have shown that DL-malate favorably alters the ruminal fermentation (Martin and Streeter, 1995; Callaway and Martin, 1996), little information is available detailing the effects of DL-malate on beef cattle performance. Therefore, the effects of DL-malate on ruminal parameters as well as the effects of supplementing finishing diets with this organic acid on feedlot cattle performance were determined (Streeter et al., 1994; Martin et al., 1999).

To determine the effects of DL-malate on ruminal metabolism, four steers equipped with ruminal cannulas were fed an 80% rolled grain (75% corn:25% wheat) diet twice daily with a DMI equal to 2.0% of BW (Streeter et al., 1994; Martin et al., 1999). DL-Malate was infused into the rumen on two consecutive d in 500 mL of phosphate buffer to provide 0, 27, 54, or 80 g DL-malate/d or final ruminal concentrations of 0, 4, 8, or 12 m*M*. When only 500 mL of phosphate buffer was infused into the rumen (0 m*M* DL-malate), ruminal pH declined to 5.49 one h after infusion and increased to 6.29 at 12 h (Figure 3). These results are consistent with the pH values associated with subclinical acidosis (Britton and Stock, 1986; Nocek, 1997; Owens et al., 1998). There was no decrease ($P < 0.05$) in ruminal pH one h after infusion of all three concentrations of DL-malate. When compared to the 0 m*M* DL-malate treatment, ruminal pH was also higher ($P < 0.05$) at 2 and 4 h in the presence of all three DL-malate concentrations. Ruminal pH was always greater than 6.0 in the presence of 12 m*M* DL-malate over the 12 h sampling

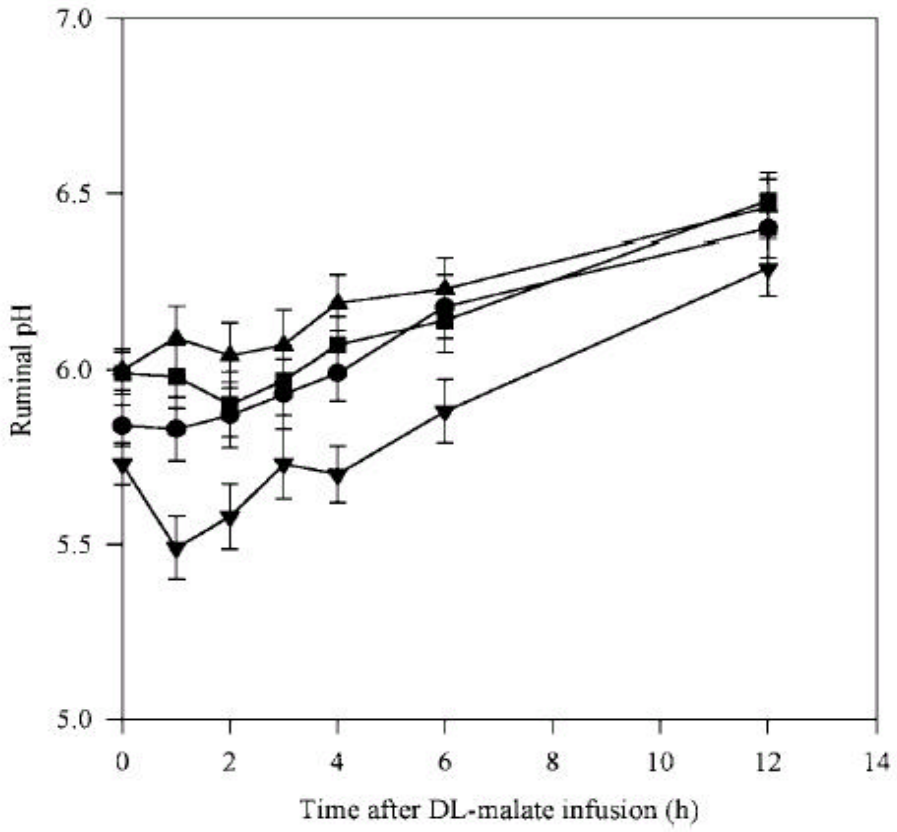

Figure 3. Effect of DL-malate treatment on ruminal pH. Treatments were 0 m*M* DL-malate (▼), 4 m*M* DL-malate (●), 8 m*M* DL-malate (■), and 12 m*M* DL-malate (▲). Error bars represent standard error of the mean (Streeter et al., 1994; Martin et al., 1999).

period. DL-Malate treatment linearly decreased total VFA and tended to linearly increase acetate concentration.

Three finishing studies were conducted to determine the effects of feeding DL-malate on growth rate and feed efficiency (Martin et al., 1999). In a 98 d experiment, 33 crossbred steers were randomly allotted to three DL-malate levels (0, 40, and 80 g/d). Steers were fed a rolled corn based diet twice daily. After 84 d on feed, feed efficiency (gain:feed) tended to improve with more DL-malate and was 8.1% greater for DL-malate compared with control. Average daily gain linearly increased with more DL-malate and was 8.6% greater for DL-malate than for the control. After 98 d on feed, ADG was linearly increased by DL-malate with the greatest increase occurring with 80 g of DL-malate. In the second performance study, 27 Angus steers were randomly allotted to three DL-malate concentrations (0, 60, and 120 g/d). Steers were fed diets used in the 98 d experiment twice daily, but DL-malate was included during the 10 d step-up period. During the 10 d step-up period, feed efficiency and ADG linearly increased with more DL-malate. DL-Malate had little effect on steer and heifer performance or plasma constituents in a 113-d finishing study. Furthermore, DL-malate had no effect on carcass characteristics in all three finishing studies. Collectively, these results suggest that feeding DL-malate to cattle consuming high grain diets alleviates subclinical acidosis and improved animal performance in two out of three finishing studies.

Forages and Organic Acids

Because the cost of supplementing diets with DL-malate is estimated to range between $0.09 to $0.19/d per head under feedlot conditions (Streeter et al., 1994; Martin et al., 1999), the inclusion of malate as

a feed additive in the diets of ruminants may not be economically feasible. Plants are a rich source of nutrients that can be utilized by both the ruminant animal and mixed ruminal microbial population. Intermediates of the citric acid cycle accumulate in plant tissue and may represent as much as 10% of the DM of grasses (Stout et al., 1967; Bohman et al., 1983). Malate can comprise up to 1.5% of the DM of mature grasses (Bohman et al., 1983). Therefore, forages that are high in organic acids might provide a vehicle for the inclusion of malate in production ruminant diets. To address the possibility of using forages as a source of malate, a study was conducted to determine the concentrations of malate present in five alfalfa varieties (Alfagraze, Apollo Supreme, Cimarron, Crockett, and Magnum III) and three bermudagrass hay (Coastal, Tifton-78, and Tifton-85) varieties at different stages of maturity (Callaway et al., 1997).

Samples were collected from replicated plots (n = 3) of each alfalfa variety at 9, 18, 28, 35, and 42 d of maturity; bermudagrass hay samples were composited from six bales of each variety from two cuttings staged to be harvested at 27 and 41 d of maturity (Callaway et al., 1997). As maturity increased, malate concentration declined in both plant species. Regression analysis indicated that malate concentrations in the alfalfa varieties decreased with increasing maturity at different rates. Cimarron had the highest rate of decline, followed by Apollo Supreme, Alfagraze, Crockett, and Magnum III. No differences in malate concentrations were observed at 9 d among the five alfalfa varieties. Malate concentrations were numerically higher in two alfalfa varieties (Crockett and Magnum III) at 35 and 42 d of maturity than in all other alfalfa varieties. Malate concentrations in bermudagrass (41 d) were lower than malate concentrations in all alfalfa varieties at 42 d of maturity. Malate declined with increasing maturity in the Coastal and Tifton-78 varieties.

Based on the presence of malate in forages, it is of interest to determine the potential ruminal concentration of malate after consumption of these forages by production ruminants. Given that malate concentrations ranged between 2.9 and 4.5% of alfalfa DM at 42 d of maturity, one can estimate how much malate might be available in the rumen of a dairy cow fed alfalfa at 42 d of maturity (Callaway et al., 1997). If the amount of alfalfa consumed per day is 6.0 kg in a TMR (West et al., 1997) and the malate concentration in Alfagraze is 2.9%, the animal consumes 174 g of malate. If the ruminal volume is approximately 70 L, then the intraruminal concentration of malate would be 2.5 g/L or 18.6 m*M* (molecular mass of malate is 134.1 g/L; Sigma Chemical Co., St. Louis, MO). Using similar calculations and 4.5% of DM malate, Magnum III would provide 29 m*M* intraruminal malate. Malate concentrations between 0.03 and 10 m*M* increased lactate uptake by *S. ruminantium* in a dose-response fashion, and the 10 m*M* level was the most stimulatory (Table 1). Therefore, all alfalfa varieties appear to provide adequate concentrations of malate to maximally stimulate lactate utilization by *S. ruminantium*. However, because of ruminal dilution rate as well as malate utilization by ruminal microorganisms (Russell and Van Soest, 1984; Callaway and Martin, 1997), it is unlikely that all malate would be readily available. In addition, release of this organic acid may be dependent on at least some plant cell wall digestion. *In vitro* studies have shown that 7.5 m*M* malate is completely fermented within 10 to 24 h by mixed ruminal microorganisms (Russell and Van Soest, 1984; Callaway and Martin, 1997). Collectively, these results suggest that selecting for and incorporating forage varieties that are high in malate into the ruminant diet may provide a vehicle for economically including malate in the diet.

Conclusions

Compared with the amount of research, both *in vivo* and *in vitro*, that has been conducted with other feed additives, very little research has been conducted with organic acids. Given the increasing concern and(or) criticism regarding the feeding of antibiotics and the potential for selection of antibiotic-resistant bacteria and resistance transfer to pathogens (Nikolich et al., 1994), alternatives to antimicrobial compounds need to be investigated. Organic acids potentially provide an alternative to currently used antimicrobial compounds by stimulating (i.e., *S. ruminantium*) rather than inhibiting specific ruminal microbial populations. By initially studying the effects of dicarboxylic acids on lactate utilization by *S. ruminantium*, we have progressed to mixed culture fermentations and limited *in vivo* studies look promising. This approach to understanding the details of the ruminal fermentation is needed if nutritionists and microbiologists hope to successfully manipulate ruminal microorganisms in the future.

Malate can be purchased in bulk quantities, and at current prices, the use of malate as a feed additive is estimated to cost $0.09 to $0.19/d per head under feedlot conditions. At present, this cost may prohibit the inclusion of malate in the diets of feedlot or dairy cattle. However, by selecting for and

incorporating forage varieties that are high in malate into the ruminant diet, ruminal fermentation might be improved. Perhaps the high malate concentrations observed in alfalfa account for some of the high quality associated with this forage. More research is needed to evaluate whether or not ensiling has any effect on forage malate concentrations.

According to Owens et al. (1998), acute and subacute acidosis continues to plague feedlot ruminants. It has been conservatively estimated that monetary losses attributed to ruminal acidosis in US feedlots are between $60 to $100 million/yr (Martin, 1992). Economic losses associated with acidosis in the dairy industry are not available, but because of the energy demands on high producing dairy cows, these losses are probably significant (Hinders, 1995; Nocek, 1997). Due to the growing focus on food safety by the national media and consumers, in my opinion, it is imperative that microbiologists and nutritionists explore alternatives to antimicrobial compounds to address the acidosis problem. Based on the results from our studies, an organic acid, like malate, would be a good alternative because there is no risk of developing antibiotic resistance or having unwanted residues appearing in either meat or milk products.

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