BRIEF COMMUNICATION: Bioactive plants inhibit bacteria that cause lactic acidosis in ruminants

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Keywords: *Eremophila glabra*; bioactive plants; lactic acidosis; rumen; *Streptococcus bovis*; secondary compounds.

INTRODUCTION

Lactic acidosis may become an animal welfare issue in New Zealand if the level of concentrates used in the feed ration increases. Lactic acidosis develops in ruminants because of the excessive production of lactic acid by starch degrading bacteria in the rumen after animals are introduced to grain–based diets containing large amounts of starch. It is important that lactic acid is converted to volatile fatty acids in the rumen rather than being absorbed into the bloodstream. Rumen bacteria that ferment starch to lactic acid are capable of rapid cell reproduction and, when starch is freely available, produce lactic acid at a higher rate than lactate is fermented to volatile fatty acids by lactate-using bacteria. Consequently lactate accumulates in the rumen causing a drop in pH and lactic acidosis develops (Nocek, 1997). *Streptococcus bovis* and *Lactobacillus spp.* are major lactate-producers and *Megasphaera elsdenii* is the major species that metabolises lactic acid and reduces its accumulation in the rumen (Gill *et al*., 2000, Nagaraja, 2002).

Antibiotics that are added to ruminant feed prevent acidosis by targeting lactic acid-producing bacteria but their use in animal feeds is under intense scrutiny (Wegener, 2003). Native plants of Australia with antimicrobial properties have been identified (Hammer *et al*., 1999, Palombo & Semple, 2001), and may be considered as a safer replacement to antibiotic feed additives.

In a previous study, we tested a range of Australian plants and essential oils (n = 110) (Hutton, 2008) in an *in vitro* batch fermentation system designed to simulate acidosis. In this screening assay test plants were added to rumen fluid at 10% of the total nutrient substrate. Five plants, namely *Eremophila glabra*, *Kennedia eximia*, *K. prorepens*, *Acacia saligna* and *A. Decurrens;* produced fermentation characteristics that were comparable to virginamycin. That is they prevented the reduction in pH without inhibiting total fermentation. The most potent plant was *E. glabra* and it was speculated that the effects we observed were due to the selective inhibition of lactate-producers. *E. glabra* is a plant that was commonly used by aboriginal people for medicinal purposes and is known to contain secondary compounds called diterpenes of which serrulatanes are the most common class (Liu *et al*. 2006). In the study here we tested ethanol extracts and purified compounds from these plants on rumen bacteria to identify plants that selectively inhibit lactate-producing bacteria but not lactate-using bacteria or other bacteria associated with normal rumen fermentation. These plants would have the potential to prevent lactic acidosis in ruminant animals and could help to reduce the use of antibiotic feed additives.

MATERIALS AND METHODS

The five plants which were selected from a previous screening assay were tested here for their selective inhibition of lactate-producing bacteria. An agar dilution method was used, modified from Hammer *et al.* (1999) and Palombo and Semple (2001), to determine the minimum inhibitory concentration (MIC) of plant extracts against three strains of *S. bovis* (AR3, AR25 and UWA), two strains of *Lactobacillus* spp. (YE08 and YE16), *M. elsdenii* (LC1), *Eubacterium ruminantium* (AR2), *Prevotella ruminicola* (AR20), *Lachnospira multiparis* (DSM 3073) and *Butyrivibrio fibrisolvens* (YE44). All preparations were under anaerobic conditions using a Coy anaerobic chamber (N_2 80%, CO_2 10%, H_2 10%). Bacterial growth medium was prepared by adding 15 g of agarose gel/L of rumen fluid based medium (Bryant & Robinson, 1961). The medium was poured into three petri dishes per treatment before filter sterilized treatments were mixed into the cooled medium. Bacterial cultures were inoculated in triplicate onto each plate using a Steer's replicator (Hammer *et al.* 1999). The experiment was repeated on a separate day.

TABLE 1: Minimum inhibitory concentration (μg/mL) of ethanol extracts from plants or virginiamycin on lactate-producing and lactate-using bacteria, and bacteria associated with normal rumen fermentation. NI = No inhibition at any of the concentrations used, - = Not tested.

Rumen bacteria	Plants with antimicrobial properties					Antibiotic (Control)
	Eremophila glabra	*Kennedia prorepens*	*Kennedia eximia*	*Acacia saligna*	*Acacia decurrens*	*Virginiamycin*
Lactate-producers						
S. bovis UWA	1,260	NI	NI	NI	NI	24
S. bovis AR3	1,260	NI	NI	NI	NI	24
S. bovis AR25	1,260	NI	NI	NI	NI	6
Lactobacillus YE08	1,260	5,000	5,000	5,000	5,000	12
Lactobacillus YE16	1,260	10,000	10,000	-	-	-
Lactate-user						
M. elsdenii LC1	10,000	NI	NI	NI	NI	48
Major anaerobes						
L. multiparis DSM 3073	-	-	-	-	-	24
B. fibrisolvens YE44	1,260	NI	NI	NI	NI	6
P. ruminicola AR20	1,260	NI	NI	NI	NI	NI
E. ruminantium AR2	-	-	-	-	-	24

TABLE 2: Selective inhibition measured as minimum inhibitory concentration (μg/mL) by unidentified serrulatane diterpenes isolated from *Eremophila* species, on lactate-producing and lactate-using bacteria. NI = No inhibition at any of the concentrations used.

Rumen bacteria	Unidentified serrulatane diterpene compounds						
	1	2	3	4	5	6	7
Streptococcus bovis	640	640	640	NI	320	320	1,077
Megasphaera elsdenii	NI	NI	NI	NI	NI	NI	1,077

The antibiotic, virginiamycin, was included as a positive control. The MIC was determined as the lowest concentration of extract that inhibited the visible growth of each organism on Bryant and Robinson agar medium (Bryant & Robinson, 1961).

In the second experiment the major secondary compounds from acetone extracts of the most promising plant, *E. glabra*, were isolated using chromatography and identified using nuclear magnetic resonance spectroscopy. The compounds were then tested in quadruplicate at concentrations of 80, 160, 320 and 1077 μg/mL in a microbroth dilution assay adapted from Hammer *et al*. (1999). The broth was then inoculated with the lactate producer, *S. bovis* (AR3) or the lactate user, *M. elsdenii* (LC1) and bacterial growth was measured by spectrophotometry. Cell proliferation was determined by measurement of the optical density at 590 nm. Cell growth was expressed as the OD_{590} after 48 h incubation.

The MIC for the broth dilutions was determined by comparing absorbance outputs for each treatment versus the controls using a one-way analysis of variance on JMP IN (SAS, 2004) and a Tukey-Kramer HSD pair-wise comparison was used when means differed by $P < 0.05$.

RESULTS AND DISCUSSION

E. glabra was identified as the most effective plant used in the agar dilution assay because it inhibited both species of lactate-producing bacteria, *S. bovis* and *Lactobacillus* spp. but not the major lactate user *M. elsdenii* (Table 1). The remaining plant extracts demonstrated selective inhibition but were not effective against *S. bovis*. The crude extracts that were recovered from the ethanol extraction procedure yielded between 17 and 25% dried extract from the original dried leaf material. The extract from *E. glabra* (17.4% extract yield) selectively inhibited the growth of the lactate-producers at 1,260 μg/mL. The MIC of *K. eximia*, *A. saligna*, *A. decurrens* and *K. prorepens* extracts against *Lactobacillus* spp. was 5,000 to 10,000 μg/mL which was not as potent as the extracts from *E. glabra*. Virginiamycin inhibited species that produce lactic acid without inhibiting the lactate-users, which is consistent with results of Ives *et al*. (2002) and provides an explanation as to why it has

been useful in the prevention of acidosis. However, in our test system and in work by Nagaraja and Taylor (1987), virginiamycin also inhibited the growth of some important rumen species including *L. multiparis* and the cellulolytic species, *B. fibrisolvens.* Virginiamycin is known to be successful in the control of acidosis, but it may restrict important aspects of microbial fermentation including fibre metabolism.

E. glabra was selected for the second experiment and serrulatane diterpenes (n = 7) were identified as the secondary compounds in *E. glabra* that inhibited lactate-producing bacteria but not lactate-using bacteria ($P < 0.05$) (Table 2). Six of the seven serrulatanes inhibited *S. bovis* in broth dilutions, although one of the six (Compound 7) also inhibited *M. elsdenii* (Table 2). The inhibition of *M. elsdenii* by Compound 7 indicates some broad-spectrum activity.

Our observations support the evidence of Liu *et al*. (2006) and Ndi *et al*. (2007) that serrulatanes inhibit the growth of specific bacteria. Our work supports our previous *in vitro* studies in which *E. glabra* prevented the development of an acidotic environment in rumen fluid challenged with readily fermentable carbohydrate. This work highlights the role that bioactive plants may have in replacing antibiotics for the prevention of lactic acidosis. The concentrations of *E. glabra* that were effective *in vitro* indicates that leaf material may prevent acidosis when included at about 10% of the total feed intake or 1 to 2% if used as an extract. However, *in vivo* studies will be needed to determine the efficacy of *E. glabra* against lactic acidosis.

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