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The effect of basal diet on lactate-producing bacteria and the susceptibility of sheep to lactic acidosis

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Abstract

The influence of a diet of either pasture or hay on the development of lactic acidosis in sheep was investigated using a grain challenge approach. Twenty-four Merino wethers with a mean live weight of 36·7 (s.e. 3·6) kg were used; 12 were adapted to grass pasture and 12 to hay (lucerne and oaten hay, 60 : 40) for 4 weeks before being given 1 kg of crushed barley via *stomach tube. Six sheep in each group were also given virginiamycin (VM; 50 mg/kg barley) with the grain to test the efficacy of this antibiotic in controlling the bacteria responsible for the development of acidosis. Changes in volatile fatty acid (VFA), pH, lactate and bacterial count in the rumen and faecal pH and dry matter (DM) were measured for a 24-h period following administration of the barley. Daily intakes of hay were measured for a 10-day period following grain engorgement. Total ruminal VFA increased* ($P < 0·01$) *over time and tended* ($P = 0·08$) *to be higher in sheep adapted to hay than in those adapted to pasture (67·5* v. *59·8 mmol/l). The molar proportions of VFA changed* ($P < 0·01$) *over time in favour of propionate in both groups. Ruminal pH was higher* ($P < 0·001$) *in pasture-adapted sheep, but declined* ($P < 0·001$) *in both groups over time following the introduction of barley. This decline in pH was associated with increases in ruminal concentration of VFA in pasture-adapted sheep and VFA and lactate in hay-adapted sheep. The addition of VM resulted in a higher* ($P < 0·001$) *proportion of propionate and a trend towards higher* ($P = 0·24$) *faecal pH and DM content. Faecal pH and DM content declined* ($P < 0·001$) *over time and was lower for the pasture-adapted sheep. The introduction of either barley alone or barley with VM from both hay and pasture diets increased* ($P < 0·05$) *the viable counts of total bacteria,* Streptococcus bovis *and lactic acid bacteria. Bacterial isolates were purified and identified by complete sequencing of the 16S rRNA gene to determine the predominant bacteria during the overfeeding of grain. Isolates from medium selective for* S. bovis *were all identified as this species when VM was not given. VM had no effect on counts of viable bacteria, but inhibited the growth of* S. bovis.

This study has shown that sheep given hay are more susceptible to lactic acidosis, the signs of which can be reduced by VM.

Keywords: *acidosis, feed grains, lactic acid bacteria,* Streptococcus bovis, *virginiamycin.*

Introduction

Adaptation of ruminants to diets containing new components is best achieved by maintaining normal rumen function and a balanced microbial population. These allow optimal utilization of food, a minimization of digestive upset and maintenance of health. A smooth transition between diets is the challenge faced by nutritionists when ruminants are given high levels of concentrate in the diet, which they have not evolved to utilize efficiently.

While the ingestion of high-fibre diets supports higher populations of cellulose- and hemicellulose-digesting bacteria, low-fibre diets that are rich in highly fermentable carbohydrates stimulate the

growth of starch-fermenters and lactate utilizers and depress the growth of fibre-digesting micro-organisms (Schwartz and Gilchrist, 1974). The introduction of starch into the rumen leads to rapid production of volatile fatty acid (VFA). As the rate of production exceeds the rate of removal, rumen pH may fall below 6·0. Such a low pH value favours the rapid growth of starch-degrading bacteria including *Streptococcus bovis* and lactobacilli. The pH continues to fall, until even the resilient *S. bovis* can no longer grow, and lactobacilli dominate the rumen microbiota, fermenting the starch to produce more lactic acid and decreasing the pH to an even lower value (e.g. below 5·5). This is the scenario often presented to describe the sequential reactions in the rumen that lead to fermentative acidosis (Huntington, 1993; Wallace, 1996).

Several methods are used to reduce the risk of acidosis, including the use of buffers such as sodium bicarbonate (Dunn *et al.*, 1979; Kezar and Church, 1979), inclusion of dietary clays such as bentonite (Huntington *et al.*, 1977; Dunn *et al.*, 1979), the use of antibiotic food additives such as virginiamycin (VM; Godfrey *et al.*, 1995; Rogers *et al.*, 1995) or certain ionophores (Nagaraja *et al.*, 1981 and 1985), use of microbial food additives, such as *Megasphaera elsdenii*, which ferment lactate (Kung and Hession, 1995; Wiryawan and Brooker, 1995) or *Saccharomyces cerevisiae* (Chaucheyras *et al.*, 1996) as well as the conventional method of gradual introduction of grains. In order to be able to prevent acidosis without further interruption to the fermentation system it is important to identify the rôle of the basal diet with regard to microbial adaptability. This is particularly important when the basal diet supplies a considerable amount of soluble carbohydrates (e.g. growing grass), and results in a microbial adaptation similar to a starch-feeding environment. It is also important to identify those bacteria responsible for the rapid fermentation of starch and other forms of readily fermentable carbohydrates and the production of lactic acid.

This experiment evaluates the influence of basal diet (grass pasture *v.* hay), and the addition of VM with barley, on the total bacterial population, the numbers of *S. bovis* and lactic acid bacteria and on some rumen and faecal parameters after a sudden introduction to a high level of barley supplement.

Material and methods

Animals and their management

Twenty-four Merino wethers of approximately 18 months of age and an average body weight of 36·7 (s.e. 3·6) kg were selected after shearing in September. They were maintained on a pasture of grasses native to the New England tableland region of NSW, Australia and had not been given grain. They were randomly divided into two equal groups. One group grazed on a paddock of approximately 2 ha with green grass and the other group were housed indoors, placed in individual pens and given a diet consisting of chopped lucerne and oaten hay, mixed in a ratio of 60 : 40, and offered at a daily rate of 900 g dry matter (DM) per head.

The experiment included a pre-treatment adaptation period of 28 days where sheep of both groups were maintained on their basal diets followed by a treatment period of 24 h for the pasture-adapted sheep and 24 h plus 10 days hay intake measurements for the hay-adapted sheep. The pasture-adapted sheep were housed indoors (in a shed) during the treatment period and had no access to pasture. For the treatment period, sheep within each group were randomly allocated to receive barley with or without VM. All sheep were given 900 g crushed barley, first mixed with 4 l water and poured into the rumen through a stomach tube. A wettable form of VM (Eskalin WP, 400 g/kg virginiamycin; Pfizer Animal Health, NSW, Australia) was used and administered as a single drench of 20 ml water containing 50 mg VM at the same time as drenching the barley. Sheep were weighed at the beginning and end of the experiment to record initial and final body weights (BW).

Sampling and measurements

Two pasture samples were collected from the paddock each week and were collected at 09:00 h and 14:00 h. A representative sample was obtained by dividing the paddock into eight blocks, then each block was sampled systematically throughout. The samples were then bulked, mixed and sub-sampled before freezing (-20ºC) prior to freeze-drying and analysis. Hay samples (100 g) were collected from each 40-kg bag and pooled for further sub-sampling and analysis. Both pasture and hay samples were analysed for DM, organic matter (OM), total nitrogen (N) (Ministry of Agriculture, Fisheries and Food, 1981), and soluble carbohydrates (Osborne and Voogt, 1978).

Samples of rumen fluid were collected, during the pre-treatment period, *via* a stomach tube at 09:00 h on day 21 for the pasture-adapted sheep and at 24 h after feeding on day 28 for the indoor hay-adapted sheep. During the treatment period, rumen fluid samples were taken immediately before giving the barley (initial, time 0 h) and then at 6, 12 and 24 h later. Roughage food was withheld for 24 h after barley feeding. Immediately after sampling an aliquot of rumen fluid sample (10 ml) was acidified

to pH 2 to 3 using concentrated sulphuric acid (3 to 4 drops) and frozen (–20ºC) for later analyses of VFA and lactate (L- and D-isomers). VFA concentrations were measured using flame ionization detection in a gas chromatograph (Hewlett-Packard) fitted with a Chromosorb 'W', acid washed and 60- to 80-mesh column coated with two liquid phases, a:o-phosphoric acid (1·5% w/w) and b : polypropylene glycol sebacate (17·5% w/w). The carrier gas was nitrogen and the temperatures of the column oven, detector and the injector were 135, 180 and 210ºC respectively. L-lactate and D-lactate were analysed by auto-analyser (Cobas Mira Autoanalyser, Roche Diagnostics Inc., Frenchs Forest, NSW) using an enzymatic procedure (Stat-Pack ™ Rapid Lactate Test, cat. no. 1112 821, Behring Diagnostics Inc., Somerville, New Jersey). The pH of rumen fluid was recorded immediately after sampling.

For microbial counts, samples of rumen fluid were immediately strained and collected in a CO_2-filled flask. Strained samples were then serially diluted 10-fold in anaerobic dilution solution (ADS) as described by Caldwell and Bryant (1966) to a final dilution of 10^{-8}. Three dilutions (10^{-6}, 10^{-7} and 10^{-8}) were used to inoculate the selective media roll tubes, in triplicate for each dilution. The tubes were incubated at 39ºC for 3 days. For bacterial enumeration the following media were used. Medium 10 (M 10) for total bacteria (Caldwell and Bryant, 1966), a modified membrane-bovis agar (m-BA) medium for *S. bovis* (Oragui and Mara, 1984) and a modified non-selective MRS medium (De Man *et al.*, 1960; Oxoid, England) for lactic acid bacteria. The MRS medium was modified by adding a freshly prepared reducing solution (1 ml containing 0·026 g cysteine-HCl and 0·026 g Na_2S. $9H_2O$ per 100 ml of media) after boiling then pre-reduced by bubbling with nitrogen on ice until cold. The pH of the medium was adjusted to 5·5 during preparation. The m-BA medium contained (g/100 ml) : 0·2 $(NH_4)_2SO_4$; 0·01 yeast extract (Oxoid); 0·4 inulin; 0·3 raffinose hydrate; 0·1 sodium beta-glycerophosphate; 0·005 sodium azide; 0·01 NaCl; 0·005 bromocresol purple; 0·02 KCl; 0·02 $MgSO_4$·7H2O; 0·01 K_2HPO_4 and 1·2 agar (Oxoid no. 1). After dissolving the ingredients, 7 ml of 8% Na_2CO_3 and 1 ml of reducing solution were added. Agar was added to Hungate tubes (about 40 mg per tube). The solution was heated gently to dissolve all ingredients and the pH was adjusted to 6·8 before being dispensed into the Hungate tubes under CO_2 (2·8 ml of medium per tube). Both media were sterilized by autoclaving at 105ºC for 45 min.

At the same time as rumen fluid was sampled, faeces were collected directly from the rectum and added to a plastic container. An amount of water equal to the weight of faeces was added to it and, after thorough mixing, the pH was measured using a portable pH meter (Activon Scientific Products Co Pty Ltd., NSW, Australia). Samples of faeces were also dried in a force draft oven at 70ºC to a constant weight for determination of moisture content. Roughage intakes were recorded for 10 days after grain over-feeding for the hay-adapted group.

Isolation and identification of high acid-producing S. bovis

The anaerobic techniques for preparing and for inoculating the bacteria into the media were those of Hungate (1969) using O_2-free CO_2. Colonies picked from the m-BA and MRS agar roll tubes were inoculated into a broth of Basal Medium 10 (BM 10) with glucose (0·2%)(Caldwell and Bryant, 1966). They were incubated anaerobically at 39ºC for 48 h then subcultured into m-BA and MRS agar roll tubes. The same procedure, comprising the picking of viable colonies and inoculating them into a broth medium and then again into roll tubes, was repeated at least twice. At 48 h of incubation a drop of the broth medium was then examined under the microscope to check the purity of the culture. Morphological and Gram staining characteristics of the cells were recorded. The ability of cultures to ferment various carbohydrates was evaluated using BM 10 broths with either arabinose, cellobiose, fructose, galactose, glucose, glycerol, inulin, lactose, maltose, mannitol, mannose, raffinose, ribose, sorbitol, starch, sucrose or xylose separately included at 2 g/l. Bacterial growth was checked by visible turbidity and optical density measured at 600 nm. Fermentation end products were measured over an incubation period of 24 h at 39ºC.

The 16S rRNA complete gene sequencing and DNA hybridization technique (Lane, 1991) were used to identify the most prevalent strains of *Streptococcus*, at the Department of Microbiology and Parasitology, University of Queensland, St Lucia, Australia, as described previously (Al Jassim and Rowe, 1999).

Statistical treatment of data

Data were analysed as a 2×2 (two basal diets, and barley with or without virginiamycin) and $2 \times 2 \times 4$ (including the effect of sampling time, 0, 6, 12 and 24 h post feeding) factorial experiment using *STATGRAPHICS* plus (1996) (Manugistics, Rockville, MD). Bacterial counts were transformed using $\log_{10}$ and initial bacterial counts were used as a covariate (when significant) for analysis of counts obtained at 6, 12 and 24 h. Log_{10} transformation was carried out primarily to stabilize the variance. Comparisons between means were made using Fisher's least

Table 1 *Chemical composition of pasture, hay, and barley* (*g/kg dry matter*)

	Diets †		
	Pasture	Hay	Barley
Dry matter	241	917	900
Ash	87	80	26
Organic matter	913	920	974
Crude protein (total N × 6·25)	175	177	112
Total soluble carbohydrates	159	74	27

† Diets, pasture: native green grasses and legumes, hay: chopped lucerne and oaten hay 60: 40.

significant differences (LSD) procedures. Values of mean viable bacterial counts were transformed back to the original scale before being presented in tables.

Results

Chemical composition of the diets

Chemical composition of pasture, hay and barley grain are presented in Table 1. Pasture provided twice as much total soluble carbohydrate as hay (159 *v.* 74 g/kg DM) and similar amounts of crude protein (175 *v.* 177 g/kg DM).

Bacterial counts

Neither the basal diet nor addition of virginiamycin (VM) had a significant effect ($P > 0{\cdot}05$) on total bacterial counts (Table 2). However there was an interaction between basal diet and VM at 24 h post barley challenge for total bacterial counts, these being higher for the pasture-adapted sheep given barley with VM, and lower for the hay-adapted sheep given barley with and without VM. There were no differences in streptococcal counts (counts on m-BA) between the two groups of sheep given different basal diets (pasture and hay) and VM had no significant effect on the numbers. There was a trend toward higher *S. bovis* counts at 24 h after feeding barley (Table 3). Lactic acid bacteria counts (Table 4) were higher ($P < 0{\cdot}01$) in the pasture-fed group at the start of the experiment and there was an increase in their counts over the 24-h sampling period. Addition of VM to barley had no effect on lactic acid bacteria (counts on MRS) but there was a significant interaction ($P < 0{\cdot}001$) between basal diet and the effect of VM on their numbers. There was an increase in lactic acid bacteria counts in the pasture-adapted sheep given barley with VM whereas there was a decline in their numbers in sheep adapted to hay and fed barley with VM.

There were major differences in the type of colonies and their capacity for acid production in m-BA roll tubes inoculated from sheep on barley plus VM as compared with barley alone. High acid producing (HAP) *S. bovis* formed mucoid, creamy, orange-centred colonies while the low acid-producing *streptococci* formed small white colonies. The

Table 3 *Counts of viable* S. bovis (*mean c. f. u.* $\times 10^7$) *in the rumen both before treatment and at various times after engorgement with barley* (*900 g dry matter per head*) *according to basal diet and addition of virginiamycin* (*VM, 50 mg/kg barley*)†

	Diets‡			
	Pasture		Chopped hay	
Time (h)	Barley	Barley + VM	Barley	Barley + VM
0 (covariates)	4·898	2·818	2·239	3·020
6	3·891	4·169	6·761	2·188
12	20·417	38·905	37·154	31·623
24	43·651	48·978	91·201	19·953

† Values are the least-squares means of six animals transformed back to the original scale.
‡ Diets, pasture: native green grasses and legumes, hay: chopped lucerne and oaten hay 60: 40.
Diet and VM had no significant effect ($P > 0{\cdot}05$).

Table 2 *Counts of total viable bacteria* (*mean c. f. u.* $\times 10^8$) *in the rumen both before treatment and at various times after engorgement with barley* (*900 g dry matter per head*) *according to basal diet and addition of virginiamycin* (*VM, 50 mg/kg barley*) †

	Diets‡						
	Pasture		Chopped hay		Significance of effects		
Time (h)	Barley	Barley + VM	Barley	Barley + VM	Diet	VM	Diet × VM
0 (covariates)	5·370	5·754	2·631	2·951	*		
6	3·388	3·981	7·244	2·570			
12	10·233	22·907	9·772	6·918			
24	16·982	30·903	19·953	8·511			*

† Values are the least-squares means of six animals transformed back to the original scale.
‡ Diets, pasture: native green grasses and legumes, hay: chopped lucerne and oaten hay 60: 40.

Table 4 *Counts of rumen viable lactic acid bacteria (mean c. f. u. $\times 10^7$) in the rumen both before treatment and at various times after engorgement with barley (900 g dry matter per head) according to basal diet and addition of virginiamycin (VM, 50 mg/kg barley)*†

	Diets‡						
	Pasture		Chopped hay		Significance of effects		
Time (h)	Barley	Barley + VM	Barley	Barley + VM	Diet	VM	Diet × VM
0 (covariates)	3·802	2·455	1·259	1·119	**		
6	3·802	7·244	6·310	2·570			
12	25·704	63·096	41·687	18·197			
24	38·019	131·826	104·713	23·442			***

† Values are the least-squares means of six animals transformed back to the original scale.
‡ Diets, pasture: native green grasses and legumes, hay: chopped lucerne and oaten hay 60: 40.

addition of VM to barley inhibited the growth of HAP strains of *S. bovis*. Although numbers of colonies in the m-BA roll tubes medium were similar in sheep challenged with barley with or without VM, changes in the bromocresol purple indicator in the media from purple to yellow revealed a higher level of acid production in tubes inoculated from sheep given barley without VM. This difference in acid production between *S. bovis* colonies was seen at all sampling times (6, 12 and 24 h).

Culture characteristics and microscopic examination
HAP *S. bovis* grew on both m-BA and MRS media where the cells were oval or spherical with an average diameter of 0·9 to 1 μm and were often encapsulated. They occurred mainly in pairs, short chains of 4 to 8 cells and singles. All were Gram-positive and the stain was more concentrated in the capsule. *S. bovis* isolates grew very quickly during the first 6 h of incubation in a broth of BM 10 with glucose (0·5%) and produced large amounts of acid, mainly L-lactate, which peaked 41 mmol/l at 12 h. Two distinguishable types of *S. bovis* (orange pigmented or white) were obtained. Both isolates were able to ferment cellobiose, fructose, galactose, glucose, inulin, lactose, maltose, mannose, raffinose, starch and sucrose but not arabinose, glycerol, mannitol, ribose, sorbitol or xylose. The orange-pigmented isolate produced most pigment on glucose, inulin and starch media.

Identification of bacteria
Complete sequences of the 16S rRNA genes of five cultures from five different purified isolates were 100% identical. They were all *S. bovis* accessions. The phylogenetic tree prepared from a dataset of 1235 nucleotides (full sequence masked according to the masking method of Lane (1991)) suggests that the *S. bovis* strains of the following database accession numbers, AF104109, AB002481 and AB002482 are the closest phylogenetic relatives. The percentage

Table 5 *Ruminal concentrations of volatile fatty acid (VFA, mmol/l) and the molar proportions of acetate, propionate, butyrate, isobutyrate, isovalerate, valerate and lactate as affected by basal diet and virginiamycin (VM, 50 mg/kg barley)*†

	Diets‡						
	Pasture		Chopped hay		Significance of effects		
Time (h)	Barley	Barley + VM	Barley	Barley + VM	Diet	VM	Diet × VM
Total VFA (mmol/l)	58·8	60·8	69·3	65·6			
Acetic acid (%)	62·7	56·0	59·2	55·5	*	***	
Propionic acid (%)	19·8	34·6	22·2	31·9		***	**
Butyric acid (%)	12·5	6·5	14·6	9·5	***	***	
Isobutyric acid (%)	2·1	1·3	0·7	1·4		*	
Isovaleric acid (%)	1·6	1·2	1·3	1·3			
Valeric acid (%)	1·3	0·6	1·3	0·8		***	
L-Lactate (mmol/l)	5·8	4·9	4·2	12·5			
D-Lactate (mmol/l)	4·5	3·9	4·5	10·9			

† Values are the least-squares means of six animals.
‡ Diets, pasture: native green grasses and legumes, hay: chopped lucerne and oaten hay 60: 40.

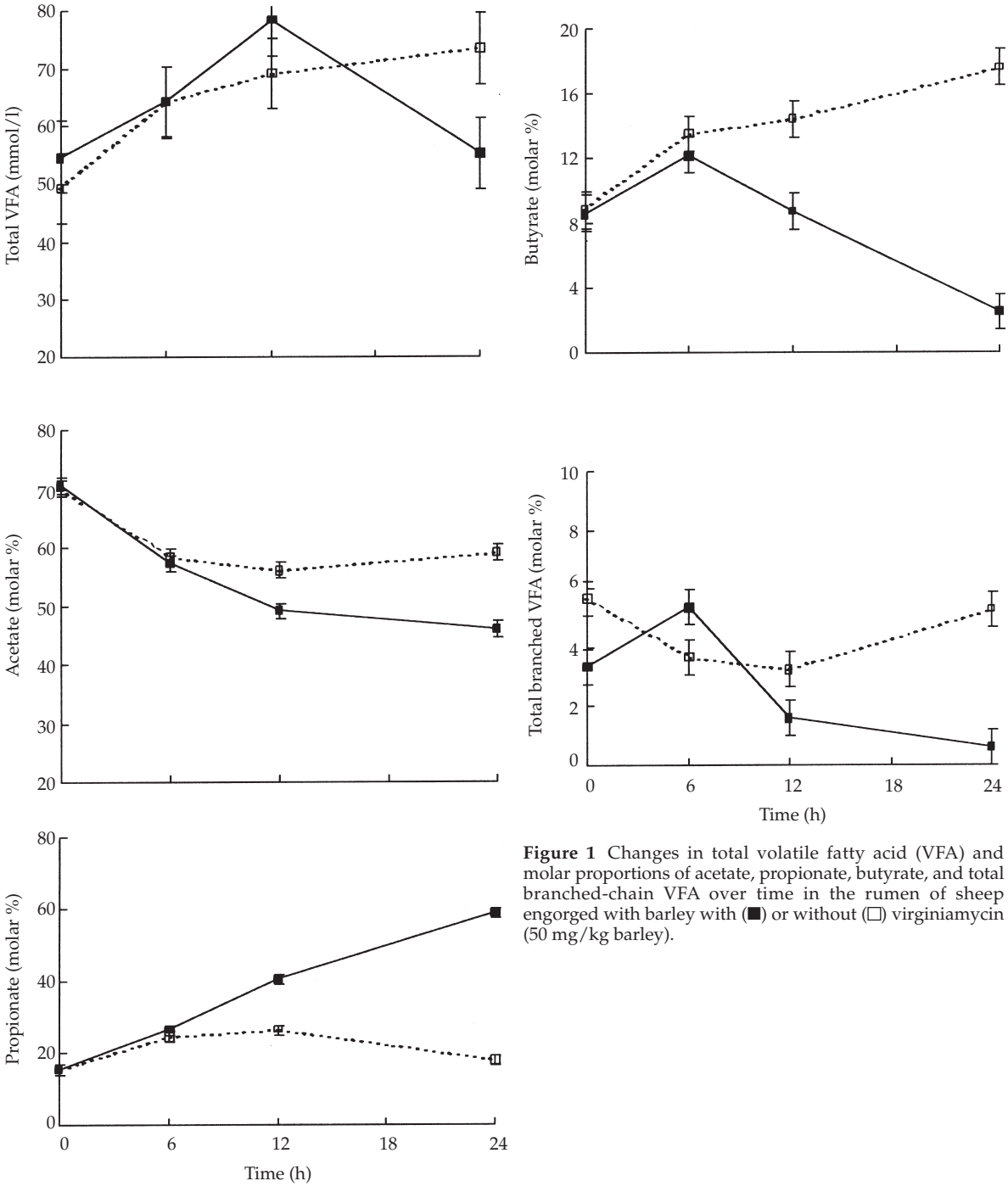

Figure 1 Changes in total volatile fatty acid (VFA) and molar proportions of acetate, propionate, butyrate, and total branched-chain VFA over time in the rumen of sheep engorged with barley with (■) or without (□) virginiamycin (50 mg/kg barley).

identities between our isolates and their closest related strains of *S. bovis* are between 99·2 and 100.

Ruminal VFA and lactate concentrations

Neither the basal diet nor the addition of VM to barley (Table 5) significantly affected the

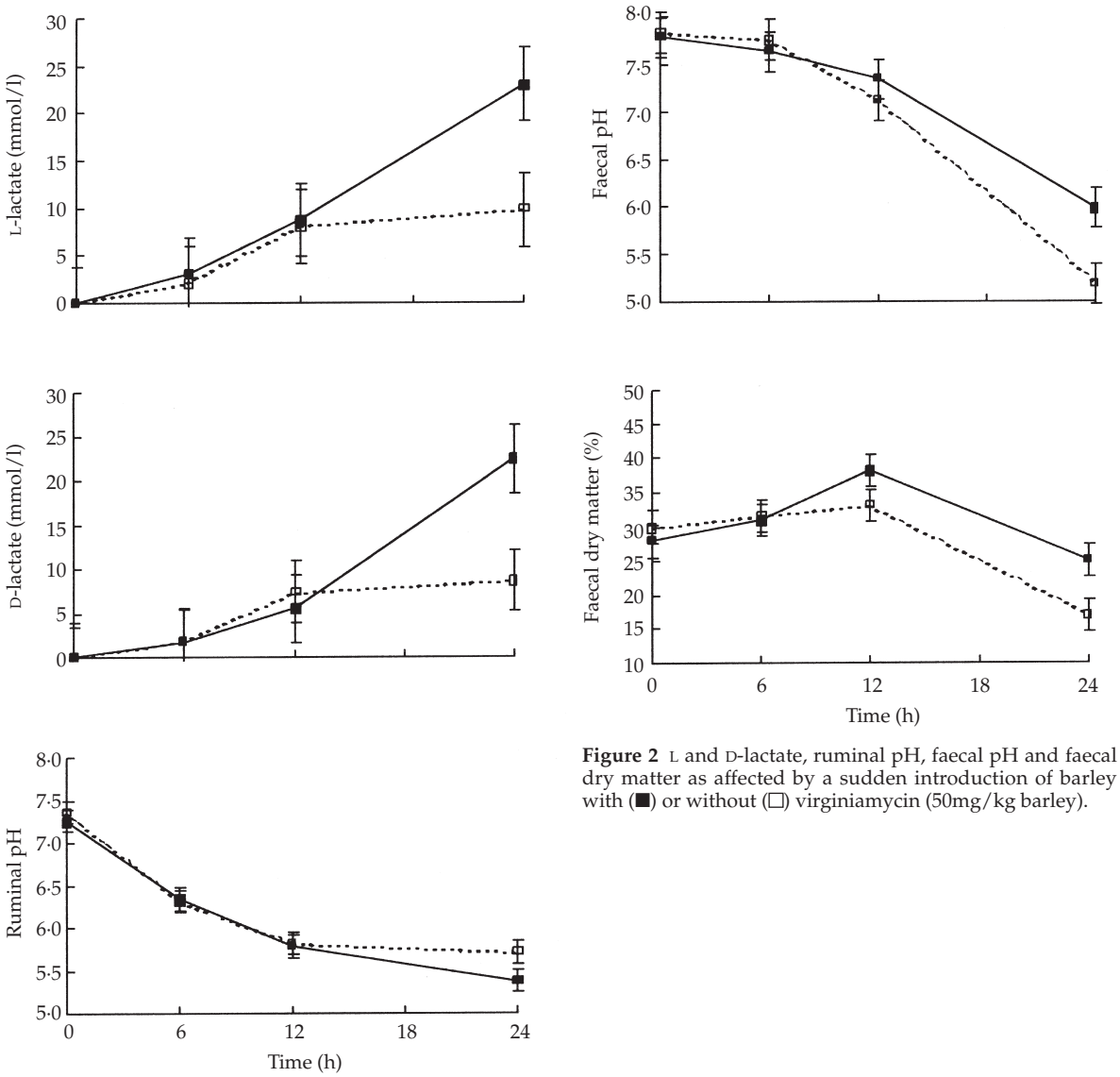

Figure 2 L and D-lactate, ruminal pH, faecal pH and faecal dry matter as affected by a sudden introduction of barley with (■) or without (□) virginiamycin (50mg/kg barley).

concentration of total VFA (mmol/l). An increase ($P < 0{\cdot}01$) in VFA concentration with time was observed for the first 12 h following barley challenge for the sheep on barley plus VM, then concentrations declined to reach the initial level by 24 h (Figure 1). The average molar proportion of acetic acid was higher ($P < 0{\cdot}05$) for the pasture-adapted sheep than for hay-adapted sheep (59·4 *v.* 57·4, Table 5) and lower ($P < 0{\cdot}001$) for sheep given barley plus VM as compared with sheep given barley alone (55·8 *v.* 61·0). There was a progressive decline ($P < 0{\cdot}01$) in acetic acid concentration in all sheep, over time following barley challenge. Interaction between addition of VM to barley and time was highly significant ($P < 0{\cdot}001$) with the rate and extent of acetate reduction being greater ($P < 0{\cdot}05$) for the sheep given barley plus VM, especially after 12 h post drenching (Figure 1). The basal diet had no effect on the concentration (molar proportions) of propionate ($P > 0{\cdot}05$) but the concentration was higher ($P < 0{\cdot}001$) for sheep on barley plus VM (Table 5) and increased ($P < 0{\cdot}001$) over time after barley drenching in all sheep. Diet × VM and VM × time interactions with respect to rumen propionate were highly significant ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively) showing a greater increase over time

Table 6 *Faecal pH both before treatment and at various times after engorgement with barley (900 g dry matter per head) according to basal diet and addition of virginiamycin (VM, 50 mg/kg barley)*†

	Diets‡				
	Pasture		Chopped hay		
Time (h)	Barley	Barley + VM	Barley	Barley + VM	Significance§ of diet
0 (covariates)	7·50	7·43	8·17	8·12	***
6	7·76	7·57	7·72	7·77	***
12	6·42	6·57	7·89	8·09	**
24	5·11	6·15	5·25	5·72	

† Values are the least-squares means of six animals.
‡ Diets, pasture: native green grasses and legumes, hay: chopped lucerne and oaten hay 60: 40.
§ VM and diet ×VM had no significant effect (P>0·05).

with VM (Figure 1). Butyric acid concentration (Table 5) was higher ($P < 0·001$) for hay-adapted sheep (12·1 *v.* 9·5) and lower ($P < 0·001$) for the sheep given barley plus VM (8·0 *v.* 13·6). Its concentration was increased with time following barley challenge to a peak concentration at 6 h post drenching, followed by a steady decline during the subsequent hours, but remained elevated at 24 h. Interaction between VM and time was significant ($P < 0·001$) indicating that the concentration of butyric acid at 24 h post barley feeding was primarily affected by the addition of VM to barley. Valeric acid concentration was also reduced ($P < 0·001$) by the addition of VM (0·7 *v.* 1·3) and the significant interaction between VM and time ($P < 0·001$) clearly demonstrates such effects. Basal diet had no effect ($P > 0·05$) on the concentration of valeric acid but there was a tendency toward a higher concentration in sheep given hay (1·1 *v.* 0·9). Concentrations of both isobutyric and isovaleric were reduced ($P < 0·01$) over time after barley challenge and by addition of VM to barley. Ruminal D-and L-lactate concentrations were increased ($P < 0·001$) over time following barley drenching (Figure 2) but only for the hay-adapted sheep. There were no changes in the concentration of D- and L-lactate over time for the pasture-adapted sheep. At 24 h after barley drenching the levels of D- and L-lactate for the sheep given barley plus VM were about double that for sheep given barley alone.

Rumen pH and faecal measurements

Addition of VM to barley did not prevent the decline in ruminal pH following barley feeding (Figure 2), and pasture-adapted sheep had higher pH than hay-adapted sheep (6·49 (s.e. 0·11) *v.* 6·00 (s.e. 0·13)). The significant interaction ($P < 0·001$) between diet and time after barley feeding showed greater decline in ruminal pH in pasture-adapted sheep compared with those adapted to hay. Faecal pH in pasture-adapted sheep was lower ($P < 0·001$) than in hay-adapted ones (Table 6). At 24 h after barley challenge, there was a trend towards a higher faecal pH for sheep on barley plus VM (Figure 2). Sheep adapted to pasture produced wetter ($P < 0·05$) faeces than hay-adapted sheep (26·2 *v.* 31·5% DM). Compared with their initial faeces, all sheep produced softer

Table 7 *Dry matter content of faeces both before treatment and at various times after engorgement with barley (900 g dry matter per head) according to basal diet and addition of virginiamycin (VM, 50 mg/kg barley)*†

	Diets ‡					
	Pasture		Chopped hay		Significance of effects§	
Time (h)	Barley	Barley + VM	Barley	Barley + VM	Diet	VM
0 (covariates)	27·38 (±3·2)	25·02 (±3·2)	32·16 (±3·19)	30·76 (±3·19)		
6	28·65 (±3·0)	27·29 (±3·0)	35·76 (±3·0)	34·53 (±3·0)	*	
12	29·22 (±3·6)	35·98 (±3·7)	38·72 (±3·7)	40·46 (±3·6)		
24	13·48 (±3·2)	22·01 (±3·3)	20·15 (±3·3)	29·10 (±3·2)		*

† Values are the least-squares means of six animals.
‡ Diets, pasture: native green grasses and legumes, hay: chopped lucerne and oaten hay 60: 40.
§ Diet × VM had no significant effect ($P > 0·05$).

faeces ($P < 0{\cdot}001$) 24 h after barley challenge, and addition of VM to the barley resulted in a higher ($P < 0{\cdot}05$) DM content (Table 7).

Sheep condition and hay intake

All sheep challenged with barley alone showed signs of distress and dullness at 12 h after barley feeding, while sheep receiving VM with barley were more alert and less distressed. One sheep from the pasture-fed group had severe diarrhoea at 24 h after barley feeding with a faecal pH of 4·7. All sheep from pasture resumed grazing after the treatment period of 24 h, although food intake was not measured after their release. Hay intake was measured over a period of 10 days following barley feeding, and was lower ($P < 0{\cdot}05$) for the sheep given VM with barley. All sheep given barley alone returned to pre-treatment hay intake levels of 900 g DM per day after 6 days. Four sheep of the six that received the VM and barley treatment returned to initial levels of roughage intake after 8 days (Figure 3), while the other two refused all food for the 10-day measurement period. These two sheep had a normal ruminal pH of 7·2 and 7·4 at 6 days after barley feeding and the rumen contents were mainly fluids. No other examinations were conducted on these sheep and they were released to pasture on day 10 after the treatment.

Discussion

It is important to note that sheep in this experiment had not previously experienced grain feeding, and the grain challenge occurred only once. Results of viable bacterial enumeration of this study are in disagreement with the general scenario often presented for the sequential events that lead to lactic acidosis in ruminants (Huntington, 1993; Wallace, 1996). Our results exhibited a similar trend to that reported by Allison *et al.* (1975), in terms of microbial changes, but the magnitude of changes were different.

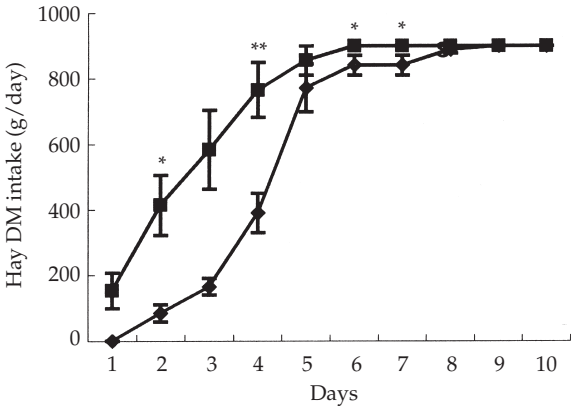

Figure 3 Daily intake of hay (g dry matter (DM) per day; ± s.e.) after engorgement with barley (900 g DM per head) with (—◆—) or without (—■—) virginiamycin (VM, 50 mg/kg barley). Two sheep of the six that received barley + VM refused all food for the 10-day measurement period and were not included in the figure above.

Counts of total bacteria, *S. bovis* (counts on m-BA medium) and lactic acid bacteria (counts on MRS medium) continued to increase for 24 h after barley challenge. The total population of lactic acid bacteria (counts on MRS), which includes *S. bovis*, increased from 16·9% of total bacteria at the commencement to about 97·3% at 24 h after barley feeding. Since m-BA medium is selective for *S. bovis* (Oragui and Mara, 1984), the increase in the numbers of this bacterial species is the major contributor to the increase in total lactic acid bacteria. The change in lactobacilli was less than expected from previous results of Allison *et al.* (1975). It is important to note here that elimination of high acid-producing (HAP) *S. bovis* did not prevent the decline in pH. This could have been caused by growth of other lactic acid producing bacteria, which benefited from substrate made available for them (Al Jassim and Rowe, 1999). However, there was a noticeable change in the proportion of HAP *S. bovis* strains in the population, as indicated by the rapid change in medium colour after incubation with an inoculum from a barley challenged sheep. The HAP *S. bovis* was isolated from both the selective m-BA and the non-selective MRS media. While these HAP *S. bovis* were the predominant ones in sheep engorged with barley alone, they were absent from sheep receiving barley with VM. The high numbers of the small white colonies counted on m-BA medium in the absence of *S. bovis* could represent other species of streptococcal bacteria such as *Enterococcus faecium* (Oragui and Mara, 1984).

It is known that sheep adapted to grain feeding undergo microbial changes leading to a ruminal ecosystem that is in balance between starch-fermenters and lactate-utilizers (Mackie *et al.*, 1978), a balance that might have been disturbed by the use of VM in this study. Although the supply of soluble carbohydrates from pasture (159 g/kg DM) was higher than hay, the initial ruminal lactate level was not affected by basal diet, which suggests the presence of an adapted microbial population. Goad *et al.* (1998) reported differences in *Lactobacillus* populations between grain-adapted and hay-adapted steers. However the supply of soluble carbohydrate from pasture is insufficient to match the quantity supplied by grains, particularly in an

unimproved native grass-based pasture that has a very short growth season. Other factors need to be considered, including rumination and salivation associated with ingestion of a roughage diet which was minimized after barley feeding, as well as rumen motility and digesta mixing.

Changes in molar proportions of VFA produced (i.e. increased propionate) as a result of adding VM to barley, as reported here, is a typical phenomenon (Godfrey *et al.*, 1993; Nagaraja *et al.*, 1995; Goad *et al.*, 1998; Thorniley *et al.*, 1998).

Ruminal and faecal pH were not affected by addition of VM to grain, and both declined over time after barley engorgement. Decreased ruminal pH was consistent with increased concentration of VFA in the pasture-adapted sheep and VFA plus lactate in the hay-adapted sheep. Ruminal pH at 24 h after barley challenge decreased to 5·7 for the sheep given barley alone and 5·4 for the sheep on barley plus VM. These pH levels indicate a state of sub-acute acidosis (Goad *et al.*, 1998). Similar responses were reported by Godfrey *et al.* (1995) when two groups of sheep, previously adapted to barley with or without VM, were divided into two sub-groups each and challenged with a greater amount of barley with or without VM. A decrease in ruminal pH was also reported by Nagaraja *et al.* (1995) in sheep gradually introduced to grains and given food at different frequencies, and by Goad *et al.* (1998) in steers adapted to either grain or hay and over-fed with grains. Although *S. bovis* is known to be less resistant to low pH (Owens *et al.*, 1998), the rapid drop in ruminal pH of this study was not associated with a decline in the *S. bovis* population. Isolates from MRS agar roll tubes' medium, which had its pH adjusted to 5·5, were also *S. bovis* as confirmed by the full gene sequence of their 16S rRNA. These isolates were considered to be the high acid producers. This indicates that pH alone is not the only limiting factor to the growth of *S. bovis* with progressive acid accumulation. There is a growing support for the hypothesis that some lactobacilli produce a bacteriocin that inhibits the growth of *S. bovis* in the rumen (Wells *et al.*, 1997).

Unlike ruminal pH, faecal pH at 24 h after barley feeding was higher for sheep challenged with barley plus VM as compared with sheep on barley alone (5·94 *v.* 5·18), and lower for sheep adapted to hay as compared with sheep adapted to pasture (5·49 *v.* 5·63). This indicates that the hindgut is an important site of action for VM. The absence of an effect with VM at the ruminal level may have been caused by the possible dilution of VM in the rumen, or by it being washed out as a result of higher outflow rate caused by using a relatively large volume of water (approximately 3 to 5 l) to facilitate drenching sheep with 0·9 kg of crushed barley. This also may not be the case since water is efficiently absorbed from the stomach and the majority of VM is not absorbed (Gottschall *et al.*, 1987). It is also possible that other lactate-producing bacteria that were not affected by VM had an established population (Al Jassim and Rowe, 1999). Therefore, it should have an effect in the rumen after the first 6 h, otherwise the effect would be evident on the hindgut environment. However, only a limited increase was recorded in faecal DM content ($P < 0·001$) and in faecal pH. It was also observed that sheep adapted to pasture exhibited signs of loose faeces and diarrhoea from 12 h after barley feeding while sheep adapted to hay required a longer time (around 24 h) to show these symptoms. This emphasizes the need for longer measurement periods in sheep given a basal hay diet in order to show the effect of barley overfeeding. The effect of basal diet was also proposed as an important factor determining the supplementary value of VM with grains for penned cattle given grain separate from roughage (Courtney and Seirer, 1996).

Conclusion

The results of this experiment show that sheep adapted to green pasture initially had higher ruminal pH than hay-adapted sheep but the pH declined in both groups following barley challenge. However, those adapted to pasture did not develop lactic acidosis when challenged with grain and the decline in ruminal and hindgut pH values was mainly due to the increase in the concentration of total VFA. Despite the effectiveness of VM against high acid producing *S. bovis*, its addition to barley neither reduced counts of lactic acid bacteria in the rumen nor prevented acidosis in sheep. Therefore, previous nutrition is a key factor in determining the type of acidosis that develops (e.g. lactic or VFA acidosis) and the hindgut of sheep is an important site for rapid fermentation of readily fermentable carbohydrates as well as for the action of VM.

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References

Al Jassim, R. A. M. and Rowe, J. B. 1999. Better understanding of acidosis and its control. *Recent Advances in Animal Nutrition in Australia* **12:** 91-97.

Allison, M. J., Robinson, I. M., Dougherty, R. W. and Bucklin, J. A. 1975. Grain overload in cattle and sheep: changes in microbial population in the cecum and rumen. *American Journal of Veterinary Research* **36:** 181-185.

Caldwell, D. R. and Bryant, M. P. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Applied Microbiology* **14:** 794-801.

Chaucheyras, F., Fonty, G., Bertin, G., Salmon, J. and Gouet, P. 1996. Effect of a strain of *Saccharomyces cerevisiae* (Levucell® SC), a microbial additive for ruminants, on lactate metabolism *in vitro*. *Canadian Journal of Microbiology* **42:** 927-933.

Courtney, D. A. and Seirer, R. C. 1996. Supplementary feeding of grain to cattle with virginiamycin to reduce the risk of acidosis. *Proceedings of the Australian Society for Animal Production* **21:** 344 (abstr.).

De Man, J. C., Rogosa, M. and Sharp, M. E. 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* **23:** 130-135.

Dunn, B. H., Emerick, R. J. and Embry, B. 1979. Sodium bentonite and sodium bicarbonate in high-concentrate diets for lambs and steers. *Journal of Animal Science* **48:** 764-769.

Goad, D. W., Goad, C. L. and Nagaraja, T. G. 1998. Ruminal microbial and fermentative changes associated with experimentally induced sub-clinical acidosis in steers. *Journal of Animal Science* **76:** 234-241.

Godfrey, S. I., Rowe, J. B., Speijers, E. J. and Toon, W. 1993. Lupin, barley, or barley plus virginiamycin as supplement for sheep at different feeding intervals. *Australian Journal of Experimental Agriculture* **33:** 135-140.

Godfrey, S. I., Rowe, J. B., Thorniley, G. R., Boyce, G. R. and Speijers, E. J. 1995. Virginiamycin to protect sheep fed wheat, barley or oats from grain poisoning under simulated drought feeding conditions. *Australian Journal of Agricultural Research* **46:** 393-401.

Gottschall, D. W., Gombatz, C. and Wang, R. 1987. Analysis of tissue residues and comparative metabolism of virginiamycin in rats, turkeys and cattle. *Journal of Agricultural and Food Chemistry* **35:** 900-904.

Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. In *Methods in microbiology, vol. 3* (ed. J. R. Norris and E. W. Ribbons), pp. 117-132. Academic Press, New York.

Huntington, G. B. 1993. Nutritional problems related to the gastro-intestinal tract: acidosis. In *The ruminant animal: digestive physiology and nutrition* (ed. D. C. Church), pp. 474-480. Waveland Press Inc., IL.

Huntington, G. B., Emerick, R. J. and Embry, L. B. 1977. Sodium bentonite effects when fed at various levels with high concentrate diets to lambs. *Journal of Animal Science* **45:** 119-125.

Kezar, W. W. and Church, D. C. 1979. Effect of thiopeptin and bicarbonate on the prevention of lactic acidosis induced in sheep. *Journal of Animal Science* **49:** 1396-1402.

Kung, L. and Hession, A. O. 1995. Preventing *in vitro* lactate accumulation in ruminal fermentation by inoculation with *Megasphaera elsdenii*. *Journal of Animal Science* **73:** 250-256.

Lane, D. J. 1991. 16S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics* (ed. E. Stackebrandt and M. Goodfellow), pp. 115-175. Academic Press, Chichester.

Mackie, R. I., Gilchrist, F. M. C., Robberts, A. M., Hannah, P. E. and Schwartz, H. M. 1978. Microbiological and chemical changes in the rumen during the stepwise adaptation of sheep to high concentrate diets. *Journal of Agricultural Science, Cambridge* **90:** 241-252.

Ministry of Agriculture, Fisheries and Food. 1981. *The analysis of agricultural materials, second edition*. A manual of the analytical methods used by the Agricultural Development and Advisory Service, pp. 1-6.

Nagaraja, T. G., Avery, T. B., Bartley, E. E., Galitzer, S. J. and Dayton, A. D. 1981. Prevention of lactic acidosis in cattle by lasalocid or monensin. *Journal of Animal Science* **53:** 206-216.

Nagaraja, T. G., Avery, T. B., Galitzer, S. J. and Harmon, D. L. 1985. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. *American Journal of Veterinary Research* **46:** 2444-2452.

Nagaraja, T. G., Godfrey, S. I., Winslow, S. W. and Rowe, J. B. 1995. Responses in ciliated protozoa and rumen fermentation in sheep with barley plus virginiamycin. *Australian Journal of Agricultural Research* **46:** 1137-1147.

Oragui, J. I. and Mara, D. D. 1984. A note on a modified membrane-Bovis agar for the enumeration of *Streptococcus bovis* by membrane filtration. *Journal of Applied Bacteriology* **56:** 179-181.

Osborne, D. R. and Voogt, P. 1978. *The analysis of nutrient in foods*. Academic Press, London.

Owens, F. N., Secrist, D. S., Hill, W. J. and Gill, D. R. 1998. Acidosis in cattle: a review. *Journal of Animal Science* **76:** 275-286.

Rogers, J. A., Branine, M. E., Miller, C. R., Wray, M. I., Bartle, S. J., Preston, R. L., Gill, D. R., Pritchard, R. H., Stilborn, R. P. and Bechtol, D. T. 1995. Effects of dietary virginiamycin on performance and liver abscess incidence in feedlot cattle. *Journal of Animal Science* **73:** 9-20.

Schwartz, H. M. and Gilchrist, F. M. C. 1974. Microbial interactions with the diet and the host animal. In *Digestion and metabolism in the ruminant* (ed. I. W. McDonald and A. C. I. Warner), pp. 165-179. University of New England Publishing Unit, Armidale, Australia.

Statgraphics Plus. 1996. *Using general linear regression models (GLM)*. Manugistics Inc., Rockville, MD.

Thorniley, G. R., Rowe, J. B., Cowcher, P. C. and Boyce, M. D. 1998. A single drench of virginiamycin to increase safety of feeding grain to sheep. *Australian Journal of Agricultural Research* **49:** 899-906.

Wallace, R. J. 1996. Rumen microbiology and efficiency of digestion. In *Milk composition, production and biotechnology* (ed. R. A. S. Welch, D. J. W. Burns, S. R. Davis, A. I. Popay and C. G. Prosser), pp. 465-487. CAB International, Wallingford.

Wells, J. E., Krause, D. O., Callaway, T. R. and Russell, J. B. 1997. A bacteriocin-mediated antagonism by ruminal lactobacilli against *Streptococcus bovis*. *FEMS Microbiology Ecology* **22:** 237-243.

Wiryawan, K. G. and Brooker, J. D. 1995. Probiotic control of lactate accumulation in acutely grain-fed sheep. *Australian Journal of Agricultural Research* **46:** 1555-1568.

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