Better understanding of acidosis and its control

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Summary

Bacterial isolates taken from rumen fluid and faeces from sheep fed hay or pasture and supplemented with wheat grain, cattle fed grain, and faeces from horses fed grain with hay or pasture have shown that *S. bovis* is a key bacterium that produces large amounts of acids, mainly as L–lactate. We have isolated two distinguishable types of *S. bovis* (orange pigmented or white) from sheep, cattle and horses. All of the *S. bovis* isolates were very sensitive to virginiamycin (VM) with a minimum inhibitory concentration (MIC) of 2 mg/ml and we have not been able to isolate this bacterium when sheep were given VM. Both types of *S. bovis* grow well on the non–selective MRS medium and their growth was not affected by the low pH of the MRS medium (5.5). Other dominant lactic acid–producing bacteria we have isolated using MRS medium have been identified as *Lactobacillus vitulinus* and *Selenomonas ruminantium*, and these appear to grow well in grain–fed sheep in both the presence and absence of VM. The population of the latter two bacteria was well established in the rumen of grass–adapted sheep when the supply of fermentable carbohydrates was plentiful. Sensitivity of *L. vitulinus* isolates to VM varied from very sensitive, with a MIC of 2 mg/ml, to being resistant to a higher concentration (6 mg/ml). While *S. bovis* produced only L–lactate, four *L. vitulinus* isolates produced D–lactate and one strain produced L– and D–lactate. Two *S. ruminantium* isolates produced L– and D–lactate and one produced only L–lactate. Only one *S. ruminantium* isolate and none of the *L. vitulinus* isolates fermented starch. *S. ruminantium* isolates were resistant to the highest VM level of 8 mg/ml. The inability of *L. vitulinus* and *S. ruminantium* to ferment starch indicates the important role of *S. bovis* and other starch fermenting bacteria in providing substrates for these bacteria and emphasizes the key role of *S. bovis* in the sequential events leading to lactic acidosis. Our results suggest that there are relatively few strains of each of *S. bovis*, *L. vitulinus* and *S. ruminantium* and that there is a close genetic homogeneity within each genus. The presence of *L. vitulinus* and *S. ruminantium* strains in the rumen suggests that the use of virginiamycin with ruminants on green feed may not always reduce fermentative acidosis.

Introduction

The introduction of starch into the rumen leads to rapid fermentation and production of VFA. As the rate of VFA production exceeds their rate of removal, rumen pH may fall below 6.0. Such low pH favours the rapid growth of starch–degrading bacteria including *Streptococcus bovis* and *Lactobacillus* spp. The pH continues to fall until even the resilient *S. bovis* can no longer grow and lactobacilli take over, fermenting the starch to produce more lactic acid and maintaining an even lower pH (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). Among the several methods for reducing the risk of acidosis, the use of antibiotic feed additives such as virginiamycin (Godfrey *et al.* 1995; Rogers *et al.* 1995) or certain ionophores (Nagaraja *et al.* 1981; Nagaraja *et al.* 1985) were very effective. However under certain feeding conditions, addition of virginiamycin has not always prevented the risk of acidosis (Godfrey *et al.* 1995; Courtney and Seirer, 1996; Thorniley *et al.* 1998; Al Jassim and Rowe, unpublished data). In the absence of information on the microbial status of the gut it was not clear why variable responses were obtained. However in horses the virginiamycin–based product Founderguard^{TM} has proved to be consistently effective against founder or laminitis in horses even under grazing conditions (Rowe 1997). Therefore we need to enhance our knowledge on the microbiology of fermentative acidosis. This work aims at identifying the key bacteria responsible for the development of acidosis and tests their sensitivity to virginiamycin (VM).

Materials and methods

Isolation, Identification and characterization of lactic acid bacteria

Rumen fluid samples were obtained via stomach tube from sheep and via rumen fistula from cattle 24 h after regular grain feeding. Faecal samples were obtained directly from the rectum of sheep and cattle and from the freshly voided faeces from horses. Samples were processed for the enumeration of lactic acid bacteria following the method of Yanke and Cheng (1998), involving one–hour exposure prior to incubation. A semi–selective MRS–agar medium, Oxoid, England (De Man *et al.* 1960) 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 CO_2 on ice until cold. The pH of the medium was adjusted to 5.5 during preparation. Viable colonies from roll tubes were picked and inoculated into a broth of a basal medium 10 (BM 10) as described by Caldwell and Bryant (1966) with glucose (0.5%) and then again cultured in roll tubes. The procedure of picking colonies and inoculating them into a broth medium followed by inoculating again into roll tubes was repeated twice. At 48 h of incubation a drop of the broth medium was examined under the microscope to check the purity of the culture. Morphology and Gram staining characteristics of the isolates were recorded. The ability of cultures to ferment various carbohydrates was evaluated using a broth of BM10 with each isolate included at 2 g per litre. *S. bovis* was distinguished from *S. equinus* on the basis of their ability to ferment starch, inulin and lactose and their ability to survive heating to 60°C for 30 minutes was also tested (Hardie 1986).

Fermentation products were measured after 24 h of anaerobic incubation of a broth consisting of BM 10 with glucose or starch (0.5%) at 39ºC. At the end of the fermentation period, samples from the media were taken for measurement of pH, then acidified with sulphuric acid for further analysis of VFA and lactate. VFA concentrations were measured using a gas chromatograph (Hewlett–Packard), fitted with a Chromosorb 'W', acid washed and 60–80 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 temperature for the column, detector and the injector were 135, 180 and 210°C respectively. L–lactate and D–lactate were analyzed by auto–analyzer (Cobas Mira Autoanalyser, Roche Diagnostics Inc., French Forest, NSW) using an enzymatic procedure (Stat–Pack^{TM} Rapid Lactate Test, Cat. No. 1112 821, Behring Diagnostics Inc., Somerville, New Jersey). The 16s rRNA complete gene sequencing and DNA hybridization techniques (Lane 1991) were used to identify the most prevalent strains of *S. bovis*, *L. vitulinus* and *S. ruminantium*.

Virginiamycin sensitivity test

Two isolates of *S. bovis* from cattle and sheep (orange pigmented and white), five *L. vitulinus* and three isolates of *S. ruminantium* were incubated in a broth of basal medium 10 with glucose (0.5%) and VM at concentrations of 0, 2, 4, 6 and 8 mg/ml. The VM solution (100 mg VM/ml) was prepared using Eskalin Wettable Powder (WP), 400 g/kg VM (Pfizer Animal Health, NSW). The WP was dissolved in distilled water (previously boiled and bubbled with nitrogen until cooled) and filter–sterilized whilst being gassed with nitrogen. This solution was used immediately after preparation. The lowest concentration of VM that resulted in no measurable growth of the test bacteria was considered as the Minimum Inhibitory Concentration (MIC). The broth–media were inoculated with 0.2 ml of the different fresh viable cultures and 0.1–0.4 ml of VM solution was added to each tube to provide the required dose. A sensitivity test was also performed when VM was added 3 and 6 h after inoculation, using the same concentrations of VM. Tests for sensitivity to VM were conducted using 24 and 4 h of anaerobic incubation.

DNA extraction, PCR amplification and cloning

Freshly grown cultures were withdrawn from screw–cap Hungate tubes by first flaming the septum with ethanol. Sub–samples (0.6 ml) of liquid culture were taken with a syringe (25G needle) and placed in sterile 1.5 ml centrifuge tubes. Cells were harvested as a pellet following centrifugation at 13500 *g* for 2 min. The supernatant was poured off and the cells resuspended in approximately 30 µL of culture fluid remaining.

DNA was extracted from the concentrated cell suspension using a Fast DNA SPIN Kit (BIO 101, Inc., CA). Extracted DNA was visualized on a 1% TAE gel amended with 0.5 µl of 10 mg/ml ethidium bromide per 50 ml agarose. PCR was used to amplify 16S rDNAs in 100 µl reactions. Each reaction tube contained 200 ng of each primer, 10 µl of 10X buffer, 6 µl of $MgCl_2$, 1U of Tth DNA polymerase (Biotech International, Perth, Australia), 10 µl of 4 x 0.5 mM dNTP's, and the remainder made up with sterile Milli–Q water and 2 µl concentrated cell suspension or DNA extract. Reactions were overlaid with sterile mineral oil and carried out in a thermocycler (Perkin–Elmer DNA Thermal Cycler 480). Thermocycling parameters employed after a 96°C denaturation for 10 min were 28 cycles of 1 min at 94°C, 1 min at an annealing temperature, and 2 min at 72°C. A further extension step involving 1 min at 48°C and 5 min at 72°C was also employed. The primers used were 27f (5'–AGAGTTTGATCCTGGCTCAG–3') and 1492r (5'–GGTTACCTTGTTACGACT–3') (Lane 1991). In some cases GeneReleaser (Bioventures, Inc., USA) was used according to the manufacturer's instructions in the reactions outlined above. PCR products were

purified using a QIAquick PCR purification kit (QIAGEN, Australia) according to the manufacturer's instructions.

Sequencing of 16S rDNA

All 16S rDNA samples were initially partially sequenced using the universal 16S rRNA primer, 530f (5'–GTGCCAGCMGCCGCGG–3') and an ABI Big Dye Terminator Cycle Sequencing Ready Reaction Mix kit (ABI, Australia). Selected 16S rDNA were subsequently fully sequenced on both strands using the following primers: 519r (5'–GWATTACCGCGGCKGCTG–3'), 27f, 907r (5'–CCGTCAATTCMTTTRAGTT T–3'), 926f (5'–AAACTYAAAKGAATTGACGG–3'), and 1492r (Lane 1991). Approximately 100 ng of purified PCR product and 25 ng of primer were used in the sequencing reactions. Thermal cycling was carried out in an MJ Research PTC–100 thermocycler with an initial denaturation step of 96°C for 2 min, followed by 25 cycles of 50°C for 15 sec , 60°C for 4 min, and 96°C for 30 sec. The resulting cycle sequencing products were purified using the ethanol plus sodium acetate method (ABI, Australia). Purified sequencing products were submitted to the Australian Genome Research Facility for analysis on an Applied Biosystems 377 automated sequencer.

Phylogenetic analysis

Phylogenetic analysis of 16S rDNAs was carried out according to Dojka *et al.* (1998). Briefly, sequences were aligned and compiled in SeqEd (Applied Biosystems, Australia). Compiled sequences were compared with those on publicly available databases by use of the BLAST (Basic Alignment Search Tool) (Altschul *et al.* 1990) to determine approximate phylogenetic affiliations. Compiled sequences were then aligned using the ARB software package (Strunk *et al.* unpublished) and refined manually. Phylogenetic trees based on comparative analysis of the 16S rRNA genes were constructed by performing evolutionary distance analyses on these alignments using the appropriate tool in the ARB database. The robustness of the tree topology was tested by performing bootstrapping (2000 replicates) in PAUP test version 4.0d65.

Results

All twenty streptococcal isolates (6 from sheep, 2 from cattle and 12 from horses) were 99% identical in sequence to each other and to *S. bovis* and *S. equinus*. These isolates therefore belong to the genus *Streptococcus* and are members of either *S. bovis* or *S. equinus*. Three of the 12 streptococcal isolates from horses were identified as *S. equinus* since they did not ferment starch or inulin and only one of them fermented lactose.

Sb R1 was the dominant *S. bovis* strain across all three animal species (cattle, sheep and horse). It grows into bright orange–centered colonies on MRS agar roll tubes and produces an orange pigment in a broth of BM 10 with glucose or starch. The cells are 0.9 –1.0 µm in diameter and often encapsulated, occurr mainly in pairs, short chains of 4–10 cells, and singles. Three streptococcal isolates from horses, identified as *S. equinus*, occurred mainly in long chains.

Table 1 shows the fermentation end products of *S. bovis*, *S. equinus*, *L. vitulinus* and *S. ruminantium* isolates. L–lactate was the main fermentation product of *S. bovis* and *S. equinus*. All of the *S. bovis* could ferment cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, starch and sucrose, but not arabinose, glycerol, mannitol, ribose, sorbitol or xylose and two of the white pigmented isolates did not ferment inulin (Table 2).

The *L. vitulinus*–like isolates have 100% sequence identity to each other, and 97% identity with *L. vitulinus*. *L. vitulinus*–like cells occurred in different shapes and sizes, mainly straight rods in singles, pairs and short chains and some cells have the tendency to branch. *L. vitulinus* isolates could not ferment starch or xylose but grew on cellobiose (except LV R3), fructose, glucose, mannose, raffinose and sucrose (Table 2). Four *L. vitulinus* isolates produced D–lactate and one (LV R3) produced L– and D–lactate at equal proportions (Table 1).

S. ruminantium was isolated from the rumen of grass–adapted sheep that received a grain supplement with or without VM. The three *Selenomonas*–like isolates formed two distinct lines of descent: Type A (isolate SR R1); and Type B (isolate SR R2 and isolate SR R3). Type A has 99% identity to *S. ruminantium* and >97% identity to Type B, and Type B has >97% identity to *S. ruminantium*. This indicates that all three isolates are members of the genus *Selenomonas,* and that Type A belongs to the species *S. ruminantium*. One isolate (SR R1) produced L–lactate while the other two (SR R2 and SR R3) produced L– and D–lactate (Table 3). A difference between SR R2 and SR R3 was that SR R3 produced 42.5% more lactic acid from starch than SR R2. *S. ruminantium* grew on most carbohydrate sources (Table 2) but their growth was noticeably high on glucose and sucrose compared with other sources and only one isolate (SR R1) did not ferment starch. The cells are 0.5–0.7 x 1.5–3.0 µm, occurred mainly in single short crescent rods, with round ends, and are Gram negative.

Similar amounts of L–lactate were produced from glucose or starch by the different *S. bovis* isolates (Table 4). All *L. vitulinus* isolates produced the same total amounts of lactate from glucose.

Incubation *in vitro* of *S. bovis*, *L. vitulinus* and *S. ruminantium* isolates in a broth of Basal Medium 10 with glucose (0.5%) with different concentrations of VM (0, 2, 4, 6 and 8 mg/ml) clearly demonstrated sensitivity of *S. bovis* to virginiamycin. *Lactobacillus* and *Selenomonas* isolates (Table 5) showed varying levels of resistance to VM. LV R1 and LV R5 were very sensitive to VM with a MIC level of 2 mg/ml. Isolates LV

Table 1 Fermentation products of *Streptococcus bovis*, *Streptococcus equinus*, *Lactobacillus vitulinus* and *Selenomonas ruminantium* isolates in a broth of basal medium 10 with glucose (0.5%).

Isolate	End products†
S. bovis	L – Lactic
S. equinus	L – Lactic
L. vitulinus (LV R1, LV R2, LV R4 and LV R5)	D – Lactic
L. vitulinus (LV R3)	L – Lactic, D – Lactic
S. ruminantium (SR R1)	L – Lactic, Acetic, Propionic
S. ruminantium (SR R2)	L – Lactic, D – Lactic, Acetic, Propionic
S. ruminantium (SR R3)	L – Lactic, D – Lactic, Acetic, Propionic

†Bold indicates main fermentation product

Table 2 Fermentation of carbohydrate substrates by *S. bovis*, *S. equinus*, *L. vitulinus* and *S. ruminantium* isolates from pasture–adapted sheep supplemented with wheat grain plus urea.

	Bacterial Isolates									
	Streptococcus		***L. vitulinus***					***S. ruminantium***		
Carbohydrate	***S. bovis***	**S. equinus**	**LV R1**	**LV R2**	**LV R3**	**LV R4**	**LV R5**	**SR R1**	**SR R2**	**SR R3**
Arabinose	–	–	–	–	–	–	–	–	–	–
Cellobiose	+	+	+	+	–	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	–	–	+	–	–	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Glycerol	–	–	–	–	–	–	–	–	–	–
Inulin	+*	–	+	+	–	+	+	–	–	–
Lactose	+	–**	–	–	+	–	–	+	+	+
Maltose	+	+	+	+	–	+	+	+	+	+
Mannitol	–	–	–	–	+	–	–	+	–	–
Mannose	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+
Ribose	–	–	–	–	–	–	–	+	+	–
Sorbitol	–	–	–	–	–	–	–	+	+	–
Starch	+	–	–	–	–	–	–	–	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Xylose	–	–	–	–	–	–	–	–	–	+

* Two of the white pigmented *S. bovis* isolates did not ferment inulin
**One *S. equinus* isolate grew on lactose

Table 3 Lactate production (mmol/l) by *S. ruminantium* (SR R1, SR R2 and SR R3) cultures after 24 h of anaerobic incubation in a broth of basal medium 10 with glucose or starch added at 0.5% at 39°C. The pH of BM 10 with glucose was 6.92 and with starch was 7.20.

	Type A		**Type B**	
Isolates →	***S. ruminantium* (SR R1)**		***S. ruminantium* (SR R2 and SR R3)**	
Carbohydrate	**Glucose**	**Starch**	**Glucose**	**Starch**
L–Lactate	23.7	0.0	20.1	15.5
D–Lactate	0.0	0.0	19.9	15.1
Total (mmol/l)	23.7	0.0	40.0	26.6
Final pH	5.41		5.35	5.59

Table 4 Lactate production (mmol/l) by *S. bovis* and *L. vitulinus* cultures after 24 h of anaerobic incubation in a broth of basal medium 10 with glucose or starch added at 0.5% at 39°C. The pH of BM 10 with glucose was 6.92 and with starch was 7.20.

Isolates	*S. bovis** (Sb R1, Sb R2, Sb R3)		*L. vitulinus* (LV R1, LV R2, LV R4, LV R5)		*L. vitulinus* (LV R3)	
Carbohydrate	**Glucose**	**Starch**	**Glucose**	**Starch**	**Glucose**	**Starch**
L–Lactate	41.1	42.8 (± 0.22)	0.0 (± 0.50)	0.0	20.7	0.0
D–Lactate	0.0	0.0	39.3	0.0 (±1.87)	20.6	0.0
Total (mmol/l)	41.1	42.8 (± 0.22)	39.3 (± 0.50)	0.0 (±1.87)	41.3	0.0
Final pH	5.04 ± 0.001	5.24 ± 0.02	5.57± 0.07		5.53	

*Mean values ± SE

Table 5 Effect of virginiamycin (VM) concentration on the growth of different bacterial isolates of *S. bovis*, *L. vitulinus* and *S. ruminantium* in a broth of basal medium 10 with glucose added at 0.5%.

Bacterial isolate	VM level (µg/ml) 0		2		4		6		8	
Incubation period (h) →	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
S. bovis and *S. equinus* (orange pigmented)	+	+	–	–	–	–	–	–	–	–
S. bovis and *S. equinus* (white pigmented)	+	+	–	–	–	–	–	–	–	–
L. vitulinus (LV R1)	+	+	–	+	–	+	–	+	–	+
L. vitulinus (LV R2)	+	+	+	+	–	+	–	–	–	–
L. vitulinus (LV R3)	+	+	+	+	–	+	–	+	–	+
L. vitulinus (LV R4)	+	+	+	+	+	+	–	+	–	+
L. vitulinus (LV R5)	+	+	–	+	–	+	–	+	–	–
S. ruminantium (SR R1, SR R2 and SR R3)	+	+	+	+	+	+	+	+	+	+

+growth; – no growth

R2 and LV R3 resisted levels up to 4 mg/ml and LV R4 up to 6 mg/ml. *S. ruminantium* isolates were resistant to the highest VM levels (8 mg/ml). Sensitivity of *Lactobacillus* to VM was different when VM was added at 3 and 6 h after inoculation. All *Lactobacillus* cultures growing for 3 h before the addition of VM had higher MIC than when VM was added to immediately after inoculation. Sensitivity to VM was also lower when assessed using a 48 h incubation. All *Lactobacillus* isolates were resistant to the highest VM levels (8 mg/ml) when cultures were allowed to grow for 6 h before the addition of VM.

Discussion

The twenty streptococcal isolates from cattle, sheep and horses show a high degree of genetic homology and despite some phenotypic differences they cannot be separated into groups; therefore they are considered to be one distinct genetic group. It is also important to note that *S. bovis* isolated from different parts of the world and from different species of animals appear to be closely related (Nelms *et al.* 1995). The closest relatives to our isolates were: JB 1 isolated from cows in California (Russell and Baldwin 1978; Russell and Robinson 1984); ATCC 33317 isolated from swine in Japan (Mori *et al.* 1997); and 26, 45S1 and 581 AXY2 from Scotland (Nelms *et al.* 1995). Our ability to isolate *S. bovis* from sheep supplemented with wheat grain but not the VM–treated wheat grain demonstrated the sensitivity of *S. bovis* to VM at the level used (40 g/tonne grain).

The presence of *L. vitulinus* and *S. ruminantium* in rumen fluid and the faeces of sheep fed diets with or without VM demonstrated the resistance of some

Lactobacillus strains to VM. This resistance of *Lactobacillus* and *Selenomonas* isolates has been confirmed by results from anaerobic *in vitro* incubation in a broth of BM 10 with glucose (0.5%).

A sensitivity test showed that *S. bovis* strains are quite sensitive to VM and this reaction seems to be irreversible and fatal. On the other hand the reaction of *Lactobacillus* cells to VM may be reversible and the initial reaction results in only bacteriostasis rather than bactericidal effects. The reason for the lesser sensitivity of *Lactobacillus* to VM measured at 48 h of anaerobic incubation as compared with 24 h would be because efficacy of VM is reduced after 24 h or because *Lactobacillus* are equipped with the enzymes that are capable of breaking down the bonding formed between VM and the specific protein. The lesser sensitivity of *Lactobacillus* to VM when VM was added at 3 or 6 h after inoculation, while the sensitivity of *S. bovis* remained unchanged, suggest that when the population of *Lactobacillus* is well established in the gut a higher level of VM may be needed to control it. It also indicates that in a grass–adapted sheep when the supply of soluble carbohydrate is plentiful the likelihood of controlling acidosis is minimal.

The fact that the Gram negative bacterium *S. ruminantium* is not sensitive to VM suggests that there will be situations where VM may not provide full protection against acidosis. Godfrey *et al.* (1995) and Nagaraja *et al.* (1995) reported that in grain–adapted sheep the inclusion of VM with grain did not prevent decreased ruminal pH and increased lactate. However, the inability of *L. vitulinus* to ferment starch and their ability to ferment glucose and maltose indicates that these bacteria benefit from their association with *S. bovis*, which provides substrates for their growth. It might also explain why it was possible to reduce the level of D–lactate but not L–lactate with VM doses over 40 mg/kg grain (Thorniley *et al.* 1998).

Populations of *S. ruminantium* may be well established in the rumen under green–grass feeding conditions, and this bacterium is known to be both a lactate producer that produces large amounts of D–lactate (Russell and Baldwin 1978) and variable in its ability to hydrolyze starch and ferment lactate (Bryant 1956). Our isolates produced either L–lactate or L– and D–lactate and two of them were able to ferment starch. These bacteria also have the advantage over *S. bovis* in having a lower energy–maintenance requirement (Russell and Baldwin 1979) which explains the ability of *S. ruminantium* to maintain their population in the rumen on diets containing low levels of soluble substrates. Therefore, controlling *S. bovis* and some strains of *Lactobacillus* alone may reduce the chances for acute acidosis, or ease the condition, but does not prevent the decline in ruminal and faecal pH due either to high production of VFA (Lana *et al.* 1998; Al Jassim and Rowe 1998 unpublished data) or the production of lactate by other bacteria.

It is interesting that in faecal material from the horse we have found no *Selenomonas* or *L. vitulinus* but rather a mixture of *S. bovis* and *S. equinus*. *S. equinus* is not able to utilize starch and only ferments sugars. It is possible that *S. equinus* strains in the horse take on an equivalent role to that played by *Selenomonas* or *L. vitulinus* in ruminant animals in complementing *S. bovis* in the fermentation of readily available carbohydrates. Both species of *Streptococcus* are sensitive to VM, which explains the consistent efficacy of Founderguard in the horse.

Conclusion

Although the role of *S. bovis* and *Lactobacillus* as major lactic acid producers is widely acknowledged, one can not rule out the involvement and the contribution of other bacteria. This is based on our basic knowledge of the complexity of the system we are dealing with, and our recent finding that *S. ruminantium* is an important lactate producer in cattle and sheep.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.

Bryant, M.P. (1956). The characteristics of strains of *Selenomonas* as isolated from bovine rumen contents. *Journal of Bacteriology* **72**, 162–167.

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.

Courtney, D.A. and Seirer, R.C. (1996). Supplementary feeding of grain to cattle with virginiamycin to reduce the risk of acidosis. *Animal Production in Australia* **21**, 344.

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.

Dojka, M.A., Hugenholtz, P., Haack, S.K. and Pace, N.R. (1998). Microbial diversity in a hydrocarbon– and chlorinated–solvent–contaminated aquifer undergoing intrinsic bioremediation. *Applied and Environmental Microbiology* **64**, 3869–3877.

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 Speijer, 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.

Hardie, J.M. (1986). Other Streptococci. In: *Bergey's Manual of Systematic Bacteriology*, Volume 2, pp. 1068–1069 (ed. Peter H.A. Sneath). Williams and Wilkins, Baltimore, USA.

Huntington, G.B. (1993). Nutritional problems related to the gastro–intestinal tract: Acidosis. In: *The Ruminant Animal: Digestive Physiology and Nutrition*, pp. 474–480 (ed. D.C. Church). Waveland Press, Inc. Illinois, USA.

Lana, P.R., Russel, J.B. and Van Amburgh, M.E. (1998). The role of pH in regulating ruminal methane and ammonia production. *Journal of Animal Science* **76**, 2190–2196.

Lane, D.J. (1991). 16S/23S Sequencing. In*: Nucleic Acids Techniques in Bacterial Systematics,* pp. 115–175 (eds. E. Stackebrandt and M. Goodfellow). John Wiley and Sons, New York.

Mori, Y., Takahashi, T., Katsumi, M., Katoh, K., Hiramune, T. and Kikuchi, N. (1997). Phylogenetic analyses of swine isolates of *Streptococcus dysgalactiae* based on 16S rDNA sequence (unpublished, submitted to the DDBJ/EMBL/ GenBank database on March 31, 1997, Accession AB002482).

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. 1(985). Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. *American Journal of Veterinary Research* **46,** 2444–2452.

Nagaraja, T.G. and Taylor, M.B. (1987). Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives*. Applied and Environmental Microbiology* **53**, 1620–1625.

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.

Nelms, L.F., Odelson, D.A., Whitehead, T.R. and Hespell, R.B. (1995). Differentiation of ruminal and human *Streptococcus bovis* strains by DNA homology and 16s rRNA probes. *Current Microbiology* **31**, 294–300.

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.

Rowe, J.B. (1997). 'Acidic gut syndrome': is it a problem for animals and humans? In: *Recent Advances in Animal Nutrition in Australia 1997,* pp. 47–55 (eds. J.L. Corbett, M. Choct, J.V. Nolan and J.B. Rowe). University of New England, Armidale NSW.

Russell, J.B. and Baldwin, R.L. (1978). Substrate preference in rumen bacteria: evidence of catabolite regulatory mechanisms*. Applied and Environmental Microbiology* **36**, 319–329.

Russell, J.B. and Baldwin, R.L. (1979). Comparison of maintenance energy expenditures and growth yields among several bacteria grown on continuous culture. *Applied and Environmental Microbiology* **37**, 537–543.

Strunk, O., Gross, O., Reichel, B., May, M., Herman, S., Struckmann, N., Nonhoff, B., Lenke, M., Vilbig, A., Ludwig, T., Bode, A., Schleifer, K–H. and Ludwig, W. Program available from http://www.mikro.biologie.tu–muenchen.de (unpublished).

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*. *Biotechnology in Agriculture Series* **18**, 465–487.

Yanke, L.J. and Cheng, K.–J. (1998). A method for the selective enumeration and isolation of ruminal *Lactobacillus* and *Streptococcus. Letters in Applied Microbiology* **26**, 248–252.