

# Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets

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## ABSTRACT

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**Aim:** To determine whether *Megasphaera elsdenii* YE34 (lactic acid degrader) and *Butyrivibrio fibrisolvens* YE44 (alternative starch utilizer to *Streptococcus bovis*) establish viable populations in the rumen of beef cattle rapidly changed from a forage-based to a grain-based diet.

**Methods and Results:** Five steers were inoculated with the two bacterial strains (YE34 and YE44) and five served as uninoculated controls. With the exception of one animal in the control group, which developed acidosis, all steers rapidly adapted to the grain-based diet without signs of acidosis (pH decline and accumulation of lactic acid).

Bacterial populations of *S. bovis*, *B. fibrisolvens* and *M. elsdenii* were enumerated using real-time *Taq* nuclease assays. Populations of *S. bovis* remained constant (except in the acidotic animal) at *ca* 10<sup>7</sup> cell equivalents (CE) ml<sup>-1</sup> throughout the study. *Megasphaera elsdenii* YE34, was not detectable in animals without grain in the diet, but immediately established in inoculated animals, at 10<sup>6</sup> CE ml<sup>-1</sup>, and increased 100-fold in the first 4 days following inoculation. *Butyrivibrio fibrisolvens*, initially present at 10<sup>8</sup> CE ml<sup>-1</sup>, declined rapidly with the introduction of grain into the diet and was not detectable 8 days after grain introduction.

**Conclusion:** *Megasphaera elsdenii* rapidly establishes a lactic acid-utilizing bacterial population in the rumen of grain-fed cattle 7–10 days earlier than in uninoculated cattle.

**Significance and Impact of the Study:** The study has demonstrated that rumen bacterial populations, and in particular the establishment of bacteria inoculated into the rumen for probiotic use, can be monitored by real-time PCR.

**Keywords:** grain feeding, lactic acidosis, *Megasphaera elsdenii*, probiotic bacteria, rumen.

## INTRODUCTION

When cattle are fed diets containing a high proportion of cereal grain, the pH of the rumen contents often falls to very low levels (pH < 5) (Dunlop 1972; Nocek 1997; Owens

*et al.* 1998). This decreases the efficiency by which substrate is converted to volatile fatty acids (VFAs) and microbial protein for animal production (Strobel and Russell 1986). The drop in pH is often associated with the accumulation of lactic acid, which can lead to acute lactic acidosis (Dawson and Allison 1988).

*Streptococcus bovis* has been implicated as the main causative agent in the syndrome as it is capable of rapid

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growth on starch-based substrates, producing lactic acid as the primary fermentation end-product (Mackie and Gilchrist 1979; Russell and Hino 1985; Owens *et al.* 1998). It is hypothesized that the rapid growth by *S. bovis* is beyond the rate that lactic acid-utilizing bacteria and other competitive starch-utilizing bacteria are capable of attaining (Russell and Hino 1985; Dawson and Allison 1988). This results in an overgrowth by the faster growing *S. bovis*, an accumulation of lactic acid, with a concomitant drop in pH, leading to acidotic ruminal conditions and reduced efficiency of feed utilization (Dawson and Allison 1988; Mackie *et al.* 2002).

For these reasons it is a widely practised management strategy to introduce cattle to grain over a number of weeks with the proportion of grain in the diet increasing over that period. This is to allow time for the resident populations of lactic acid-utilizing and other starch-fermenting bacteria to keep up with the growth of *S. bovis* and prevent acidosis from occurring. Alternative preventative measures that have been suggested include the use of antibiotics (Godfrey *et al.* 1995; Owens *et al.* 1998), probiotic bacteria (Kung and Hession 1995; Wiryawan and Brooker 1995; Owens *et al.* 1998) and immunization against *S. bovis* (Shu *et al.* 1999).

Here we discuss the probiotic approach whereby lactic acid-utilizing and competitive starch-utilizing bacteria are selected from the rumens of cattle adapted to a high-grain diet, and introduced to cattle entering a feedlot. This should augment indigenous populations of these bacteria thereby preventing acute lactic acidosis, allowing a faster introduction to grain and improving the efficiency of starch utilization.

This paper reports the isolation of bacteria with the potential for use as probiotics and their establishment in the rumen of cattle being introduced to a high-grain diet.

## MATERIALS AND METHODS

### Isolation, selection and characterization of bacteria for probiotic use

Bacteria capable of growth on either wheat starch (Sigma) or lactic acid (85%, Univar, Sydney, Australia) as the primary substrate for growth, were isolated from samples of rumen contents from cattle adapted to a high-grain diet (barley or sorghum). Samples of rumen contents were those used for the isolation of the Sb series of *S. bovis* strains reported previously (Klieve *et al.* 1999). The isolated bacteria were evaluated for probiotic use based on VFA and organic acid (OA) profiles, growth rate and cell density (based on optical density) and total cellular nitrogen content, following 24 h incubation (results not shown), as an estimate of microbial protein yield. A single bacterial isolate from each substrate-utilizing group was selected for further evaluation and characterized on phenotypic criteria, VFA and OA profiles and comparative DNA sequence analysis of the 16S rRNA

gene, as previously reported (Ouwkerk and Klieve 2001; Ouwkerk *et al.* 2002). The selected starch-utilizing bacterium was a strain of *Butyrivibrio fibrisolvens* (designated YE44) and the lactic acid-utilizing bacterium was a strain of *Megasphaera elsdenii* (designated YE34), as previously reported (Ouwkerk *et al.* 2002).

### Pen trial

**Experimental design.** Animal ethics approval was obtained from NSW Agriculture's North Coast Animal Care and Ethics Committee, and was in accordance with the Australian code of practice.

A pen trial was conducted to determine whether *M. elsdenii* YE34 and *B. fibrisolvens* YE44, when inoculated into the rumen, would establish viable populations and prevent pH decline in the rumen and hindgut, and lactic acid accumulation in the rumen of beef cattle that were rapidly switched from a forage-based diet to a grain-based diet.

Ten rumen cannulated *Bos taurus* (Hereford) steers were used in the pen trial. Steers were individually penned and allocated to two groups of five steers. One group was inoculated with *M. elsdenii* YE34 and *B. fibrisolvens* YE44 (see below), the other group was the uninoculated control.

All steers received the same feed. For the first 6 days in pens they were fed a medium quality paspalum (*Paspalum dilatatum*) hay (8.8 MJ ME kg<sup>-1</sup> DM, 78 g N kg<sup>-1</sup> DM) supplemented with Molafos [molasses-based mineral supplement, Ridley AgriProducts (Aust) Pty Ltd, Wacol, Queensland] (8% w/w of total ration), protein meal (4.5%), urea (1%), lime (1%) and bicarbonate (0.5%). Over the next 5 days they were introduced to a high-grain (rolled barley) diet in the following steps: days 7 and 8, 45% rolled barley (balance being paspalum hay); days 9 and 10, 60% rolled barley (balance as before); and days 11–22, 75% rolled barley (balance as before). Day 22 was the final day of the trial.

**Ruminal inoculation with *M. elsdenii* YE34 and *B. fibrisolvens* YE44.** Ten starter cultures (5 ml) were incubated at 39°C overnight. Five of the cultures were *M. elsdenii* YE34 [in rumen fluid (RF) lactate broth] and five were *B. fibrisolvens* YE44 (RF starch broth). Under anaerobic conditions (Coy Laboratory anaerobic chamber, Coy Laboratory Products Inc., Ann Arbor, MI, USA; 95% CO<sub>2</sub>, 5% H<sub>2</sub>), each 5 ml culture was added to one of 10 serum bottles (Wheaton, Millville, NJ, USA) containing 500 ml of broth. *Megasphaera elsdenii* was placed in RF lactate broth and *B. fibrisolvens* in RF starch broth. The medium in the serum bottles was prewarmed by overnight incubation at 39°C. Following inoculation with either *M. elsdenii* or *B. fibrisolvens*, serum bottles were resealed and transported at room temperature to the site of the pen trial (duration *ca* 5 h). Upon arrival pressure was released from

the serum bottles by inserting a 26-gauge needle through the butyl rubber septum, the needle was left in place to prevent pressure build-up during incubation. The serum bottles were incubated at 39°C overnight.

Before the cattle were inoculated with the bacteria, samples (2 ml) were removed from the serum bottles for enumeration of bacterial numbers and determination of the dose of the inoculum. Samples were taken, using a needle and syringe, from six bottles (three of each species) and transferred to sterile, sealed and pregassed (95% CO<sub>2</sub>, 5% H<sub>2</sub>) Hungate tubes. These were placed on ice, transported back to the laboratory following inoculation of the steers and immediately enumerated using Hungate's roll-tube technique (Hungate 1969). A subsample of each was also visually examined (Olympus BH-2 microscope, Olympus Optical Co. Ltd, Japan; mag. 400×) to ensure that the bacteria appeared morphologically sound and that no contamination had occurred.

Immediately after samples were removed from the culture vessels and prior to the morning feeding (08.00 hours), five steers were inoculated with a 500 ml culture of each bacterial species. Inoculation was directly into the rumen via the cannula. The remaining five steers were the untreated control group.

**Sampling procedure.** Samples of ruminal fluid for pH determination, analysis of VFA and OA, and for enumeration of specific bacterial populations, were obtained by siphoning contents from the rumen directly through the rumen cannula. Separate tubing was used for each animal throughout the trial. Samples of faeces for determination of faecal pH were from freshly voided material or from 'grab' samples, directly from the rectum.

Ruminal contents (10–20 ml) were sampled and pH measured within 30 s of aspiration, on four occasions each day [08.00 hours (immediately prior to feeding), 11.00, 14.00 and 17.00 hours] from each animal throughout the experiment. Faecal pH was measured twice per day (08.00 and 14.00 hours).

Samples of ruminal contents (10 ml) were also collected for VFA and OA analysis.

These samples were placed into screw-capped vials to which two drops of saturated HgCl<sub>2</sub> had been added previously and air-dried (to arrest fermentation immediately). The samples were stored frozen at –20°C until analysed. Analysis was as previously described (Ouwkerk and Klieve 2001).

Samples of ruminal contents for enumeration of *M. elsdenii*, *B. fibrisolvens* and *S. bovis* were treated as follows. From each animal a 200-ml sple of rumen contents was collected into a beaker and placed on ice. Each sample was strained through a single layer of nylon stocking to remove large particulate matter. From each sample 1 ml subsamples were placed into each of four 1.5 ml microfuge tubes and centrifuged at 12 000 *g* for 15 min. The supernatants were removed and discarded, and the bacterial pellets were stored at –20°C, prior to DNA extraction.

**Enumeration of bacterial populations by real-time Taq nuclease assay.** The development and application of a real-time Taq nuclease assay (TNA) for the enumeration of *M. elsdenii* has been reported previously (Ouwkerk *et al.* 2002).

The development of real-time TNAs for *B. fibrisolvens* and *S. bovis* was based on that developed for *M. elsdenii*, and followed the same developmental steps in terms of DNA extraction, PCR amplification, sequencing and sequence analysis of 16S rRNA genes, PCR calibration and verification of specificity and statistical analyses.

For *B. fibrisolvens* YE44, primers and probes were initially selected from 16S rRNA gene sequence data using HYB-simulator v4 (RNAture Inc., West Irvine, CA, USA), then refined using ABI PRISM, Primer Express software to fit the specifications of the Taqman Universal Master Mix Protocol (PE Applied Biosystems, Foster City, CA, USA). Identified primers and probe were checked for specificity using the programs Probe Match version 2.1 from the Ribosomal Database Project (Maidak *et al.* 2000) and the BLAST program (Altschul *et al.* 1990) at the National Centre for Biotechnology Information (NCBI) site. The primers and probe sequences used were: BfibF, 5'-ACA-CACCGCCCGTCACA-3' (*E. coli* numbering system 1427–1443); BfibR, 5'-TCCTTACGGTTGGGTCACAGA-3' (1469–1490) and Bfibprobe 5'-TCGGGCATTCCTCCCATG-3' (1445–1467).

For *S. bovis*, primers and probes were initially selected from 16S rRNA gene sequence data, as previously reported (Reilly *et al.* 2002), then refined as described for the *B. fibrisolvens* assay above. The primers and probe sequences used were: SbovisF, 5'-ATGTTAGATGCTTGAAAGG-AGCAA-3' (222–243); SbovisR, 5'-CGCCTTGGTGAGC-CGTTA-3' (294–12) and Sbovisprobe, 5'-CTCACCAAC-TAGCTAATACAACGCAGGTCCA-3' (262–292).

The *M. elsdenii* and *S. bovis* probes were end-labelled at the 5'-end with the fluorescent reporter dye 6-carboxyfluorescein (6FAM) and at the 3'-end with the quencher dye 6-carboxy tetramethylrhodamine (TAMRA). The *B. fibrisolvens* probe was end-labelled at the 5'-end with the fluorescent reporter dye VIC<sup>TM</sup> (PE Applied Biosystems) and at the 3'-end with the quencher dye TAMRA.

Real-time TNA was performed as previously described (Ouwkerk *et al.* 2002).

## RESULTS

### Pen trial

**Feeding behaviour.** All animals, except steer 2, a control group animal, settled into the pens well and ate the feed on offer from the start of the trial and throughout the

changeover to grain period. Steer 2 ate poorly prior to grain introduction, consuming only 300 g [dry matter (DM) basis] on day 5 and refusing all feed on offer on day 6. On the first day of grain feeding (day 7, 45% grain), steer 2 consumed all feed offered (9 kg) but on day 8 it reduced its intake to 3 kg (DM). This reduced intake, together with a low ruminal pH on days 8 and 9 (see below) was suggestive of acute lactic acidosis, therefore the animal was removed from the experiment on welfare grounds. The later biochemical and microbial analyses confirmed acidosis had occurred.

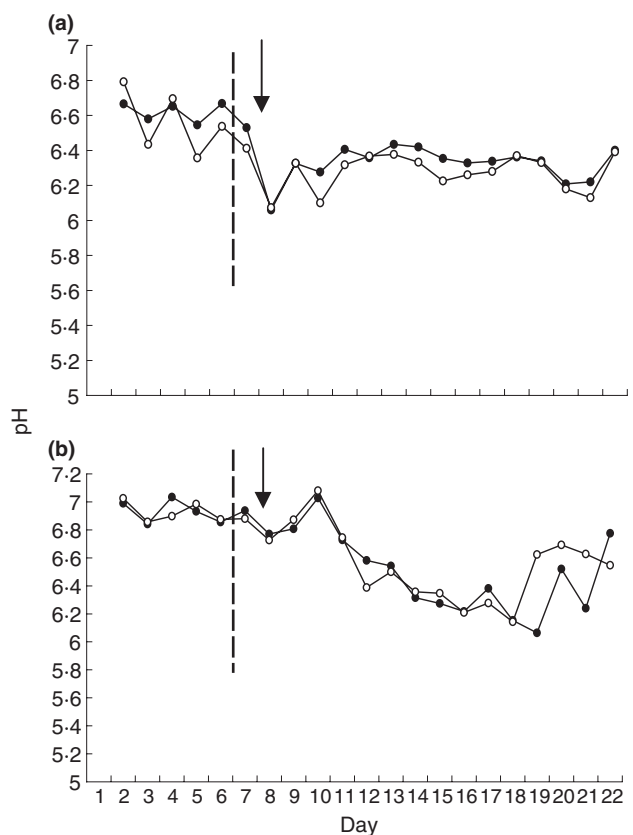
**Bacterial inoculation, ruminal and faecal pH measurements and ruminal fermentative acid production.** From roll-tube enumeration each inoculated steer received  $5.5 \times 10^{11}$  colony-forming units (CFU) of *M. elsdenii* YE34 and  $3 \times 10^{12}$  CFU of *B. fibrisolvens* YE44.

Steer 2, as described previously, developed acute lactic acidosis, the results of which will be discussed separately from the remaining animals in the control group.

Ruminal and faecal pH measurements (08.00–08.30 hours samples only) are presented in Fig. 1, for all steers except steer 2. With the exception of steer 2, there was little difference between the control and treated animals in pH measurements. No other animals appeared to experience acidotic rumen or hindgut conditions with the ruminal pH remaining above 5.6 and the faecal pH above 5.8 throughout the trial. Generally, pH was reduced by approximately half a unit following the introduction to grain. The ruminal pH of steer 2 dropped to 4.8 at 10.00 hours on the second day of grain feeding (45% grain) and remained below 5 throughout the day. Although the pH had risen to 5.8 by 10.00 hours on day 9 (third day of introduction to grain) the animal had eaten very little in the preceding 2 days.

The major VFAs produced from fermentation (viz. acetate, propionate and butyrate) are presented in Fig. 2 for all steers except steer 2. There appeared to be no differences in VFA concentrations between the inoculated and the control groups. A change in VFA profile on introduction to grain was evident in all animals with a minor decrease in acetate and a doubling of the concentration of propionate and butyrate. The concentrations of the minor VFAs (valerate etc. not shown) also increased.

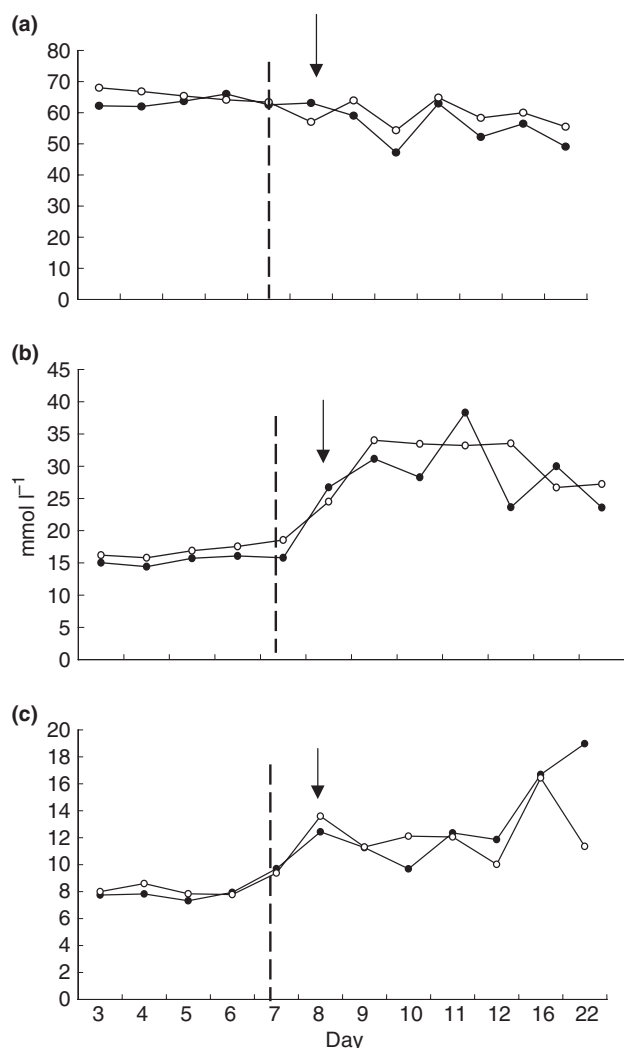
The only OAs detected were lactate and succinate. With the exception of steer 2, at no stage of the trial did the ruminal lactate concentration rise above  $2 \text{ mmol l}^{-1}$ . Lactate was detected in both treatment groups on 43 occasions (23 times in the treated cattle). Steer 2 had a ruminal lactate concentration of  $103.5 \text{ mmol l}^{-1}$  on the second day of grain feeding (45% grain) and despite not feeding that day or the next, the lactate concentration was  $38.5 \text{ mmol l}^{-1}$  on the third day of grain feeding (day 9 of the trial). Succinate was only detected sporadically (12 occasions in total – 10 times in



**Fig. 1** Ruminal (a) and faecal (b) pH throughout the pen trial at 08.00–08.30 hours. Series (●) represent the inoculated animals and series (○) represent the uninoculated control animals. Dashed lines indicate the first day that grain was introduced into the diet. Arrows indicate day of inoculation

the treated animals and twice in control animals) in the fermentation mix and then at low concentrations, only once being above  $0.3 \text{ mmol l}^{-1}$ . This occasion was on day 8 in steer 2 when the lactate concentration in this animal was above  $100 \text{ mmol l}^{-1}$ .

**Real-time TNA calibration and specificity.** Calibration curves of the relationship between cell numbers and fluorescence at the cycle threshold ( $C_T$ ), set at 40 cycles, gave a straight line relationship with correlation coefficients approximating to unity and similar to published values for *M. elsdenii* (Ouwkerk *et al.* 2002). Standard curves for diluted DNA series from pure cultures of *B. fibrisolvens* YE44 and *S. bovis* 2B and the dilution series of known numbers of these bacteria in samples of rumen contents prior to DNA extraction were parallel to, but above, the curve established for background contents of these bacteria pre-existing in the samples. It was assumed that, as with *M. elsdenii* (Ouwkerk *et al.* 2002), the parallel relationship would be maintained at all bacterial densities. Unlike



**Fig. 2** Ruminal concentrations of the volatile fatty acids (VFAs) acetate (a), propionate (b) and *n*-butyrate (c) throughout the pen trial. Series (●) represent the inoculated animals and series (○) represent the uninoculated control animals. Dashed lines indicate the first day that grain was introduced into the diet. Arrows indicate day of inoculation

the case with *M. elsdenii*, the RF in which cells of *B. fibrisolvens* YE44 and *S. bovis* 2B were added for calibration contained a background of these species and it was not possible to use the curve with cells added to rumen contents to calibrate samples with unknown numbers of bacteria. Instead, pure culture calibration curves were used for this purpose and corrected for the quenching caused by rumen contents as determined by the difference between parallel calibration curves, as reported previously (Ouwkerk *et al.* 2002).

With the *B. fibrisolvens* TNA, using Probe Match (Maidak *et al.* 2000) and BLAST (Altschul *et al.* 1990), the DNA sequence of primer BfibF was less specific than

the reverse primer, BfibR, and the probe, Bfibprobe, showing 100% sequence similarity to most bacteria and some euryarchaeota. BfibR was highly specific with 100% sequence similarity with *B. fibrisolvens* DNA. Bfibprobe had 100% sequence similarity with members of the *B. fibrisolvens* subgroup including *B. fibrisolvens*, *Pseudobutyrvibrio ruminis* A12-1 (DSM9787) and *Eubacterium rectale* (ATCC 33656). However, the combined primers and probe set provided sufficient specificity to differentiate the target bacterium, *B. fibrisolvens*, from all other bacterial species.

With the *S. bovis* TNA, the forward primer SbovisF was highly specific with 100% sequence similarity to only *S. bovis* and *S. infantarius*, which has never been detected in the rumen. The reverse primer SbovisR was nonspecific matching to most members of the domain Bacteria. The probe Sbovisprobe showed 100% sequence similarity with the following Streptococci subgroups, *S. pyogenes*; *S. thoraltensis*; *S. salivarius*; *S. constellatus*; *S. suis* and *S. pneumoniae*. The combination of primers and probe provided sufficient specificity to differentiate the target bacterium *S. bovis*. Only *S. bovis* (*S. equinus*) and *S. gallolyticus* (*S. caprimus*) (Brooker *et al.* 1994; Osawa *et al.* 1995), which are closely related, are commonly isolated from the rumen. A ruminal isolate of *S. intermedius* (strain AR36) was not amplified (Table 1).

All bacterial DNA used in the evaluation of the TNAs was first shown to be suitable for use in PCR by testing in a universal PCR and this PCR resulted in all DNA samples giving the correct, expected positive band, as previously reported (Ouwkerk *et al.* 2002). Results from the real-time amplification of the bacterial panel genomic DNA, with both the *B. fibrisolvens* and *S. bovis* TNA are presented in Table 1.

In the *B. fibrisolvens* TNA, the five strains of this bacterium all amplified strongly indicating the presence of *B. fibrisolvens* at numbers between  $1.1 \times 10^7$  (*B. fibrisolvens* AR27) and  $2.6 \times 10^8$  (*B. fibrisolvens* AR12) cells. Nonspecific amplification was evident with most of the bacteria in the panel but was minor being generally below  $10^4$  *B. fibrisolvens* CE (BfCE). Four species were above the threshold level of  $10^4$  cells, as previously set by Ouwkerk *et al.* (2002); *Bacteroides fragilis* ( $2.3 \times 10^5$  BfCE), *Clostridium butyricum* ( $1.1 \times 10^4$  BfCE), *M. elsdenii* ( $2.1 \times 10^6$  BfCE) and *S. bovis* ( $1.8 \times 10^5$  and  $2 \times 10^4$  BfCE).

In the *S. bovis* TNA, the four strains of *S. bovis* all amplified strongly indicating numbers of the bacterium between  $4.2 \times 10^8$  (*S. bovis* AR25) and  $2.3 \times 10^9$  (*S. bovis* 2B) *S. bovis* CE (SbCE). Nonspecific amplification was evident but at a very low level, with most of the bacteria in the panel. Two strains, *Ruminococcus flavefaciens* AR 45 and *Bact. fragilis* 683, amplified as high as the threshold minimum of  $10^4$  SbCE.

Bacteria	<i>B. fibrisolvens</i> cycle threshold ( $C_T$ )	<i>B. fibrisolvens</i> ( $\text{ml}^{-1}$ )	<i>S. bovis</i> cycle threshold ( $C_T$ )	<i>S. bovis</i> ( $\text{ml}^{-1}$ )
<i>Bacteriodes fragilis</i> 683	25-25	$2.3 \times 10^5$	31-2	$1 \times 10^4$
<i>B. fibrisolvens</i> AR12	14-25	$2.6 \times 10^8$	33-4	$3 \times 10^3$
<i>B. fibrisolvens</i> AR27	19-2	$1.1 \times 10^7$	38-85	74
<i>B. fibrisolvens</i> AR73	15-15	$1.4 \times 10^8$	33-25	$3.6 \times 10^3$
<i>B. fibrisolvens</i> ATCC 19171	14-55	$2.1 \times 10^8$	34	$2.2 \times 10^3$
<i>B. fibrisolvens</i> YE44	16-55	$5.8 \times 10^7$	33-2	$3.4 \times 10^3$
<i>Clostridium butyricum</i> YE12	36-35	$2 \times 10^2$	35-1	$9.8 \times 10^2$
<i>Cl. butyricum</i> YE15	30-05	$1.1 \times 10^4$	34	$2.1 \times 10^3$
<i>Escherichia coli</i> K13 (ATCC 15766)	31-65	$3.9 \times 10^3$	37-65	$2 \times 10^2$
<i>Eubacterium ruminantium</i> AR2	38-45	52	39-75	41
<i>Fusobacterium necrophorum</i> AR4	40	0	37-6	$1.7 \times 10^2$
<i>Lactobacillus</i> sp. YE07	37-25	$1.1 \times 10^2$	35-95	$6.2 \times 10^3$
<i>Lactobacillus</i> sp. YE08	35-8	$2.8 \times 10^2$	35-05	$1.1 \times 10^3$
<i>Lactobacillus</i> sp. YE16	35-35	$3.8 \times 10^2$	32-75	$5 \times 10^3$
<i>Megasphaera elsdenii</i> YE34	21-75	$2.1 \times 10^6$	36-55	$3.6 \times 10^2$
<i>Prevotella ruminicola</i> AR20	28-5	$3.4 \times 10^3$	37-35	$2.8 \times 10^2$
<i>P. ruminicola</i> AR29	34-25	$7.4 \times 10^2$	35-95	$5.3 \times 10^2$
<i>R. flavefaciens</i> AR45	33-85	$9.6 \times 10^2$	31-55	$1.1 \times 10^4$
<i>Selenomonas ruminantium</i> AR55	35-45	$3.6 \times 10^2$	37-65	$2 \times 10^2$
<i>Streptococcus bovis</i> AR25	25-6	$1.8 \times 10^5$	15-9	$4.2 \times 10^8$
<i>S. bovis</i> Sb15	29-1	$2 \times 10^4$	15-15	$7.2 \times 10^8$
<i>S. bovis</i> YE01	36-25	$2.3 \times 10^2$	14-95	$8.2 \times 10^8$
<i>S. bovis</i> 2B	34-65	$6.7 \times 10^2$	13-3	$2.3 \times 10^9$
<i>S. intermedius</i> AR36	33-3	$1.5 \times 10^3$	36-65	$3.6 \times 10^2$

**Table 1** Cycle thresholds and the *Butyrivibrio fibrisolvens* or *Streptococcus bovis* cell equivalent per millilitre of the bacterial panel in the real-time TNAs. Each figure is the average of two determinations

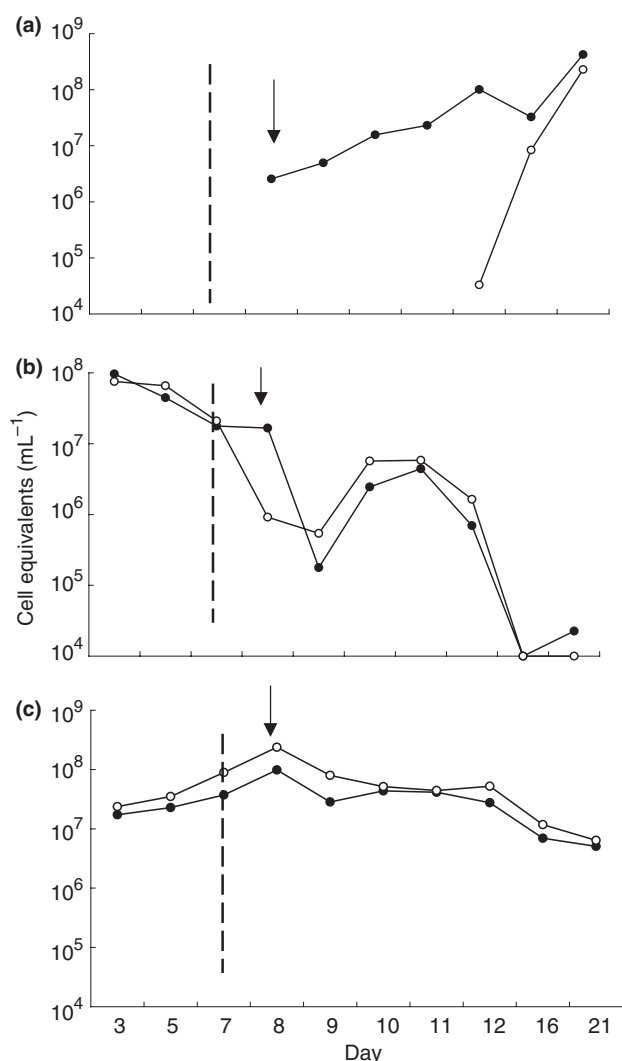
*Population changes in ruminal populations of M. elsdenii, B. fibrisolvens and S. bovis.* Changes in ruminal populations of *M. elsdenii*, *B. fibrisolvens* and *S. bovis* throughout the pen trial are presented in Fig. 3. The results for steer 2 are presented separately in Fig 4.

*Megasphaera elsdenii* (Fig. 3a) was not detectable in any of the animals prior to either inoculation with *M. elsdenii* YE34 or the introduction to grain. Immediately following inoculation on day 8, *M. elsdenii* was present in the treated animals at an average density of  $>10^6$  *M. elsdenii* CE (MeCE)  $\text{ml}^{-1}$ . In the inoculated group, *M. elsdenii* was thereafter present in all animals throughout the experiment. The population density of *M. elsdenii* in inoculated animals steadily increased throughout the trial, to reach densities in excess of  $10^8$  MeCE  $\text{ml}^{-1}$  by the end of the trial. In the uninoculated control group, *M. elsdenii* was not detected at an average density above  $10^4$  MeCE  $\text{ml}^{-1}$  before day 12, although for 3 days prior to this it was occasionally above this threshold in individual animals within the group. Thereafter, the density of *M. elsdenii* in control animals steadily increased and by day 21 was similar to that in the inoculated group.

The population of *B. fibrisolvens* followed a similar pattern in both the inoculated and control groups (Fig. 3b). In both groups of animals population density was high

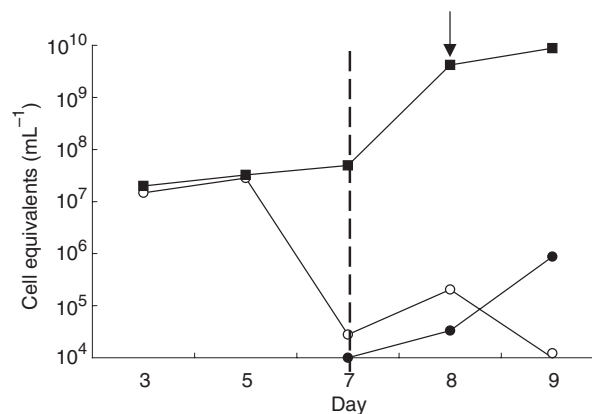
( $10^8$  BfCE  $\text{ml}^{-1}$ ) at the start of the trial when animals came off pasture. Numbers decreased slightly prior to the introduction of grain and they dropped rapidly to  $ca$   $10^5$  BfCE  $\text{ml}^{-1}$  during the introduction. There was an increase in numbers after the initial decrease (up to almost  $10^7$  BfCE  $\text{ml}^{-1}$ ) but once the diet had increased to the maximum grain content of 75% on day 11 there was a rapid decrease in numbers and by day 16 numbers were generally below the threshold of  $10^4$  BfCE  $\text{ml}^{-1}$ . The only noticeable variation between treatments was on day 8 when the average density of *B. fibrisolvens* in the inoculated group was  $1.7 \times 10^7$  BfCE  $\text{ml}^{-1}$  compared with the control group at  $9.2 \times 10^5$  BfCE  $\text{ml}^{-1}$ . However, prior to sampling on this day the inoculated group had been ruminally inoculated with *B. fibrisolvens* YE44. The number of bacteria introduced by the inoculum ( $3 \times 10^{12}$  CFU per animal) could account for the increased BfCE in the inoculated group on that day.

Unlike the populations of *M. elsdenii* and *B. fibrisolvens*, the *S. bovis* population (Fig. 3c) remained remarkably stable, in both the inoculated animals and the controls, throughout the pen trial. Group averages were generally between  $10^7$  and  $10^8$  SbCE  $\text{ml}^{-1}$ . Numbers in both groups peaked on day 8 of the trial, the second day of grain feeding (45% grain).



**Fig. 3** Ruminal densities of (a) *Megasphaera elsdenii*, (b) *Butyrivibrio fibrisolvens* and (c) *Streptococcus bovis* throughout the pen trial. Series (●) represent the inoculated animals and series (○) represent the uninoculated control animals. Dashed lines indicate the first day that grain was introduced into the diet. Arrows indicate day of inoculation. The inoculated group were ruminally inoculated with *M. elsdenii* YE34 ( $5.5 \times 10^{11}$  CFU) and *B. fibrisolvens* YE44 ( $3 \times 10^{12}$  CFU) prior to feeding on the second day of grain feeding (day 8 of the trial, 45% grain)

Unlike the other nine animals that progressed from a forage-based to a high-grain diet without acidotic problems arising, steer 2 suffered from an acute lactic acidosis and this was reflected in dramatic fluctuations in ruminal populations of *M. elsdenii*, *B. fibrisolvens* and *S. bovis* (Fig. 4). Within 24 h of grain introduction (day 8) the population of *S. bovis*, which had been stable around  $10^7$  SbCE ml<sup>-1</sup>, increased about 100-fold from  $4.9 \times 10^7$  to  $4.2 \times 10^9$  SbCE ml<sup>-1</sup>. On day 9, when the animal was removed from the trial, the



**Fig. 4** Ruminal densities of *Megasphaera elsdenii* (●), *Butyrivibrio fibrisolvens* (○) and *Streptococcus bovis* (■) in steer 2 prior to removal from the pen trial. Dashed lines indicate the first day that grain was introduced into the diet. Arrows indicate day of inoculation

*S. bovis* population had further increased to  $8.8 \times 10^9$  SbCE ml<sup>-1</sup>. The *B. fibrisolvens* population declined earlier and more rapidly than in the other animals. The decline began before grain was introduced to the diet and fell 1000-fold between days 5 and 7 ( $2.8 \times 10^7$  to  $2.8 \times 10^4$  BfCE ml<sup>-1</sup>) and by day 9 *B. fibrisolvens* was not detectable. The *M. elsdenii* population increased earlier and more rapidly than in the other control group steers. The population was above the  $10^4$  MeCE ml<sup>-1</sup> threshold on day 8 and the population had increased to a density of  $10^6$  MeCE ml<sup>-1</sup> by the following day.

## DISCUSSION

The putative probiotic strains were evaluated *in vivo* in order to determine their survival and establishment in the rumen, and their impact on the prevention of pH decline and lactic acid accumulation in beef cattle when offered a grain-based diet. The real-time TNA technique was used to enumerate specific populations of the two probiotic bacterial species, *M. elsdenii* and *B. fibrisolvens*, and *S. bovis*, the ruminal bacterium commonly associated with lactic acidosis.

As with *M. elsdenii* (Ouwkerk *et al.* 2002) a threshold for enumeration of  $10^4$  cells ml<sup>-1</sup> of sample was set for each bacterial species. With total ruminal bacteria numbering  $10^9$ – $10^{10}$  cells ml<sup>-1</sup> (Hungate 1966), it could be expected that a ruminal population below  $10^4$  cells ml<sup>-1</sup> would have a negligible effect on the metabolism of major substrates in feed material. In addition, this threshold would exclude any ambiguity caused by  $C_T$  values obtained from unrelated bacteria because of nonspecific amplification, as observed with the pure culture genomic DNA panel.

Although the primers and probe were designed to be, in combination, specific to *B. fibrisolvens* and *S. bovis*,



respectively, and this was confirmed by comparison of the DNA sequences with other sequences in international databases, some nonspecific amplification of DNA from pure cultures of unrelated bacteria was observed. The  $C_T$  values produced were generally low (below  $10^4$  CE) and were from genomic (g)DNA template obtained from bacterial cultures with cell numbers in the range of  $10^8$ – $10^9$ . In comparison, gDNA template from true positives gave CE of  $10^8$ – $10^9$ , as expected. The bacteria in the panel that produced a low  $C_T$  value, did so late in the PCR reaction and suggested CE *ca*  $10^5$ – $10^6$  times fewer than for known positives with the same amount of template DNA. It is highly unlikely these nonspecific products interfered with the assays, as the rumen would have to contain a pure culture of one or more of these bacteria, at densities  $>10^{10}$  cells  $\text{ml}^{-1}$ , for them to register at the detection threshold ( $10^4$  cells  $\text{ml}^{-1}$ ). All the nonspecific reactions in the *S. bovis* TNA were  $\leq 10^4$  SbCE  $\text{ml}^{-1}$  but three bacteria were above this level in the *B. fibrisolvens* TNA [*Bact. fragilis* 683 ( $2.3 \times 10^5$  BfCE  $\text{ml}^{-1}$ ), *M. elsdenii* ( $2.1 \times 10^6$  BfCE  $\text{ml}^{-1}$ ) and *S. bovis* AR25 ( $1.8 \times 10^5$  BfCE  $\text{ml}^{-1}$ )]. None of these bacteria is related to *B. fibrisolvens*, and *Bact. fragilis* is not a normal inhabitant of the rumen, nor has it been isolated from that environment. While it is possible that the nonspecific amplification of *M. elsdenii* and *S. bovis* can interfere with enumeration of *B. fibrisolvens*, when the population is at a low density, no interference was detected *in vivo*. The *B. fibrisolvens* population rapidly disappeared upon introduction to grain and despite the *S. bovis* population being maintained at  $10^7$  SbCE  $\text{ml}^{-1}$  and *M. elsdenii* increasing to above  $10^9$  MeCE  $\text{ml}^{-1}$  throughout this period, BfCE remained undetectable.

With the exception of steer 2, the cattle in the pen trial unexpectedly adapted readily to their rapid introduction to grain, with no indication of acidosis. Ruminal and faecal pH remained above 5.0, ruminal lactic acid concentration remained very low and the feed was fully consumed. In these animals the *S. bovis* population remained stable at around  $10^7$  SbCE  $\text{ml}^{-1}$  throughout the experiment. Steer 2 on the other hand, did not adjust to pen feeding conditions reflected in the small daily intakes of the hay diet prior to introduction of grain. This steer then consumed all the grain offered on the first day of changeover and showed classic symptoms of acute lactic acidosis [pH below 5, accumulation of lactic acid ( $>100$  mmol  $\text{l}^{-1}$ ) and a 100-fold increase in the population density of *S. bovis* (Nocek 1997; Owens *et al.* 1998)], although the concentration of grain was only 45% of the ration.

The probiotic use and establishment of *M. elsdenii*, and *B. fibrisolvens* in the rumen of grain-fed steers indicated that while *M. elsdenii* rapidly established useful populations, *B. fibrisolvens* was not an important species in starch fermentation *in vivo*.

Initial numbers of *M. elsdenii* detected in the inoculated animals were in reasonable agreement with the numbers present in the inoculum. The inoculum contained  $5.5 \times 10^{11}$  CFU of *M. elsdenii* YE34. The volume of the contents in the adult steers in the trial would be likely to approximate to 100 l, therefore the original density following inoculation could be estimated at  $5.5 \times 10^6$  CFU  $\text{ml}^{-1}$ . The population as measured by the TNA averaged above  $10^6$  MeCE  $\text{ml}^{-1}$ . There appeared to be no lag in growth of *M. elsdenii* following inoculation. As expected, wild populations of *M. elsdenii* were established in the control animals following their introduction to grain but took 5–7 days longer to establish populations of similar density to those with probiotically introduced *M. elsdenii*. This advantage in establishing a population of *M. elsdenii* could be highly protective in animals that have been predisposed to the onset of acute acidosis, given the speed of onset of the condition, as demonstrated by steer 2, and knowing that *M. elsdenii* is responsible for metabolizing  $>70\%$  of the lactate produced in the rumen (Counotte *et al.* 1983a,b).

*Butyrivibrio fibrisolvens* did not establish in the rumen at all but on the contrary a dense population that was present in the rumen whilst the steers were on pasture-based diets was rapidly lost from the ecosystem when the steers' diet changed to grain. Although *B. fibrisolvens* YE44 was selected *in vitro* as an alternate starch degrader to *S. bovis* because of its rapid and dense growth when wheat starch was the sole carbon source and energy substrate in the growth medium, this ability was not evident *in vivo*. In addition, the TNA for *B. fibrisolvens* was genetically quite broad and appears to detect across the spectrum of *B. fibrisolvens* strains (Table 1). The disappearance of *B. fibrisolvens* would therefore indicate the disappearance of all 'strains' of the genetically variable group of bacteria known as *B. fibrisolvens* (Forster *et al.* 1996, 1997) and it appears that no *B. fibrisolvens* strains could persist for long when the animals were eating 75% barley. We speculate that the temporary rise in *B. fibrisolvens* numbers, 2–4 days after grain introduction may have been because of the presence of a range of *B. fibrisolvens* strains in the rumen, some of which were not favoured when the diet was roughage based but found growth conditions more suitable when the amount of grain being fed was relatively small.

The *S. bovis* population was stable throughout the experiment, which was surprising considering its supposedly pivotal role in starch utilization and acidosis (Mackie and Gilchrist 1979; Russell and Hino 1985; Dawson and Allison 1988). Not only was *Streptococcus bovis* not problematic in this study but the lack of increase in population density with a major increase in starch into the diet also suggests that it does not appear to be a major starch-utilizing bacterium under these conditions. A general lack of lactate in the fermentation mix tends to confirm this belief and



comparison with steer 2 shows the marked difference that is expected when *S. bovis* predominates in the fermentation. However, the data do show a rapid increase in propionate (in particular) indicating that the starch was being effectively utilized within the rumen. A question that arises from the study is, what were the major starch-utilizing bacteria in these cattle? Obviously, it was not *B. fibrisolvens* or *S. bovis* and it is unlikely to be *M. elsdenii*, despite the fact that it might play a role, because in the control cattle the *M. elsdenii* population would not have been sufficiently numerous, at the time grain feeding began, to utilize effectively the amount of available starch. The identity of the predominating starch utilizers in these animals, rapidly introduced to, and effectively utilizing a high-grain diet, would be of considerable interest as potential probiotic organisms to improve the efficiency of starch utilization in grain-fed ruminants.

With the movement away from the use of chemicals and antibiotics in modern animal agriculture it is imperative that alternative processes are found that will maintain levels of production and feed utilization efficiency. The use of live microbes as inocula to manipulate the rumen ecosystem is one such process and the success of the microbial inocula containing *Synergistes jonesii* to protect cattle feeding on *Leucaena leucocephala*, from mimosine poisoning (Klieve *et al.* 2002), demonstrates that it can be a practical solution.

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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.
- Brooker, J.D., Odonovan, L.A., Skene, I., Clarke, K., Blackall, L. and Muslera, P. (1994) *Streptococcus caprinus* sp. nov., a tannin-resistant ruminal bacterium from feral goats. *Letters in Applied Microbiology* **18**, 313–318.
- Counotte, G.H.M., Lankhorst, A. and Prins, R.A. (1983a) Role of DL-lactic acid as an intermediate in rumen metabolism of dairy cows. *Journal of Animal Science* **56**, 1222–1235.
- Counotte, G.H.M., Prins, R.A., Jansen, R.H.A.M. and Debie, M.J.A. (1983b) Role of *Megasphaera elsdenii* in the fermentation of DL-[2-<sup>13</sup>C] lactate in the rumen of dairy cattle. *Applied and Environmental Microbiology* **42**, 649–655.
- Dawson, K.A. and Allison, M.J. (1988) Digestive disorders and nutritional toxicity. In *The Rumen Microbial Ecosystem* ed. Hobson, P.N. pp. 445–459. London: Elsevier Science Publishers Ltd.
- Dunlop, R.H. (1972) Pathogenesis of ruminant lactic acidosis. *Advances in Veterinary Science and Comparative Medicine* **16**, 259–302.
- Forster, R.J., Teather, R.M., Gong, J. and Deng, S.-J. (1996) 16S rDNA analysis of *Butyrivibrio fibrisolvens*: phylogenetic position and relation to butyrate-producing anaerobic bacteria from the rumen of white-tailed deer. *Letters in Applied Microbiology* **23**, 218–222.
- Forster, R.J., Gong, J. and Teather, R.M. (1997) Group-specific 16S rRNA hybridisation probes for determinative and community structure studies of *Butyrivibrio fibrisolvens* in the rumen. *Applied and Environmental Microbiology* **63**, 1256–1260.
- Godfrey, S.I., Rowe, J.B., Thorniley, G.R., Boyce, M.D. 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.
- Hungate, R.E. (1966) *The Rumen and its Microbes*. New York, NY: Academic Press.
- Hungate, R.E. (1969) A roll-tube method for the cultivation of strict anaerobes. In *Methods in Microbiology* ed. Norris, J.R. and Ribbons, D.W. pp. 117–132. London: Academic Press.
- Klieve, A.V., Heck, G.L., Prance, M.A. and Shu, Q. (1999) Genetic homogeneity and phage susceptibility of ruminal strains of *Streptococcus bovis* isolated in Australia. *Letters in Applied Microbiology* **29**, 108–112.
- Klieve, A.V., Ouwkerk, D., Turner, A. and Robertson, R. (2002) The production and storage of a fermentor-grown bacterial culture containing *Synergistes jonesii*, for protecting cattle against mimosine and 3-hydroxy-4 (1H)-pyridone toxicity from feeding on *Leucaena leucocephala*. *Australian Journal of Agricultural Research* **53**, 1–5.
- Kung, L.J. and Hession, A.O. (1995) Preventing *in vitro* lactate accumulation in ruminal fermentations by inoculation with *Megasphaera elsdenii*. *Journal of Animal Science* **73**, 250–256.
- Mackie, R.I. and Gilchrist, F.M.C. (1979) Changes in lactate-producing and lactate-utilising bacteria in relation to pH in the rumen of sheep during stepwise adaptation to a high-concentrate diet. *Applied and Environmental Microbiology* **38**, 422–430.
- Mackie, R.I., Mcsweeney, C.S. and Klieve, A.V. (2002) Microbial ecology of the ovine rumen. In *Sheep Nutrition* ed. Freer, M. and Dove, H. pp. 71–94. Oxon New York: CAB International.
- Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, J.C.T., Saxman, P.R., Stredwick, J.M., Garrity, G.M., Li, B. *et al.* (2000) The RDP (Ribosomal Database Project) continues. *Nucleic Acids Research* **28**, 173–174.
- Nocek, J.E. (1997) Bovine acidosis: implications on laminitis. *Journal of Dairy Science* **80**, 1005–1028.
- Osawa, R., Fujisawa, T. and Sly, L.L. (1995) *Streptococcus gallolyticus* sp. nov.; gallate degrading organisms formerly assigned to *Streptococcus bovis*. *Systematic and Applied Microbiology* **18**, 74–78.
- Ouwkerk, D. and Klieve, A.V. (2001) Bacterial diversity within feedlot manure. *Anaerobe* **7**, 59–66.

- Ouwerkerk, D., Klieve, A.V. and Forster, R.J. (2002) Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. *Journal of Applied Microbiology* **92**, 753–758.
- 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.
- Reilly, K., Carruthers, V.R. and Attwood, G.T. (2002) Design and use of 16S ribosomal DNA-directed primers in competitive PCRs to enumerate proteolytic bacteria in the rumen. *Microbial Ecology* **43**, 259–270.
- Russell, J.B. and Hino, T. (1985) Regulation of lactate production in *Streptococcus bovis*: a spiralling effect that contributes to rumen acidosis. *Journal of Dairy Science* **68**, 1712–1721.
- Shu, Q., Gill, H.S., Hennessey, D.W., Leng, R.A., Bird, S.H. and Rowe, J.B. (1999) Immunisation against lactic acidosis in cattle. *Research in Veterinary Science* **67**, 65–71.
- Strobel, H.J. and Russell, J.B. (1986) Effect of pH and energy spilling on bacterial protein synthesis by carbohydrate-limited cultures of mixed rumen bacteria. *Journal of Dairy Science* **69**, 2941–2947.
- 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.