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Increased efficiency of microbial protein production in the rumen through manipulation of nutrients and rumen microbial populations

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Abstract

This project examined strategies to increase microbial protein production (MCP) in cattle and the response of bacterial species within the rumen of cattle fed various pastures and supplements. Supplementation with Spirulina algae markedly increased MCP, efficiency of MCP (EMCP) and live weight gain of weaner steers, similarly to cottonseed meal. Supplementation with casein or branch chain amino acids did not markedly affect MCP and EMCP. Weaners selected for differences in live weight gain had no differences in intake, rumen function or dominant bacterial species. The dominant rumen bacterial species were relatively stable across forages and supplement treatments but specific species changes did occur in response to differences in diet digestibility, supplement N level and between individual animals. MCP appeared to respond to rumen conditions affecting growth of bacteria in general without major shifts in the population of dominant bacterial species.

Executive summary

This project examined strategies to increase microbial protein (MCP) production within the rumen of cattle. It did this by examining the effect of specific nutrients and nutrient intake on the population of the dominant bacterial species in the rumen and the subsequent flow of MCP from the rumen. Microbial protein contributes on average 72% (and up to 100% with low quality forages) of the total protein supply to cattle. Temperate forages have high efficiency of microbial protein production (EMCP) within the rumen and tropical forages, with low crude protein (CP) content, have low EMCP which can be increased to the lower values used in the feeding standards with urea based supplements but never reach the higher values found with temperate forages. It was hypothesized that limiting supply of specific nutrients (peptides, amino acids and branch chain fatty acids) and low dilution rate accounted for these differences through their effect on the bacterial species present. If EMCP on tropical forages could be increased to the higher levels achieved on temperate pasture, then liveweight gain would increase significantly. An algal supplement was investigated as a novel means of providing this package of nutrients which has the potential to be supplied via the drinking water.

Five experiments were conducted to examine the effect on EMCP of different forages, different nutrient supplement types, the use of algae and the selection of animals for higher post-weaning growth rate on low CP forages. In all experiments, intake, rumen function and microbial genetic profiles (MGP) (dominant bacterial species) were determined.

The main findings and conclusions of this project were:

- Supplements of algae (*Spirulina platensis*) significantly increased MCP production and EMCP. This was associated with increased dilution rate (lower retention time) within the rumen, which was greater than that achieved by other strategies reported in the literature.
- Supplements of algae increased liveweight gain of weaner steers from -0.2 (control) to 0.75 kg/d when supplemented with 4 g Spirulina/kg W/d. At this intake, the response was similar to cottonseed meal but, at lower intakes of supplement, the response to cottonseed meal was higher and algae was similar to urea based supplements. These experiments suggested that algae CP was highly degraded within the rumen and that algal supplements could be provided at a much higher intake of nitrogen (N) than urea based supplements and provide a source of extra metabolisable energy as well as N. There were some specific changes in the species of dominant bacteria present in the rumen at high but not low intake of algae. The identity of these species will be determined in due course.
- There was no effect of large amounts of degradable protein (casein) or a mixture of branch chain amino acids plus phenylalanine (BCAA) on MCP production or EMCP. These nutrients were supplied in higher amounts than previously used. There were no major changes in the presence of the dominant bacterial species in the rumen in response to casein or BCAA.
- There were significant effects of forage type on MCP production and EMCP which appeared to be related to the supply of N and fermentable organic matter to the rumen. There were no major changes in the bacterial species which dominated the population within the rumen. This suggested that the changes in EMCP across forage types was more related to the conditions affecting growth of microbes (nutrient supply and dilution rate) rather than shifts in the microbial population.
- Animals selected on the basis of post-weaning liveweight gain (low or moderate growth rate) had no difference in intake, EMCP, rumen function and dominant bacterial species when fed

Mitchell grass hay in pens. They did not differ in response to supplements of urea but there were different responses to cottonseed meal. They did differ in the concentration of plasma Insulin-like growth factor-1 (IGF-1) suggesting the differences in liveweight gain were due to metabolic differences between these selected groups of animals rather than differences in rumen function.

- The rumen bacterial community was stable between animals and over time, despite quite large differences in basal diet (hay) quality, particularly CP content, across all experiments. Throughout the experiments it was apparent that a "core" of dominant bacterial species was always present and most likely are important for the digestion of tropical pastures by *Bos indicus* cross cattle in northern Australia. The identity of these species still requires definition.
- It is apparent that extreme populations of protein-scavenging bacteria can occur in the rumen once a high intake of N supplementation has been removed. These bacteria are Spirochaetes and a member of the Bacteroidetes phylum. While they are entirely associated with the liquid fraction, they do not appear to increase EMCP.
- While there was a profile of bacterial species dominating the population in the rumen which was generally similar between animals, profiles differed slightly on an animal by animal basis, indicating that the genetics or physiology of the individual animal exerted a dominant influence on the rumen ecosystem. However, in specific cases, where either high or low intakes of N (as urea or Spirulina algae) were imposed in the diet, the individual animal effect was over-ridden by the diet effect.
- Bacterial profiles at high and low intakes of N appeared to cluster relative to high and low EMCP values and this suggested a correlation between bacterial community structure and EMCP at the extremities of the range.
- Quantitatively, strategies aimed at increasing EMCP from tropical forages to the high levels found with temperate forages are important for increasing liveweight gain. However, increasing EMCP in cattle given forages with low CP and digestibility and associated with low dilution rate within the rumen appears very difficult to achieve through the addition of simple nutrients (peptides, amino acids, branch chain fatty acids) in addition to N. The feeding of algae, which supplied a package of nutrients and changed dilution rate and, at a high intake, some species of microbes, was the only successful strategy to increase EMCP in the present study. Conditions affecting bacterial growth, especially N supply and rumen dilution rate, rather than species of bacteria, appear most important in determining EMCP.

Aside from gaining a much better understanding of the impact of rumen ecology on factors impacting on cattle performance, the immediate significance of this work is that algae are a valuable supplement for cattle grazing low CP tropical pastures by which to increase liveweight gain. Various algal sources may become increasingly available as a consequence of biofuel and C sequestration industries and will provide an alternative protein meal for the beef industry which could potentially be provided within water medicators and licks. Only *Spirulina platensis* was investigated here and the value of other species needs to be investigated. Identifying the dominant rumen bacterial species will be accelerated with new 454-pyrosequencing methods and the linkages of bacterial species with EMCP in the rumen have yet to be fully elucidated. However, the dominant bacterial species appear relatively constant across the wide range of forages and supplements studied, which suggests that factors which influence growth of microbes is more important in affecting MCP production and EMCP than the profile of bacterial species which dominate the rumen bacterial population.

Contents

1 Background

1.1 Introduction

Growth rate in cattle is a function of both metabolisable energy (ME) intake and metabolisable protein (MP) supply. Metabolizable protein supply is largely influenced by MCP supply which supplies on average 72% of MP supply but up to approximately 90-100% on many tropical forages. Microbial protein supply is largely influenced by EMCP, expressed as g MCP/kg digestible organic matter (DOM) or g MCP/MJ fermentable ME. The EMCP is low in tropical forages (as low as 33 g MCP/kg DOM) largely as a consequence of N supply to the rumen but also because of rumen function with respect to dilution rate, specific nutrients and possibly the microbial species present in the rumen. The accepted EMCP from tropical forages when N is adequate is approximately 130 g MCP/kg DOM but temperate forages reach approximately 170 g MCP/kg DOM. Increasing EMCP from tropical forages will result in a modest (ca. 300 g/d) increase in liveweight gain but this is a significant change in liveweight gain from rangeland pastures where there is no opportunity to change intake through new pasture species or energy supplements. There is limited knowledge of the microbial species present in and passing from the rumen which contribute to MCP. It has been estimated that only 5-10% of the microbe species present in the rumen have been identified to date. New methods of microbial genetic profiling to track the changes in microbial populations are now available and these will expedite the identification of microbe species within the rumen.

Grazing animals in Australia are faced with feed of varying quality (digestibility (DMD) and N or CP content) throughout the year leading to variable feed intake. Generally there is high intake over the growing (wet) season and low intake (often below maintenance) over the dry season. The opportunities to change this through grazing management or introduction of grasses is limited and non-existent for the extensive native pasture areas, which contrasts with strategies available in temperate, higher rainfall areas. A more practical approach in the rangelands is to utilise as much of the pasture resource as possible through stocking rate and strategic supplementation of limiting nutrients, e.g. N and S, to increase MCP production from the existing resources.

1.2 Microbial crude protein production

The factors affecting MCP production have been reviewed by Poppi and McLennan (1995) and Poppi *et al*. (1997). Briefly, fermentable ME intake or DOM intake and rumen degradable N (RDN) are the main factors which affect MCP production but factors within the rumen are also important. These relate to the supply of specific nutrients (e.g. branch chain fatty acids (BCFA), nucleic acids, minerals and vitamins) but also conditions within the rumen such as dilution rate and the type of carbohydrate being fermented (e.g. sugars, starches, fibre). The various feeding standards throughout the world are in general agreement that when RDN is adequate, EMCP will at least reach 130 g MCP/kg DOM (e.g. Freer *et al*.,2007,, AFRC 1993, NRC 1996) although each system uses different units. However, with tropical forages the values are often much lower due largely to the N content of the forage. Similarly with temperate forages the values can be much higher, up to 170 g MCP/kg DOM, where there is a rapid rumen dilution rate and high intakes of rumen degradable protein (RDP). There is a theoretical upper value of 200 g MCP/kg DOM (SCA, 1990). Thus the range in EMCP achieved from forages is quite large and this will have an impact on the liveweight gain of cattle. Poppi and McLennan (1995) and Poppi *et al*. (1997) have calculated that increasing the EMCP from 80 (observed in low CP pasture) to 130 g MCP/kg DOM would result in an increase in liveweight gain of about 300 g/d.

The supply of N to the rumen is the main factor affecting EMCP and this is directly related to the CP content of the pasture. However some other factors of protein degradation in the rumen are important. Most N used by microbes to synthesize MCP comes from ammonia, the final degradation product of protein, but peptides and amino acids can also be used directly by the microbes (Rook and Armstrong, 1989) and BCFA, derived from BCAA, are a specific requirement of cellulolytic microbes (Hume, 1970a,b). These nutrients are supplied in large amounts with forages high in CP (e.g. temperate forages) but not with forages low in CP (e.g. tropical forages). Many feeding standards indicate that EMCP will reach 130 g MCP/kg DOM under simple non-protein N (NPN) sources, e.g. urea, but suggest that approximately 25% of the RDN should come from peptides and amino acids (i.e. RDP) if higher values of EMCP are to be achieved. The significance of these changes is that increased EMCP will result in increased total MP supply to the animal.

Dijkstra *et al*. (2002) have modelled the range of nutrients required by microbes and indicated that nutrients other than N, e.g. S, nucleic acids, BCFA, cell wall degradation products (e.g. phenyl acetate), vitamins and other co-factors, are important for microbial growth. Nitrogen supplements based on urea do not supply these nutrients and protein meals, depending on their source and processing method, may provide variable amounts. Single cell protein sources (e.g. algae and yeasts) are alternative means of providing this package of nutrients, and sources of these single cell proteins are increasingly becoming more available, e.g. C sequestration and biofuel industries. Some of these sources may be provided in the drinking water and so would have ready application for cattle production systems within the rangelands.

Dilution rate within the rumen is a major factor affecting EMCP (Dijkstra *et al*. 2002) and is highly variable between forage types. High dilution rates are associated with high quality temperate forages and high intake, while tropical forages have a low dilution rate. The AFRC (1992) uses this observation to modify EMCP such that EMCP increases with intake. Supplements which increase intake and dilution rate are associated with higher EMCP.

1.3 Microbial genetic profile within the rumen

It has been estimated that only 5-10% of the microbe species within the rumen have been identified (Mackie *et al.* 2002). Thus the major microbial species which make up the bulk of MCP flowing from the rumen are not known. Earlier work (Tolosa *et al*., 2004; Dinh *et al*., 2004) indicated that novel microbial species could be identified. Techniques are now available to identify and quantify the microbial species and whether they change in response to specific nutrients. For example, cellulolytic species are known to have a specific requirement for BCFA and phenylacetate but there are no studies which show if and how they respond to supplements which provide these nutrients. It is important in any study seeking to alter MCP production that the species of microbe which are being targeted are identified and also to understand how microbe species change in response to rumen conditions of nutrient supply and dilution rate.

This project aimed to link MCP production with rumen microbial genetic profiles where diets and supplements were varied so as to alter rumen function. The desired outcome was to increase liveweight gain of cattle by increasing EMCP with the use of supplements targeted towards specific microbe species.

2 Project objectives

1. Determine the effects on animal production and microbial protein production of key nutritional manipulations and correlate these with changes in rumen microbial populations;
2. From a knowledge of these changes in rumen ecology, target those bacterial species associated with high efficiencies by using specific nutrient manipulations and quantify these effects in terms of MCP production and animal production;
3. Develop nutritional strategies based on these findings to increase microbial protein production, intake and hence liveweight gain of cattle at pasture

3 Methodology

There were 5 experiments planned and completed. These are reported as separate sections under each experimental title. The microbial genetic profile studies from these experiments are reported under the one, separate title so as to better discuss the work and methodology.

The original 5 experiments were:

- Experiment 1. The effect of forage species on microbial genetic profiles and rumen function.
- Experiment 2. The effect of urea N or rumen degradable protein on microbial genetic profiles and rumen function.
- Experiment 3. The effect of rumen degradable protein or single cell organism on microbial genetic profiles and rumen function.
- Experiment 4. Rumen function in animals selected for growth rate on low quality diets.
- Experiment 5. Liveweight gain of animals consuming different forms of N supplement.

All procedures were conducted in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were reviewed and approved by the University of Queensland Animal Ethics Committee.

3.1 Experiment 1. The effect of forage species on rumen function

This experiment has been written as a draft paper entitled, “Effect of forage type on intake, digestibility, retention time, microbial protein production and the efficiency of microbial protein production in cattle”, Panjaitan, T., Quigley, S.P., McLennan, S.R., Swain, T. and Poppi, D.P.

3.1.1 Animals and experimental design

The experiment was conducted at the Mt. Cotton Research Farm, University of Queensland, Queensland. Eight rumen-cannulated Brahman crossbred steers (424 ± 37 kg) were used in the experiment. The steers were weighed prior to the commencement of the experiment and randomly allocated to treatments, floor pens and metabolism crates and remained in the same floor pens and metabolism crates throughout the experiment. The experimental design consisted of two 4 x 4 Latin squares, with 4 treatments (i.e. forage type) and 1 replicate (i.e. steer) per treatment, represented in each Latin square. The experiment was conducted over 4 runs, resulting in 8 replicates per treatment (i.e. 4 runs x 2 steers per treatment per run). Each experimental period was conducted

over 26 d, consisting of a 14 d preliminary feeding period, comprised of 10 d in floor pens and 4 d in metabolism crates, followed by a 7 d collection period in metabolism crates. At the completion of each collection period, all steers were relocated to floor pens and the rate of digestion of low and high quality forages was then measured by incubation in nylon bags in the rumen of each of the animals from a single 4 x 4 Latin square, over 4 d. Liveweight of each steer was recorded on d 1, on d 10, prior to shifting into the metabolism crate, on d 21, upon completion of the collection period, and on d 26, upon completion of the rate of digestion period. Upon completion of the nylon bag study all steers were moved to a paddock for 2 weeks, with *ad libitum* access to pangola (*Digitaria eriantha*) dominant pastures and mineral blocks (Go-Block, Olsson's Pty Ltd; Australia) before the commencement of the next run.

3.1.2 Diets and feeding management

The treatments used in the present study were low quality tropical grass hays typical of the rangeland environments in western Queensland and the Northern Territory, speargrass (*Heteropogon contortus*) and Mitchell grass (*Astrebla* spp.); a medium quality tropical grass hay, pangola grass; and a high quality temperate grass hay, ryegrass (*Lolium multiflorum*. Cv. Aristocrat). All hays were chopped to 2-10 cm in length before feeding. In the floor pens the steers were fed once daily at 08:00 h. In the metabolism crates, steers were fed equal amounts of hay at hourly intervals by an automatic feeder (Minson and Cowper, 1977). For the first 12 d of the preliminary period the animals were offered hay *ad libitum* (previous day's intake plus 20%) to determine average daily feed intake. For the final 2 d of the preliminary period and the 7 d collection period, feed was offered at 110% of average feed intake over the first 12 d of the preliminary period. Steers were fed *ad libitum* during the rate of digestion period and had *ad libitum* access to water.

3.1.3 Sampling procedures, measurements, analytical techniques and statistical analysis

The sampling procedures, measurements, analytical techniques and statistical analysis are described in detail in Appendix 1. Only those methods that are specific to this experiment or vary from those presented in Appendix 1, are described here.

Dry matter, OM, ash-free neutral detergent fibre (NDF), ash-free acid detergent fibre (ADF), N and lignin content were determined in feed offered, feed residues and faeces. The proportion of leaf and stem was determined in samples of feed offered. Digestibility was determined over a 7 d collection period. Purine derivatives (PD) were determined in urine samples collected during the collection period for the calculation of MCP production. The pH and concentration of volatile fatty acids (VFA) and ammonia-N (NH_3-N) was determined in rumen fluid collected at the end of the collection period. Retention time of liquid and particles within the rumen was determined using Chromium-EDTA (Cr-EDTA) and Ytterbium chloride ($YbCl_3$) markers, respectively, during the collection period, with samples collected prior to dosing (0 h) and 3, 6, 9, 12, 24 and 32 h after dosing. Retention time of DM, OM, NDF and lignin was also measured by complete evacuation of the rumen after the collection period. Plasma urea-N concentration was determined in blood samples collected at the end of the collection period. Rate of digestion was determined after the collection period by incubating nylon bags containing ryegrass or speargrass hay in the rumen of steers (n=4) from one of the latin-squares for 0, 3, 7, 10, 14, 24, 48, 72 and 96 h.

The statistical significance of treatment effects on each variable was tested by analysis of variance (ANOVA), with terms for hay type (treatment), steer type (used in nylon bag study or not), interaction between hay type and steer type, steer and run. The significant pair-wise differences between hay types were tested using the protected least significant difference (LSD) procedure, if the ANOVA test

of the hay type effect was significant. For missing data, the statistical significance of the effects was tested by fitting general linear models with the same terms as used in the ANOVAs. If the steer effect and the interaction between hay type and steer type were not significant, the data were reanalysed with terms for steer type, hay type and run, otherwise log transformation of all data was used to avoid any inconsistency in variability. Dry matter disappearance of incubated materials in the rumen was determined by measuring the residual substrate of the sample. Dry matter disappearance at each time (0 to 96 h) was plotted against time and the disappearance data was fitted to the degradation curve. Each variable derived from the fitted curve was then analysed with a mixed model using restricted maximum likelihood (REML). The fixed effects were diet, hay type and their interaction. An approximate F-test was used to assess the significance of the fixed effects. If the interaction was not significant ($P > 0.05$) it was removed and the model refitted. Approximate pair-wise comparisons were made between means for significant fixed effects. The 5% level was used to assess statistical significance in all cases. All data was analysed using the statistical package GenStat 2007 (GenStat for windows, 10th edition, VSN International Ltd.).

3.2 Experiment 2. The effect of urea N or rumen degradable protein on microbial genetic profiles and rumen function

This experiment was modified by the supply of increasing amounts of a mixture of amino acids as a treatment and the experimental design was altered slightly from the original proposal on the advice of the statistician. Originally 2 low quality and 1 medium quality hay were to be used in response curve experiments to supplements of a single N supplement. A more powerful design was used incorporating more treatments (urea, casein and branch chain amino acids) and only 1 low quality hay and 1 medium quality hay was used so as to be able to accommodate all treatments within budget. It compared the intake and rumen function of weaners supplemented with various levels (over a wide range) of urea and S, casein (source of RDP) and BCAA plus phenylalanine.

3.2.1 Animals and experimental design

Thirteen cannulated Brahman crossbred steers were used for Experiments 2.1 and 2.2. The steers were from Swans Lagoon Research Station (QPIF) and underwent surgical insertion of cannula (Bar diamond, US, 8 cm i.d.) at approximately 8 months of age and 218 kg liveweight, with a larger cannula (Bar diamond, 10 cm i.d.) inserted 6 weeks later. The steers were approximately 9 months of age and 219 ± 9.3 kg liveweight at the commencement of Experiment 2.1, and approximately 12 months of age and 224 ± 11.4 kg liveweight at the commencement of Experiment 2.2. The steers were allocated into 3 groups based on ranked full liveweight. The steers were allocated to concrete floor pens and metabolism crates. Steers remained in the same floor pens and metabolism crates throughout the experiment but in each run the steers were randomly allocated to a different supplement type and supplement intake.

An incomplete Latin square design was employed in each experiment, consisting of 13 steers and 3 types of supplements in 3 runs. Each run lasted 26 d, consisting of a 14 d preliminary feeding period comprising of 13 d in the floor pens and 1 d in the metabolism crates, followed by a 7 d collection period in the metabolism crates. At the completion of each collection period, all steers were relocated to the floor pens. In both Experiments 2.1 and 2.2, 7 of the steers were used to measure rate of digestion in a nylon bag study over a further 5 d. Upon completion of Experiment 2.1, steers grazed a pangola dominant pasture with access to mineral blocks for 3 weeks before commencing Experiment 2.2.

Steers were offered either a basal hay diet (control) or control plus increasing amounts of one of three different supplements. The basal diets given to steers were medium quality pangola grass hay (pangola hay; 15.8 g N/kg DM, 784 g NDF/kg DM) for Experiment 2.1 and low quality Mitchell grass hay (Mitchell hay; 7.2 g N/kg DM, 799 g NDF/kg DM) for Experiment 2.2. The 3 supplements were urea-ammonia sulphate (US), a fixed level of US plus increasing levels of leucine, isoleucine, valine and phenylalanine (BCAA) and casein. Each supplement provided 4 amounts of RDP/DOM, with one replicate (steer) per treatment per run and one control (steer) per run. The sources of N and amounts supplied were:

- Control + US. Urea (Incitec Pivot Ltd; Australia) and ammonium sulphate (AMSUL, Hifert Ltd; Australia) were mixed 4:1, with an estimated N:S of 10:1. The US treatment was supplied in increasing amounts to provide 130, 170, 210 and 250 g RDP/kg DOM by providing 3, 13, 22 and 31 g US DM/kg pangola hay DM, respectively, and 12, 19, 26 and 33 g US DM/kg Mitchell hay DM, respectively. The treatments are described as US130, US170, US210 and US250.
- Control + casein (Sodium caseinate, Murray Goulburn Co-operative Co Ltd; Australia). The casein was supplied in increasing amounts to provide 130, 170, 250 and 300 g RDP/kg DOM by providing 10, 38, 107 and 160 g casein DM/kg pangola hay DM, respectively, and 36, 61, 119 and 162 g casein DM/kg Mitchell hay, respectively. The treatments are described as C130, C170, C210 and C300.
- Control + BCAA. Urea-ammonium sulphate was supplied in a fixed amount to provide 170 g RDP/kg DOM by providing 13 g US DM/kg pangola hay DM and 19 g US DM/kg Mitchell hay DM. In addition to the fixed level of US, the BCAA plus phenylalanine mixture (Ajinomoto Co., INC, Tokyo-Japan for Experiment 2.1; Ningbo Create Biological Project Co., Ltd, China for Experiment 2.2) was provided in the same proportion and level as that present in the casein treatment provided at 130, 170, 250 and 300 g RDP/kg DOM. The BCAA plus phenylalanine mixture was supplied at 3, 10, 26 and 35 g DM/kg pangola hay DM, and 9, 15, 30 and 41 g DM/kg Mitchell hay DM, respectively. The treatments are described as USAA130, USAA170, USAA210 and USAA300.

3.2.2 Diets and feeding management

Pangola grass hay used in Experiment 2.1, was grown and harvested during the summer of 2006 at Mt. Cotton Research Farm, while the Mitchell grass hay used in Experiment 2.2, was obtained from the Barkly Tablelands, Northern Territory. The hay was chopped to 2-10 cm in length before feeding. Hay was offered *ad libitum* during the preliminary feeding period and fixed to 90% of *ad libitum* during the collection period. Steers were fed at 08:30 h each day.

The casein and BCAA supplements were administered through the cannula immediately before feeding hay each day. The US supplement was dissolved in water at a rate of 1 part US to 3 parts water, to make a 25% US solution. The solution was manually sprinkled on the hay using a 1 L plastic container with small holes in the lid and thoroughly mixed to incorporate throughout the chopped hay. A similar approach was used to add water, in an amount equal to that provided in the highest US treatment, to the hay for the other treatments so that the moisture content of the hay was approximately equal for all treatments. The composition of the BCAA treatment was similar to the four AA in casein of each treatment. The proportion of each amino acid in the total AA was leucine (33.6%), isoleucine (22.1%), valine (26.2%) and phenylalanine (18.0%). Steers had access to fresh drinking water at all times.

3.2.3 Sampling procedures, measurements, analytical techniques and statistical analysis

The sampling procedures, measurements, analytical techniques and statistical analysis are described in detail in Appendix 1. Only those methods that are specific to this experiment or vary from those presented in Appendix 1, are described here.

Dry matter, OM, NDF, ADF, N and lignin content were determined in feed offered, feed residues and faeces. The proportion of leaf and stem was determined in samples of feed offered. Digestibility was determined over a 7 d collection period. Purine derivatives were determined in urine samples collected during the collection period for the calculation of MCP production. The pH and concentration of VFA and NH_3-N was determined in rumen fluid collected on d 5 of the collection period. Retention time of liquid within the rumen was determined using Cr-EDTA as a marker, during the collection period, with rumen fluid samples collected prior to dosing (0 h) and 3, 6, 9, 12, 24, 32 and 48 h after dosing. Retention time of DM, OM, NDF and lignin was also measured by complete evacuation of the rumen after the collection period. Plasma urea-N concentration was determined in blood samples collected at the end of the collection period. Rate of digestion was determined after the collection period by incubating nylon bags containing Mitchell grass, pangola grass and ryegrass hay in the rumen of steers (n=7; control, US170, US250, C170, C300, USAA170, USAA300) for 0, 3, 6, 9, 12, 24, 48, 72 and 96 h.

For each variable, the data were initially summarised by fitting a general linear mixed model, with run, supplement type, supplement level and interaction between type and level as fixed effects and steer as a random effect. Graphs of the residuals were used to check for outliers and the assumption of homogeneity of variance. A sequence of general linear mixed models was then fitted to determine an appropriate low order polynomial model to describe the responses to RDP (g/kg DOM) for each variable. All models included run as a fixed effect and steer as a random effect and all RDP values had the mean RDP for control subtracted from them. Initially quadratic models for the response to RDP for each supplement type were compared to linear responses, then the responses between types compared, based on either linear or quadratic response curves depending on the result of the initial test. Where differences between types were not significant, a common function was fitted. Finally, if the slope for linear responses was not significant the term was removed; i.e. a constant response was used in the model. The resulting model was illustrated by plotting the fitted curves with means from the initial summary included on the plot. An approximate R^2 for the model was calculated from the reduction in the sum of squares of residuals from the corresponding model with no RDP terms. The pattern of change in rumen NH_3-N over time of measurement within a day (0, 4, 8 and 12h) was estimated by fitting a general linear mixed model with run, supplement type, supplement level, type by level interaction and time as fixed effects and steer and steer by run as random effects. DM disappearance of incubated materials in the rumen was determined by measuring residual substrate of the sample. Dry matter disappearance at each time (3 to 96 h) was plotted against time and the disappearance data was fitted to the degradation curve. Each variable derived from the fitted curve was then analysed with a mixed model using restricted maximum likelihood (REML) in GenStat. The fixed effects were supplements, hay type and their interaction. Approximate F-tests were used to assess the significance of the fixed effects. If the interaction was not significant ($P > 0.05$) it was removed and the model refitted. Approximate pair-wise comparisons were made between means for significant fixed effects. The 5% level was used to assess statistical significance in all cases. All analyses were done using the statistical package GenStat 2007 (GenStat for Windows, 10th edition).

3.3 Experiment 3. The effect of rumen degradable protein or single cell organism on microbial genetic profiles and rumen function

This experiment has been written as a draft paper entitled, “Spirulina (*Spirulina platensis*) algae supplementation increases microbial protein production and feed intake and decreases particle and liquid retention time in the rumen of cattle”, Panjaitan, T., Quigley, S.P., McLennan, S.R., Swain, T. and Poppi, D.P. The original application proposed that 1 low quality hay and 1 medium quality hay would be evaluated within a response curve design. Given the results of the first experiment, with a low quality hay, it was postulated that no additional useful information would be obtained if the same design was repeated with a medium quality hay. Of more interest was the evaluation of potential new sources of algal species to be developed for C sequestration procedures by MBD Biodiesel Ltd. (Townsville). Unfortunately the supply of adequate amounts of algae did not occur at the time of preparation of this report and this experiment will be completed by a PhD student. A report will be submitted to MLA when this experiment has been completed.

3.3.1 Animals and experimental design

Nine Brahman crossbred steers with rumen fistula were used. The steers were used in experiments 2.1 and 2.2 and were approximately 18 months of age and 271 ± 17 kg in liveweight at the commencement of the experiment. The animals had never been exposed to any form of algae under experimental conditions, prior to the experiment. Steers were allocated to floor pens and metabolism crates, treatments and treatment levels on a stratified random weight basis at the commencement of the experiment and remained in the same floor pens and metabolism crates throughout the experiment. An incomplete Latin square design consisting of 9 steers, 2 supplement types over 3 experimental runs was used. The basal diet was Mitchell grass (*Astrebla* spp.) hay (5.1 g N/kg DM; 766 g NDF/kg DM). The two supplement types were US (9 parts Urea, Incitec Pivot Ltd., Australia, mixed with 1 part AMSUL, Hifert Ltd., Australia) and Spirulina (Life Stream International; New Zealand). Each supplement was provided at 4 levels with 1 replicate (steer) per level per treatment per run and 1 control (steer) per run. The control steer was offered Mitchell grass hay only, with no supplement. The treatment levels for US supplemented steers were Mitchell grass hay plus 6, 13, 19 and 33 g US DM/kg hay DM (US90, US130, US170 and US210, respectively). The treatments for Spirulina supplemented steers were Mitchell grass hay plus 0.5, 1.4, 2.5 and 6.1 g Spirulina DM/kg W/d (S90, S130, S170 and S290, respectively). The intention was to supply approximately equivalent amounts of increasing RDP/kg DOM, for both supplements (90, 130, 170 and 210 g RDP/kg DOM for US and 90, 130, 170 and 290 g RDP/kg DOM for Spirulina). Urea-ammonium sulphate was not supplied at the highest comparable amount of Spirulina due to risks of toxicity associated with feeding the required amount of US to provide an equivalent of 290 g RDP/kg DOM.

The duration of each experimental run was 30 d, consisting of a 14 d preliminary feeding period, a 9 d collection period, a 1 d rumen emptying and equilibration period and a 6 d rate of digestion period. The preliminary feeding period consisted of 13 d in floor pens and 1 d in metabolism crates. The 9 d collection period consisted of a 7 d faeces and urine collection period and a 2 d rumen sampling and retention time measurement period in metabolism crates. Upon completion of the collection period the animals were returned to floor pens for the rate of digestion period, involving seven of the nine animals. *Ad libitum* feed intake was established during the first 13 d of each experimental run and was thereafter fixed at 90% of this value for the remainder of the experimental run.

3.3.2 Diets and feeding management

Mitchell grass hay, obtained from the Barkly Tableland, Northern Territory, contained 35% Mitchell grass, 55% Flinders grass (*Iseilema* spp.) plus a mixture of other native grasses. The hay was chopped to 2-10 cm in length prior to feeding. Hay was offered *ad libitum* (10% refusal or at least 500 g of refusal each day) during the 13 d preliminary feeding period and was fixed at 90% of *ad libitum* intake during the collection and rate of digestion periods. Steers were fed once each day at 08:30 h for the first 13 d of the preliminary feeding period while in the floor pens and then in equal amounts at hourly intervals by an automatic feeder (Minson and Cowper, 1977) in the metabolism crates for the remaining 1 d of the preliminary period and throughout the 9 d collection and rumen measurement period. They were fed once each day at 8:30 h during the rate of digestion period. Spirulina was dissolved in water to make a 25% Spirulina solution and administered through the cannula immediately before feeding hay each day during the preliminary period and twice daily (30% at 08:30 h and 70% at 14:30 h) during the collection period. Water, equal to that supplied in the highest Spirulina treatment (~10 L), was administered through the cannula of all steers at the same times as above. During the measurement of rumen retention time, Spirulina solution was administered in equal amounts at hourly intervals over a 24 h period prior to rumen emptying. The US supplement was mixed with water to make a 25% US solution which was sprayed onto, and mixed thoroughly with, the hay. Water was sprayed onto the hay of all treatments in an amount equal to the highest US treatment so that the moisture content of the hay offered was approximately equal across all treatments. Steers had access to fresh drinking water at all times.

3.3.3 Sampling procedures, measurements, analytical techniques and statistical analysis

The sampling procedures, measurements, analytical techniques and statistical analysis are described in detail in Appendix 1. Only those methods that are specific to this experiment or vary from those presented in Appendix 1, are described here.

Dry matter, OM, NDF, ADF, N and lignin content were determined in feed offered, feed residues and faeces. The proportion of leaf and stem was determined in samples of Mitchell grass hay offered. Digestibility was determined over a 7 d collection period. Purine derivatives were determined in urine samples collected during the collection period for the calculation of MCP production. The pH and concentration of VFA and NH_3-N was determined in rumen fluid collected at the end of the collection period and prior to rumen emptying. Retention time of liquid and particles within the rumen was determined using Cr-EDTA and $YbCl_3$ markers, respectively, during the collection period, with samples collected prior to dosing (0 h) and 3, 6, 9, 12, 24 and 32 h after dosing. Retention time of DM, OM, NDF and lignin was also measured by complete evacuation of the rumen after the collection period. Plasma urea-N concentration was determined in blood samples collected at the end of the collection period. Rate of digestion was determined after the collection period by incubating nylon bags containing Mitchell grass, pangola grass and ryegrass hay in the rumen of steers (n=7; Control, US90, US170, US210, S90, S170, S290) for 0, 3, 6 9, 12, 24, 48 and 96 h, while Mitchell grass and pangola grass were also incubated for 120 and 144 h.

For each variable the data were initially summarised by fitting a general linear mixed model, with run, supplement type, supplement level and interaction between type and level as fixed effects and steer as a random effect. Graphs of the residuals were used to check for outliers and the assumption of homogeneity of variance. A sequence of general linear mixed models was then fitted to determine an appropriate low order polynomial model to describe the responses to level of Spirulina supplement or RDP (g/kg DOM) for each variable. All models included run as a fixed effect and steer as a random effect and all RDP values had the mean RDP for control subtracted from

them. Initially, quadratic models for the response to RDP for each supplement type were compared to linear responses, and then the responses between types were compared, based on either linear or quadratic response curves depending on the result of the initial test. Where the relationship was not consistent with a straight line, it has been described by a quadratic equation, especially when comparing between the responses between the two supplements. Where differences between types were not significant, a common function was fitted. Finally, if the slope for a linear response was not significant the term was removed; i.e. a constant response was used in the model. The resulting model was illustrated by plotting the fitted curves with means from the initial summary included on the plot. An approximate R^2 for the model was calculated from the reduction in the sum of squares of residuals from the corresponding model with no RDP terms. Dry matter disappearance in polyester bags at each time (0 to 144 h or to 96 h for ryegrass) was plotted against time and the disappearance data was fitted to the degradation curve, so as to obtain estimates of 'a' (initial DM disappearance at time zero), 'b' the slowly degradable DM fraction and 'c' the fractional digestion rate of DM (Ørskov and McDonald, 1979). Each variable derived from the fitted curve was then analysed with a mixed model using restricted maximum likelihood (REML) in GenStat. The fixed effects were supplements, hay type and their interaction. Approximate F-tests were used to assess the significance of the fixed effects. If the interaction was not significant ($P > 0.05$) it was removed and the model refitted. Approximate pair-wise comparisons were made between means for significant fixed effects. The 5% level was used to assess statistical significance in all cases. All analyses were done using the statistical package GenStat 2007 (GenStat for Windows, 10th edition).

3.4 Experiment 4. Rumen function in animals selected for growth rate on low quality diets

This experiment examined the intake and rumen function of weaners selected for moderate and low growth rates after grazing dry season spear grass (*Heteropogon contortus*) based pastures after weaning and the response of these parameters to supplements of US or cottonseed meal (CSM). The experiment was modified slightly on the advice of a statistician so that response curves to US and CSM were developed for both groups after a period of the animals grazing together for a short period over the wet season. Rumen fluid was not exchanged between the groups based on the fact that they were grazing together over this period in a small group and from a microbiological view it was agreed that the exchange of such a small amount of rumen fluid was irrelevant. The design was further modified in that a response curve to both US and CSM meal was developed rather than just US alone as it was felt that statistically and biologically more useful conclusions could be established.

3.4.1 Animals and experimental design

Brahman crossbred steers (n=100; 182 ± 2.3 kg in liveweight) were weaned in July 2007 onto a common area of low quality black spear grass dominant native pasture and given a molasses-urea supplement at Swans Lagoon Research Station. After 90 d the steers were weighed and selected on growth rate post-weaning so that 8 lowest (Low) and 8 highest (Moderate) growth rate steers were paired on initial weight; the average liveweight and growth rates were 172 ± 3.5 kg and -70 g/d for Low, and 197 ± 4.3 kg and 210 g/d for the Moderate groups, respectively. Venous jugular blood and rumen fluid samples were taken at this time and the steers were immediately transported to the Centre for Advanced Animal Science (CAAS) at Gatton in Southeast Queensland. The experiment consisted of four runs. Each run consisted of a 21 d preliminary period, in which *ad libitum* intake was determined from 7 to 21 d, followed by a 7 d collection period in metabolism crates, during which animals were fed at 90% of *ad libitum* intake. During the first run, all steers were offered Mitchell grass hay (Mitchell hay; 6.4 g N/kg DM, 610 g NDF/kg DM) from Longreach, Queensland.

Upon completion of run 1, the steers were grazed as one group on medium quality tropical pastures at UQ Gatton for 40 d. The animals were then returned to CAAS and within each group (Low and Moderate) allocated randomly in an 8 x 3 incomplete latin-square design, with 8 treatments and 3 runs. Steers were offered either a basal hay diet (control) or control plus increasing amounts of either US or CSM. The basal diet given to steers was the low quality Mitchell hay used in run 1. The US supplement provided 3 amounts of RDP/DOM and the CSM supplement provided 4 amounts of RDP/DOM, with one replicate (steer) per group (Low and Moderate) per run and one control (steer) per run. The amounts of supplements and RDP used were:

- Control + US. Urea and ammonium sulphate were mixed 4:1, with an estimated N:S of 10:1. The US provided 100, 130 and 170 g RDP/kg DOM by supplying 5, 10 and 17 g US DM/kg Mitchell hay DM, respectively.
- Control + CSM. The CSM provided 2.5, 5, 10 and 15 g CSM/kg W/d (as fed), respectively.

3.4.2 Diets and feeding management

Mitchell grass hay, used in all runs, was chopped to 2-10 cm in length prior to feeding. Hay was offered *ad libitum* (10% refusal or at least 500 g of refusal each day) during the 21 d preliminary feeding period and was fixed at 90% of *ad libitum* intake during the 7 d collection period. Steers were fed once each day at 08:00 h throughout the experiment. The US supplement was mixed with water to make a 25% US solution which was sprayed onto and mixed thoroughly with the hay. Water was sprayed onto the hay of all animals in an amount equal to the highest US treatment so that the moisture content of the hay offered was approximately equal across all treatments. Cottonseed meal was offered at the same time as the hay in a separate container in the feed trough. Steers had access to fresh drinking water at all times.

3.4.3 Sampling procedures, measurements, analytical techniques and statistical analysis

The sampling procedures, measurements, analytical techniques and statistical analysis are described in detail in Appendix 1. Only those methods that are specific to this experiment or vary from those presented in Appendix 1, are described here.

Hay and supplement intake were determined daily and liveweight was measured weekly. Dry matter, OM, NDF, ADF, N and lignin content were determined in feed offered, feed residues and faeces. Digestibility was determined over a 7 d collection period. Purine derivatives were determined in urine samples collected during the collection period for the calculation of MCP production. The pH and concentration of VFA and NH_3-N was determined in rumen fluid collected by stomach tube 0 and 3 h after feeding on the day after the collection period Retention time of liquid and particles within the rumen was determined using Cr-EDTA and $YbCl_3$ markers, respectively, during the collection period, with faecal grab samples collected prior to dosing (0 h) and 12, 24, 32, 48, 56, 72 and 96 h after dosing. Serum IGF-1 was determined on samples collected at the end of the post-weaning period and the day after the collection period, using the IGF-1 RIA Kit (Biolcone, Australia) following the manufacturer's instructions.

For each variable the data was initially summarised by fitting a general linear model with period, growth group, supplement type, supplement level and interaction between supplement type and level as fixed effects and animal as a random effect. Initially quadratic models for variable response to N intake (g/kg W/d) were compared to linear models and the responses between supplements and between growth rate groups were compared based on linear or quadratic response curves. An approximate R^2 was calculated from the reduction in sum of squares of residuals for each model and

the significance of the fixed effects was assessed using the F test. They were assumed to be significant if $P < 0.05$. All analyses were done using the SAS statistical package (SAS, 2001).

3.5 Experiment 5. Liveweight gain of animals consuming different forms of N supplement

This experiment examined the liveweight gain response of weaner steers to US, Spirulina algae and CSM supplements.

3.5.1 Animals and experimental design

Brahman crossbred steers, approximately 9 months of age and 236.3 ± 1.8 kg liveweight (at the commencement of the experiment) were used. The experimental design was a completely randomised block design where each animal was considered an experimental unit. Steers were allocated to individual pens and to one of four levels of 3 different supplements or to a control treatment, with 3 replicates for each level of each supplement and 6 replicates for the control treatment, making a total of 42 steers. The experiment consisted of a 7 d equilibration period followed by a 10 week experimental period. During week 8 of the experimental period, total faecal output was collected over 7 consecutive days to determine digestibility. During the equilibration period steers were fed pangola grass hay (*Digitaria eriantha*) *ad libitum* in groups, with 3 steers per pen. At the end of the equilibration period the steers were weighed full and fasted (24 h off feed, 16 h off water), ranked on liveweight and allocated to individual pens and treatments on the basis of the fasted liveweight (0 d). Steers were divided into three blocks according to weight classes at allocation with each block representing a replicate. Steers were then moved to their allocated pens in which they stayed for the remainder of the experiment.

3.5.2 Diets and feeding management

The basal feed was speargrass (*Heteropogon contortus*) hay chopped to approximately 5 cm in length in a tub grinder. The supplements were CSM (BEC feed solutions, Kingaroy, Queensland), *Spirulina platensis* algae (AL; Phytofoods Ltd., Labrador, Queensland) and stockfeed urea (46% N) plus granulated ammonium sulphate (20.2% N, 24% S) (US; Incitec Pivot, Childers, Queensland, Australia) mixed in the ratio of 1 kg urea with 0.222 kg ammonium sulphate to provide an estimated N:S of 9.5:1. The control treatment was speargrass hay offered alone *ad libitum*. True protein supplements (CSM and AL) were offered at 0.08, 0.16, 0.32 and 0.48 g N/kg W/d (i.e. CSM08, CSM16, CSM32 and CSM48; and AL08, AL16, AL32 and AL48) with speargrass hay provided *ad libitum*. The non-protein nitrogen supplement (US) was offered at 0.04, 0.08, 0.14 and 0.20 g N/kg W/d (i.e. US04, US08, US14 and US20) with speargrass hay provided *ad libitum*. The highest amount of N supplied from US was lower than the other treatments to avoid risks associated with the high ingestion of urea. During the experimental period, hay was offered at 8:00 am each day and residues were collected once each week. Prior to feeding each morning the amount of residual hay was assessed for each steer and the amount of hay to be offered was estimated to provide about 10% in excess of the previous day's intake. Supplement allowances were adjusted weekly for each steer based on the most recent liveweight measurement. The CSM supplement was offered daily in a 32 L container, at the same time as the hay. The AL supplement was mixed with water to a final concentration of approximately 20% in a 20 L container to produce a slurry and offered to animals daily, approximately 1 h prior to the hay. The US was mixed with water (approximately 333 g US/L) and sprayed onto and mixed thoroughly throughout the hay offered to steers each day.

3.5.3 Sampling procedures, measurements, analytical techniques and statistical analysis

The sampling procedures, measurements, analytical techniques and statistical analysis are described in detail in Appendix 1. Only those methods that are specific to this experiment or vary from those presented in Appendix 1, are described here.

Steers were weighed, unfasted, prior to feeding once each week. Liveweight gain was estimated from the regression of liveweight over time. Hay and CSM intake were determined weekly; AL intake was determined daily. Dry matter, OM, N, NDF and ADF content were determined in feed offered. Digestibility was determined by total faecal collection over a 7 d collection period conducted during week 7 of the experiment. The pH and concentration of VFA and NH_3-N was determined in rumen fluid collected 3 h after feeding on 50 d of the experiment. Plasma urea-N concentration was determined in blood samples collected on 50 d of the experiment.

Statistical analysis was performed on the data in a completely randomised block design where each animal was considered an experimental unit, comprising 3 replications for each treatment and 6 for the control treatments. Animals were grouped into blocks based on their initial body weight. The 5% level was used to assess statistical significance in all cases. All analyses were done using the statistical package GenStat 2007 (GenStat for Windows, 10th edition).

3.6 Microbial genetic profiling across all experiments

This series of experiments used samples collected from the rumen of cattle used in Experiments 1 to 5 and analysed their microbial genetic profiles. This was done to varying degrees of complexity depending on the animal data which was obtained and the question of interest. The key issues were to identify the bacteria species present in the rumen, whether they changed in response to treatment, whether there were novel bacteria present and whether across experiments there were consistencies of species and changes which could be associated with observed changes in EMCP. The DGGE patterns provided a means by which bacterial profiles could be assessed. The use of next generation sequencing technologies (454 sequencing) will be used by two PhD students over the next 2 years to identify bacterial species of interest and to expand on the interpretation of shifts in and clusters of bacterial species over and beyond that originally outlined in the objectives and methodology of the original application.

3.6.1 Sample collection and processing

In experiments where fistulated cattle were used, solid digesta and rumen fluid samples were collected from the rumen via the cannula. In experiments where intact animals were used, rumen fluid samples were collected by stomach tube under mild vacuum. Digesta samples were processed and separated into four fractions: the liquid planktonic phase, the plant associated phase and both loosely and tightly attached populations. Each solid digesta phase was achieved by a progressive process of both physical and chemical dissociation to remove bacteria that were associated with plant material, described in detail in Appendix 1.

3.6.2 Extraction, amplification and denaturing gradient gel electrophoresis

Fractionated samples were bead-beaten and genomic DNA extracted. The V2V3 region of 16S ribosomal DNA was amplified via polymerase chain reaction (PCR) and the resulting bacterial community samples were analysed using denaturing gradient gel electrophoresis to create a population profile for each sample. The DGGE gel band patterns and intensity were visualised to examine the effect of hay type on the rumen microbe populations of the same animal fed different

diets and of different animals fed the same diet. Dominant bands and bands of interest were selected for DNA amplification, purification, cloning and sequencing. These methods are described in detail in Appendix 1.

4 Results and discussion

4.1 Experiment 1. The effect of forage species on rumen function

4.1.1 Results

Chemical composition of the hays. The nutrient composition of the hays varied between treatments, confirming that the forages were of low, medium and high quality (Table 1). The CP content was highest for ryegrass hay and lowest for speargrass hay. However, the high CP content of the ryegrass hay was also associated with a high potassium nitrate (KNO_3) concentration (11 g/kg DM). In addition, ryegrass hay had a higher proportion of leaf than the other treatments.

Table 1. Nutrient composition of speargrass, Mitchell grass, pangola grass and ryegrass hays.

Parameter	Speargrass	Mitchell grass	Pangola grass	Ryegrass
Dry matter (g/kg)	910	917	893	877
Organic matter (g/kg DM)	921	907	934	889
Crude protein (g/kg DM)	25.7	29.7	75.5	199.8
Neutral detergent fibre (g/kg DM)	709	692	691	584
Lignin (g/kg DM)	71.8	42.5	66.2	24.6
Leaf (%)	49	63	64	94

Microbial protein production. Microbial protein production was greatest in steers fed ryegrass hay, which was 2-fold, 3.6-fold and 7.8-fold greater than MCP production in steers fed pangola grass, Mitchell grass and speargrass hays, respectively (Table 2). Microbial protein production was related to DOMI (Figure 1). The EMCP between forage types was only significantly different when two outlying data points were removed from the analysis; the difference was not significant when the two outlying data points were included in the analysis or when the data was analysed as back transformed means.

Table 2. Microbial protein (MCP) production and the efficiency of microbial protein production of steers fed speargrass, Mitchell grass, pangola grass and ryegrass hay *ad libitum* at hourly intervals. Values are means and standard error of the difference of the means (SEM). Within a row, means without a common superscript differ ($P < 0.05$).

Parameter	Speargrass	Mitchell grass	Pangola grass	Ryegrass	SEM
		Microbial crude protein production			
(g/d)	80.4[a]	172.0[b]	328.1[c]	626.7[d]	31.3
(g/kg W/d)	0.2[a]	0.4[b]	0.8[c]	1.5[d]	0.07
		Efficiency of microbial crude protein production			
(g MCP/kg DOM)[A]	107.7	110.2	102.3	135.0	23.14
(g MCP/kg DOM)[B]	77.7[a]	78.6[a]	102.3[b]	135.0[c]	6.77

[A]all data included.

[B]outlying data not included.

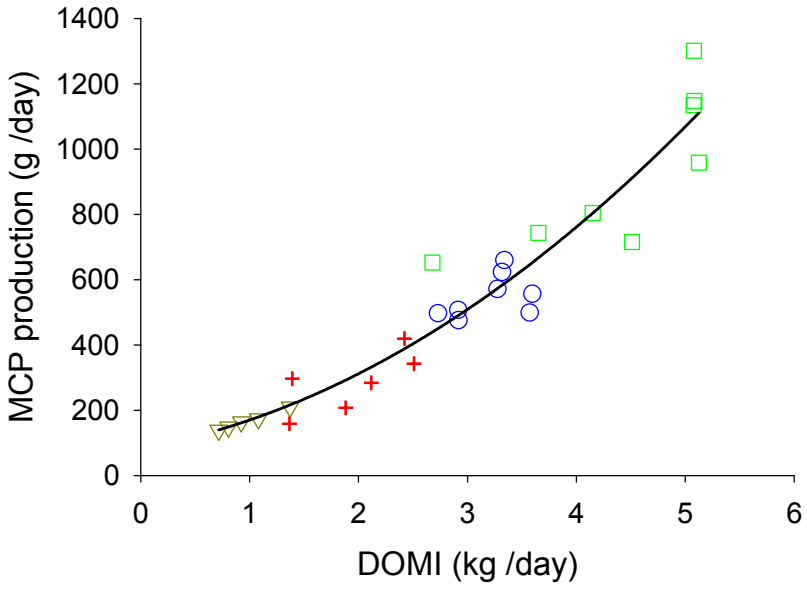

Figure 1. Relationship between microbial crude protein (MCP) production and digestible organic matter intake (DOMI) in steers fed speargrass (▽), Mitchell grass (+), pangola grass (O) and ryegrass (□) hays. Points represent individual steers and are measured, unadjusted values which have not undergone statistical analysis. The relationship is represented by the equation, $Y = 83.4 + 59x + 27.6x^2$, adjusted $R^2 = 0.91$.

Intake and digestibility. Steer type, experimental period and the interaction between treatment and steer type had no effect on intake, digestibility and digestible intake parameters. Dry matter intake of ryegrass and pangola grass hays was similar and both were greater than speargrass and Mitchell grass hays (Table 3). In addition, DM intake of Mitchell grass hay was greater than speargrass hay. The DM digestibility of ryegrass hay was greater than that of pangola grass and speargrass hays, which were in turn greater than Mitchell grass hay. Total DOMI differed between each of the hay

types, with the sequence from the highest to the lowest being ryegrass, pangola grass, Mitchell grass and speargrass.

Table 3. The dry matter (DM) intake, digestibility of DM, organic matter (OM) and neutral detergent fibre (NDF), the digestible organic matter (DOM) intake and the crude protein (CP) to DOM and rumen degradable protein (RDP) to DOM ratios of speargrass, Mitchell grass, pangola grass and ryegrass hays fed to steers *ad libitum* at hourly intervals. Values are means and standard error of the difference of the means (SEM). Within a row, means without a common superscript differ ($P < 0.05$).

Parameter	Speargrass	Mitchell grass	Pangola grass	Ryegrass	SEM
		Intake			
DM (g/d)	1908[a]	4083[b]	6367[c]	7243[c]	0.574
DM (g/kg W/d)	5.5[a]	10.1[b]	15.6[c]	17.6[c]	1.358
		Digestibility			
DM (%)	46.5[ab]	40.7[a]	54. 6[b]	69.7[c]	3.79
OM (%)	50.4[a]	46.2[a]	54.7[a]	71.0[b]	3.98
NDF (%)	54.8[a]	50.8[a]	56.8[a]	79.7[b]	3.72
		Digestible intake			
OM (g/d)	839[a]	1821[b]	3233[c]	4682[d]	0.267
OM (g/kg W/d)	2.4[a]	4.5[b]	7. 9[c]	11.4[d]	0.596
CP:DOM (g/kg)	56.7	64.1	137.4	285.4	
RDP/DOM [A] (g/kg)	42.6	48.1	103.1	214.1	

[A]assumes a crude protein degradability of 75% (McLennan *et al*., 1997).

Rumen parameters. Mean rumen fluid pH was 6.7 and was unaffected by treatment (Table 4). The plasma urea-N concentration was higher in steers fed ryegrass hay compared with steers fed the three tropical forage hays, with no differences observed between the tropical forages. Rumen NH_3-N concentration of steers fed ryegrass hay was greater than the other treatment hays. Steers fed pangola grass hay had a higher rumen NH_3-N concentration than steers fed Mitchell grass hay, while rumen NH_3-N concentration of steers fed speargrass hay was similar to those fed both pangola grass and Mitchell grass hays. The rumen NH_3-N concentration of steers fed Mitchell grass and speargrass hays were both below the minimal level suggested for microbial function (50 mg/L). Steers fed ryegrass hay had the highest molar concentration of VFA, followed by pangola grass, Mitchell grass and speargrass hays. The molar proportion of acetate was greater in the rumen fluid of steers fed speargrass and Mitchell grass hays than in steers fed pangola grass hay, which in turn was greater than in steers fed ryegrass hay. In contrast, the molar proportion of propionate and total BCFA were greater in the rumen fluid of steers fed ryegrass hay than in steers fed the tropical forage hays.

Table 4. The pH, ammonia-nitrogen (NH_3-N) and volatile fatty acid (VFA) concentrations and the molar percentage of individual VFA in the rumen, and the plasma urea nitrogen (N) concentration of steers fed speargrass, Mitchell grass, pangola grass and ryegrass hay *ad libitum* at hourly intervals. Values are means and standard error of the difference of the means (SEM). Within a row, means without a common superscript differ ($P < 0.05$).

Parameter	Speargrass	Mitchell grass	Pangola grass	Ryegrass	SEM
Rumen pH	6.7	6.7	6.7	6.7	0.065
Rumen NH3-N (mg/L)	48.5^{ab}	31.3^{a}	57.1^{b}	191.0^{c}	10.70
Total VFA (mM)	57.15^{a}	61.97^{b}	81.13^{c}	90.6^{d}	5.06
Acetate (%)	78.6^{a}	78.8^{a}	74.4^{b}	70.7^{c}	0.787
Propionate (%)	14.3^{ab}	13.5^{a}	15.2^{b}	17.2^{c}	0.511
Butyrate (%)	5.6^{a}	6.9^{ab}	8. 6^{c}	8.0^{bc}	0.613
Iso-butyrate (%)	0.8^{a}	0.4^{a}	0.6^{a}	1.4^{b}	0.171
Iso-valerate (%)	0.4^{a}	0.3^{a}	0.7^{b}	1.8^{c}	0.102
Valerate (%)	0.3^{a}	0.2^{a}	0.5^{b}	0.9^{c}	0.067
BCFA (%)	1.2^{ab}	0.8^{a}	1.3^{b}	3.2^{c}	0.214
Plasma urea-N (mg/dL)	16.0^{a}	9.4^{a}	11.1^{a}	48.0^{b}	3.43

Retention time. Retention time of the fluid marker, Cr-EDTA, in the rumen of steers consuming ryegrass hay was lower than it was for pangola grass, Mitchell grass and speargrass hays (Table 5). Retention time of Cr-EDTA in pangola grass fed steers was lower than in speargrass and Mitchell grass hays, with no difference between the latter two hays. Retention time of the particle marker, $YbCl_3$, in the rumen of steers consuming ryegrass hay was lower than it was in steers fed pangola grass, Mitchell grass and speargrass hays. There was no difference in retention time of $YbCl_3$ in the rumen between pangola grass and Mitchell grass hays and Mitchell grass and speargrass hays. However, the retention time of digesta DM and NDF measured by emptying the rumen was different between pangola grass, Mitchell grass and speargrass. Steers fed speargrass hay had the lowest total digesta load in the rumen (Table 6). However, total DM digesta load in the rumen did not differ between the four forages and the differences in total rumen load between diets was due to differences in the proportion of liquid in the total digesta load.

Table 5. The retention time of chromium (Cr-EDTA; fluid marker) and Ytterbium ($YbCl_3.6H_2O$ particle marker) and dry matter (DM), neutral detergent fibre (NDF) and lignin by emptying the rumen, of steers fed speargrass, Mitchell grass, pangola grass and ryegrass hay *ad libitum* at hourly intervals. Values are means and standard error of the difference of the means (SEM). Within a row, means without a common superscript differ ($P < 0.05$).

Parameter	Speargrass	Mitchell grass	Pangola grass	Ryegrass	SEM
		Retention time of markers			
Fluid phase (Cr-EDTA) (h)					
	33.7[a]	31.7[a]	13.7[b]	10.2[b]	2.94
Particle phase ($YbCl_3.6H_2O$) (h)					
	57.5[a]	41.6[ab]	28.9[b]	13.2[c]	4.94
		Retention time by gravimetric measurement			
DM (h)	72.1[a]	47.7[b]	28.6[c]	19.1[c]	4.59
NDF (h)	69.8[a]	47.8[b]	29.1[c]	20.7[c]	4.77
Lignin (h)	129.6[a]	120.5[a]	53.4[b]	N/D[A]	8.73

[A]N/D, lignin content was below the level accurately detected by the method used.

Table 6. Total digesta load, dry matter (DM) load and water load in the rumen of steers fed speargrass, Mitchell grass, pangola grass and ryegrass hay *ad libitum* at hourly intervals. Values are means and standard error of the difference of the means (SEM). Within a row, means without a common superscript differ ($P < 0.05$).

Parameter	Speargrass	Mitchell grass	Pangola grass	Ryegrass	SEM
Total digesta load (kg)	43.7[a]	58.0[b]	70.0[b]	62.9[b]	5.89
Total digesta load (% W)	11.9[a]	14.4[ab]	17.0[b]	15.4[b]	1.38
DM load (kg)	7.3	8.0	7.4	5.9	0.85
DM load (% W)	2.0	2.0	1.8	1. 5	0.20
Water load (kg)	36.4[a]	50.1[b]	62.5[c]	57.0[bc]	5.52
Water load (% W)	9.9[a]	12.4[b]	15.2[b]	13.9[b]	1.31
Water load (% Total load)	81.4[a]	86.5[ab]	89.4[b]	90.8[b]	3.11

Rate of digestion. The potential degradability (a + b) of the ryegrass hay substrate was higher than the speargrass hay substrate ($P < 0.05$). There were no differences in the slowly fermentable fraction (b) and potential degradability (a + b) of each of the substrates between the four forage diets. There was a significant interaction between diet and substrate in the immediately soluble fraction (a), slowly fermentable fraction (b), degradation rate (c) and the effective degradability (ED) of DM estimated at a rumen outflow rate of 0.05/h (Table 7). In general, (a), (b), (c) and (ED) of DM, estimated at a rumen outflow rate of 0.05/h, of the ryegrass hay substrate were higher in steers fed better quality forages; these parameters did not vary for the speargrass hay substrate with the exception of (ED).

Table 7. The immediately soluble fraction (a), slowly fermentable fraction (b), fractional degradation rate of b (c) and effective degradability (ED) of speargrass and ryegrass hay substrates incubated in the rumen of steers fed speargrass, Mitchell grass, pangola grass and ryegrass hay *ad libitum*. Values are means and standard error of the difference of the means (SEM). Within a column, means without a common superscript differ ($P < 0.05$).

Diet	Substrate	a (% DM)	b (% DM)	c (h-1)	ED[A] (% DM)
Speargrass	Spear grass	8.5[b]	67.8[bc]	0.0143[d]	22.2[e]
Mitchell grass	Spear grass	7.7[b]	62.8[c]	0.0214[d]	26.5[d]
Pangola grass	Spear grass	7.5[b]	58.4[c]	0.0263[d]	27.7[d]
Ryegrass	Spear grass	7.6[b]	55.5[c]	0.0207[d]	23.0[e]
Speargrass	Ryegrass	15.9[a]	77.8[b]	0.0752[c]	63.8[c]
Mitchell grass	Ryegrass	15.0[a]	79.9[b]	0.1011[b]	69.3[b]
Pangola grass	Ryegrass	-0.9[c]	95.0[a]	0.1737[a]	74.7[a]
Ryegrass	Ryegrass	11.9[ab]	81.2[ab]	0.0777[c]	62.8[c]
SEM		2.13	4.51	0.00682	1.02

[A]effective degradability at rumen outflow rate 0.05/h.

4.1.2 Discussion

Microbial protein, as influenced by DOMI and EMCP, supplies 72% of metabolisable protein to ruminants, on average, and up to 100% of metabolisable protein from low CP, low digestibility forages (Poppi and McLennan, 1995). An increase in microbial protein supply results in an increase in liveweight gain (Poppi and McLennan, 1995). This experiment determined the effect of forage quality on MCP production and EMCP and investigated the underlying mechanisms responsible for variations in MCP production in cattle. In the present study, steers fed speargrass and Mitchell grass hays had lower daily feed intake, due to lower digestibility and CP content and slower rumen retention times, than steers fed ryegrass and pangola grass hays. These differences were associated with lower MCP production but little difference in EMCP was observed in steers consuming speargrass and Mitchell grass hays compared with steers consuming ryegrass and pangola grass hays.

In the present study, MCP production, determined by the measurement of purine derivatives in the urine, varied markedly between diets and was highest in animals consuming ryegrass hay, followed by pangola grass, Mitchell grass and speargrass hays. The actual MCP production values measured in the present experiment appear to be within the range reported elsewhere for temperate and tropical grasses (Prior *et al*., 1998; Lee *et al*., 2002). There was a trend for EMCP to be highest in animals consuming ryegrass hay, followed by pangola grass, Mitchell grass and speargrass hays, although there was no significant difference in EMCP between diets when all data were included. The mean EMCP value among the tropical forages in the present study was lower than the minimum value of 130 g MCP/kg DOM suggested for forage based diets (Freer *et al*., 2007), which was expected given that the availability of RDP in all tropical forages used in the present study was below 130 g RDP/kg DOM, recommended by Freer *et al*. (2007). In contrast, ryegrass hay provided 214 g RDP/kg DOM, which was above the recommended range (130 to 170 g RDP/kg DOM; Freer *et al*., 2007). However, the actual EMCP value of ryegrass hay (135 g MCP/kg DOM) observed in the present study was approximately 37% below the predicted value, which may indicate inefficient N utilisation in the rumen (Egan, 1974).

In the present study, the mean concentration of NH_3-N in the rumen increased from 31 to 191 mg NH_3-N/L with increasing CP content of the forage. Steers fed ryegrass hay had the greatest concentration of ammonia-N in the rumen and the highest levels of plasma urea-N. The mean concentration of rumen NH_3-N in steers fed ryegrass hay was in excess of both the suggested minimum level for optimal MCP production and for maximum EMCP (Satter and Slyter, 1974; Van Soest, 1994) which resulted in high MCP production and EMCP. Ammonia-N concentration in the rumen of steers fed the Mitchell grass and speargrass hays was lower than the minimum amount (50 mg NH_3-N/L) for optimal rumen function and resulted from low RDP supply, due to low feed intake and the low CP content of the diets. Total VFA concentration, in the present study, increased with increasing forage quality, associated with increasing intake and DOMI. Feeding ryegrass hay resulted in an increase in the molar percentage of propionate and BCFA and a decrease in the molar percentage of acetate, which may stimulate bacterial growth and improve fermentation within the rumen (Van Soest, 1994; Wallace, 1997).

An increase in the availability of DOM in the rumen increases total microbial protein yield from the rumen (Owens and Goetsch, 1988). The highest DOMI in the present study was with steers fed ryegrass hay and this was associated with the highest MCP production, followed by the pangola grass, Mitchell grass and speargrass hays. In the present study, increasing CP content and digestibility of the forage was associated with an increase in voluntary feed intake. Differences in intake between forages have previously been related to the physical regulation of intake, where rumen fill is at a constant high level and intake is directly related to retention time of digesta in the rumen (Blaxter *et al*., 1956; Thornton and Minson, 1973; Poppi *et al*., 1981b). However, Egan (1977) suggested that when animals are fed low CP diets their intake is below this potential physical level because the protein to energy ratio (P:E) of absorbed substrates is low and there is a metabolic mechanism for intake regulation. Since MCP production is the major source of metabolisable protein from low CP diets, it follows that MCP production and the EMCP are the major factors affecting the P:E of absorbed substrates. A simple physical regulation of intake nor a P:E regulation of intake appeared consistent in these experiments. One quantitative outcome, irrespective of the intake mechanism, is the very long retention time of digesta DM, OM and NDF and water (Cr-EDTA) or digesta ($YbCl_3$) markers in the rumen of steers consuming speargrass and Mitchell grass compared to pangola grass and ryegrass which has implications for the EMCP, where dilution rate is a key factor controlling microbial growth (Djikstra *et al*., 1998). For example, retention time of DM in the rumen ranged from 19.1 hrs (ryegrass) to 72.1 hrs (speargrass) whilst retention time of Cr-EDTA, a marker associated with the water phase whose turnover in the rumen has most impact on growth and species of microbes, ranged from10.2 hrs (ryegrass) to 33.7 hrs (speargrass). The implications of this for the growth and composition of microbial species within the rumen is discussed in section 4.6.1.

Turnover or passage rate of digesta from the rumen (retention time) has an important synergistic role with MCP production and EMCP (Cole *et al*., 1976). Increased digesta passage rate results in decreased energy used for maintenance of the bacteria, decreased bacterial lysis and increased escape of bacteria from protozoal predation (Owens and Goetsch, 1988; Baker and Dijkstra, 1999). Digesta retention time, measured either by emptying the rumen or using the $YbCl_3$ or Cr-EDTA marker techniques differed between forages of different quality. This, coupled with the fractional growth rate estimates (10%/h) (Dijkstra *et al*., 1998), means that forages with short retention times promote conditions for the rapid growth of microbes, whilst forages with long retention times provide conditions for the slow growth of microbes. Both low RDP and slow turnover of water and digesta appear to contribute to the low MCP production and the EMCP observed for steers fed speargrass

and Mitchell grass hays. These retention time values are related to the rate of breakdown of large particles and the turnover of water in the rumen (Poppi *et al*., 1981a).

In conclusion, MCP production in steers consuming tropical forages was below that measured in steers fed a temperate forage and was well below the minimum amounts suggested by the feeding standards. Inadequate RDP supply in the rumen appeared to be the main factor limiting MCP production and EMCP. This, coupled with the physical structure of the tropical forages, resulted in low voluntary feed intake, long digesta retention time and low rumen ammonia-N concentrations. There did not appear to be a consistent physical or metabolic mechanism regulating intake of the three tropical forages. These 4 forages provide an excellent model by which to examine rumen ecology and microbial species, using microbial genetic profile methodology, where rumen function differs markedly and to identify those microbes which contribute the bulk of the microbial protein within the rumen. These issues are discussed in section 4.6.1.

4.2 Experiment 2. The effect of urea N, rumen degradable protein or branch chain amino acids and phenylalanine on rumen function

4.2.1 Results

Chemical composition of the diets. The composition of the pangola and Mitchell grass hays is presented in Table 8 and the actual RDP intake from the intended RDP/DOM treatments is presented in Table 9. The N content (and hence CP content) of the pangola grass hay was approximately twice that of the Mitchell grass hay, with the S content of the two hays being 1 and 1.7 g/kg DM, respectively, and little difference in the other chemical components. The pangola grass hay had a higher proportion of leaf (67%) compared with the Mitchell grass hay (55%).

Table 8. Nutrient composition of pangola grass or Mitchell grass hay and urea-ammonium sulphate (US[A]), US plus amino acids (USAA[B]) and casein used in Experiments 2.1 and 2.2.

Feeds	DM	OM	N	NDF	ADF	Lignin
	(g/kg)	---------- (g/kg DM) ----------				
			Experiment 2.1			
Pangola grass	898	926	15.8	784	441	44
			Experiment 2.2			
Mitchell grass	916	898	7.2	799	464	56
			Experiments 2.1 and 2.2			
US	993	999	456.8	n/m	n/m	n/m
Amino acids (AA)	993	999	101.7	n/m	n/m	n/m
Leucine	993	n/m	108.9	n/m	n/m	n/m
Isoleucine	993	n/m	108.7	n/m	n/m	n/m
Valine	993	n/m	119.8	n/m	n/m	n/m
Phenylalanine	993	n/m	86.2	n/m	n/m	n/m
Casein	954	958	145.9	n/m	n/m	n/m

[A]mixture of urea (90%) and ammonium sulphate (10%).

[B]mixture of leucine (33.6%), isoleucine (22.1%), valine (26.2%) and phenylalanine (18.0%).

n/m, not measured.

Table 9. Actual rumen degradable protein (RDP) intake (g/kg DOM[A]) of pangola grass hay and Mitchell grass hay alone or supplemented with urea-ammonium sulphate (US[B]), US plus amino acids (USAA)[c] and casein at intended RDP intakes of 130, 170, 210, 250 and 300 g/kg DOM.

Feeds	RDP/DOM (g/kg)[D]					
	Control	130	170	210	250	300
			Experiment 2.1			
Pangola grass	121	n/t	n/t	n/t	n/t	n/t
US	n/t	132	171	208	236	n/t
USAA	n/t	175	176	n/t	194	199
Casein	n/t	135	171	n/t	246	303
			Experiment 2.2			
Mitchell grass	76	n/t	n/t	n/t	n/t	n/t
US	n/t	144	184	225	266	n/t
USAA	n/t	189	196	n/t	209	220
Casein	n/t	147	187	n/t	274	329

[A]RDP was estimated from previous values in this laboratory and DOM was calculated from measured OM intake and OM digestibility in this experiment.

[B]mixture of urea (90%) and ammonium sulphate (10%).

[C]US supplied to provide 170 g RDP/kg DOM plus mixture of amino acids, described in sections 3.2.1 and 3.2.2.

[D]calculated based on actual measured DOM. n/t, no treatment at this level.

n/t, no treatment.

Microbial protein production. The effects of supplement type and intake on MCP production and EMCP for the two forage types are presented in Table 10. For control steers, the MCP production and EMCP were 178 g/d and 94 g/kg DOM, respectively, for pangola grass hay and 156 g/d and 112 g/kg DOM, respectively, for Mitchell grass hay. In Experiment 2.1 (pangola grass) MCP production increased linearly with increasing RDP supplied by casein supplementation but was unaffected by the US and USAA supplements. In Experiment 2.2 (Mitchell grass), a linear increase in MCP production was observed with increasing RDP supplied by USAA and casein supplementation but there was no effect of the US supplement alone. The MCP production response was similar for USAA and casein. However, EMCP was not affected by increasing RDP intake from any of the supplements for steers consuming either pangola grass or Mitchell grass hays.

Table 10. Relationship between supplement rumen degradable protein (RDP) intake (x; g/kg DOM) and the microbial crude protein (MCP) production and the efficiency of MCP production (EMCP) of steers fed either pangola grass hay (Experiment 2.1) or Mitchell grass hay (Experiment 2.2) at 90% *ad libitum* with different supplements (see text for supplement treatments). A single relationship is shown for two or more supplements where there were no significant difference between supplement types ($P > 0.05$), and no relationship is shown where the relationship was not significant for a supplement type.

Parameter	Equation	R^2	RSD	*P* value
	Experiment 2.1, pangola grass			
MCP production (g/d)				
US[A], USAA[B]	NSR, mean value = 174 ± 11.5		26.52	
Casein	Y = 163.8 + 0.09x	0.36	29.87	0.001
EMCP (g MCP/kg DOM)				
US, USAA, casein	NSR, mean value = 96 ± 4.5		14.63	
	Experiment 2.2, Mitchell grass			
MCP production (g/d)				
US	NSR, mean value = 141 ± 12.4		0.03	
USAA, casein	Y = 122 + 0.24x	0.22	30.08	0.001
EMCP (g MCP/kg DOM)				
US, USAA, casein	NSR, mean value = 102 ± 5.7	0.00	19.53	

[A]mixture of urea (90%) and ammonium sulphate (10%).
[B]US supplied to provide 170 g RDP/kg DOM plus mixture of amino acids, described in sections 3.2.1 and 3.2.2.
NSR, no significant relationship.

Intake and digestibility. The effect of supplement type and supplement intake on hay intake, total DM and NDF intake for the pangola and Mitchell grass hays are shown in Table 11. Daily hay intake of control steers was 14.7 ± 0.5 g/kg W/d and 13.7 ± 0.6 g/kg W/d for pangola grass and Mitchell grass hays, respectively. In Experiment 2.1, a linear decrease in pangola grass hay intake was observed with increasing RDP intake for all supplements, with no significant difference between supplements (Figure 2). Total DM and NDF intake increased linearly with increasing RDP intake from casein but there was no effect of US and USAA; total DM and NDF intake from US and USAA supplements was 15 ± 0.3 g/kg W/d and 11 ± 0.2 g/kg W/d, respectively. In Experiment 2.2 (Mitchell grass), hay intake, total DM and NDF intake increased quadratically with increasing RDP intake from casein. However, increasing RDP intake from US and USAA did not affect hay intake (14 ± 0.5 g/kg W/d), total DM (15 ± 0.5 g/kg W/d) and NDF (12 ± 0.4 g/kg W/d) intake.

The effects of increasing RDP intake from US, USAA or casein on the digestibility of OM and NDF and total DOM intake for two forage types are presented in Table 12. The OMD and NDFD were 60.8 and 69.2%, respectively for pangola grass hay alone and 46.5 and 52.5% for Mitchell grass hay alone. In Experiment 2.1, (pangola grass hay) the OMD and NDFD increased linearly with increasing RDP intake from all supplements (Figure 3). However, the rate of increase in OMD and NDFD was greater for steers supplemented with casein than for those supplemented with US and USAA, which were not different to each other. Casein increased total DOM intake in a linear fashion but there

were no effects of other supplements (8 ± 0.2 g/kg W/d) (Figure 4). In Experiment 2.2 (Mitchell grass hay), there was a similar linear increase in OMD and NDFD with increasing RDP intake (Figure 3). The response in OMD to USAA and casein was similar and was greater than that for US. There was no difference in the rate of increase in NDFD between the supplements. Total DOM intake increased linearly with the US and USAA supplements and quadratically with the casein supplement (Figure 4).

Table 11. Relationship between supplement rumen degradable protein (RDP) intake (x; g/kg DOM) and the intake of hay[A], total dry matter (DM) and neutral detergent fibre (NDF) of steers fed pangola grass hay (Experiment 2.1) or Mitchell grass hay (Experiment 2.2) at 90% *ad libitum* with different supplements (see text for supplement treatments). A single relationship is shown for two or more supplements where there were no statistical differences ($P < 0.05$) between supplement types, and no relationship is shown where the relationship was not significant for a supplement type.

Parameter	Equation	R^2	RSD	*P* value
	Experiment 2.1, pangola grass			
Hay DM intake (g/kg W/d)				
US[B], USAA[C], casein	Y = –5 - 0.004x	0.07	0.76	0.17
Total DM intake (g/kg W/d)				
Casein	Y = 13 + 0.009x	0.34	0.73	< 0.001
Total NDF intake (g/kg W/d)				
Casein	Y = 11 + 0.007x	0.34	0.57	< 0.001
	Experiment 2.2, Mitchell grass			
Hay DM intake (g/kg W/d)				
Casein	$Y = 12 + 0.03–x - 0.00009x^2$	0.54	0.82	0.025
Total DM intake (g/kg W/d)				
Casein	$Y = 12 + 0.03–x - 0.00006x^2$	0.76	0.90	0.096
Total NDF intake (g/kg W/d)				
Casein	$Y = 10 + 0.02–x - 0.00005x^2$	0.76	0.72	0.096

[A]*ad libitum* intake was determined over the 0-13 d preliminary period and then fixed at 90% *ad libitum* during the collection period.

[B]mixture of urea (90%) and ammonium sulphate (10%).

[C]US supplied to provide 170 g RDP/kg DOM plus mixture of amino acids, described in sections 3.2.1 and 3.2.2.

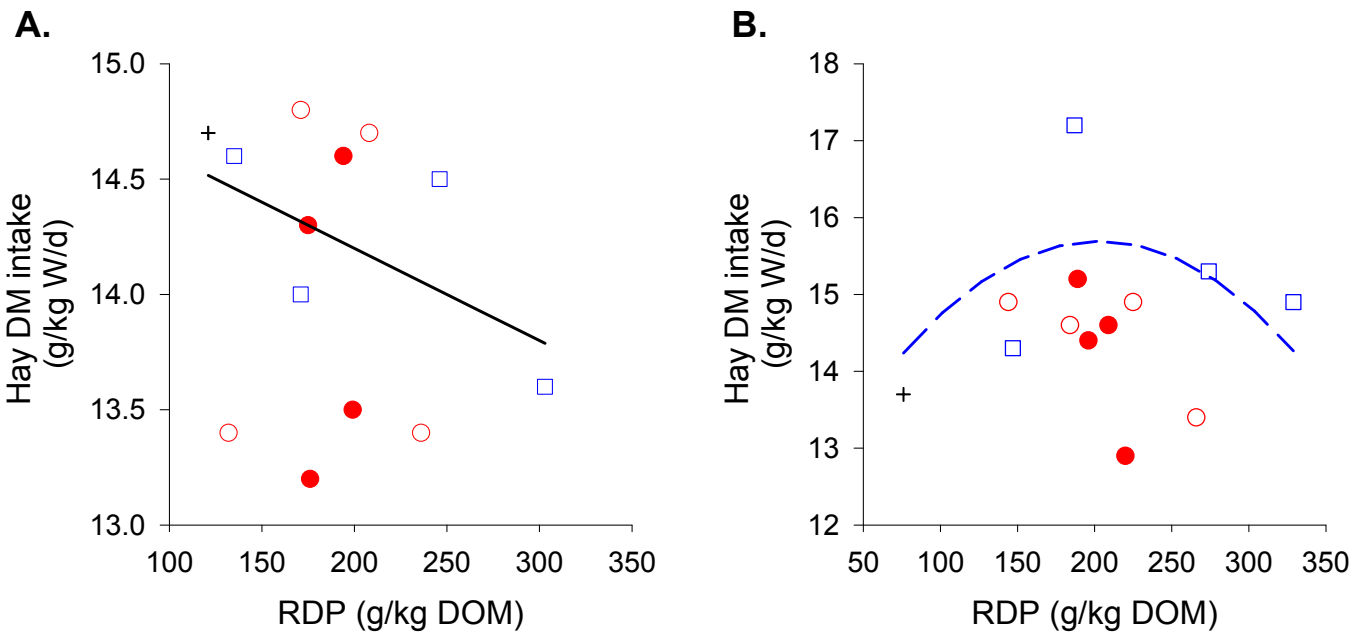

Figure 2. Effect of increasing rumen degradable protein (RDP) intake from urea-ammonium sulphate (US; ○), US plus amino acids (USAA; ●) and casein (□) on hay dry matter (DM) intake of steers fed pangola grass (A.) and Mitchell grass (B.) hays. The response equations and *P* values are presented in Table 11. A significant response to increasing RDP for US, USAA, casein or a common response is indicated by (. . .), (.-.-.), (- - -) and (━), respectively. Points represent mean of 3 steers.

Table 12. Relationship between supplement rumen degradable protein (RDP) intake (x; g/kg DOM) and the digestibility of organic matter (OM) and neutral detergent fibre (NDF) and the total digestible organic matter (DOM) intake of steers fed either pangola grass hay (Experiment 2.1) or Mitchell grass hay (Experiment 2.2) at 90% *ad libitum* with different supplements (see text for supplement treatments). A single relationship is shown for two or more supplements where there were no statistical differences ($P < 0.05$) between supplement types, and no relationship is shown where the relationship was not significant for a supplement type.

Parameter	Equation	R^2	RSD	*P* value
	Experiment 3, pangola grass			
OMD (%)				
US[A], USAA[B]	Y = 60 + 0.018x	0.18	1.33	0.012
Casein	Y = 59 + 0.029x	0.75	1.39	< 0.001
NDFD (%)				
US, USAA	Y = 67 + 0.015x	0.22	1.05	0.019
Casein	Y = 66 + 0.025x	0.75	1.20	< 0.001
Total DOM intake (g/kg W/d)				
Casein	Y = 7 + 0.083x	0.64	0.50	< 0.001
	Experiment 4, Mitchell grass			
OMD (%)				
US	Y = 46 + 0.012x	0.50	0.95	0.060
USAA, casein	Y = 45 + 0.022x	0.62	1.53	< 0.001
NDFD (%)				
US, USAA, casein	Y = 50 + 0.027x	0.59	1.69	< 0.001
Total DOM intake (g/kg W/d)				
US, USAA	Y = 6 + 0.004x	0.32	0.08	0.031
Casein	Y = 4 + 0.02–x - 0.00003x^2	0.87	0.10	0.014

[A]mixture of urea (90%) and ammonium sulphate (10%).

[B]US supplied to provide 170 g RDP/kg DOM plus mixture of amino acids, described in sections 3.2.1 and 3.2.2.

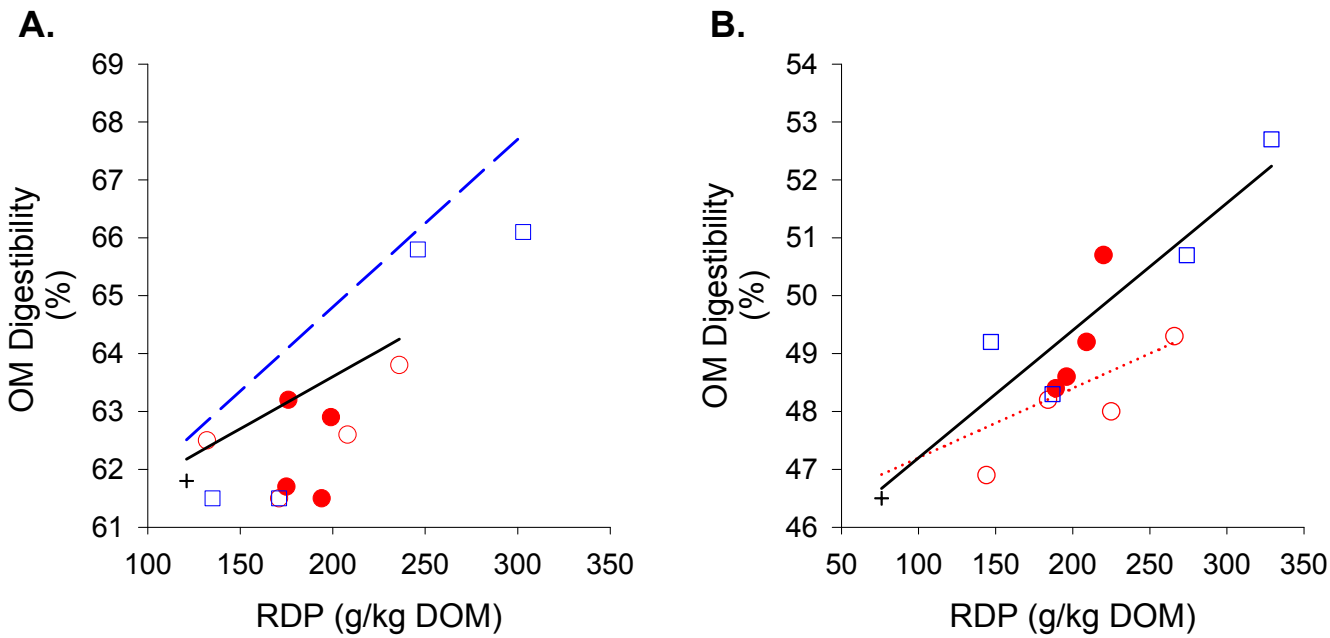

Figure 3. Effect of increasing rumen degradable protein (RDP) intake from urea-ammonium sulphate (US; o), US plus amino acids (USAA; ●) and casein (□) on the organic matter (OM) digestibility of pangola grass (A.) and Mitchell grass (B.) hays fed to steers. The response equations and *P* values are presented in Table 12. A significant response to increasing RDP for US, USAA, casein or a common response is indicated by (. . .), (.-.-.), (- - -) and (━), respectively. Points represent mean of 3 steers.

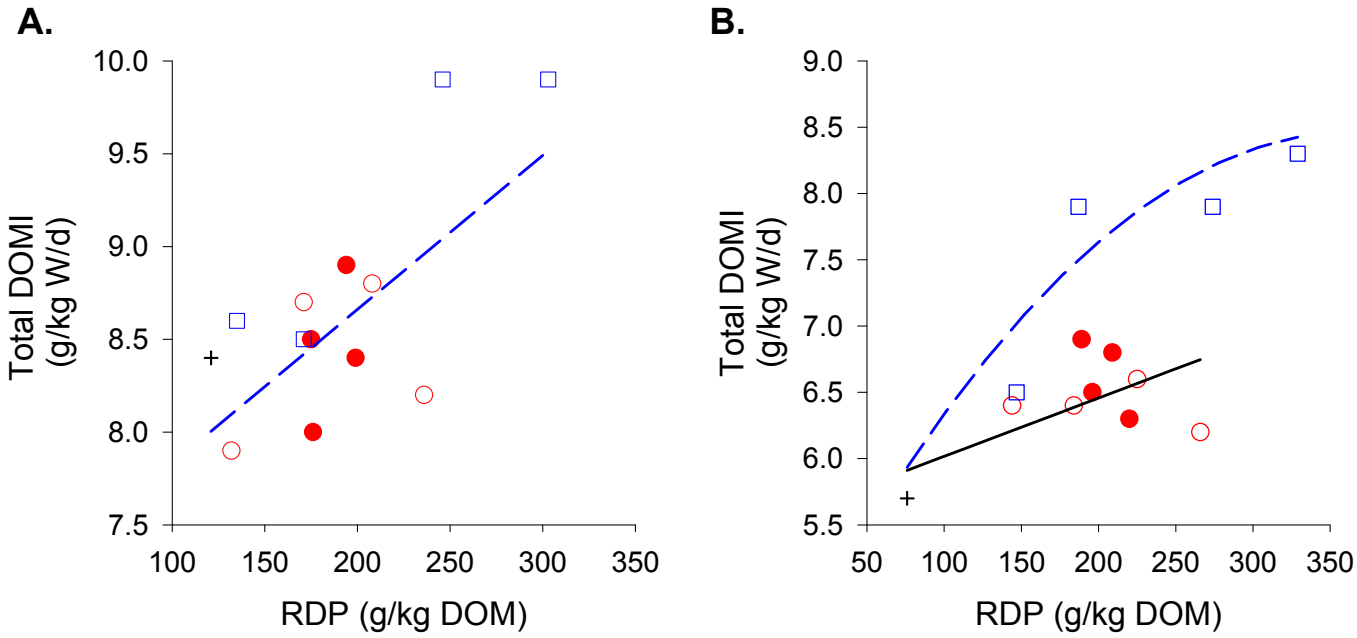

Figure 4. Effect of increasing rumen degradable protein (RDP) intake from urea-ammonium sulphate (US; o), US plus amino acids (USAA; ●) and casein (□) on the total intake of digestible organic matter (DOMI) of steers fed pangola grass (A.) and Mitchell grass (B.) hays. The response equations and *P* values are presented in Table 12. A significant response to increasing RDP for US, USAA, casein or a common response is indicated by (. . .), (.-.-.), (- - -) and (━), respectively. Points represent mean of 3 steers.

Rumen parameters and serum urea-N. The effects of increasing supplement intake on rumen pH and NH_3-N concentration, and serum urea-N concentration for the two forage types are presented in Table 13. In the absence of supplement, rumen NH_3-N concentration was 52 and 37 mg NH_3-N/L for pangola grass hay and Mitchell grass hay, respectively. Supplementing with US, USAA and casein in Experiment 2.1 (pangola grass hay), increased the NH_3-N concentration in a quadratic fashion but the response was not different between supplements. In Experiment 2.2 (Mitchell grass hay), there was a similar single quadratic increase in NH_3-N concentration to increasing RDP intake provided by all supplements, with no difference between supplements (Figure 5). Rumen pH was not affected by supplement type and intake in Experiment 2.1 (pangola grass hay). However, a small linear increase (from 6.9 to 7) was observed for pH with increasing RDP intake from all supplements in Experiment 2.2 but the response was not different between supplements and could be represented by a single relationship. The rumen pH of steers fed pangola grass and Mitchell grass hay alone was 6.7 ± 0.1 and 6.9 ± 0.1, respectively. The serum urea-N in unsupplemented steers was 13.1 ± 2.08 mg N/dL and 11.6 ± 2.66 mg N/dL for pangola grass and Mitchell grass hay, respectively. In Experiment 2.1 (pangola grass hay), serum urea-N increased quadratically with increasing RDP intake from all supplements but the response to casein was greater than that for US and USAA, with no difference between the latter two supplements. In Experiment 2.2 (Mitchell grass hay), there was a linear increase in serum urea-N with increasing RDP intake for all supplements and there were no difference in response between the supplements.

Total VFA concentration was 64.4 ± 2.70 and 65.7 ± 2.72 mM in control steers fed pangola grass and Mitchell grass hays. The effect of increasing levels of RDP on VFA concentration and the molar proportion of BCFA for the two forage types are presented in Table 13. In Experiment 2.1 (pangola

grass hay), increasing RDP intake from casein increased total VFA concentration linearly. In contrast, a linear decrease was observed in response to increasing RDP intake from USAA and there was no effect of US supplement. The total concentration of BCFA increased quadratically with USAA supplement, linearly with casein but was not affected by US supplementation (Figure 6). In the absence of supplement, the molar percentage of BCFA was 1.9 ± 0.18% and 1.5 ± 0.41% for pangola grass and Mitchell grass hays, respectively. In Experiment 2.2 (Mitchell grass hay), total VFA concentration decreased linearly with increasing RDP intake from US and USAA, with no difference between the two, but there was no response to casein supplementation. Similarly a quadratic and a linear increase were observed for total BCFA concentration with increasing RDP intake from USAA and casein, respectively, but increasing RDP intake from US did not affect total BCFA concentration (Figure 6). The total BCFA proportion, in response to USAA, peaked at 6.6%.

Table 13. Relationship between supplement rumen degradable protein (RDP) intake (x; g/kg DOM) and the concentration of rumen NH_3-N and serum urea-N and the pH of steers fed either pangola grass hay (Experiment 2.1) or Mitchell grass hay (Experiment 2.2) at 90% *ad libitum* with different supplements (see text for supplement treatments). A single relationship is shown for two or more supplements where there were no statistical differences ($P < 0.05$) between supplement types, and no relationship is shown where the relationship was not significant (NSR) for a supplement type.

Parameter	Equation	R^2	RSD	*P* value
	Experiment 2.1, pangola grass			
LnNH_3-N concentration[A](mg/L)				
US[B], USAA[C], casein	$Y = 1.2 + 0.0–x - 0.00005x^2$	0.95	0.12	< 0.001
Total VFA concentration (mM)				
USAA	$Y = 66–8 - 0.035x$	0.15	3.24	0.072
Casein	$Y = 60.0 + 0.022x$	0.25	3.42	0.050
BCFA[D] (% of total VFA)				
US	NSR, mean value = 2.0 ± 0.2		0.33	
USAA	$Y = –8 - 0.025x + 0.0009x^2$	0.81	0.84	< 0.001
Casein	$Y = -0.4 + 0.020x$	0.97	0.29	< 0.001
Serum urea-N concentration (mg/dL)				
US, USAA	$Y = -32 + 0.–x - 0.001x^2$	0.80	3.16	0.044
Casein	$Y = -37 + 0.–x - 0.0009x^2$	0.97	2.66	0.001
	Experiment 2.2, Mitchell grass			
LnNH3-N concentration C(mg/L)				
US, USAA, casein	$Y = 1.8 + 0.02–x - 0.00004x^2$	0.82	0.29	< 0.001
Total VFA concentration (mM)				
US, USAA	$Y = 67–7 - 0.031x$	0.25	3.54	0.006
Casein	NSR, mean value = 65.3 ± 1.2		4.20	
BCFA (% of total VFA)				
US	NSR, mean value = 1.4 ± 0.2		0.16	
USAA	$Y = –0 - 0.162x + 0.0007x^2$	79.36	1.12	< 0.001
Casein	$Y = -0.2 + 0.020x$	94.96	0.45	< 0.001
Rumen pH				
US, USAA, casein	$Y = 6.8 + 0.0005x$	21.3	0.07	0.022
Serum urea-N concentration (mg/dL)				
US, USAA, casein	$Y = - 5 + 0.173x$	92.9	3.43	< 0.001

[A]NH_3-N concentration is actual mean concentration prior to feeding and 4, 8, and 12 h after feeding; Ln is the natural logarithm.
[B]mixture of urea (90%) and ammonium sulphate (10%).
[C]US supplied to provide 170 g RDP/kg DOM plus mixture of amino acids, described in sections 3.2.1 and 3.2.2.
[D]BCFA (isobutyrate + isovalerate).

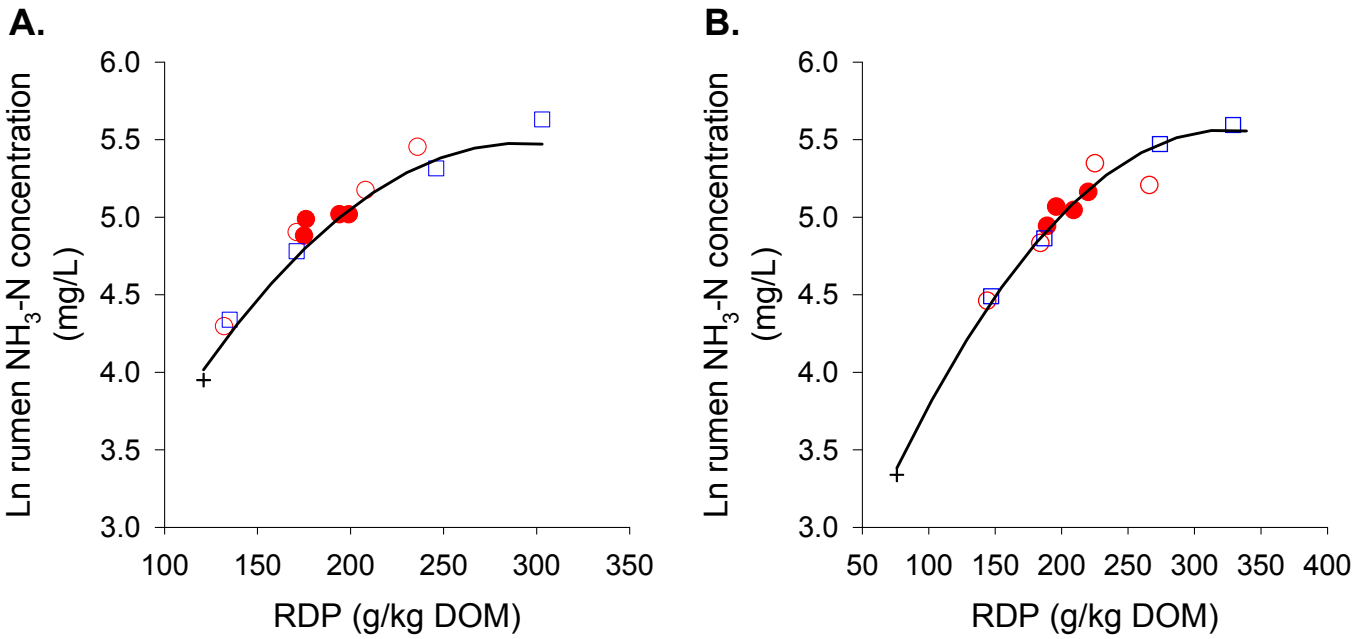

Figure 5. Effect of increasing rumen degradable protein (RDP) intake from urea-ammonium sulphate (US; o), US plus amino acids (USAA; ●) and casein (□) on the concentration of ammonia-N (Ln NH_3-N) in the rumen fluid of steers fed pangola grass (A.) and Mitchell grass (B.) hays. The response equations and *P* values are presented in Table 13. A significant response to increasing RDP for US, USAA, casein or a common response is indicated by (. . .), (.-.-.), (- - -) and (━), respectively. Points represent mean of 3 steers.

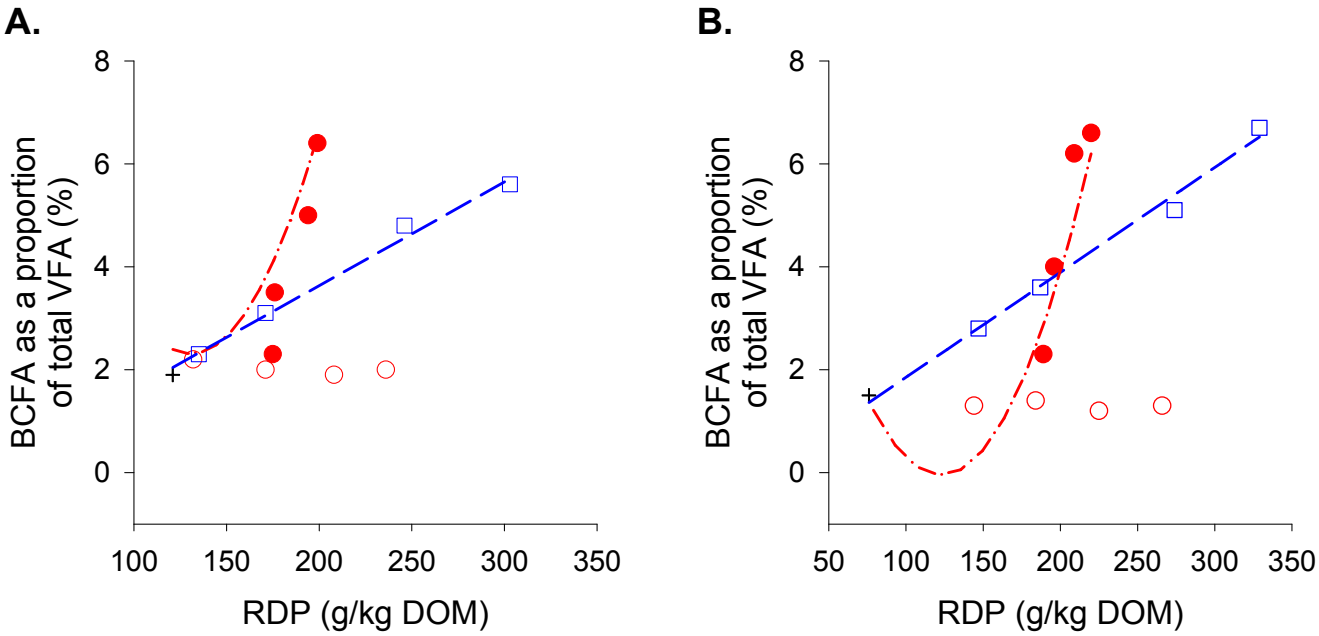

Figure 6. Effect of increasing rumen degradable protein (RDP) intake from urea-ammonium sulphate (US; o), US plus amino acids (USAA; ●) and casein (□) on the amount of branch chain fatty acids (BCFA), as a percentage of total volatile fatty acids (VFA), in the rumen fluid of steers fed pangola grass (A.) and Mitchell grass (B.) hays. The response equations and *P* values are presented in Table 13. A significant response to increasing RDP for US, USAA, casein or a common response is indicated by (. . .), (.-.-.), (- - -) and (━), respectively. Points represent mean of 3 steers.

Retention time. Retention time of Cr-EDTA for pangola grass alone was 17 h. This was not affected by increasing RDP intake from all supplements and averaged 16 h. Retention time of Cr-EDTA for Mitchell grass alone was 26 h. This decreased linearly with increasing RDP intake from casein (Y = -6 - 0.016x; R^2 = 0.33, RSD = 2.98, $P < 0.001$) but the other supplements had no effect. The lowest Cr-EDTA retention time was 20 h in the casein treatment and averaged 25 h for the US and USAA treatments.

Rate of digestion. The potential degradable fraction (a + b), degradation rate (c) and effective degradability (ED) were greater in ryegrass, followed by pangola grass and Mitchell grass substrates incubated in nylon bags in the rumen of unsupplemented steers consuming pangola grass or Mitchell grass hays ($P < 0.05$; Table 14). For steers consuming pangola grass, the immediately soluble fraction (a), showed a similar trend to a + b, c and ED. However, the slowly fermentable fraction (b) was higher in pangola grass than in the ryegrass substrate and both were higher than the Mitchell grass substrate ($P < 0.05$). The slowly fermentable fraction of ryegrass was greater than, pangola grass and Mitchell grass substrates incubated in the rumen of steers fed Mitchell grass but the immediately soluble fraction was not different between Mitchell grass and pangola grass substrates which were both lower than the ryegrass substrate. Increasing RDP intake from all supplements had no effect on the rate of digestion parameters for any substrates incubated in the rumen of steers consuming either pangola grass or Mitchell grass hay ($P > 0.05$).

Table 14. The effect of increasing supplement rumen degradable protein (RDP) intake on the immediately soluble fraction (a), slowly fermentable fraction (b), potential degradability (a +

b), fractional degradation rate of b (c) and effective degradability (ED) of Mitchell grass, pangola grass and ryegrass hay substrates incubated in the rumen of steers fed either pangola grass hay (Experiment 2.1) or Mitchell grass hay (Experiment 2.2) at 90% *ad libitum* with different supplements. Values are means and standard error of the difference of the means (SEM). Within a column, means without a common superscript differ ($P < 0.05$).

Feed type	a (% DM)	b (% DM)	a + b (% DM)	c (h^{-1})	ED[A] (% DM)
		Experiment 3, pangola grass			
		Substrates			
Mitchell grass	12.9[c]	48.6[c]	63.9[c]	0.0291[c]	31.6[c]
Pangola grass	17.2[b]	55.6[a]	76.3[b]	0.0461[b]	45.6[b]
Ryegrass	26.8[a]	53.3[b]	83.7[a]	0.0828[a]	62.2[a]
SEM	1.20	0.69	1.22	0.00223	1.36
		Treatment (RDP g/kg DOM)[B]			
Control	18.7	53.5	75.7	0.0521	47.1
US[C]170	18.8	52.2	74.4	0.0541	46.5
US250	18.9	52.1	74.3	0.0519	46.4
USAA[D]170	18.9	51.9	74.0	0.0566	46.9
USAA300	19.1	53.0	75.1	0.0512	46.0
Casein170	19.0	52.6	74.7	0.0513	46.1
Casein300	19.5	52.3	74.4	0.0518	46.1
SEM	1.24	0.85	1.39	0.00305	1.43
		Experiment 4, Mitchell grass			
		Substrates			
Mitchell grass	7.4[b]	40.2[c]	52.7[c]	0.0436[c]	28.8[c]
Pangola grass	7.4[b]	52.6[b]	70.5[b]	0.0555[b]	41.3[b]
Ryegrass	10.3[a]	56.7[a]	86.9[a]	0.1030[a]	63.1[a]
SEM	0.62	0.45	0.51	0.00253	0.53
		Treatment (RDP g/kg DOM)			
Control	9.4	50.3	70.4	0.0617	43.5
US170	7.7	49.4	69.8	0.0655	43.5
US250	7.3	50.8	71.0	0.0703	45.4
USAA170	9.1	49.4	69.7	0.0691	44.8
USAA300	8.1	49.6	69.9	0.0665	43.9
Casein170	8.1	50.3	70.6	0.0699	45.1
Casein300	8.7	48.7	69.0	0.0686	44.8
SEM	0.96	0.65	0.67	0.0037	0.66

[A]effective degradability (ED) at outflow rate of 0.05/h.

[B]actual RDP supply is listed in Table 9.

[C]mixture of urea (90%) and ammonium sulphate (10%).

[D]US supplied to provide 170 g RDP/kg DOM plus mixture of amino acids, described in sections 3.2.1 and 3.2.2.

4.2.2 Discussion

There was no significant effect of casein or BCAA plus phenylalanine on EMCP for steers fed either Mitchell grass or pangola hay. It has been suggested in the past that RDP (peptides, amino acids) and BCFA are limiting factors to rumen microbial growth on high fibre diets when ammonia-N is adequate. Further, it has been proposed that the level of BCFA supply needs to be high to meet the cellulolytic bacterial requirements to increase MCP production and EMCP through an increase in growth rate. Hume *et al*. (1970) showed the increase in MCP production with one level of BCFA (12.3 g/kg DM) in sheep. The current experiment was designed to determine the response of rumen microbes to higher levels of RDP or BCFA by measuring MCP production and EMCP to increasing casein or AA supplement when supplied with adequate NPN and S in the rumen. The high levels supplied in the present experiment, which were greater than Hume *et al*. (1970), was to determine if an additional response could be achieved in addition to that commonly observed with a simple mix of US. This was tested by supplementing with US to supply 170 g RDP/kg DOM (so as to ensure enough RDP for a response) and AA (BCAA; leucine, isoleucine and valine plus phenylalanine) at high levels. High levels of NPN sources will only increase EMCP to about 130 g MCP/kg DOM (Hume *et al*., 1970; Koster *et al*., 1997; Mullik, 1999; Marsetyo, 2004) and it was postulated that by providing a mix of USAA, thought to provide limiting BCFA to the microbes, that EMCP would be increased above 130 g MCP/kg DOM. A similar hypothesis was proposed for casein but here it was the broad spectrum of degradable protein and the subsequent supply of peptides and amino acids which were tested. The highest levels of casein supplementation were designed to reach the RDP/DOM ratios seen with temperate forages (Cruickshank *et al*. 1992) and the objective in both treatment supplements was to increase EMCP to values observed in temperate forages. The hypothesis was that peptides, amino acids and BCFA are needed at high levels to enable high EMCP to be achieved. The amount of each AA provided was the same as the amount of each AA present in casein for each treatment level. The RDP levels were not the same as casein but a high RDP level was supplied from US (170 g RDP/kg DOM) and equivalent USAA mix adjusted so as to be similar to that provided from casein. Thus the effect of specific USAA could be distinguished from a high RDP level. Two hays markedly different in CP content and digestibility were used to examine the hypotheses. One hay (Mitchell grass) was deficient in CP content by accepted standards (4.5% CP). The results indicated that neither increasing US supplement nor increasing BCAA, with adequate RDP from US, nor increasing casein had any effect on EMCP when expressed against g RDP/kg DOM.

This experiment had high variability in EMCP and also low EMCP values, as did Experiment 1. Considerable additional evaluation of the method of measuring PD was done both by HPLC and spectrophotometric methods. Both methods agreed very closely. Additionally, storage methods of acid and the effect of the crystal formation observed in urine collection trays were evaluated. None of these features affected the results. When there is low intake, as observed here, then the PD excretion in the urine from the microbes passing from the rumen is low and the effect of animal variation in endogenous PD excretion (required in the calculation) may have a large effect on the estimation of MCP production. We investigated this by recalculating data from Bowen *et al*. (2006). Microbial protein production throughout this study was measured by the indirect method by measuring urinary excretion of PD (Chen and Gomes, 1995) and this relies on an estimate of endogenous PD excretion to calculate PD from microbial nucleic acids. Bowen *et al*. (2006) have shown that *Bos indicus* cross animals have a much lower endogenous purine excretion than *Bos taurus* animals. The value of 190 µmol/kg $W^{0.75}$ for *Bos indicus* animals (Bowen *et al*., 2006) was used in the calculations in this report. However, there is variation in the endogenous value. The standard error of the endogenous value from the five Brahman crossbreed steers used by her was ± 23.8 µmol/kg $W^{0.75}$ or a standard deviation of ± 53.1 µmol/kg $W^{0.75}$ (M. Bowen, personal

communication). An estimate of the potential error in estimating MCP production and EMCP may be made. Table 15 shows the magnitude of change associated with using a constant endogenous PD excretion for all animals. Thus the method is good for detecting changes in MCP production but the quantitative values are relative rather than precise and the discussion here recognises that. However despite this the values estimated will still indicate if they are low or high values and should be able to detect the changes we anticipated might occur under the supplements. To further improve precision, we fixed intakes at 90% of *ad libitum* intake determined during the previous 7-14 d. Experiments 2.1 and 2.2 showed more variability in the data than any previous studies of ours or the other experiments in this report.

Table 15. Microbial crude protein (MCP) production and efficiency of MCP production (EMCP) estimated using the mean values for endogenous purine derivative excretion proposed by Bowen *et al*. (2006) or ± 1 standard deviations (SD) for a pangola grass hay diet with dry matter intake of 16 g/kg W/d, organic matter digestibility of 55% and crude protein of 7.5% (Experiment 2.1).

	Assumed endogenous value	Recalculated values with ± 1 SD of Bowen endogenous PD	
Endogenous purine	Bowen *et al*. (2006)	+	-
DOM intake (kg/d)	3.2	3.2	3.2
EMCP (g MCP/kg DOM)	101	-	-
MCP (g/d)	322	-	-
PD excretion (mM/d)[A]	71	71	71
New MCP (g/d)	as above	295	347
New EMCP (g MCP/kg DOM)	as above	92	108

[A]calculated from the equation of Chen and Gomes (1995) with mean endogenous PD excretion of 0.190 mmol/kg $W^{0.75}$ from Bowen *et al*. (2006), maximum and minimum value at 1 standard deviation (SD).

Rumen ammonia concentration increased under all forms of supplement. Microbial crude protein production and EMCP are determined by the amount and type of substrate available to the rumen microbes and the passage of microbes exiting the rumen (Dijkstra *et al*., 2002). In this experiment increasing RDP intake from a US supplement was associated with an increase in the level of NH_3-N concentration in the rumen. This is a straightforward relationship as US is degraded quickly in the rumen. The rumen NH_3-N concentration increased from 52 to 234 mg NH_3-N/L for steers fed pangola grass supplemented with US. The rumen NH_3-N concentration increased from 37 to 210 mg NH_3-N/L for steers fed Mitchell grass hay with increasing RDP intake from US supplementation. Furthermore, serum urea-N concentration was increased quadratically and linearly with increasing US intake in pangola grass and Mitchell grass hay, respectively. Serum urea-N correlates with urea synthesis in the liver and rumen NH_3-N is a primary source of this N, hence, increasing serum urea-N reflects NH_3-N status in the rumen. Thus rumen NH_3-N concentration, especially in supplemented animals, would not be expected to be limiting under conventional concepts (Satter and Slyter, 1974). Such a high rumen NH_3-N concentration under supplementation ensures that NH_3-N should be accessible to microbes within isolated niches within the rumen (Owens and Zinn, 1988). However, the rumen NH_3-N concentration in the basal diets examined in the present study already approximated the minimum level required for microbial growth. Some literature suggests that the minimum NH_3-N concentration depends on the available fermentable energy source (Pisulewski *et al.*, 1981; Van Soest, 1994; Maeng *et al*., 1997), but for both these low quality hays of quite different

digestibility the rumen NH_3-N concentration may have been adequate. Dijkstra *et al*. (1998; 2002) suggested that NH_3-N uptake follows the kinetics of enzyme saturation with any incremental NH_3-N concentration above a critical level resulting in a marginal response in fractional growth rate. This may partly explain the lack of response in EMCP even though the rumen NH_3-N concentration increased with increasing US supplement. It may be concluded that there is no additional advantage to EMCP associated with increasing rumen NH_3-N concentration above the critical level. This is despite the accepted means of ration formulation (RDP/DOM) indicating that Mitchell grass hay was deficient in RDP and a response to N addition was expected. Such a calculation ignores recycling of N to the rumen which can be substantial, as a proportion of total N intake, with low CP forages (Nolan, 1993).

Factors other than NH_3-N, which can enhance the growth rate of rumen microbes (e.g. true protein) have been investigated previously (Hume, 1970; Hume *et al*., 1970; Van Soest, 1994; Wallace *et al*., 1997). True protein sources can supply the rumen microbes with peptides, AA and BCFA. Branch chain amino acids (leucine, isoleucine and valine) are deaminated to produce BCFA (isovalerate, 2-methylbutyrate, and isobutyrate). Many bacteria, particularly cellulolytic bacteria, methanogenic bacteria and some amylolytic bacteria, use NH_3-N and have a specific additional requirement of BCFA for growth (Mackie and White, 1990; Van Soest, 1994). In addition, Salter *et al*. (1979) indicated that phenylalanine is likely to be the first limiting amino acid for rumen microbial growth in steers fed diets low in protein and high in NPN. Furthermore, Morrison *et al*. (1990) reported that the addition of phenylacetate in growth medium improved cellulose hydrolysis by fibre digesting bacteria *Ruminococcus albus*. Bowen (2003) has indicated that a high EMCP may only be achieved when a very high RDP/DOM is provided in the diet, e.g. with temperate pastures (Cruickshank *et al*., 1992). The objective of the current experiment was to provide specific BCAA plus phenylalanine at very high levels, with adequate N from inorganic sources. This has not been done previously and may account for the variable responses in the literature. However, there was no effect of increasing USAA supplement or casein supplement on EMCP for either forage type.

The molar percentage of BCFA in the two forages was low, 1.9% and 1.5% of total VFA in pangola grass and Mitchell grass hay, respectively. The diets thus provided a good test of the effect of USAA and casein on MCP production and EMCP when there is an excess of RDN and rumen ammonia-N. The molar percentage of BCFA increased 3 to 4-fold over and above the control diets with increasing intake of the USAA supplement or casein. Thus, neither NH_3-N supply nor the supply of BCFA and phenylacetate could be expected to be limiting to the rumen microbes under the high levels of supplementation. The rate of increase in BCFA was not different compared to casein for the two forage types despite differences in RDP levels. It may be concluded that factors other than BCFA and degradable true protein may limit microbial growth when NH_3-N supply is adequate.

Microbial protein production and EMCP are influenced by dilution rate (Owens and Goetsch, 1988). Microbes leave the rumen in fluid and attached to particles. Dilution rate affects MCP production and EMCP through the amount of time microbes are retained in the rumen which affects energy maintenance, microbial lysis and protozoal predation (Van Soest, 1994). In the present experiment, neither quantity nor type of supplement (US or USAA) had any effect on Cr-EDTA retention time in the rumen for either of the hay diets, except for level of casein supplement with the Mitchell grass diet where there was a negative linear relationship. The retention time of Cr-EDTA, with increasing RDP intake with USAA supplementation ranged from 16-20 h for pangola grass hay, and from approximately 24-28 h for Mitchell grass hay. Casein supplementation of Mitchell grass reduced retention time of Cr-EDTA from 26 to 21 hrs. Many studies in the past reported that the Cr-EDTA retention time of tropical forages ranged from 11-25 h (Poppi *et al*., 1981a; Mullik, 1999; Marsetyo, 2004). In addition, the retention time value in the present study is within the range reported in

Experiment 1. The long retention time of fluid (from Cr-EDTA) has been recognised as one of the factors which limit the intake of tropical grasses (Poppi *et al*., 1981a). Fractional outflow rate (FOR; inverse of retention time) of water by passage from the rumen is determined by the osmolarity within the rumen which is largely a function of rate of digestion and production of osmotically active compounds (Beever *et al*., 1981; Faichney *et al*., 1981). Tropical forages have a low fractional outflow rate or long retention time (14 to 25 h) whilst temperate forages have very low (6 h) retention times (Poppi *et al*., 1981; Cruickshank *et al*., 1992). With such a long retention time, there is inevitably a high turnover of microbes within the rumen (Cottle, 1982). Dijkstra *et al*. (2002), in their simulation, indicated that when recycling of microbial N occurred at 20% of gross production, 3.8 g fermentable OM is required to produce 1 g MCP flowing from the rumen. However, if the recycling of microbial N increased to 90% of gross production the amount of fermentable OM required to produce 1 g MCP flowing from the rumen increased to 30 g. This may impose an upper limit to EMCP, irrespective of nutrient supply. These conditions provide a unique test of the microbial species and their response to the various supplements (US, USAA and casein). The implications of this for the growth and composition of microbial species within the rumen is discussed in section 4.6.2.

There were marked differences in rate of digestion between the three grass substrates, as expected. Supplements had no significant effect on rate of digestion of DM of Mitchell grass, pangola grass and ryegrass hays in the rumen of steers fed either a low CP (4.5% CP) Mitchell grass hay or an adequate CP (9.9% CP) pangola grass hay. The lack of response in rate of digestion may be related to the NH_3-N concentration in the rumen, where both control hays had values of approximately 45 mg NH_3-N/L (Boniface *et al*., 1986). Therefore, any increase in NH_3-N concentration may have no effect on rate of digestion. The addition of casein or USAA markedly increased the molar percentage of BCFA, in addition to rumen NH_3-N concentration. This would provide ideal conditions to test the response in rate of digestion to these nutrients. There was no response indicating no limitation of BCFA and phenylacetate on rate of digestion under these circumstances. Various authors (Jouany *et al*., 1988; Van Soest, 1994; Dijkstra *et al*., 1998) have suggested that bacterial turnover and lysis within the rumen would provide adequate BCFA for the cellulolytic microbes. Hunter and Siebert (1985) and Panjaitan *et al*. (2006) demonstrated that the rate of digestion increases with an increase in NH_3-N concentration in the rumen. However, once the NH_3-N concentration reached a critical level, no additional increase in the rate of digestion would occur. Ortiz-Rubio *et al*. (2007) reported no difference between inorganic N and organic N sources in affecting rate of digestion. However, fractional degradation rate itself depends on the amount of microbes present in the rumen and their activity (Dijkstra *et al*., 2002). The lack of any significant effect of the US, USAA or casein treatments on the rate of digestion suggests that intake and liquid retention time are important limiting factors for EMCP and that rate of digestion was already at maximal rates for these substrates under these rumen conditions.

The mean total DM intake in control steers fed pangola grass and Mitchell grass hays was 14.7 ± 0.5 g/kg W/d and 13.7 ± 0.6 g/kg W/d, respectively. The DM intake in pangola grass was numerically higher than Mitchell grass hay but not greatly so. This was likely to be associated with the higher CP content, lower fibre content and lower stem proportion in pangola grass resulting in higher digestibility and lower water retention time in the rumen. There was only a small change in intake associated with any of the supplements with the changes unlikely to be of any biological significance.

Organic matter digestibility was numerically higher in steers fed pangola grass (61.8%) than in those fed Mitchell grass (46.5%) and this was associated with higher CP, rate of NDF digestion, potential digestibility and leaf proportion. Organic matter digestibility linearly increased with increasing RDP

intake from US, USAA or casein in either hay but the increase was biologically small except for casein which was much larger. These changes are due to the high digestibility (100%) of the various supplements and the higher value for casein just reflects the higher amount of digestible organic matter provided as casein compared to the other two supplements.

The pH of rumen fluid from steers consuming the pangola grass diet did not differ for all supplements and averaged 6.8 across all treatments. This pH would have provided favourable conditions for cellulolytic bacteria in the rumen (Owens and Goetsch, 1988) and did not limit MCP production and EMCP.

In conclusion, the provision of increasing amounts of BCAA plus phenylalanine or casein to cattle resulted in no additional response in MCP production and EMCP over and above that obtained from increasing RDP through an inorganic N source alone. There were changes in rumen function especially the supply of BCFA, ammonia and dilution rate and this situation provides an excellent model to examine the microbial genetic profiles of the resident microbial populations which evolve under these vastly different conditions.

4.3 Experiment 3. The effect of rumen degradable protein or single cell organism on rumen function

4.3.1 Results

Chemical composition of the diets. The chemical composition of the Mitchell grass hay and Spirulina algae is given in Table 16. Mitchell grass hay used in this experiment was low in N and high in fibre, with a leaf proportion of 52% and a S content of 3.4 g/kg DM. The ether extract content of Spirulina was 100 g/kg DM. The estimated RDP/DOM which resulted from the levels of urea or Spirulina supplement offered and calculated from the measured values of CP content and OM digestibility are presented in Table 17.

Table 16. Nutrient composition of Mitchell grass hay and Spirulina.

Composition	Mitchell grass hay	Spirulina
Dry matter (g/kg)	916	921
Organic matter (g/kg DM)	887	912
Nitrogen (g/kg DM)	6.1	114.3
Neutral detergent fibre (ash free) (g/kg DM)	746	35
Acid detergent fibre (ash free) (g/kg DM)	481	18
Lignin (g/kg DM)	65	n/d[A]
Ether extract (g/kg DM)	not analysed	100

[A]n/d, not detected.

Table 17. Estimated rumen degradable protein (RDP)/digestible OM (DOM) intake of steers fed Mitchell grass alone or supplemented with increasing levels of urea-ammonium sulphate mix (US, 90% urea and 10% ammonium-sulphate) or Spirulina at pre-planned treatment RDP levels of 90, 130, 170, 210 and 290 g RDP/kg DOM.

	Mitchell grass hay	US	Spirulina
Pre-planned treatment level	Actual feeding level[A] (g RDP/kg DOM)		
Control	62	n/t[B]	n/t

90 (g RDP/kg DOM)	n/t	109	116
130 (g RDP/kg DOM)	n/t	138	155
170 (g RDP/kg DOM)	n/t	175	199
210 (g RDP/kg DOM)	n/t	245	n/t
290 (g RDP/kg DOM)	n/t	n/t	358

[A]calculation based on measured CP, estimated CP degradability and measured DOM. The estimated CP degradability was 70% for Mitchell grass (McLennan *et al*., 1997), 85% for Spirulina (Devi *et al*., 1981) and 100% for urea.

[B]n/t, no treatment at this intake.

Effect of level of Spirulina intake on microbial protein production, intake and digestibility. Microbial protein production and EMCP increased in a quadratic fashion in response to increasing Spirulina intake (Table 18). Microbial protein production was predicted to reach a peak of 593 g MCP/d at a Spirulina intake of 10.1 g Spirulina DM/kg W/d. The EMCP was predicted to reach a peak of 176 g MCP/kg DOM at a Spirulina intake of 8.7 g Spirulina DM/kg W/d. There was a quadratic increase in hay intake and total DM intake with increasing Spirulina intake. Hay DM intake was predicted to peak at 22 g/kg W/d at 3.7 g Spirulina DM/kg W/d and total DM intake was predicted to peak at 26 g/kg W/d at 4.6 g Spirulina DM/kg W/d. A linear and a quadratic increase were observed for the digestibility of OM and total DOM intake respectively, in response to increasing Spirulina intake.

Table 18. Predicted microbial crude protein (MCP) production and the efficiency of MCP production (EMCP), hay and total dry matter (DM) intake, digestibility of dry matter (DMD) and total digestible organic matter intake (DOMI), of steers fed Mitchell grass hay at 90% *ad libitum* intake in response to Spirulina intake (x is g Spirulina DM/kg W/d).

Parameter	Equation	R^2	RSD	*P* value
MCP production (g/d)	$Y = 103.3 + –7.1x - 4.8x^2$	0.98	27.1	0.020
EMCP (g MCP/kg DOM/d)	$Y = 64.5 + –5.7x - 1.5x^2$	0.96	8.38	0.078
Hay intake (g DM/kg W/d)	$Y = 14.7 +–3.9x - 0.5x^2$	0.89	1.19	< 0.001
Total DM intake (g DM/kg W/d)	$Y = 14.7 +–4.9X - 0.5x^2$	0.99	0.77	< 0.001
DMD (%)	$Y = 42.9 + 1.14x$	0.79	1.49	< 0.001
Total DOMI (g DOM/kg W/d)	$Y = 5.2 +–2.8x - 0.3x^2$	0.99	0.33	< 0.001

Effect of level of Spirulina and urea-ammonium sulphate intake on microbial protein production. The MCP production and EMCP in control steers was 66 ± 15 g MCP/d and 54 ± 6.5 g MCP/kg DOM, respectively. Microbial protein production increased in a quadratic fashion in response to increasing RDP intake from both Spirulina and US supplements (Figure 7). The EMCP increased in a linear fashion in response to increasing RDP intake from both the Spirulina and US supplements (data not shown). The variability of the EMCP increased at higher levels of RDP with the Spirulina supplement, so data were transformed by the natural logarithm (Ln) and re-analysed. The Ln EMCP increased quadratically with increasing RDP intake from Spirulina and linearly with increasing RDP intake from US (Figure 7).

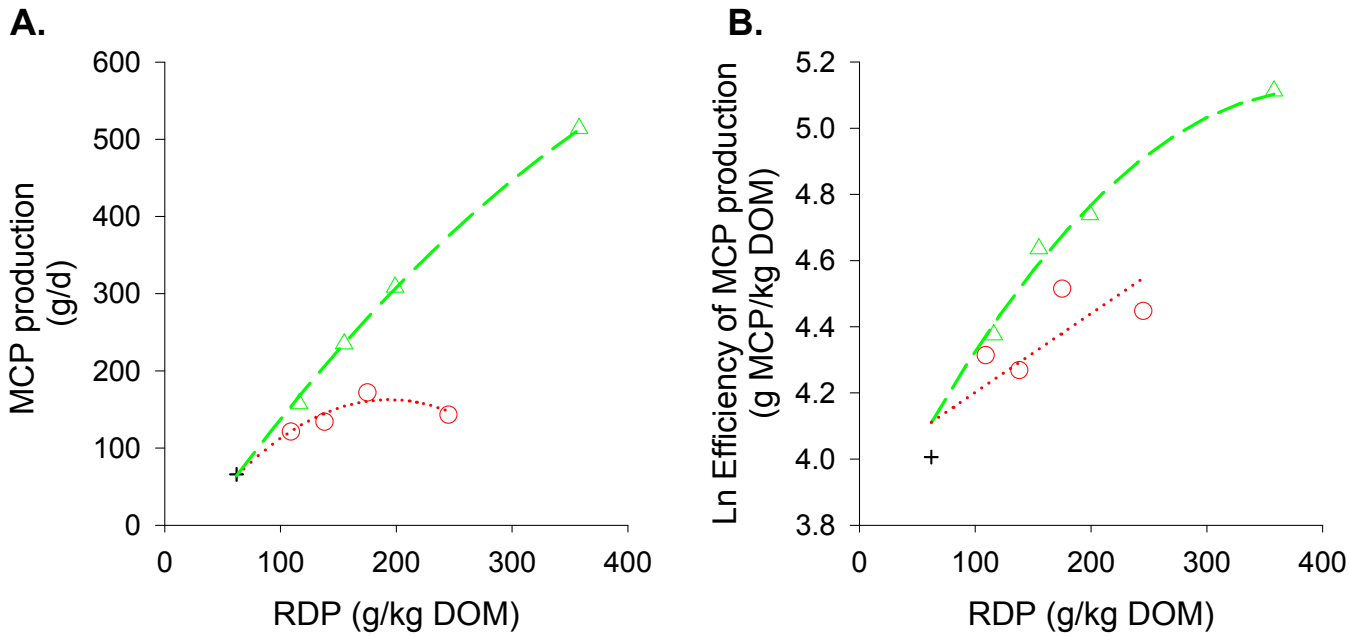

Figure 7. Microbial protein (MCP) production (A.) and the natural logarithm (Ln) of the efficiency of MCP production (B.) in response to rumen degradable protein (RDP) in digestible organic matter (DOM) supplied by Mitchell grass hay alone (+) or by Mitchell grass hay supplemented with increasing amounts of urea-ammonium sulphate (US; o) or Spirulina (Δ). The responses to US (. . .) were, MCP production = -50.4 +–2.2x - 0.006x^2, R^2 = 0.81, RSD = 20.8, *P* = 0.002; Ln efficiency of MCP production = 3.96 + 0.002x, R^2 = 0.42, RSD = 0.22, *P* < 0.001. The responses to Spirulina (- - -) were, MCP production = -65.6 +–2.2x - 0.002x^2, R^2 = 0.99, RSD = 21.0, *P* = 0.018; Ln efficiency of MCP production = 3.70 + 0–007x - 0.00001x^2, R^2 = 0.93, RSD = 0.13, *P* = 0.035. Points represent mean of 3 steers.

Effect of level of Spirulina and urea-ammonium sulphate intake on intake and digestibility. Mean intake of hay in control steers was 13 ± 0.7 g DM/kg W/d. The digestibility of DM, OM and NDF in control steers was 44 ± 0.9, 46 ± 1.0 and 43 ± 1.5%, respectively and the total DOM intake was 5 ± 0.3 g DOM/kg W/d. Hay (Figure 8) and total DM intake (data not shown) increased in a quadratic response to increasing US and Spirulina supplements. Hay intake peaked at 200 g RDP/kg DOM (18.5 g DM/kg W/d) for the US supplement and at 300 g RDP/kg DOM (24.5 g/kg W/d) for the Spirulina supplement. There was a linear increase in organic matter digestibility in response to increasing RDP intake from US (Y = 45+0.01x; R^2 = 0.38; RSD = 1.40; *P* = 0.024) and Spriulina (Y = 44+0.03x; R^2 = 0.78; RSD = 1.61; *P* < 0.001). There was a quadratic increase in total DOM intake to both supplements (Figure 8).

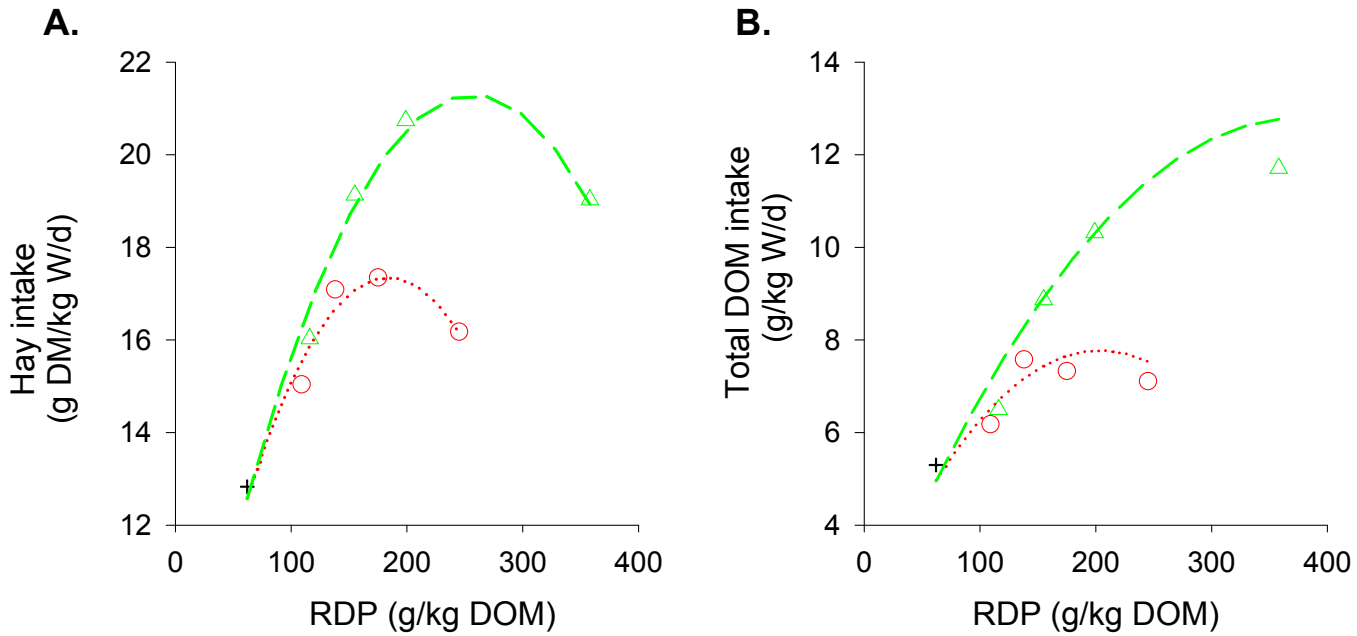

Figure 8. Hay (A.) and total digestible organic matter (DOM) (B.) intake in response to rumen degradable protein (RDP) in DOM supplied by Mitchell grass hay alone (+) or by Mitchell grass hay supplemented with increasing amounts of urea-ammonium sulphate (US; o) or Spirulina (Δ). The responses to US (...) were, Hay intake = -6.5 + –.12x - 0.0003x^2, R^2 = 0.91, RSD = 0.71, *P* < 0.001; DOM intake = 1.97 + 0.057x-0.00014x^2, R^2 = 0.81, RSD = 0.52, *P* = 0.002. The responses to Spirulina (- - -) were, Hay intake = 6.5 + –.12x - 0.0002x^2, R^2 = 0.97, RSD = 0.74, *P* < 0.001; DOM intake = 1.54 + –.06x - 0.0008x^2, R^2 = 0.98, RSD = 0.48, *P* < 0.001. Points represent mean of 3 steers.

Effect of level of Spirulina and urea-ammonium sulphate intake on rumen parameters. There was no difference in rumen pH, rumen NH_3-N concentration and the total concentration and molar proportion of VFA in the rumen of steers, between the mean of samples collected over a 24 h period or samples collected at rumen emptying. The results presented and discussed are based on the mean values collected over the 24 h sampling period. Mean rumen pH was 6.9 ± 0.1 in steers fed Mitchell grass hay alone. There was a linear decline in rumen pH in response to increasing levels of Spirulina and US (pH = 6.86-0.0008x, R^2 = 0.25, RSD = 0.12, *P* = 0.011) with no significant difference between the supplements; the decline was very small and was not biologically significant (from 6.9 to 6.6). The control steers had very low NH_3-N concentration in the rumen (5 ± 1.3 mg NH_3-N/L). A quadratic increase was observed for rumen NH_3-N concentration in response to increasing RDP intake from both supplements (Figure 9). The highest rumen NH_3-N concentration was 128 (Ln 4.9) mg NH_3-N/L from US at 223 g RDP/kg DOM and 264 (Ln 5.6) mg NH_3-N/L from Spirulina at 296 g RDP/kg DOM, respectively. Total VFA concentration in the rumen of control steers was 65 ± 5 mM. Increasing RDP intake resulted in a linear increase in total rumen VFA concentration, which was not significantly different between supplements (Figure 9). There was a linear decrease in the molar percentage of acetate from 77 to 72% of total VFA (Figure 10) whilst the molar percentage of BCFA increased linearly from 1.2 to 4.2% of total VFA in response to increasing Spirulina intake. Neither acetate nor BCFA were affected by increasing RDP intake from US. There was a linear increase in the molar percentage of propionate from 13.5 to 14.6% of total VFA in

response to increasing levels of Spirulina and US with no significant difference between the supplements. There was no change in the molar percentage of butyrate with increasing RDP from Spirulina or US (7.4 ± 0.26%).

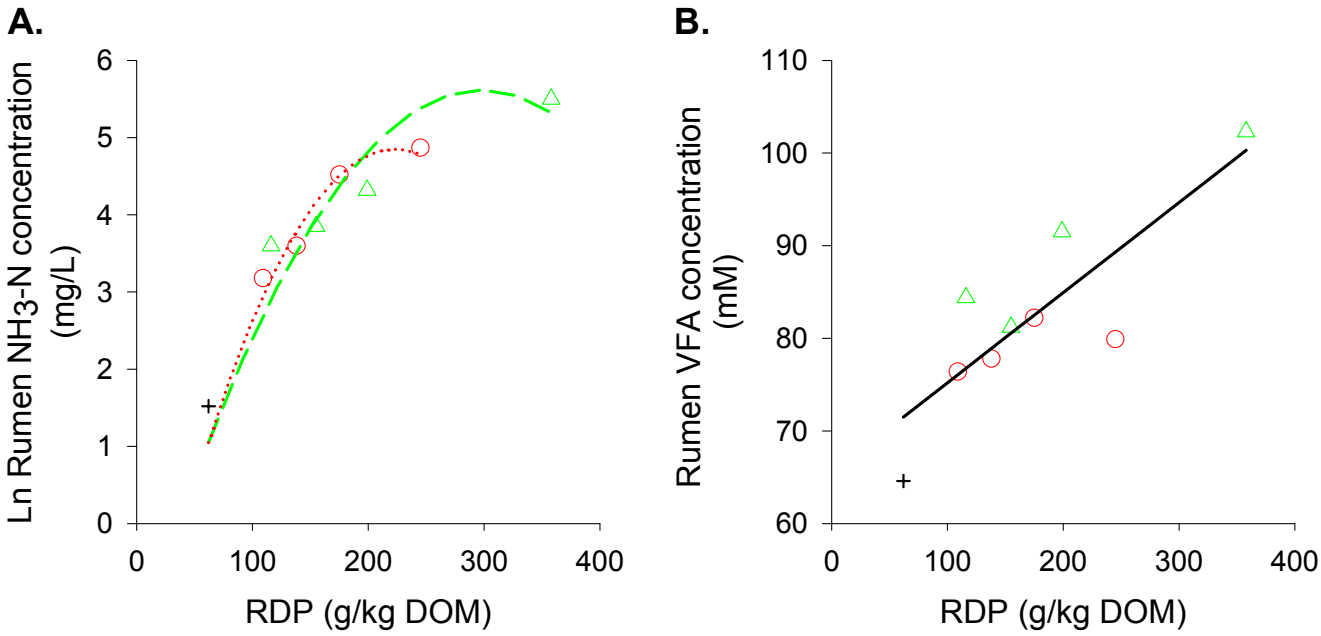

Figure 9. The natural logarithm (Ln) of ammonia (NH_3-N) (A.), and volatile fatty acids (VFA) (B.) concentration in the rumen in response to rumen degradable protein (RDP) in digestible organic matter (DOM) supplied by Mitchell grass hay alone (+) or by Mitchell grass hay supplemented with increasing amounts of urea-ammonium sulphate (US; o) or Spirulina (Δ). The responses to US (**. . .**) were, rumen NH_3-N concentration = -2.437827 + 0.06–261x - 0.000146x^2, R^2 = 0.95, RSD = 0.37, P < 0.001. The responses to Spirulina (**- - -**) were, rumen NH_3-N concentration = -1.663321 + 0.04–869x - 0.000082x^2, R^2 = 0.92, RSD = 0.53, P < 0.001). A similar response of VFA concentration in the rumen occurred for both supplements (**—**), Rumen VFA concentration = 65 + 0.10x, R^2 = 0.43, RSD = 8.95, P < 0.001. Points represent mean of 3 steers.

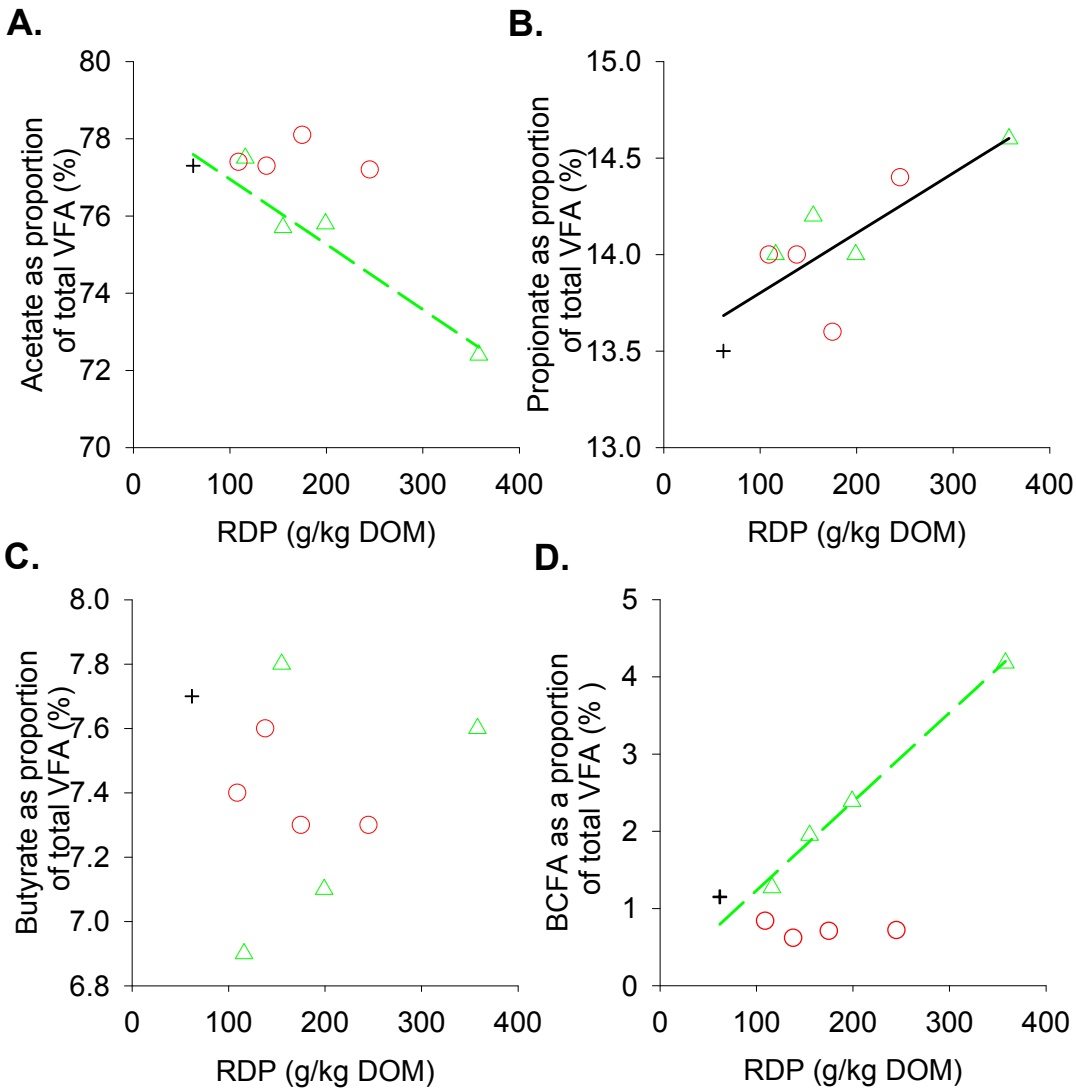

Figure 10. Molar percentage of acetate (A.), propionate (B.), butyrate (C.) and branch chain fatty acids (BCFA) (D.) in total volatile fatty acids (VFA) in response to rumen degradable protein (RDP) in digestible organic matter (DOM) supplied by Mitchell grass hay alone (+) or by Mitchell grass hay supplemented with increasing amounts of urea-ammonium sulphate (US; o) or Spirulina (Δ). The responses to US (…) were, acetate = 77.5 ± 0.4%, no significant relationship; Butyrate = 7.5 ± 0.3%, no significant relationship; BCFA = 0.70 ± 0.06%, no significant relationship. The responses to Spirulina (- - -) were, acetate–= 79 - 0.02x, R^2 = 0.89, RSD = 0.75, P < 0.001; Butyrate = 7.4 ± 0.3%, no significant relationship; BCFA = 0.08 + 0.01x, R^2 = 0.94, RSD = 0.32, P < 0.001. A similar response to the molar percentage of acetate in total VFA occurred for both supplements (━), propionate = 13 + 0.003x, R^2 = 0.20, RSD = 0.59, P = 0.024. Points represent mean of 3 steers.

Effect of level of Spirulina and urea-ammonium sulphate on retention time of Cr-EDTA and Ytterbium markers, and dry matter, NDF and lignin in the rumen. The retention time of Cr-EDTA and $YbCl_3$ in the rumen of steers consuming Mitchell grass alone was 24 ± 0.9 and 34 ± 2.3 h, respectively. Retention time of Cr-EDTA declined quadratically in response to increasing RDP intake from US and Spirulina (Figure 11). The minimum retention time of Cr-EDTA was predicted to occur

at 326 g RDP/DOM for Spirulina (12.5 h) and at 164 g RDP/kg DOM for US (23 h). Retention time of $YbCl_3$ was not affected by increasing RDP intake from US. In contrast, retention time of $YbCl_3$ decreased linearly in response to increasing RDP intake from Spirulina and at the highest level (358 g RDP/kg DOM or 6.1 g Spirulina DM/kg W/d) it declined to 22 h. The retention time of DM, NDF and lignin in the rumen of steers consuming Mitchell grass hay alone was 44 ± 2.0, 45 ± 2.0 and 79 ± 3.7 h, respectively. Dry matter, NDF and lignin retention time determined by emptying of the rumen (data not shown) declined quadratically in response to increasing RDP intake from both supplements. The lowest retention time of DM, NDF and lignin for the US supplement was predicted to occur at 187, 179 and 192 g RDP/kg DOM and the values were 32 and 33 and 56 h, respectively. The lowest retention time of DM, NDF and lignin for the Spirulina supplement was predicted to occur at 303, 273 and 268 g RDP/kg DOM and the values were 24, 29 and 45 h, respectively.

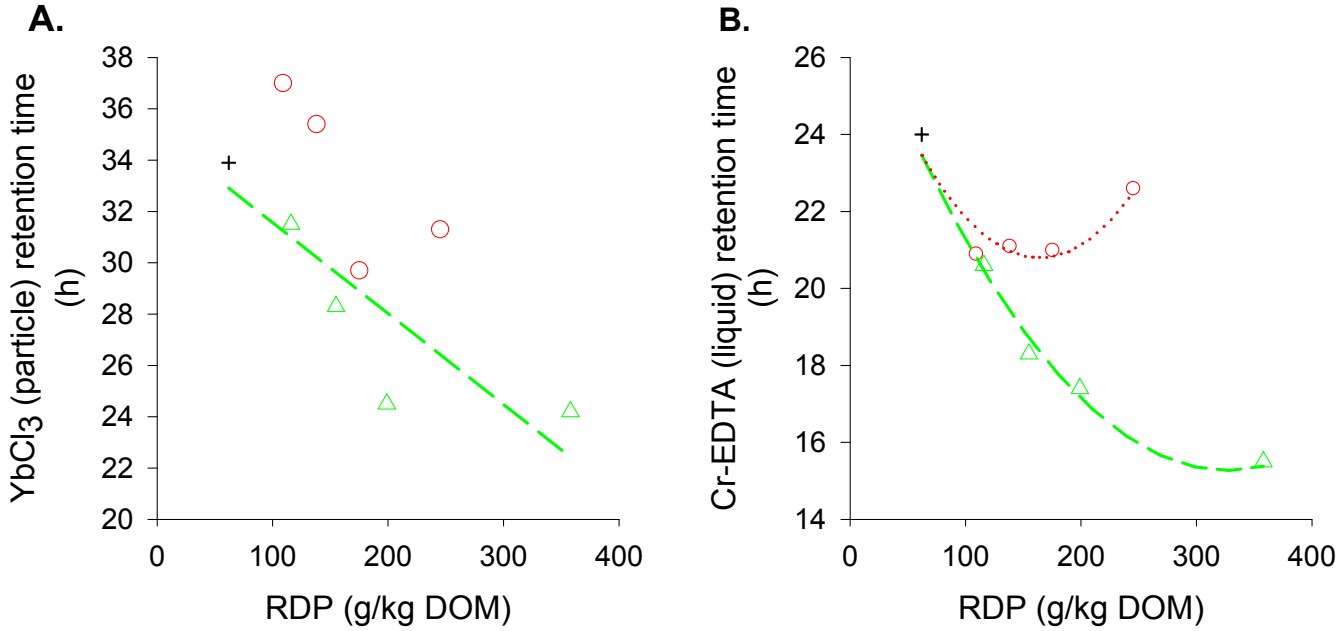

Figure 11. Retention time of Ytterbium Chloride ($YbCl_3$) (A.) and Chromium-EDTA (Cr-EDTA) (B.) in response to rumen degradable protein (RDP) in digestible organic matter (DOM) supplied by Mitchell grass hay alone (+) or by Mitchell grass hay supplemented with increasing amounts of urea-ammonium sulphate (US; o) or Spirulina (Δ). The responses to US (…) were, $YbCl_3$ = 33.1 ± 1.3 h, no significant relationship; Cr-EDTA–= 28 - 0.08x + 0.0003x^2, R^2 = 0.32, RSD = 1.62, *P* = 0.018. The responses to Spirulina (- - -) were $YbCl_3$–= 35 - 0.04x, R^2 = 0.46, RSD = 4.15, *P* < 0.001; Cr-EDTA–= 28 - 0.08x + 0.0001x^2, R^2 = 0.87, RSD = 1.31, *P* = 0.009. Points represent mean of 3 steers.

Effect of level of Spirulina and urea-ammonium sulphate intake on the rate of digestion. Both US and Spirulina supplementation increased the rate of digestion of Mitchell grass, pangola grass and ryegrass incubated in the rumen, above the rate of digestion in control steers (Table 19). There was an interaction between substrates and supplements in degradation rate (Table 20). The highest degradation rate of the ryegrass substrate was recorded for Spirulina at the 290 g RDP/kg DOM level but did not differ to the US treatment level of 210 g RDP/kg DOM. The highest rate of digestion of the pangola and Mitchell grass substrates were for Spirulina at the 290 g RDP/kg DOM level which was similar to the US treatment level of 170 g RDP/kg DOM.

Table 19. The effect of level of urea and ammonium-sulphate mixture (US, 90% urea and 10% ammonium-sulphate) or Spirulina supplementation on the immediately soluble fraction (a), slowly fermentable fraction (b) and the potential degradability (a + b) for Mitchell grass, pangola grass and ryegrass substrates incubated in the rumen of steers fed Mitchell grass hay at 90% *ad libitum* intake. Values are means and standard error of the difference of the means (SEM). Within a column and a main effect, means without a common alphabetical superscript differ ($P < 0.05$). See materials and methods for description of treatments.

Feed type	a (% DM)	b (% DM)	a + b (% DM)	Lag time (h)
Incubation substrate				
Mitchell grass	8.6	45.4^{f}	54.0^{f}	1.94^{e}
Pangola grass	7.3	64.3^{e}	71.7^{e}	3.37^{d}
Ryegrass	6.9	70.0^{d}	76.9^{d}	3.31^{d}
SEM	0.43	0.61	0.41	0.62
Supplement type and level				
Control	9.7^{d}	59.5	68.9^{d}	4.14^{d}
US90	8.6^{de}	59.7	68.8^{d}	2.70^{e}
US170	6.3^{f}	60.4	66.6^{e}	2.98^{e}
US210	7.3^{ef}	59.3	66.6^{e}	2.49^{e}
S90	6.6^{f}	60.9	67.7^{de}	2.75^{e}
S170	8.3^{de}	58.5	66.7^{d}	2.52^{e}
S290	6.2^{f}	61.2	67.6^{de}	2.54^{e}
SEM	0.53	0.81	0.56	0.657

Table 20. The effect of level of urea and ammonium-sulphate mixture (US, 90% urea and 10% ammonium-sulphate) or Spirulina supplementation on the fractional digestion rate (c) for Mitchell grass, pangola grass and ryegrass substrates incubated in the rumen of steers fed Mitchell grass hay at 90% *ad libitum* intake. Values are means and standard error of the difference of the means (SEM). Within a column and a main effect, means without a common alphabetical superscript differ ($P < 0.05$). See materials and methods for description of treatments.

Supplement	Substrate	c (h^{-1})
S290	Ryegrass	0.095[a]
US210	Ryegrass	0.087[ab]
S170	Ryegrass	0.081[b]
US170	Ryegrass	0.079[b]
S90	Ryegrass	0.078[b]
US90	Ryegrass	0.060[c]
Control	Ryegrass	0.052[cd]
S290	Pangola grass	0.048[d]
US 170	Pangola grass	0.045[de]
S170	Pangola grass	0.044[de]
US 210	Pangola grass	0.044[def]
S90	Pangola grass	0.043[defg]
US90	Pangola grass	0.036[efgh]
Control	Pangola grass	0.029[hi]
S290	Mitchell grass	0.038[efgh]
US170	Mitchell grass	0.037[efgh]
S170	Mitchell grass	0.035[fgh]
S90	Mitchell grass	0.034[gh]
US90	Mitchell grass	0.0291[hi]
US210	Mitchell grass	0.029[hi]
Control	Mitchell grass	0.023[i]
SEM		0.004

4.3.2 Discussion

Spirulina algae are a novel nutrient source which can provide a range of nutrients such as nucleic acids, vitamins, minerals and fatty acids in addition to ammonia and amino acids for the rumen microbes. Spirulina has the potential to be included in the drinking water and, as such, is a potential source of N for ruminants. This appears to be the first experiment to use Spirulina algae as a source of RDP in ruminant animals consuming low quality tropical pastures. It was hypothesized that Spirulina may have a stimulatory effect on MCP production and EMCP over and above that achieved by non-protein N supplementation. This experiment was conducted to compare Spirulina to US supplementation for their effects on MCP production, EMCP and intake in steers fed low quality tropical pasture. The results demonstrated that Spirulina supplementation increased MCP production and EMCP above that of US supplementation. The increase in MCP production and EMCP was associated with an increase in intake, digestibility, DOM intake, digestion rate and passage rate and a decrease in digesta retention time in the rumen.

Increasing Spirulina supply. Responses to supplements are best evaluated when expressed as intake of supplement/kg W/d as that provides a practical basis to assess the response and make decisions about amount of supplement which is required to achieve the desired response. Spirulina supplementation showed the classical response curve for the effect of a protein supplement on intake, digestibility, intake substitution and MCP production. In the present study, a relatively low amount of supplement (4.9 g Spirulina DM/kg W/d) maximised DOM intake and hence expected liveweight gain. The primary mode of action appears to be the increase in MCP production and EMCP which affects the protein/energy ratio for absorbed substrates and hence increases intake with these low quality forages (Egan, 1977). There was also a significant increase in the rate of digestion as increasing Spirulina supply increased rumen ammonia-N concentration from approximately 5 (control) up to 270 mg NH_3-N/L (at the highest amount of Spirulina supply). The higher Spirulina supply was associated with a much lower retention time of digesta within the rumen, determined by both rumen emptying and marker dilution methods. The change in these values for such a low amount of supplement was very significant quantitatively and we are unaware of any other treatment which has caused such a big change. Thus the combination of higher rumen dilution rate and higher rumen ammonia has resulted in a much higher MCP production, EMCP (and hence protein/energy ratio) and intake at relatively low amounts of supplementation. Similar results might be expected from other protein meals such as cottonseed meal (McLennan, unpublished).

Algal by-products from biofuel or C sequestration industries (Hu *et al*., 2008) will be available as powder or pellets and can be supplemented in the same manner as other protein meals. However, algal ponds associated with water supply of range cattle offer a novel way in which to provide algae in a similar manner to urea water medicators (Entwistle and Jephcott, 2005). Such ponds are common in aquaculture. Steers of a comparable liveweight to those used in the present study (i.e. approximately 300 kg) would be expected to drink approximately 51.6 L water/d in a tropical environment (172 mL/kg W/d; Siebert and MacFarlane, 1969). To provide 4.9 g Spirulina DM/kg W/d (1.47 kg/d) there would need to be 28.5 g Spirulina DM/L water. Simple pond systems may only achieve 0.4-1.23 g algae DM/L water (Costa *et al*., 2003; Radmann *et al*., 2007). Thus such algal ponds could only provide approximately 0.07-0.21 g algal DM/kg W/d. Based on the results of the present study this amount of algae intake would not result in large increases in intake, digestibility and MCP production. Nevertheless, in combination with a urea water medicator system, this may provide a practical means of providing a mix of N compounds to enhance MCP production in cattle managed under extensive rangelands conditions.

Comparison of algal and urea based supplements. The main supplementation requirement for animals consuming low CP forages, typical of those present in the extensive rangelands of tropical regions, is for N in the rumen. Provision of N and S through mixes of US is a common practical method to supplement grazing cattle (Winks *et al*., 1970). Provision of N, via NPN sources, will increase EMCP to the minimum value reported in the various feeding standards (equivalent to approximately 130 g MCP/kg DOM) but higher values (equivalent to approximately 170 g MCP/kg DOM) may only be reached where there are higher intakes (associated with higher rumen dilution rate) (AFRC, 1993) or a proportion of the N comes from degradable true protein (NRC, 1996). Algae provide a means of providing a range of substrates (peptides, amino acids, nucleic acids, minerals and vitamins) known to be associated with higher EMCP (Dijkstra *et al*., 1998). A comparison of US and algae based on the RDP/DOM supply from both supplement types showed that algae resulted in greater MCP production, EMCP and DOMI in cattle than a US supplement. The mechanism responsible for the increased MCP production, EMCP and DOMI in cattle supplied with Spirulina compared with US is not certain. The obvious mechanisms relate to the mix of nutrients supplied to the rumen microbes in the Spirulina and seen in the elevated rumen ammonia-N concentration, the

higher concentration of BCFA, the lower retention time within the rumen and the higher intake of forage and total DOMI.

In the present study, the highest EMCP of animals supplemented with US was below the minimum value (130 g MCP/kg DOM) quoted in the feeding standards (Freer *et al*., 2007). In contrast, Spirulina supplementation increased EMCP to the higher values quoted in the feeding standard range (130-170 g MCP/kg DOM; Freer *et al*., 2007) which suggests a beneficial effect of Spirulina on MCP production compared to the US supplement. While the actual values measured in the present study may be influenced by the indirect urinary PD excretion method used, the results do indicate an upper level of MCP and EMCP from the US supplement below that achieved by the Spirulina supplement.

The retention time of digesta in the rumen has an important role in MCP production and EMCP (Van Soest 1994; Dijkstra *et al*., 2002; Firkins *et al*., 2007). Increasing dilution rate or decreasing retention time of rumen fluid and digesta, increases MCP supply to the host animal and increases EMCP (Firkins *et al*., 1986, 2007; Owens and Goetsch, 1988; AFRC, 1993). This is associated with reduced maintenance requirement of bacteria and recycling within the rumen (Owens and Goetsch, 1988; Van Soest, 1994; Baker and Dijkstra, 1999). Owens and Goetsch (1988) estimated that the ATP used for maintenance decreased from 65% to 32% of the ATP when digesta retention time decreased from 50 to 17 h. The results reported here indicate that water retention time (from the Cr-EDTA marker results), digesta retention time (from $YbCl_3$ marker results) and retention time of DM, NDF and lignin (gravimetrically measured by emptying the rumen) declined with increasing RDP intake from both supplements, generally with a greater decline in retention time with Spirulina. Van Soest (1994) suggested that a reduction in retention time of water in the rumen by approximately 50% (from 25 to 12.5 h) can increase EMCP by approximately 35% (from 130 to 200 g MCP/kg DOM). In the current experiment, increasing RDP intake from Spirulina decreased water retention time by 38% from 24 to 15 h.

The highest amounts of Spirulina supplementation in the present study resulted in a 2-fold increase in EMCP from the control of 64 to approximately 133 g MCP/kg DOM compared to US of approximately 90 g MCP/kg DOM, at an RDP of 250g RDP/kg DOM or a Spirulina intake of approximately 4.3 g/kg W/d. For a 300 kg steer, this would result in an extra 360 g MCP/d under Spirulina supplementation compared to an extra 105 g MCP/d under US supplementation. This increase would have a significant impact on the nutrient status and liveweight gain of the animal. Mbongo *et al*. (1994) reported that for Belmont Red steers, of similar liveweight to those used in the present study, an extra 150 g/d of intestinal protein supplied in the form of formaldehyde treated casein resulted in an increase in liveweight gain of 250 g/d above the control steers grazing a setaria (*Setaria sphacelata*) dominant pasture with 13% CP content.

The intake response to the Spirulina supplement was much greater than that to the US supplement. The Spirulina supplement markedly increased hay intake by increasing the rate of digestion and the rate of passage. Substitution started to occur when Spirulina supplementation exceeded 3.7 g/kg W/d. This is similar to Marsetyo (2004) who found that intake of low CP diets was stimulated with supplementation up to approximately 5 g DM/kg W/d and declined thereafter. In the current study, very high amounts of Spirulina were not used so the substitution rate could not be determined and compared. Nevertheless a plateau in total DM intake occurred at 4.6 g Spirulina DM/kg W/d and the highest Spirulina supplementation used in this experiment was 6.1 g DM/kg W/d. At these higher amounts of Spirulina supplementation (i.e. 4.6 and 6.1 g DM Spirulina/kg W/d) total DOMI was more than 2.3-fold higher than control total DOMI. This is a major increase in DOMI, ME and

metabolizable protein supply to cattle consuming low quality native grass hay and, as calculated previously, would be expected to result in a major increase in liveweight gain.

Low rate of digestion often affects intake of low quality forage (Minson, 1990). The rate of DM digestion here was improved when incubated in the rumen of steers receiving either US or Spirulina. However, the rate of DM digestion of forage different in degradable characteristics responded differently to supplement type and level. The fractional degradation rate of ryegrass substrate was optimised at an RDP intake of 245 and 358 g RDP/kg DOM for US and Spirulina, respectively but there was no difference between supplements. This optimum value was associated with a rumen ammonia-N concentration of 130 and 244 mg NH_3-N/L for US and Spirulina, respectively. In contrast, there was no further change in fractional degradation rate of pangola grass and Mitchell grass when ammonia-N concentration was close to 50 mg NH_3-N/L and there was no difference between supplements. This would suggest that the concentration of ammonia-N rather than BCFA was the dominant factor controlling rate of digestion.

Total VFA concentration in the rumen increased with increasing RDP intake for both supplement types but they were not different to one another, despite the higher total DOMI in response to Spirulina supplementation. Rumen VFA concentration is the balance between rate of production and of removal from the rumen, together with inter-conversion between individual VFA. In the current experiment, Spirulina supplementation decreased the molar percentage of acetate and increased the molar percentage of BCFA from 1.2 to 4.2% of total VFA, presumably as a result of increased degradation of BCAA. An increase in BCFA supply has been associated with an increase in MCP production and EMCP (Hume, 1970).

In conclusion, supplementation of low quality Mitchell grass hay with Spirulina increased MCP production and EMCP to the highest values reported in the feeding standards and this response was greater than that measured for US supplement. Spirulina supplementation stimulated hay intake and intake substitution of the hay started to occur when supplementation exceeded 3.7 g Spirulina DM/kg W/d. Overall, Spirulina increased DOMI and MCP production much greater than a US supplement and, as such, it has the potential to be used as a novel protein source for cattle grazing low quality tropical grasses. The practical advantage of using an algae source as a supplement is that it may be supplied or suspended in the drinking water of animals.

4.4 Experiment 4. Rumen function in animals selected for growth rate on low quality diets

4.4.1 Results

Rumen function in divergent growth steers consuming low CP Mitchell grass. After grazing speargrass pasture for 90 d after weaning, steers were selected based on liveweight change as either highest growth rate (moderate growth; 0.21 $\pm$ 0.02 kg/d) or lowest growth rate (low group; negative 0.07 $\pm$ 0.01 kg/d). After transport to CAAS, steers were then tested for differences in intake and rumen function when consuming a low CP Mitchell grass hay (6.4 g N/kg DM). Dry matter intake, digestibility and DOMI of the Mitchell grass hay diet did not differ between the moderate and low growth groups (Table 21).

Table 21. Dry matter (DM), organic matter (OM), ash free neutral detergent fibre (NDF), digestible organic matter (DOM) intake and apparent digestibility of Mitchell grass hay of steers selected based on divergent post-weaning growth rate (moderate or low) on protein deficient pasture. Values are means and standard error of the difference of the means (SEM).

Parameter	Moderate	Low	SEM	*P* value
	Intake (g/kg W/d)			
DM[A]	19.3 ± 0.40	19.5 ± 0.60	0.50	0.70
DOM[A]	7.4 ± 0.10	7.5 ± 0.30	0.28	0.77
DM[B]	14.6 ± 0.47	15.4 ± 0.52	0.57	0.22
OM[B]	13.0 ± 0.42	13.7 ± 0.46	0.52	0.23
NDF[B]	9.4 ± 0.30	9.9 ± 0.34	0.38	0.21
DOM[B]	6.1 ± 0.15	6.4 ± 0.28	0.35	0.45
	Apparent digestibility (g/kg)			
DM[B]	430 ± 11	422 ± 12	18.2	0.67
OM[B]	470 ± 10	465 ± 12	16.9	0.77
NDF[B]	510 ± 9	496 ± 12	12.5	0.28

[A]*Ad libitum* intake determined during preliminary period.

[B]Determined during the collection period in metabolism crates.

There were no differences in rumen parameters between the two groups (Table 22). Microbial protein production tended to be greater in moderate group compared to low group steers (*P* = 0.09) but there was no difference in EMCP between the two groups. Serum IGF-1 concentration was significantly greater for the moderate group than the low group both prior to transport from Swan's Lagoon and at the end of the Mitchell grass hay experiment at CAAS.

Table 22. Rumen parameters, microbial protein production and serum insulin-like growth-factor-1 (IGF-1) concentration of steers fed Mitchell grass hay selected based on divergent post-weaning growth rate (moderate or low) on protein deficient pasture. Values are means and standard error of the difference of the means (SEM).

Parameter	Moderate	Low	SEM	*P* value
Rumen parameters				
pH	7.6 ± 0.07	7.5 ± 0.05	0.06	0.19
Ammonia-N (mg/L)	18.1 ± 2.9	16.3 ± 2.2	3.61	0.64
Total volatile fatty acids (VFA) (mmol/L)	56.8 ± 1.8	56.6 ± 2.9	4.14	0.95
VFA % Acetate	77.1 ± 0.2	77.5 ± 0.5	0.64	0.62
Propionate	13.7 ± 0.1	13.5 ± 0.3	0.32	0.73
Butyrate	7.9 ± 0.3	7.6 ± 0.3	0.41	0.50
BCFA	1.3 ± 0.03	1.4 ± 0.1	0.09	0.45
Acetate:Propionate	5.7 ± 0.15	5.7 ± 0.2	0.18	0.63
Retention time of Cr-EDTA (h)	34.7 ± 1.6	35.8 ± 1.5	2.66	0.68
Retention time $YbCl_3$ (h)	42.6 ± 6.5	36.4 ± 4.0	7.24	0.42
Microbial protein production				
Microbial protein production (MCP) (g/d)	74.1 ± 8.6	53.4 ± 5.5	10.62	0.09
Efficiency of MCP (g/kg DOM)	68.3 ± 7.1	59.9 ± 11.7	14.52	0.58
Serum IGF-1 concentration				
IGF-1 after divergent growth on pasture (ng/mL)[A]	42.1 ± 2.0	28.9 ± 4.2	4.51	0.02
IGF-1 after Mitchell grass feeding at CAAS (ng/mL)[B]	45.3 ± 5.0	32.2± 3.2	5.88	0.06

[A]samples were collected at time of selection, after animals grazed a speargrass dominant pasture for 90 f post-weaning.

[B]samples were collected at the end of 28 d Mitchell grass feeding experiment at CAAS.

Response to nitrogen supplementation with CSM or urea of steers selected for low or moderate growth rates on protein deficient pasture.

There was a linear increase in NH_3-N concentration in the rumen to increasing N intake of steers supplemented with US, although there was no difference between L or M steers. There was no significance response in MCP production and EMCP (Figure 12), hay DM and DOM intake (Figure 13), VFA concentration in the rumen (Figure 14), the molar proportion of VFA (Figure 15) and retention time of particle and liquid markers in the rumen (Figure 16) of L or M steers to increasing N intake when supplemented with US. There was a linear increase in MCP production in response to increasing N intake when animals were offered increasing amounts of CSM (Figure 12) and this response was different between L and M steers. However, there was no change in EMCP in response to increasing N intake from CSM, and no difference in EMCP between L and M steers. Total DOMI, rumen NH_3-N concentration and the molar proportion of butyrate and BCFA increased and the molar proportion of acetate decreased in response to increasing N intake from CSM, with a similar response for L and M steers for each parameter. Hay DM intake and retention time of a particle marker within the rumen of M steers fed CSM responded in a quadratic fashion to increasing N intake, with no response observed for L steers. There was a significant response of the molar proportion of propionate to increasing N intake from CSM and this response was different for L and M steers. Total VFA and the retention time of a liquid marker within the rumen were not influenced by increasing N intake of either L or M steers supplemented with CSM.

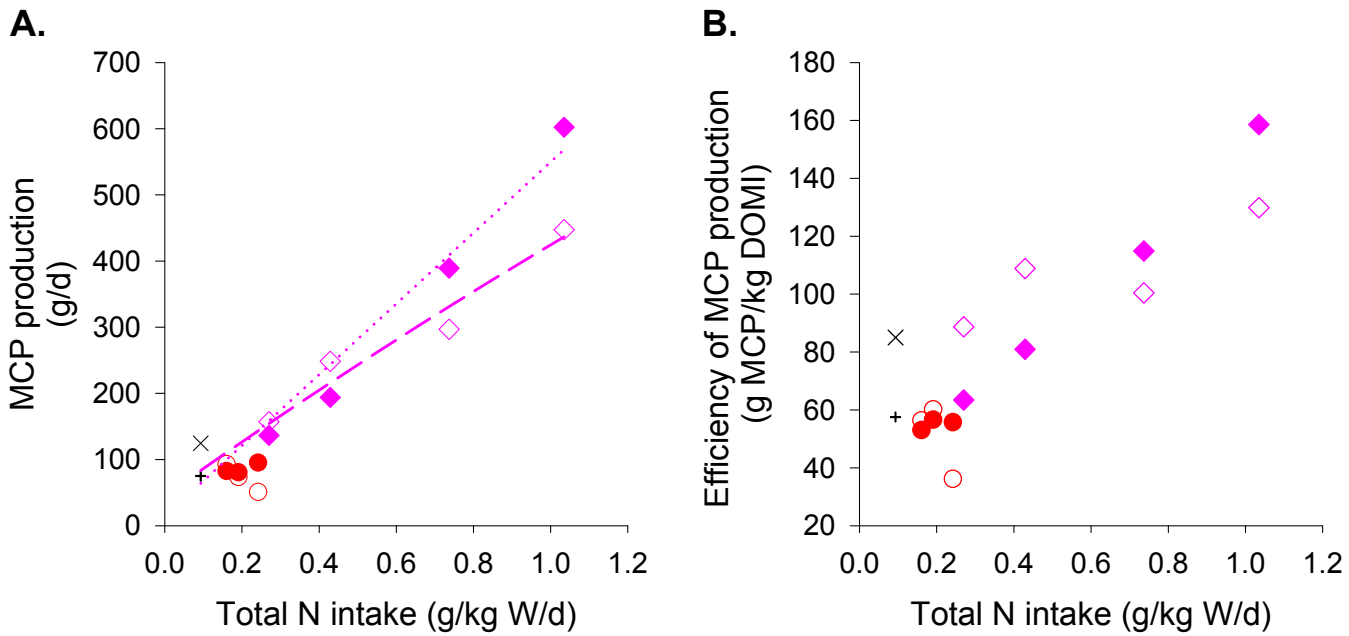

Figure 12. Microbial protein (MCP) production (A.) and the efficiency of MCP production (B.; EMCP) of steers of low (L) and moderate (M) post-weaning growth in response to nitrogen intake supplied by increasing amounts of urea-ammonium sulphate (US; ○, ●; L and M, respectively) or cottonseed meal (CSM; ◊, ♦; L and M, respectively). The response of MCP production (Y) to total N intake was, Y = 73.2 ± 8.6; Y = 95.9 ± 10.1; Y = 45.4 + 412.1x – 33.2x^2, R^2 = 0.56, *P* < 0.05 (- - -); Y = 14.0 + 535.7x, R^2 = 0.69, *P* < 0.0001(. . .), for L-US, M-US, L-CSM and M-CSM, respectively. The response of EMCP (Y) to total N intake was, Y = 52.6 ± 5.5; Y = 62.6 ± 7.5; Y = 97.1 ± 12.0; Y = 100.6 ± 16.7, for L-US, M-US, L-CSM and M-CSM, respectively. Points represent mean of 2 or 3 steers.

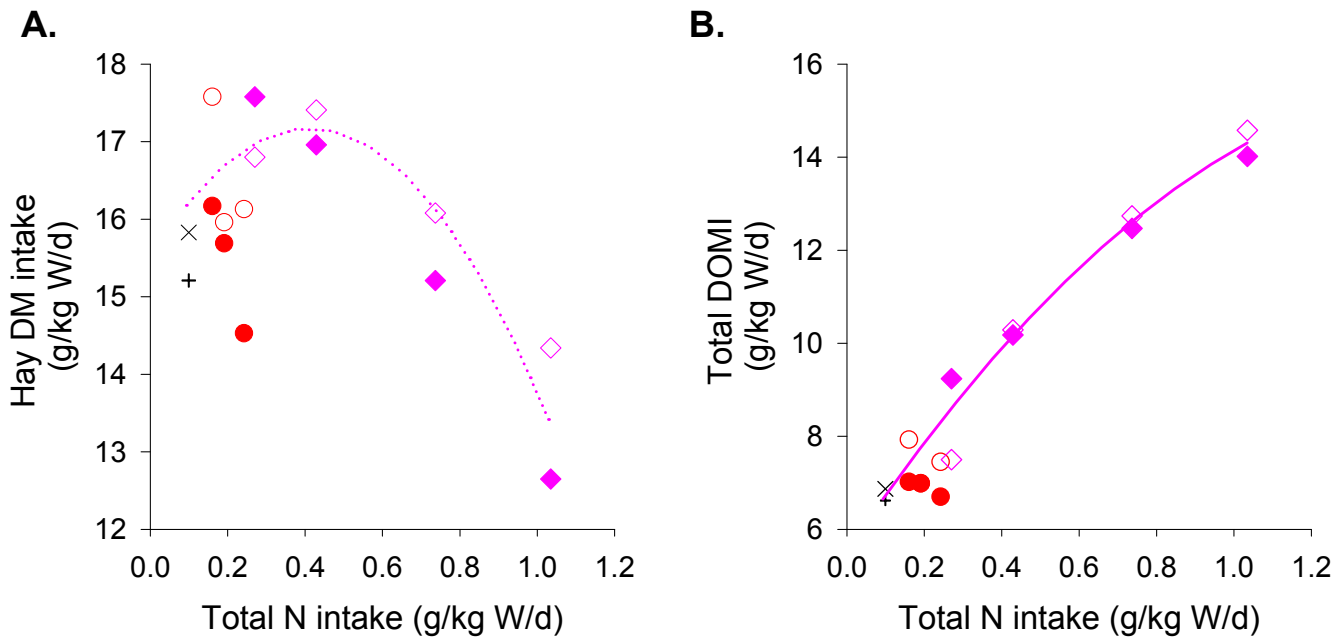

Figure 13. Hay dry matter (A.; DM) intake and digestible organic matter intake (B.; DOMI) of steers of low (L) and moderate (M) post-weaning growth in response to nitrogen intake supplied by increasing amounts of urea-ammonium sulphate (US; ○, ●; L and M, respectively) or cottonseed meal (CSM; ◊, ♦; L and M, respectively). The response of Hay DM intake (Y) to total N intake was, Y = 16.2 ± 0.5; Y = 15.6 ± 0.4; Y = 16.0 ± 0.6; Y = 15.6 + 8.1x - 9.8x^2, R^2 = 0.56, *P* < 0.05 (. . .), for L-US, M-US, L-CSM and M-CSM, respectively. The response of Total DOMI (Y) to total N intake was, Y = 7.3 ± 0.3; Y = 6.9 ± 0.1; Y = 5.5 + 12.4x – 3.8x^2, R^2 = 0.88, *P* < 0.0001(—); for L-US, M-US and CSM, respectively, with no difference between L and M for CSM. Points represent mean of 2 or 3 steers.

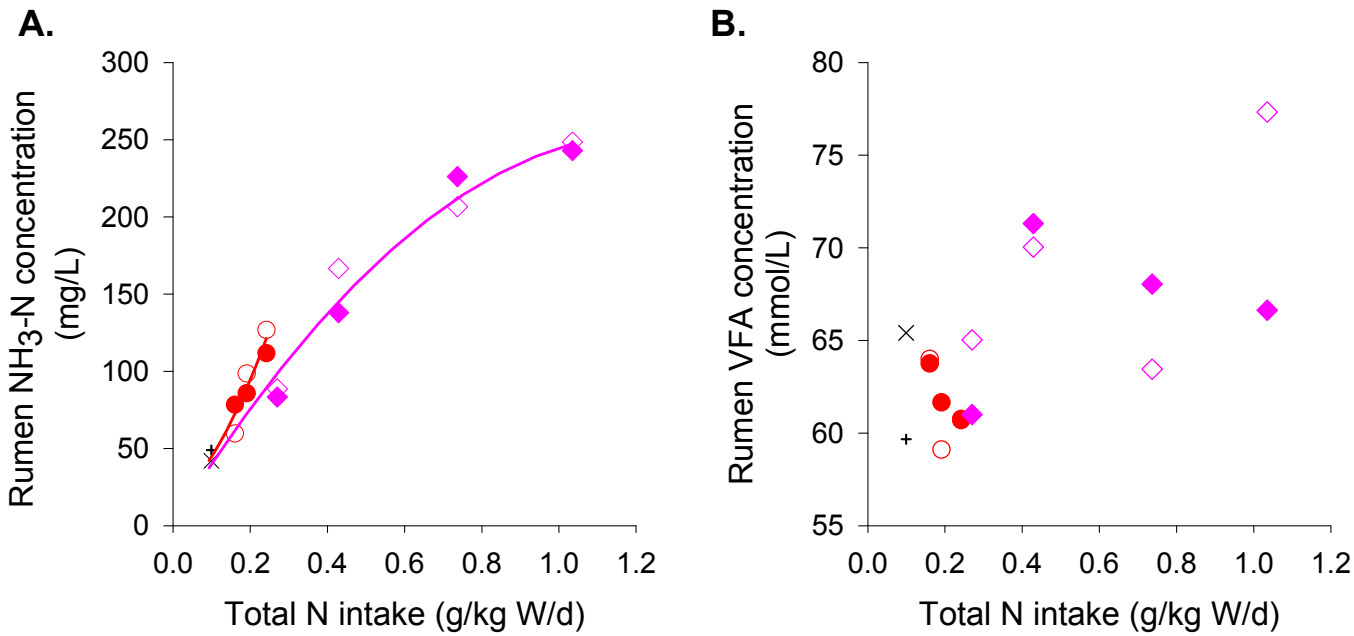

Figure 14. Concentration of ammonia-N (A.; NH_3-N) and volatile fatty acids (B.; VFA) in the rumen fluid of steers of low (L) and moderate (M) post-weaning growth in response to nitrogen intake supplied by increasing amounts of urea-ammonium sulphate (US; ○, ●; L and M, respectively) or cottonseed meal (CSM; ◊, ♦; L and M, respectively). The response of NH_3-N concentration (Y) to total N intake was, $Y = 15.2 + 196.4x + 1002.4x^2$, $R^2 = 0.62$, $P < 0.001$ (━); $Y = 0.999 + 405.8x – 162.2x^2$, $R^2 = 0.90$, $P < 0.0001$(━), for US and CSM, respectively, with no difference in response for L and M within a supplement. The response of VFA (Y) to total N intake was, Y = 60.9 ± 1.1; Y = 62.9 ± 1.1; Y = 67.1 ± 3.1; Y = 66.5 ± 1.7, for L-US, M-US, L-CSM and M-CSM, respectively. Points represent mean of 2 or 3 steers.

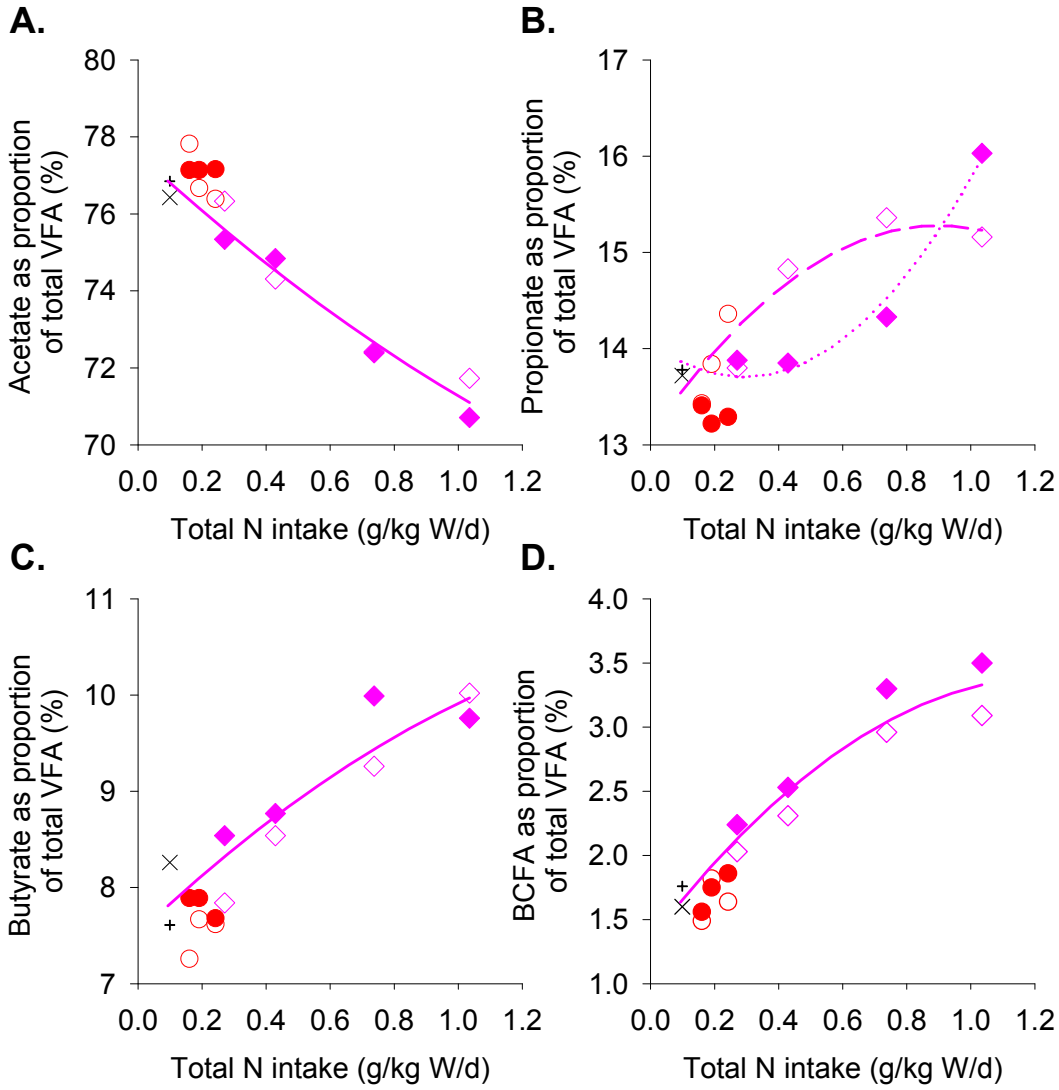

Figure 15. Volatile fatty acid molar % in rumen of steers of low (L) and moderate (M) post-weaning growth in response to nitrogen intake supplied by increasing amounts of urea-ammonium sulphate (US; o, ●; L and M, respectively) or cottonseed meal (CSM; ◊, ♦; L and M, respectively). The response of acetate (Y) to total N intake was, Y = 76.9 ± 0.3; Y = 77.0 ± 0.2; Y = 77.6 – 7.7x + 1.4x^2, R^2 = 0.79, *P* < 0.001 (—), for L-US, M-US and CSM, respectively, with no difference between L and M for CSM. The response of propionate (Y) to total N intake was, Y = 13.9 ± 0.2; Y = 13.4 ± 0.1; Y = 13.1 + 4.8x – 2.7x^2, R^2 = 0.58, *P* < 0. 01 (- - -); Y = 14.1 - 2.4x + 4.1x^2, R^2 = 0.86, *P* < 0. 0001 (. . .); for L-US, M-US, L-CSM and M-CSM, respectively. The response of butyrate (Y) to total N intake was, Y = 7.5 ± 0.1; Y = 7.9 ± 0.1; Y = 7.5 + 3.1x – 0.8x^2, R^2 = 0.57, *P* < 0.0001 (—), for L-US, M-US and CSM, respectively, with no difference between L and M for CSM. The response of BCFA (Y) to total N intake was, Y = 1.7 ± 0.1; Y = 1.7 ± 0.1; Y = 1.3 + 3.3x - 1.3x^2, R^2 = 0.74, *P* < 0.0001 (—), for L-US, M-US and CSM, respectively, with no difference between L and M for CSM. Points represent mean of 2 or 3 steers.

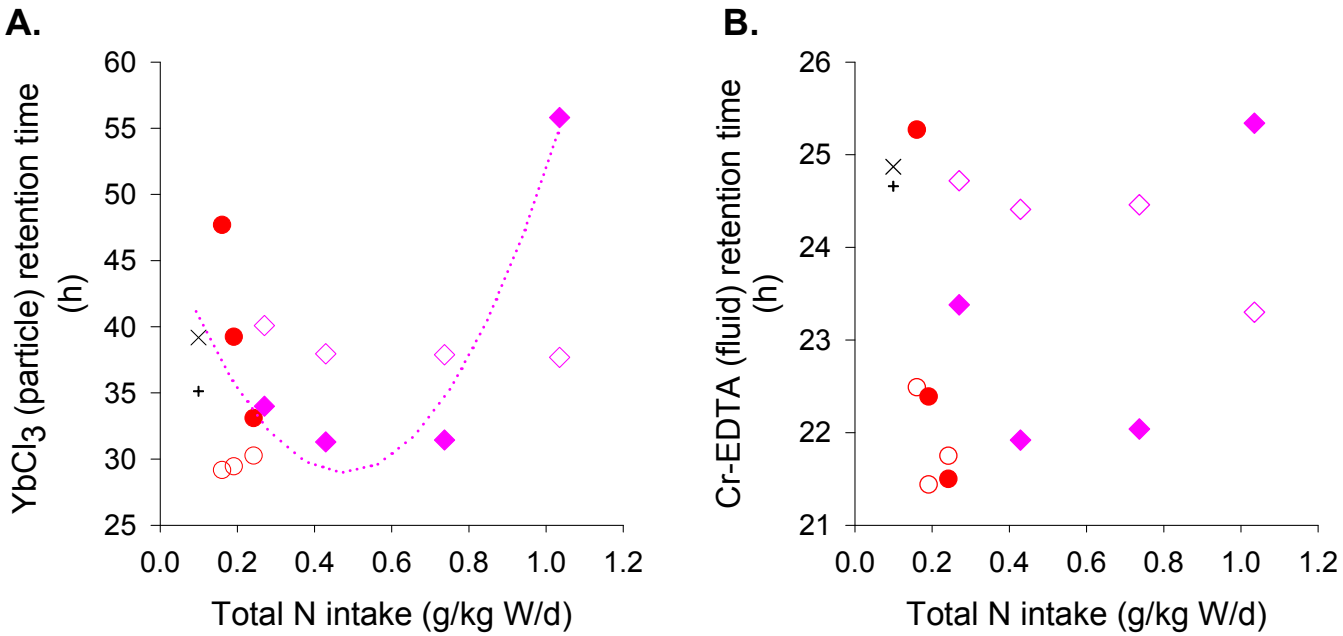

Figure 16. Retention time of Ytterbium Chloride ($YbCl_3$) (A.) and Chromium-EDTA (Cr-EDTA) (B.) in the rumen of steers of low (L) and moderate (M) post-weaning growth in response to nitrogen intake supplied by increasing amounts of urea-ammonium sulphate (US; ○, ●; L and M, respectively) or cottonseed meal (CSM; ◊, ♦; L and M, respectively). The response of $YbCl_3$ retention time (Y) to total N intake was, Y = 31.0 ± 1.4; Y = 39.8 ± 3.0; Y = 37.8 ± 0.8; Y = 47.8 + 79.3x + 83.5x^2, R^2 = 0.66, P < 0.01 (…), L-US, M-US, L-CSM and M-CSM, respectively. The response of Cr-EDTA retention time (Y) to total N intake was, Y = 22.6 ± 0.7; Y = 23.5 ± 0.9; Y = 24.3 ± 0.3; Y = 23.5 ± 0.7, for L-US, M-US, L-CSM and M-CSM, respectively. Points represent mean of 2 or 3 steers.

4.4.2 Discussion

The results from this study indicate that the differences in liveweight gain of steers at pasture post-weaning are not related to differences in intake and rumen function and that other factors are involved. Further, it appears that there is little difference in intake and rumen function between L and M steers fed diets low in N, high in N and high in CP. The differences in plasma IGF-1 concentration post-weaning might suggest metabolic differences account for the observed differences in liveweight gain. This may include differences in maintenance energy requirement as has been proposed as the possible reason for differences in residual feed intake of different lines of Angus cattle selected for growth (Oddy *et al*., 1998). Alternative reasons for the differences in post-weaning growth rate of steers may include genetics, disease status and grazing behaviour. Reasons for this difference in post-weaning liveweight gain warrant further investigation.

4.5 Experiment 5. Liveweight gain of animals consuming different forms of N supplement

4.5.1 Results

Chemical composition of the diets. The chemical composition of the hay and supplements offered in this experiment are described in Table 23. Speargrass hay used in this experiment was low in N (33 g CP/kg DM) and high in fibre (689 g NDF and 403 g ADF/kg DM). The CP content of Spirulina used in this experiment was 708 g/kg DM and the CP content of the CSM was 503 g/kg DM which is higher than typical values reported elsewhere of between 380 to 420 g/kg DM. The lipid content of the algae was lower than expected.

Table 23. Nutrient composition of Speargrass hay, Cottonseed meal and Spirulina.

Parameter	Speargrass	Cottonseed meal	Spirulina
OM[A] (g/kg DM)	911	925	908
N[A] (g/kg DM)	5.3	80.5	113.3
NDF[A] (g/kg DM)	689	138	n/m[B]
ADF[A] (g/kg DM)	403	97	n/m
EE[A] (g/kg DM)	n/m	31	20

[A]Organic matter (OM); Nitrogen (N); ash free Neutral detergent fibre (NDF); ash free Acid detergent fibre (ADF); Ether Extract (EE).
[B]n/m, not measured.

Liveweight gain. Liveweight gain increased in response to increasing supplement N intake from all three supplements. Cottonseed meal had a quadratic response curve for liveweight gain with better responses than Spirulina at low levels of supplementation. At higher levels of supplementation there was little difference between Spirulina and CSM. The response curves of both supplements were significantly different. Algae and US followed the same linear response curve but higher liveweight gains were achieved with algae because higher amounts of supplement N intake were offered (with associated ME) in the form of algae compared to US; the amount of US offered was restricted due to the risks of urea toxicity (Figure 17; Table 24). This is similar to Experiment 3 where the response to US and Spirulina were similar at low levels but much higher levels of Spirulina supplementation could be safely provided to animals. Thus Spirulina could be safely used suspended in the drinking water without the toxicity issues associated with urea feeding via water medicators and so achieve higher liveweight gains than US. Control steers lost 0.20 kg/d reflecting the low quality of the speargrass hay. The response curves were developed over a low supplementation range, approximately 0 to 5.6 g DM/kg W/d for CSM and 0 to 4 g DM/kg W/d for Spirulina. Nevertheless liveweight gain increased for both supplements from -0.2 kg/d to 0.75 kg/d, which were biologically significant changes for such low levels of supplementation. McLennan (1997) achieved similar results with CSM previously. Cottonseed meal on a similar N basis would provide greater ME intake and probably more undegraded protein than Spirulina and this may account for the difference in liveweight gain at low levels of supplementation. In Experiment 3, Spirulina supplementation of Mitchell grass occurred over the range 0-6 g Spirulina/kg W/d with a 2.5-fold increase in DOM intake, similar to the results estimated from DM values with speargrass in this experiment. The rumen ammonia concentrations from Sprirulina were much higher than for CSM when compared on the same supplemental N intake suggesting that Spirulina is much more extensively degraded in the rumen.

There was poor acceptance of the algae supplement by some steers, particularly early in the experiment, which restricted the range of intakes for this treatment. There was only one steer in the high Algae treatment (AL48) which consumed a high proportion of its allocated supplement. This was unexpected, given that similarly naïve steers readily consumed high levels of algae in an associated experiment (Panjaitan, 2009).

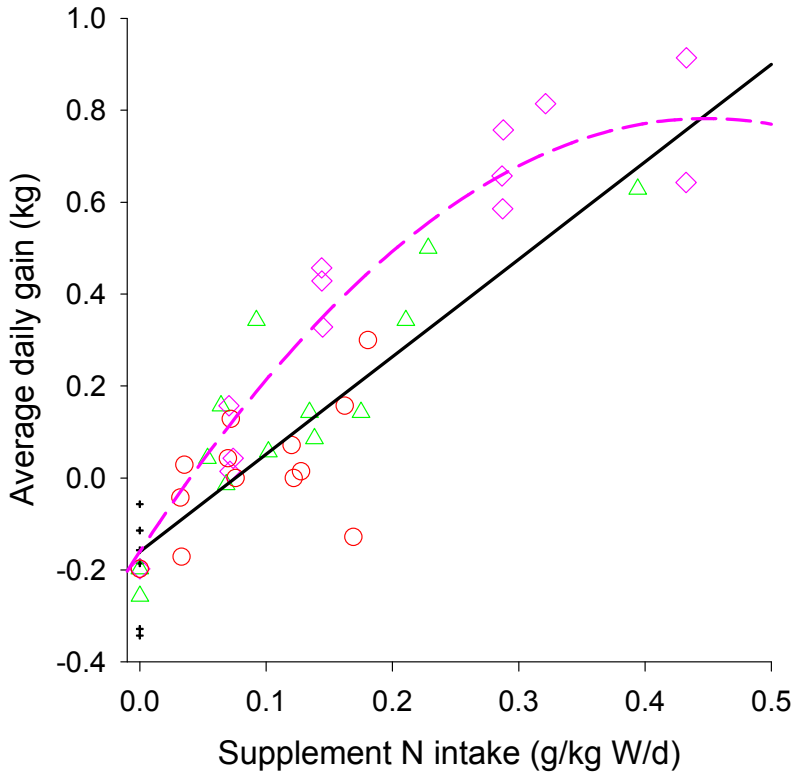

Figure 17. Effects of supplement N intake on average daily gain of steers (un-fasted weight) receiving speargrass hay *ad libitum* alone (+) or speargrass hay *ad libitum* with urea-ammonium sulphate (US; o), Spirulina (AL; Δ) or cottonseed meal (CSM; ◊) supplements over 70 d. Points on the curves represent individual steers. Response equations to US and AL (━) and CSM (- - -) are presented in Table 24.

Table 24. Effects of supplement N intake (x; g N/kg W/d) on liveweight gain of steers (un-fasted weight) receiving speargrass hay *ad libitum* with urea-ammonium sulphate (US), Spirulina (AL) and cottonseed meal (CSM) supplements over 70 d.

Parameter	Supplement	Equation	R^2	RSD	*P* value
Liveweight gain (kg/d)	US, AL	$Y = -0.161 + 2.121x$	0.71	0.12	< 0.001
Liveweight gain (kg/d)	CSM	$Y = -0.161 + 4.205x - 4.69x^2$	0.95	0.10	< 0.01

Intake and digestibility. The effects of supplement type and level on hay intake and the total DM intake are presented in Table 25 and Figure 18. Mean intake of total DM and hay did not differ statistically between treatments ($P < 0.001$). Hay DM intake peaked at 1.7% W/d (17 g/kg W/d) at a supplement intake of 0.26 g N/kg W/d (or at approximately 2.3 g Spirulina and 3.2 g CSM/kg W/d) and total DM intake peaked at 2.0 %W/d (20 g/kg W/d) at a supplement intake of either Spirulina or CSM of 0.42 g/kg W/d. These intakes may be compared to experiment 3 with Mitchell grass where hay intake increased from approximately 12 to 21 g Mitchell grass DM/kg W/d with Spirulina supplementation. Spirulina, like CSM, increases hay intake at low levels but substitution starts to occur at higher levels, a result not dissimilar to the experiments collated by McLennan (1997). The difference in liveweight gain between CSM and Spirulina, without a similar difference in intake, suggests that more undegraded protein passes from the rumen of animals fed CSM leading to the better response at low levels of inclusion. Panjaitan (2009) found that 20% of Spirulina in drinking water bypassed the rumen on drinking the solution but CSM has an undegradable value of 36% (McLennan, 1997). It is clear that like any other protein supplement, Spirulina first causes an increase in intake, until it reaches a peak, where substitution starts and a reduction in forage intake is observed while total intake continues to rise. There was a significant increase in DMD associated with the combined relationship of all supplements (Table 25). However, the increase was small representing about 3% units of DMD over the full range of supplement intake. This is to be expected even for a high digestibility supplement such as CSM as the level of supplement intake in proportion to hay intake is small and an additive effect would not result in a shift in DMD. The digestibility of the hay was very low at approximately 48% DMD and the US supplement had no effect on DMD as has been found previously.

Table 25. Effects of supplement N intake (x; g N/kg W/d) on intake and digestibility of steers receiving speargrass hay *ad libitum* with urea-ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM) supplements over 70 d. Equations are given separately for each supplement where the response is different for each supplement type or for combinations of supplements where there was no difference between the combined supplements. No relationship for a particular supplement indicates there was no significant relationship.

Parameter	Supplement	Equation	R^2	RSD	*P* value
Hay DM intake (%W/d)	US, AL, CSM	$Y = 1.238 + 3.403x - 6.63x^2$	0.46	0.16	< 0.001
Total DM intake (%W/d)	US, AL, CSM	$Y = 1.236 + 3.975x - 5.09x^2$	0.73	0.16	< 0.01
Dry matter digestibility (%)	US, AL, CSM	$Y = 47.87 + 8.45x$	0.2	2.15	0.01

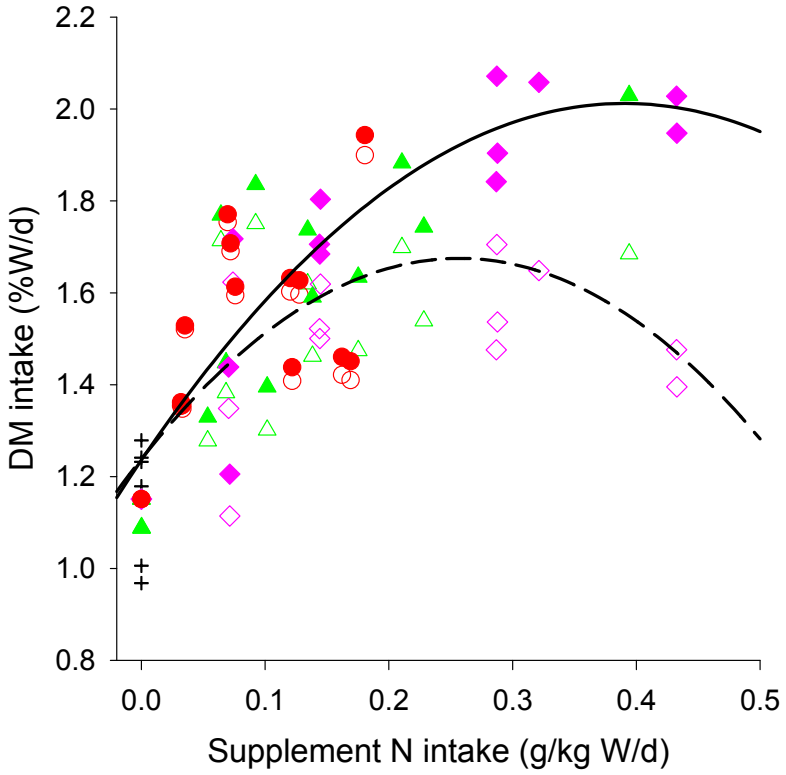

Figure 18. Effects of supplement N intake on hay (- - -) and total (—) dry matter (DM) intake of steers receiving speargrass hay *ad libitum* alone (+) or speargrass hay *ad libitum* with urea-ammonium sulphate (US; o, ●; hay and total, respectively), Spirulina (AL; Δ, ▲; hay and total, respectively) or cottonseed meal (CSM; ◊, ♦; hay and total, respectively) supplements over 70 d. Points on the curves represent individual steers. Response equations are presented in Table 25.

Rumen parameters and plasma urea-N. Rumen ammonia-N concentrations increased with supplement N intake but the increase was much greater for AL than for CSM (Table 26). This is a reflection of the generally rapid intake of algae which was supplied as a slurry at the start of feeding. It appears highly degradable given the rumen ammonia-N concentration. In contrast, the CSM, which was also quickly eaten, did not result in such a high rumen ammonia-N concentration, suggesting a slower degradability. Urea-ammonium sulphate was mixed with the hay and would have a slow rate of intake in this type of feeding as rate of hay intake is slow over the day. Figure 19 shows the concentration of rumen ammonia-N of the three supplements in the rumen fluid collected 3 h after feeding. Even at low intakes, all supplements increased the rumen ammonia-N concentration rapidly to provide adequate NH_3-N for microbial protein production (> 50 mg ammonia-N/L). Total VFA concentration in the rumen fluid of steers increased with intake of all supplements, resulting in a single response relationship (Table 26). Figure 20 shows the molar concentration of BCFA in the rumen fluid of steers, 3 h after feeding. Branch chain fatty acid concentration increased quadratically with increasing Spirulina intake. There was no effect on BCFA concentration when cattle were supplemented with US or CSM. The higher concentration of BCFA in the rumen fluid of steers fed Spirulina suggests that there is extensive degradation of protein within the rumen of steers fed Spirulina. The molar % of VFA did not change significantly with supplement intake except

for butyrate which declined markedly with high intakes of Spirulina corresponding with an associated increase in BCFA concentration. The molar % for acetate, propionate and butyrate were: Control: 78, 13, 7.5; AL48: 74, 13.4, 0.1; and CSM48: 74, 14.6, 9.9, respectively. These indicate that fermentation of Spirulina did not unusually alter VFA patterns except for the increase in BCFA which is an advantage for digestion of high fibre forage diets. Plasma urea-N concentrations increased linearly with supplement N intake and differences between supplement types were small but still indicated the high rate of N absorption from the Spirulina treatment relative to the other supplements (Table 26; Figure 21).

Table 26. Effects of supplement N intake (x; g N/kg W/d) on rumen parameters and plasma urea-N of steers receiving speargrass hay *ad libitum* with urea-ammonium sulphate (US), spirulina (AL) or cottonseed meal (CSM) supplements over 70 d. Equations are given separately for each supplement where the response is different for each supplement type or for combinations of supplements where there was no difference between the combined supplements. No relationship for a particular supplement indicates there was no significant relationship.

Parameter	Supplement	Equation	R^2	RSD	*P* value
Rumen ammonia-N (mg/L)	US	$Y = 13.47 + 613x$	0.67	29.5	< 0.001
Rumen ammonia-N (mg/L)	AL	$Y = 13.47 + 1679x - 2682x^2$	0.87	40.2	< 0.001
Rumen ammonia-N (mg/L)	CSM	$Y = 13.47 + 338.5x$	0.73	35.9	< 0.001
Total VFA (mM)	US, AL, CSM	$Y = 64.14 + 35.3x$	0.14	11.1	< 0.05
Branch chain FA (molar%)	AL	$Y = 0.93 + 21.64x - 37.29x^2$	0.86	0.51	< 0.001
Plasma urea-N (mg/dL)	US	$Y = 1.683 + 66.91x$	0.77	2.50	< 0.001
Plasma urea-N (mg/dL)	AL	$Y = 1.683 + 97.8x - 110.9x^2$	0.91	2.45	< 0.05
Plasma urea-N (mg/dL)	CSM	$Y = 1.683 + 38.23x$	0.91	2.15	< 0.01

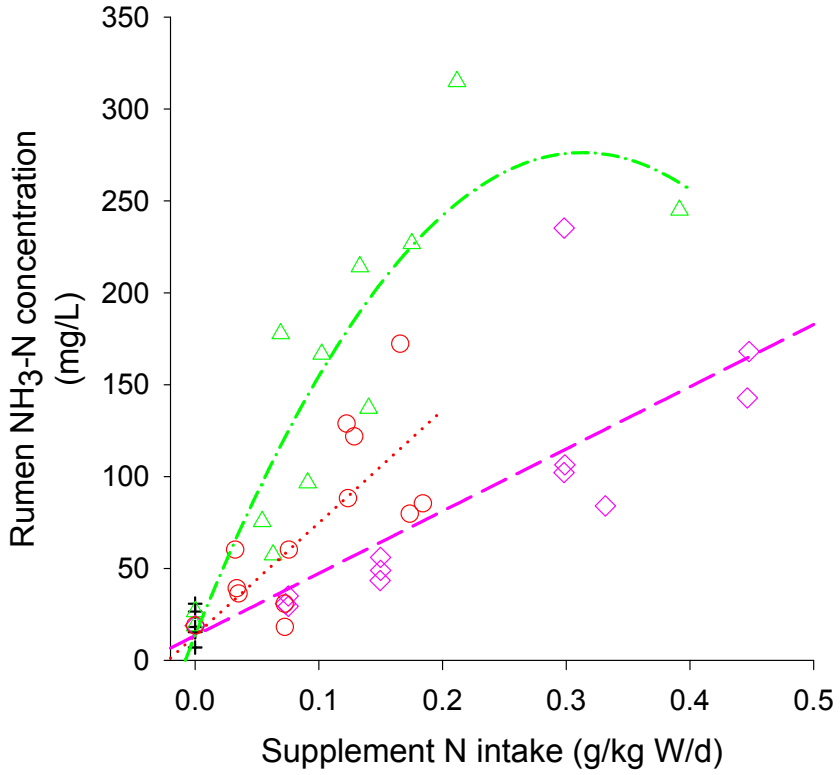

Figure 19. Effects of supplement N intake on the concentration of ammonia-N (NH_3-N) in the rumen fluid, 3 h after feeding, of steers receiving speargrass hay *ad libitum* alone (+) or speargrass hay *ad libitum* with urea-ammonium sulphate (US; o), Spirulina (AL; Δ) or cottonseed meal (CSM; ◊) supplements over 70 d. Points on the curves represent individual steers. Response equations to US (...), AL (_._.) and CSM (- - -) are presented in Table 26.

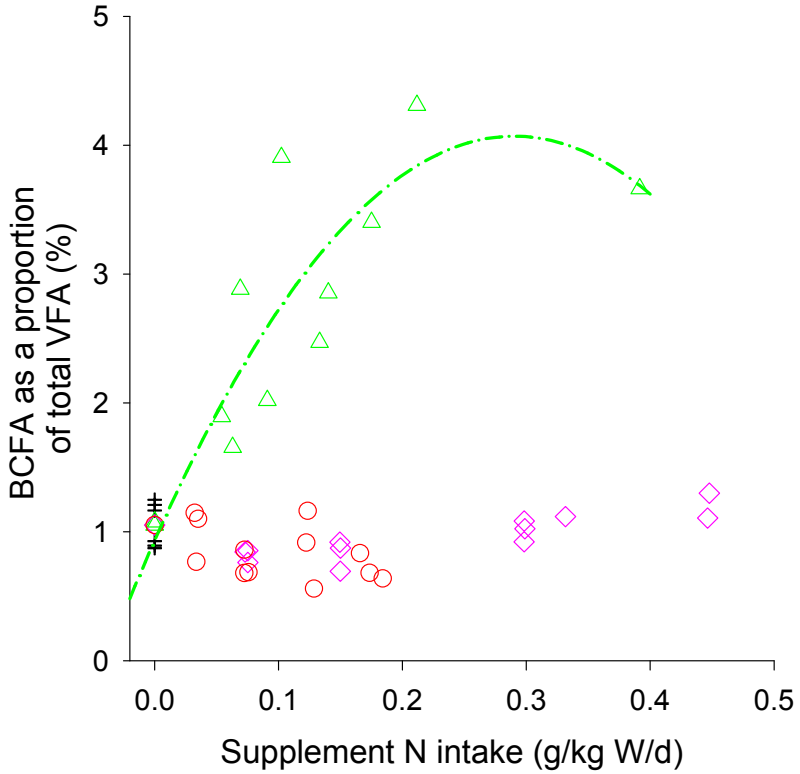

Figure 20. Effects of supplement N intake on the concentration of branch chain fatty acids (BCFA) in the rumen fluid, 3 h after feeding, of steers receiving speargrass hay *ad libitum* alone (+) or speargrass hay *ad libitum* with urea-ammonium sulphate (US; o), Spirulina (AL; Δ) or cottonseed meal (CSM; ◊) supplements over 70 d. Points on the curves represent individual steers. The response equation to AL (_.-._.) is presented in Table 26.

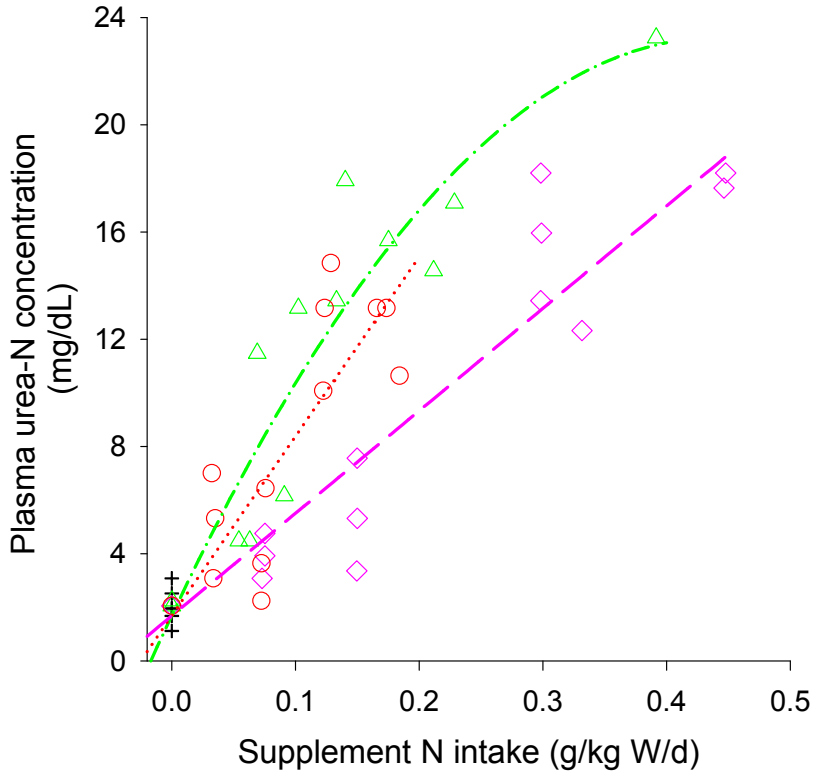

Figure 21. Effects of supplement N intake on the plasma urea-N concentration, 3 h after feeding, of steers receiving speargrass hay *ad libitum* alone (+) or speargrass hay *ad libitum* with urea-ammonium sulphate (US; o), Spirulina (AL; Δ) or cottonseed meal (CSM; ◊) supplements over 70 d. Points on the curves represent individual steers. Response equations to US (…), AL (_._._) and CSM (- - -) are presented in Table 26.

4.5.2 Discussion

Supplementation of low quality speargrass hay with Spirulina increased liveweight gain of weaner steers similar to that of a US supplement, at low levels of supplementation. However, Spirulina could be fed at much higher intakes than urea and at these higher intakes the response of liveweight gain approached that of CSM. Cottonseed meal gave a higher liveweight gain response compared to Spirulina at intakes less than approximately 4-5 g supplement/kg W/d and the response curve to CSM was significantly different to the response curve from Spirulina. Spirulina ponds have a maximum concentration of approximately 1.2 g DM/L (Costa *et al*., 2003; Radmann *et al*., 2007). and with water intake of 172 mL/kg W/d (Siebert and MacFarlane, 1969) this could provide approximately 0.2 g Spirulina/kg W/d which, based on our results, would give a similar response to urea (Figure 17). However, it would probably be safer to supply N in the drinking water in this form compared to urea. To achieve the higher liveweight gain response, similar to CSM, algal ponds would need to deliver approximately 23 g DM/L water and under bloom conditions this has never been achieved. Thus external sources of algae would need to be added to drinking water or it could be provided as a dry supplement as is done with CSM in the form of a lick, block or pellet. Nevertheless with the potential for the supply of algal by-products to increase from the C sequestration, biofuel and fatty acid industries, algal protein meals or total algal products could

become a valuable protein source. It is possible for producers to grow algae on farm but growing and harvesting the product requires specialised expertise at present. The current commercial price of algal products is high, based on their application as human supplements, and these sources are not commercially viable for animal production systems. However, the growth of algae for animals does not need the same stringent conditions as for humans and cheaper sources could become available as part of other industries, e.g. C sequestration and biofuel production. There is an attraction to develop algal ponds on the rangelands in association with water medicators so as to supply protein to cattle but the technology at present will not achieve that. The current results show that, if simple algal ponds were developed and algal yields could be increased, the technology would have significant effects on increasing liveweight gain of cattle in the dry season.

In conclusion, liveweight gain of steers increased with increasing N in the diet, as supplied by each of the supplements. The liveweight gain response to CSM was quadratic, while the response to Spirulina and US was linear, with no difference between the two supplements. This experiment suggests that algae or algal by-products may have use as a supplement for cattle grazing low CP pastures in the future, providing low cost sources are available.

4.6 Microbial genetic profiling across all experiments.

4.6.1 Experiment 1. The effect of forage species on microbial genetic profiles.

There was little difference in the MGP in the liquid, squeezed, solid associated and solid attached phases of fractionated rumen digesta. However, some species appeared more closely associated with plant material than others (Figure 22). There was little difference in MGP in the pre- and post-rumen emptying samples (Figure 23). This finding has resulted in an adjustment to the sample collection methodology. There was little change in the dominant bacterial species of samples collected from the same animal fed different diets (Figure 24) indicating a relatively stable rumen microbe population when an animal is fed a range of forages of variable quality. However, some variation in the less dominant bacterial species was evident. There was little difference in the dominant bacterial species of samples collected from different animals fed the same diet (Figure 25).

The dominant species (represented as bands 4, 18, 13 (band B) and 21 (band A) in Figure 25) have been cloned, sequenced and identified (Table 27). Band 4 was chosen because it was only present on Speargrass diets. The other three bands were selected because they remained common and dominant throughout all dietary treatments. The bands chosen for phylogenetic analysis formed three distinct clades within the produced neighbour-joining dendrogram (Figure 26). Clade I contains all sequences for band 13 and band 21 which branch apart from each other deeply, indicating a close phylogenetic relationship between the two detected bacteria. A search of the Ribosomal Database Project (RDP) was able to determine the nearest related cultured and characterised species. The search found that both bands cluster with *Anaerovorax odorimutans*, a putrescine fermenting anaerobe that utilises 4-aminobutyrate and 4-hydroxybutyrate as growth substrates to produce acetate, butyrate, hydrogen and ammonia, and two members of the *Sedimentibacter* genus (*S. saalensis* and *S. hydroxybenzoicum*) who display no ability to ferment carbohydrates and instead ferment amino acids and pyruvate to produce acetate and butyrate. A search of the BLAST database found that sequences obtained from Band 13 matched to sequences obtained from the liquid phase of rumen of Holstein heifers in high temperature and humidity conditions (Tajima *et al*., 2007) and from yak rumen contents. Band 21 was related also to both sources, but identical sequences were also recovered from the rumen of sheep feeding on pasture supplemented with flavomycin, in steers adapting to a high-grain diet and also in the rumen of African herbivore Grant's gazelle. Clade II contains 3 sequences obtained from band 18 and all 5 sequences from band 4. The

sequences recovered from band 18 clustered closest with *Clostridium* sp. strain P6, an isolate obtained from an upward-flow anaerobic sludge blanket (UASB) reactor but not characterised. A BLAST search revealed that identical unpublished sequences had been obtained from bacteria inhabiting sheep, swamp buffalo and yak rumen in addition to swine and human gut microflora. The sequence obtained from Band 4 matched with 100% identity to that of Rumen Bacterium R-25, an uncharacterised bacterium isolated from the attached fraction of an incubation of rumen fluid with added cellulose powder. Also included in this clade is Bacterium str. *Rautii*, a gram-positive rod isolated from human urine, and *Fastidiosipila sanguinis*, a gram-positive cocci isolated from human blood, but both were too distant to be considered closely related. A number of identical sequence matches were uncovered by a BLAST search mainly originating from ruminants including buffalo, yak, Thompson's gazelle and both the liquid and solid phase of rumen contents from Holstein heifers. The appearance of Band 4 in the spear grass diets suggests that these bacteria are most likely to be cellulolytic and may make up a larger proportion of the rumen population during the feeding of speargrass hay due to the reduction in readily fermentable carbohydrates and protein in speargrass diets compared with that of ryegrass. If this is the case, it would be expected that these bacteria involved in primary fibre degradation would be responsible for the release of downstream nutrients for bacterial growth. Clade 3 contained two divergent sequences obtained from Band 18. Clone 16 (B18 C16) clustered with a number of Clostridia species known to readily ferment carbohydrates and cellulose compounds in human and swine gut communities. BLAST results indicated that identical sequences were obtained from other ruminants including water buffalo and Speke's Gazelle. Additionally, a match was seen to sequences detected from rumen bacterium during the addition of concentrates to a hay diet. Clone 11 and 44 formed a cluster with a number of acetogenic bacteria from herbivorous gastrointestinal tracts, including *Ruminococcus productus* and both *Blautia* spp. A subsequent BLAST search revealed complete sequence matches to those recovered from rabbit faeces and Hoatzin crop communities. Additionally, representatives from all bands were detected in a recent paper outlining the metagenomics of the fibre-adherent fraction of the bovine rumen microbial community (Brulc *et al*., 2009).This suggests that all bands and corresponding species identified from this study could be considered to be normal rumen inhabitants and may be involved with fibre degradation in the rumen.

This was the first time that MGP had been examined in cattle fed a range of forages of varying quality. Other studies have demonstrated changes in the dominant microbial species in cattle, but only in association with significant changes in the rumen environment, including diets containing high levels of molasses (Tolosa *et al*., 2004), roughage compared with concentrate based diets (Kocherginskaya *et al*., 2001) and bloated compared with non-bloated steers (Min *et al*., 2006). The dominant microbial species, likely to be the main contributors to MCP production, were closely associated with plant material on all diets examined in this study and remained stable in response to forage quality.

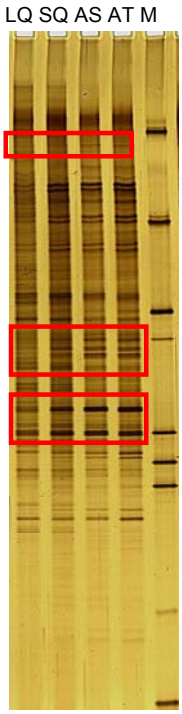

Figure 22. Microbial population profiles for animal 283 demonstrating differences between fractions. Exemplar species that differ in band intensity across fractions are indicated in red boxes. LQ = Liquid, SQ = Squeezed, AS = Associated, AT = Attached, M = Marker.

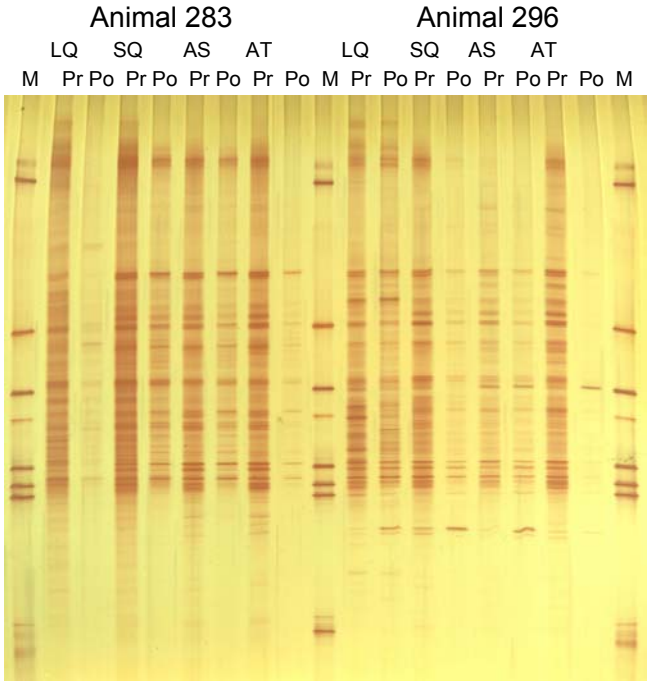

Figure 23. Microbial population profiles of digesta from animal 283 (speargrass treatment) and animal 296 (Mitchell grass treatment) for all fractions, collected pre- (Pr) and post- (Po) rumen emptying. M = Marker, LQ = Liquid, SQ = Squeezed, AS = Associated, AT = Attached.

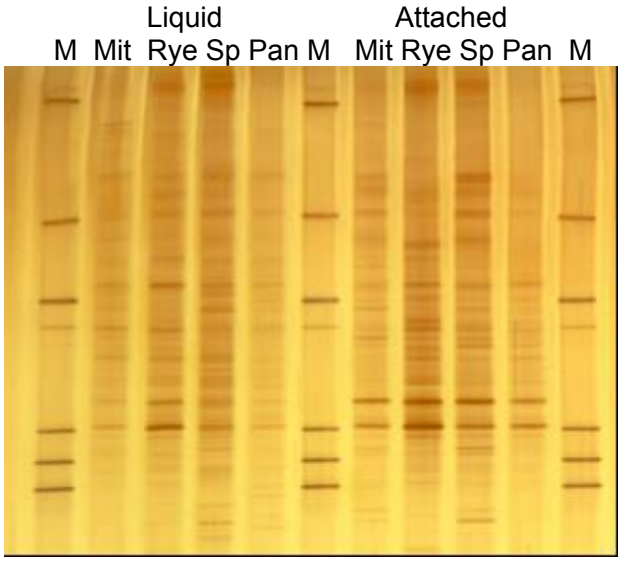

Figure 24. Microbial population profiles of digesta from animal 283 consuming all forage types. M = Marker, Mit = Mitchell grass, Rye = ryegrass, Sp = speargrass, Pan = pangola grass.

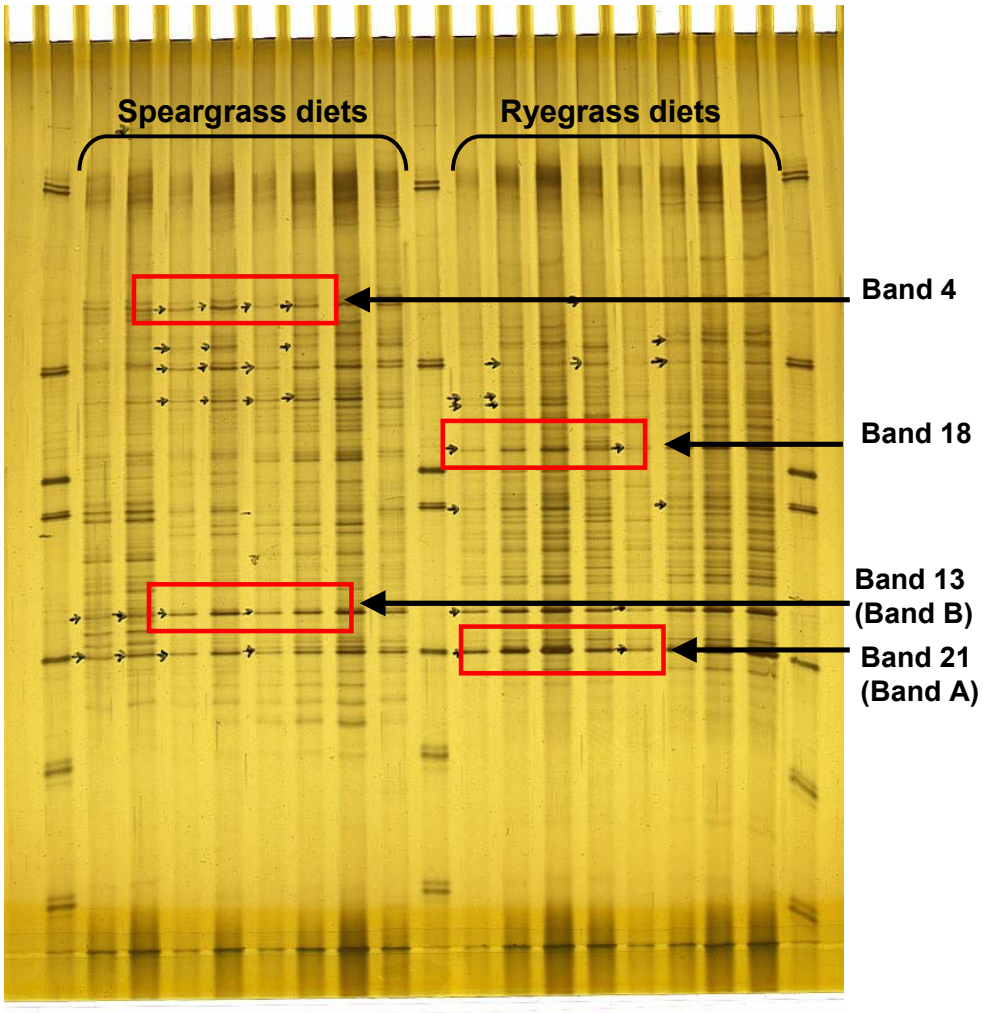

Figure 25. Microbial population profiles of steers fed speargrass (n=8) and ryegrass (n=8) hay diets. Dominant bands are indicated in red boxes.

Table 27. Nearest cultured relatives and percentage match to sequences obtained from band stabs and clone libraries of rumen bacteria from *Bos indicus* steers consuming basal hay diets as obtained from RDPII public database.

Band I.D.	No. of Clones	Sequence	Closest cultured relative(s)	Source	% match
Band A	5	16 S	Rumen bacterium R-7	Sheep rumen	87.1% / 1508 bp
			YE 57	Eastern Grey Kangaroo	65.1% / 1512 bp
Band B	5	16 S	Rumen bacterium R-7	Sheep rumen	95.6% / 1508 bp
			YE 57	Eastern Grey Kangaroo	64.9% / 1512 bp
Band 4 At/Spear	5	V2V3	Rumen bacterium R-25	Sheep rumen	100% / 147 bp
			Fastidiosipila bacterium str. Rautii	Human urine	74.8% / 147 bp
Band 18 At/Rye #1[A]	3	V2V3	Clostridium sp. strain P6	UASB reactor	77.9% / 149 bp
Band 18 At/Rye #2[A]	2	V2V3	Blautia producta	Rumen	91.1% / 147 bp
Band 21 At/Rye (Band A)	6	V2V3	Rumen bacterium R-7	Rumen	96.6% / 147 bp
Band 13 At/Spear (Band B)	4	V2V3	Rumen bacterium R-7	Rumen	100% / 147 bp

[A]Two separate sequences were obtained for Band 18 indicating co-migration of sequences to the same position on a DGGE gel.

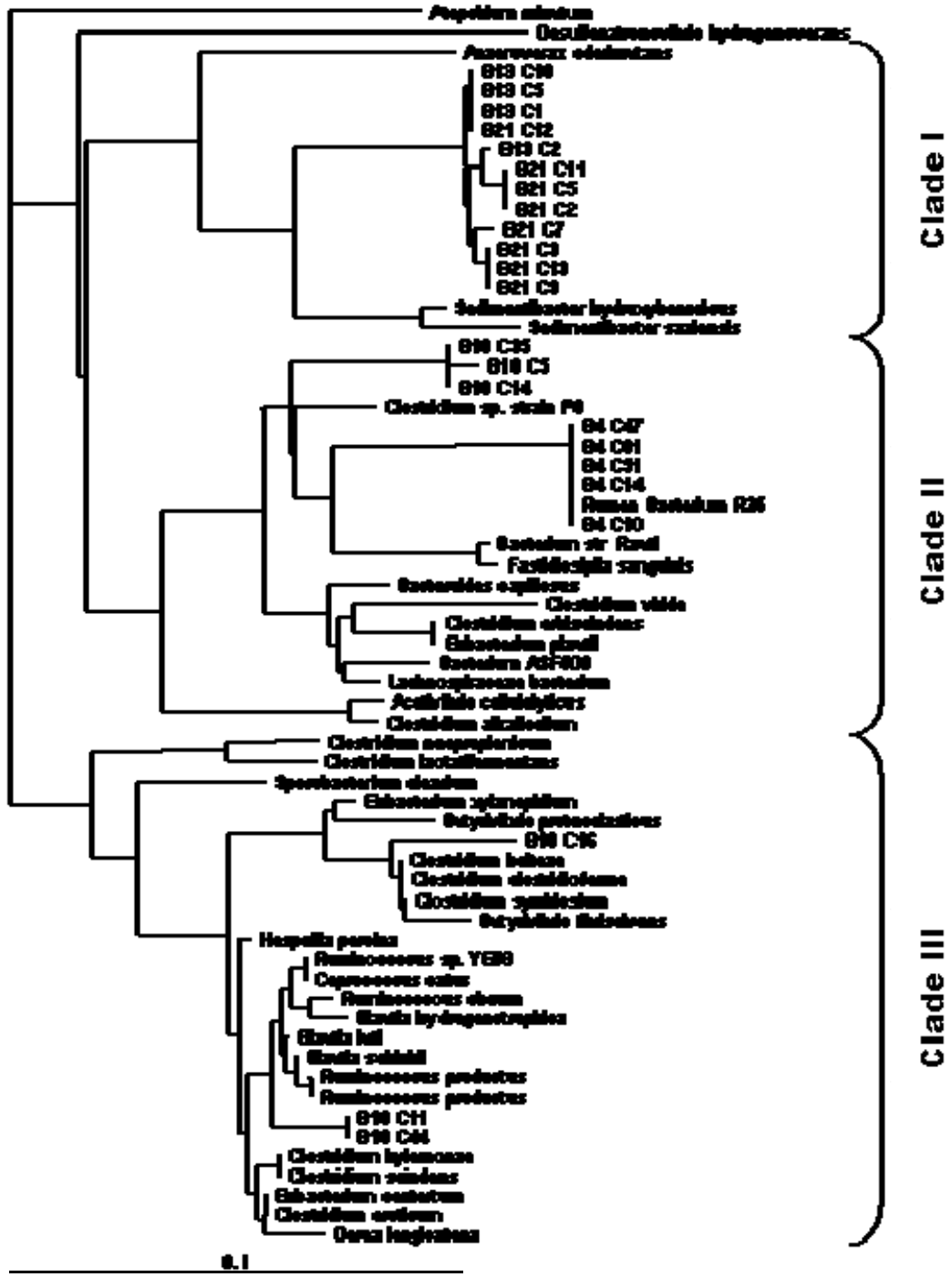

Figure 26. Neighbour joining dendrogram demonstrating phylogenetic relationships of 16S V2V3 sequences obtained from band-stabs of DGGE gels. Sequences are labelled with band number and clone number.

DGGE Analysis of Microbial Community Dynamics. DGGE profiles obtained from each dietary treatment within all steers were compared to each other to assess the bacterial species dynamics

that occurred within the rumen microbial community between hay types. Figure 27 presents the resulting cluster analysis from the Pearson correlation algorithm of all 40 DGGE fingerprints obtained from both liquid and attached phases of rumen contents from five steers consuming the four (speargrass, Mitchell grass, pangola grass and ryegrass) hay types. The resulting dendrogram demonstrates an initial clustering pattern that closely reflects an effect of gel upon similarity coefficients. There are four major clusters that appear above a 60% similarity value, three of which group samples together from a single gel. Importantly, within these larger clusters fingerprints from attached and liquid fractions grouped together at >80% similarity, but these groups contained no more than three profiles in each. The exceptions were animal 282 during run 1 consuming ryegrass, which clustered both liquid and attached fractions together and the profile from the liquid fraction of animal 287 consuming speargrass, which clustered with attached fraction profiles of other animals from the same run. Also, in the majority of groups, samples from animals consuming low-quality tropical forages (speargrass and Mitchell grass hay) grouped together, whilst ryegrass hay profiles clustered either by itself or with medium-quality pangola hay, with only two ryegrass profiles appearing with profiles from low-quality diets in the lower groups on the dendrogram. Preliminary Principle Co-ordinate analysis (PCO) of data revealed that the standard number of three principle components utilised in the analysis explained less than 40% of variation between profiles. As a consequence, the PCO analysis was extended to include four components which accounted for a total of 45.4% of total variability. The subsequent PCO plots are illustrated in Figure 28. Once again, there was a strong gel effect and components accounting for the majority of gel effect have been removed from the analysis. ANOVA of the PCO revealed that there were significant effects of phase seen for components 1 and 2, whilst there were significant effects observed in component 3 however some interaction of gel effect remained. Clustering of data points within the PCO plots, also demonstrated limited clustering or separation between samples. Visual inspection of profiles revealed a number of dominant bands that appeared within fingerprints remaining constant throughout the experiment. Three bands, as indicated by arrowheads in Figure 27, appeared in all steers and fractions regardless of dietary treatment. Two of these bands were heavily dominant throughout the profiles, and appeared to be of greater intensity within fingerprints associated with attached fractions, when compared to other bands within the profiles.

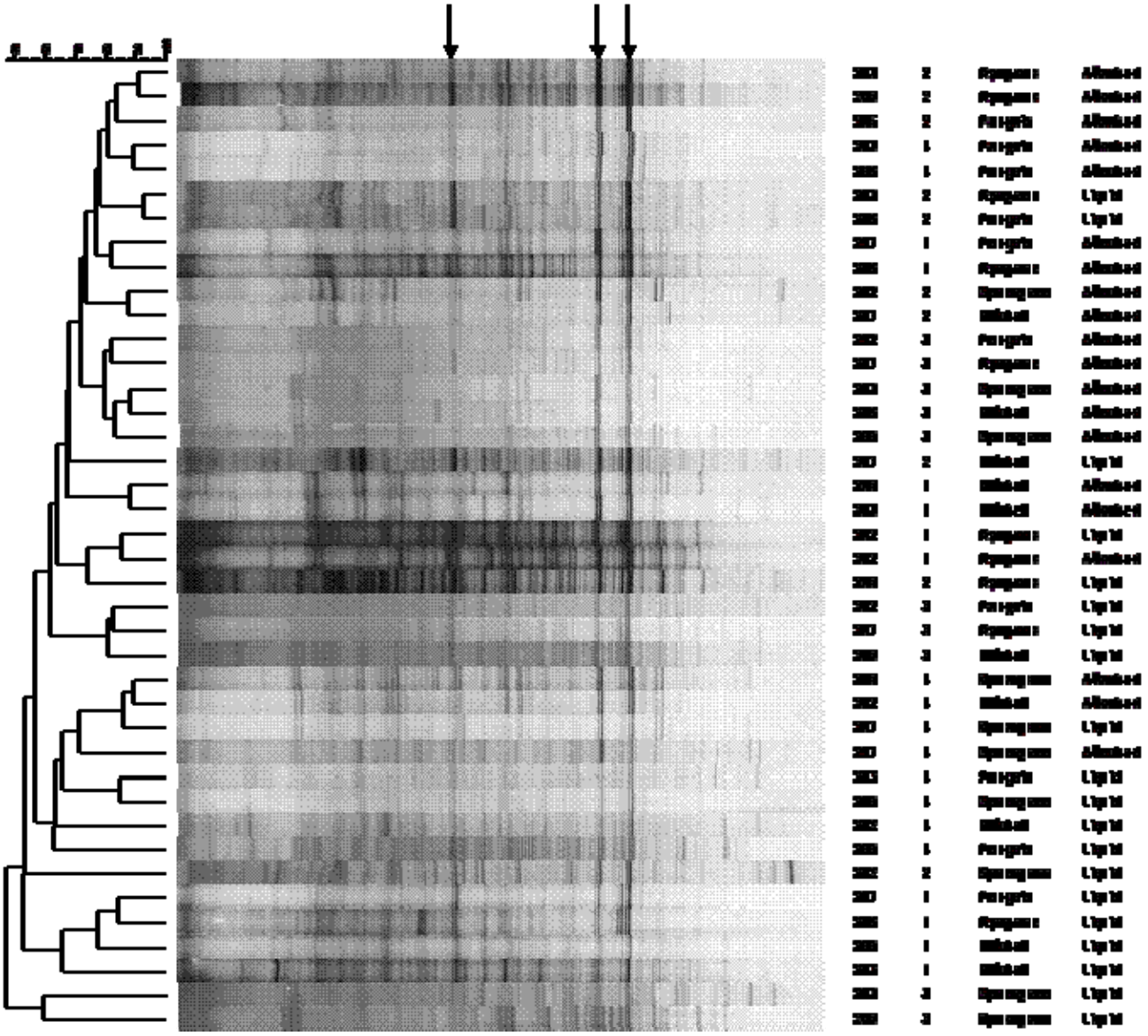

Figure 27. Cluster analysis of 40 rumen bacterial community profiles obtained from the liquid and solid fraction of digesta from steers in Experiment 1 receiving four basal hay diets. Profiles are labelled accordingly with animal identification number, basal hay type, gel/run number and fraction. The scale indicated at the top of the dendrogram indicates the level of similarity determined between profiles (%) and the vertical line indicates the 80% similarity threshold as determined by marker lane similarity.

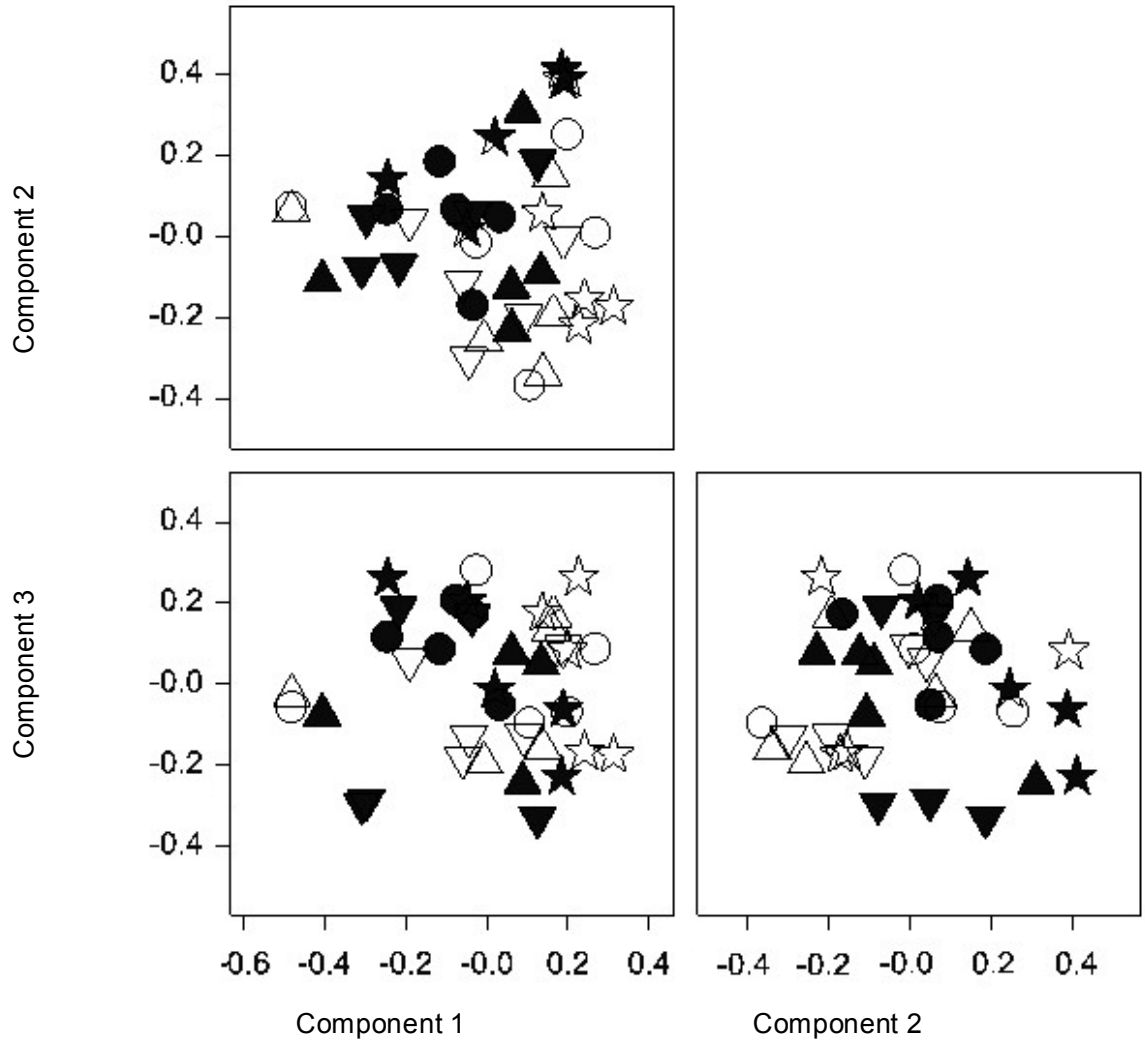

Figure 28. Principle coordinate analysis (PCO) of DGGE profiles obtained from both liquid and solid rumen digesta fractions obtained from steers receiving basal hay diets during Experiment 1. Symbols indicate diet (★ = Speargrass, ● = Mitchell, ▲ = Pangola, ▼= Ryegrass) and digesta phase (Filled = Attached; Unfilled = Liquid).

Bacterial diversity indices and evenness measures. Bacterial diversity measures and mean evenness values obtained from densitometric curve data for all DGGE profiles are presented in Table 28. Once again, the analysis of these measures was subject to a significant level of gel variation and again any effects due to gel differences were removed. The results of regression analysis showed that the average number of bands present in each profile was significantly affected by phase, with a reduction in the number seen in liquid fractions to those detected in fibre attached fractions. The calculated indices (Shannon and Simpson) agreed, with both predicting a significant effect of phase on bacterial species diversity. Additionally, the Simpson diversity index also detected a significant effect of diet on the diversity coefficient of profiles, but this was not reflected in the calculated Shannon diversity index. Finally, evenness values across profiles demonstrated significant effects of both diet and phase, in addition to a diet-phase interaction. Pangola demonstrated the lowest evenness values, whereas ryegrass was the highest and attached fractions

showed lower evenness values than liquid phases. There was no significant effect of steer observed on any calculated diversity of evenness coefficient.

Table 28. Diversity measures (bacterial species richness estimates, Simpson and Shannon diversity indices and evenness values) obtained from Denaturing gradient gel electrophoresis profiles of liquid (L) and attached (A) fractions and the diet mean of the rumen bacterial community in *Bos indicus* steers fed four hay diets in Experiment 1. Different alphabetical superscripts within a column indicate significant differences in diversity measures for L and A fractions; different alphabetical superscripts across a row indicate differences between hays within a diversity measure ($P < 0.05$).

Diversity measures	Fraction	*Diet*				Fraction mean
		Speargrass	Mitchell	Pangola	Ryegrass	
Richness (# of bands)	L	51.87	49.80	41.43	47.10	47.55[a]
	A	42.07	32.80	33.63	47.30	38.95[b]
	Mean	46.97	41.30	37.53	47.20	
Simpson Index	L	27.36	29.92	22.63	26.95	26.71[a]
	A	23.75	17.44	14.37	25.54	20.28[b]
	Mean	25.55[b]	23.68[ab]	18.50[a]	26.25[b]	
Shannon Index	L	3.439	3.661	3.359	3.494	3.476[a]
	A	3.378	3.104	2.984	3.445	3.228[b]
	Mean	3.408[b]	3.358[ab]	3.172[a]	3.496[b]	
Evenness	L	0.9039	0.9287	0.9040	0.9156	0.9130[a]
	A	0.9102	0.8974	0.8627	0.9089	0.8948[b]
	Mean	0.9070[b]	0.9130[b]	0.8833[a]	0.9123[b]	

Denaturing gradient gel electrophoresis analysis of bacterial community structure and dynamics between diets. A comprehensive analysis of all 40 DGGE profiles from samples collected from five steers across four experimental runs was conducted to investigate the effect of basal hay diets upon the rumen bacterial assemblage. This fingerprint analysis demonstrated some degree of variability across profiles, with PCO analysis required to be extended to four principle components to cover approximately 45% of variability. Much of the initial clustering observed within the dendrogram is due to gel effects, but within these clusters, profiles show grouping by digesta phase and diet. These results were confirmed by PCO analysis with, aside from the aforementioned gel effects, basal hay type and fraction accounting for significant variability between profiles in the dendrogram for two principle components each of the four plotted. The influence of diet type upon species richness, diversity and evenness indicators displayed a number of differences between profiles. Both the number of bands detected and the Shannon diversity index were significantly reduced between fractions, reinforcing previous results from this experiment. No consistent effect of diet was observed in these measures, despite the large nutritional difference in the basal hay fed to steers. Contrary to these results, significant effects of diet appeared in addition to that of phase within the Simpson diversity index and in species evenness measures. Simpson diversity index calculations concluded that ryegrass hay produced the greatest mean number of resolvable bands within a profile, but the next highest quality diet, pangola hay, produced the lowest mean number of bands. Additionally, pangola diets produced the most uneven band intensity across the experiment whilst the greatest evenness was seen when steers were fed Mitchell grass hay. There seems to be no apparent pattern that can be attributed to these differences, however the variation in the amount of available protein and energy provided by higher quality diets is most likely to support greater levels of

microbial growth, faster rates of digesta passage and improved animal performance. Kocherginskaya *et al*. (2001) established that steers receiving corn-based diets possessed a more diverse and species rich bacterial population based upon DGGE fingerprint information than those receiving a hay only diet, but more recent work by Larue *et al.* (2005) and Sadet *et al.* (2007) have both found no response in terms of diversity indices to an improvement in diet quality with the addition of concentrates in either liquid or solid phases. The result obtained in the study by Kocherginskaya *et al*. (2001) can be attributed to the large amount of readily fermentable non-structural carbohydrate (NSC) provided by the corn diet, a result that has been confirmed by previous work by Tolosa (2006) where a population shift and diversity effects were seen with molasses supplementation of a forage diet. Other studies, which provided a range of higher quality diets, did not provide sufficient levels of NSC to elucidate the same response. At this point, it is also important to consider the limitations of DGGE when examining microbial diversity, particularly with regards to less-abundant species. Only approximately 1% of all species abundance within microbial communities is able to be resolved within a DGGE gel and there remains a significant portion of the assemblage whose contribution to diversity is underestimated (Fromin *et al*. 2002) although their contribution to overall ecosystem functionality may be limited (Pedros-Alio, 2006) as would, therefore, their contribution to microbial protein. This being the case, there may have been changes in speciation and diversity present within the lower orders of the rumen communities studied, but in terms of the most dominant species detected within DGGE profiles, no definitive response was seen. Visual inspection of all profiles reveals a number of bands common across all diets and fractions. Of these, two of the bands are heavily dominant in all fractions and diets regardless of hay quality or animal. These were identified as belonging to the genus Anaerovorax and Clostridium as outlined above. Recently, the intensity of bands within DGGE profiles has been directly correlated to cell abundance within gut microbial ecosystems (Milinovich *et al*. 2008), where increasing intensity of given bands within DGGE profiles produced over a period of time was directly comparable to the increase in cell numbers as demonstrated by fluorescent in-situ hybridisation (FISH). The greater intensity of these two bands within the profiles are therefore contributed by species that may constitute a considerable proportion of the rumen microbial ecosystem within hay-fed *Bos indicus* steers and thus contribute extensively to the amount of microbial protein passing into the duodenum.

In conclusion, this body of work has utilised 16S DGGE molecular fingerprints to examine the effects of basal hay diets upon the rumen microbial population in *Bos indicus* steers. The analysis of DGGE profiles and subsequent PCO analysis has demonstrated, in agreement with previously published studies, that distinct microbial populations and species distribution exists within the liquid and solid phases of rumen community. Furthermore, it has been shown that hay type and quality demonstrates limited significant effects upon the structure and diversity of species detectable by DGGE within these niche communities, with common and dominant bands most likely to be significant contributors to microbial protein being maintained across all treatments. Further study into the metabolic actions of bacteria within these microbiomes should be considered to elucidate the true impacts of improved pasture quality to the rumen bacterial community.

4.6.2 Experiment 2. The effect of urea N or rumen degradable protein on microbial genetic profiles

The results of DGGE analysis of the effects of high levels of various RDN supplements upon the rumen bacterial community of steers consuming basal tropical hay diets are presented in this section. The effects of these supplements were analysed within the context of two basal tropical hays (Mitchell grass and pangola). Evidence suggested that the type of supplement had little effect on the genetic profile of the rumen microbe population of animals consuming Mitchell grass (Figure 29) and pangola grass (Figure 30) hays.

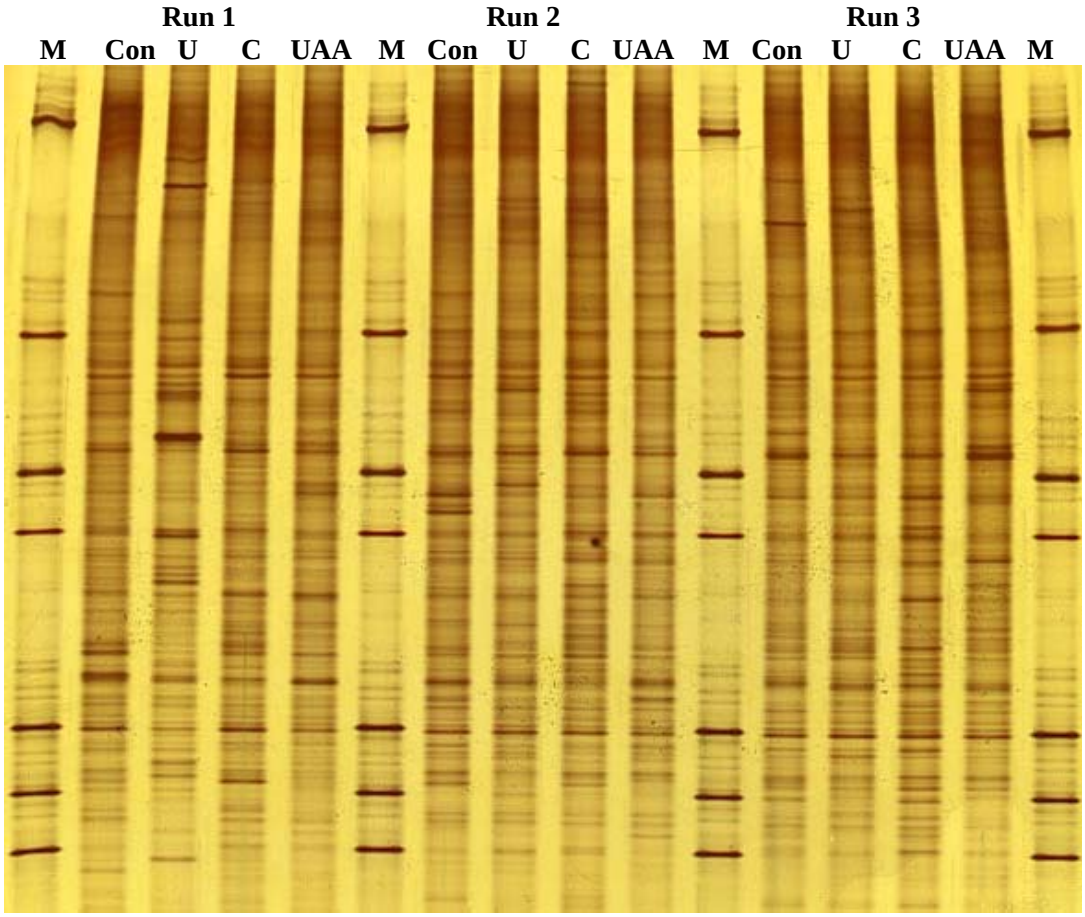

Figure 29. Microbial population profiles of animals fed Mitchell grass hay alone (Con; 70 g RDP/kg DOMI) or Mitchell grass hay supplemented with urea (U; 250 g RDP/kg DOMI), casein (C; 300 g RDP/kg DOMI) and urea plus branch chain amino acids (UAA; 300g RDP/kg DOMI). Profiles are from different animals over three consecutive runs.

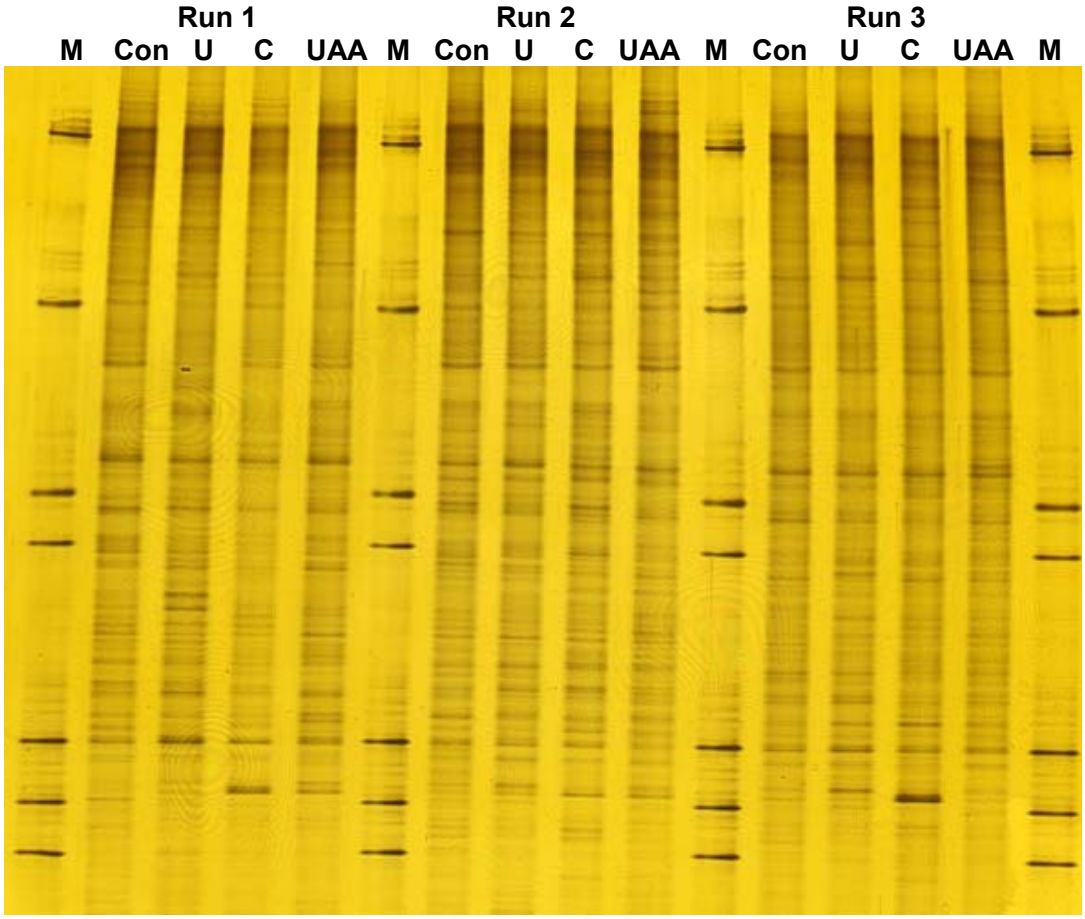

Figure 30. Microbial population profiles of animals fed pangola grass hay alone (Con; 120g RDP/kg DOMI) or pangola grass hay supplemented with urea (U; 250g RDP/kg DOMI), casein (C; 300g RDP/kg DOMI) and urea plus branch chain amino acids (UAA; 300g RDP/kg DOMI). Profiles are from different animals over three consecutive runs.

Bioinformatic analysis of rumen bacterial communities. Total rumen bacterial communities from all animals receiving RDN supplementation were analysed through the amplification and separation of the V2V3 region of 16S gDNA by DGGE. As identical marker lane profiles were determined to be approximately 80% similar by analysis software, a threshold of 80% was decided upon which to evaluate relatedness of DGGE fingerprints. Cluster analysis of 12 rumen bacterial community profiles from 9 animals receiving Mitchell grass hay supplemented with a high rate of urea, BCAA plus urea, casein or no supplement (control) is presented in Figure 31. Three runs were completed and all DGGE analysis was carried out within a single gel. The dendrogram shows no definitive clustering pattern, with no sub-clustering occurring at a similarity above the 80% threshold. However, some overall similarity between profiles was observed with initial branching occurring at approximately 54%, where a small sub-cluster of two animals supplemented with casein separated, with the next cluster being formed at approximately 58% similarity by two samples (control and urea treatments from Steer 686 and 809 respectively) collected during run 4. Overall, a minor trend towards profiles clustering by run occurs, however all of these clusters fall outside the threshold limits. Figure 32 shows the resulting graphical output of PCO analysis of DGGE data, where no strong clustering trends are apparent. All data appears scattered throughout plots and no separation by diet occurred. Subsequent PCO analysis (Figure 32) and ANOVA of contributing components

showed that no significant differences occurred between profiles from supplement treatments. Similarly, cluster analysis of 12 V2V3 16S DGGE bacterial community profiles from a second group of 6 steers receiving the same supplemental regime with basal pangola hay is presented in Figure 33. Again, three experimental runs were completed and all DGGE analysis of profiles occurred within a single DGGE gel. The resulting dendrogram demonstrates a clustering pattern with no sub-cluster formation above the 80% threshold limit. Once again, no obvious trends emerged with regards to diet or run effects. One steer (871) receiving a urea supplement formed a distinct branch at a low level of approximately 35% similarity, whilst the remainder are, once again, similar at a level greater than 60%. Figure 34 shows the PCO analysis of DGGE profile data from pangola hay plus supplement treatments. No strong separation resulting from dietary treatments is seen for any components however the separation of one steer receiving urea, as seen in the dendrogram, is accounted for by the first component. All samples from both casein and BCAA supplemented animals appeared to cluster closer together than those receiving control or urea supplemented diets. No significant difference between supplementation was demonstrated by PCO analysis and subsequent ANOVA of PCO scores.

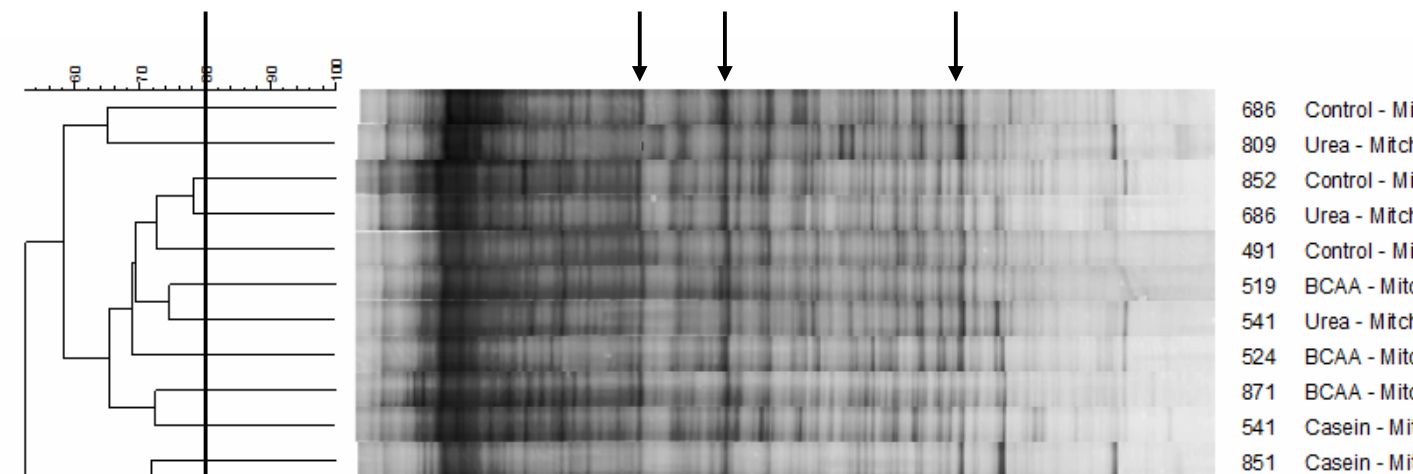

Figure 31. Cluster analysis of 12 rumen bacterial community profiles obtained from steers in Experimen receiving Mitchell grass hay supplemented with various RDP sources. Profiles are labelled accordingly with su and animal identification number. The scale indicated at the top of the dendrogram indicates the level of similarity determin between profiles (%) and the vertical line indicates the 80% similarity threshold as determined by marker lane similarity. Th indicate some of the common and dominant bands present across diets and animals.

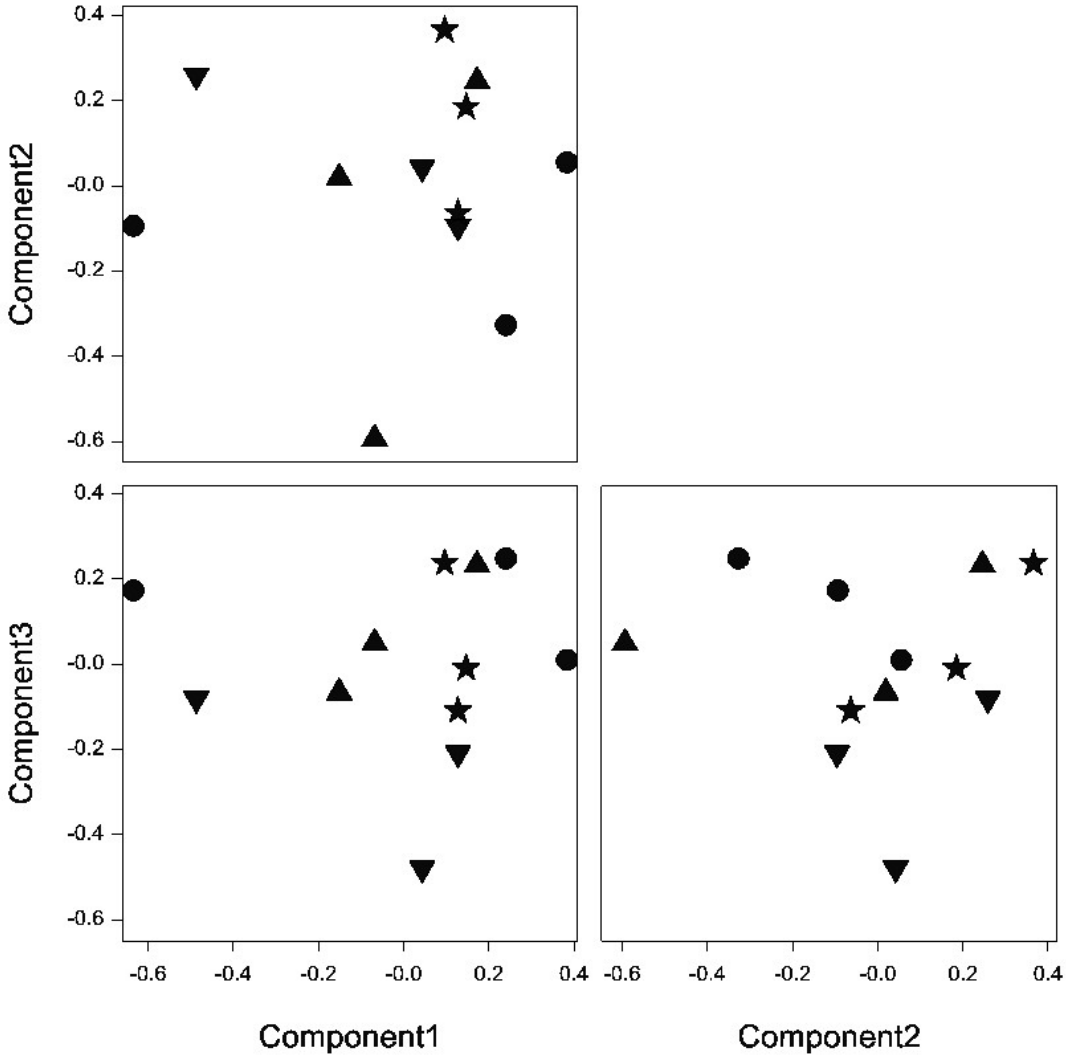

Figure 32. Principle coordinate analysis (PCO) of DGGE profiles obtained from steers receiving Mitchell grass hay supplemented with various RDP sources during Experiment 2. Symbols indicate supplement treatments (● = Control, ▲ = Urea, ▼= BCAA, ★ = Casein).

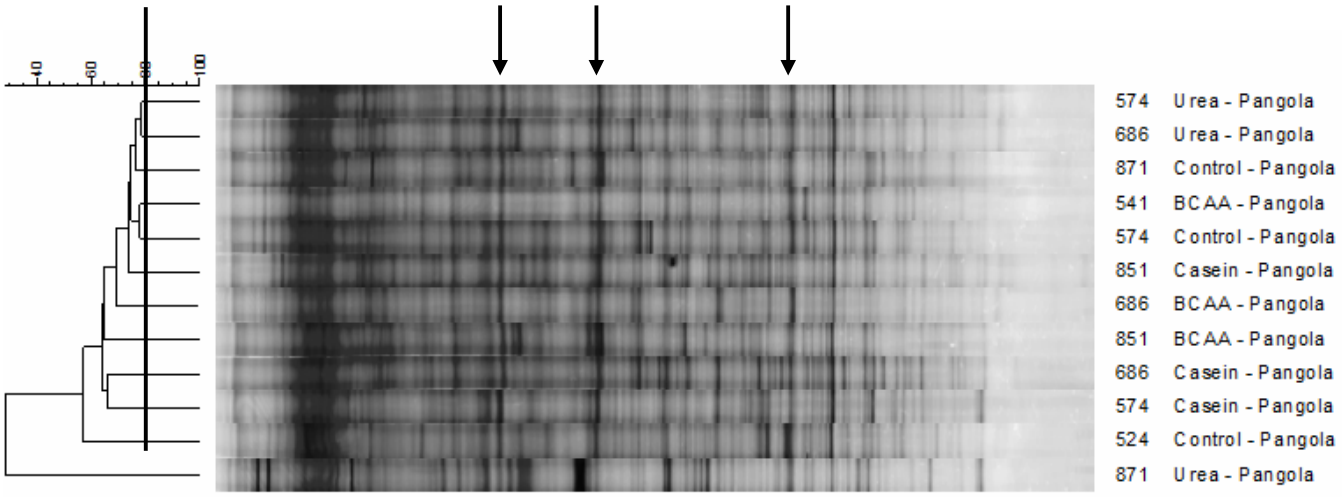

Figure 33. Cluster analysis of 12 rumen bacterial community profiles obtained from steers in Experiment 2 receiving pangola hay supplemented with various RDP sources. Profiles are labelled accordingly with supplement and animal identification number. The scale indicated at the top of the dendrogram indicates the level of similarity determined between profiles (%) and the vertical line indicates the 80% similarity threshold as determined by marker lane similarity. The arrows indicate some of the common and dominant bands present across diets and animals.

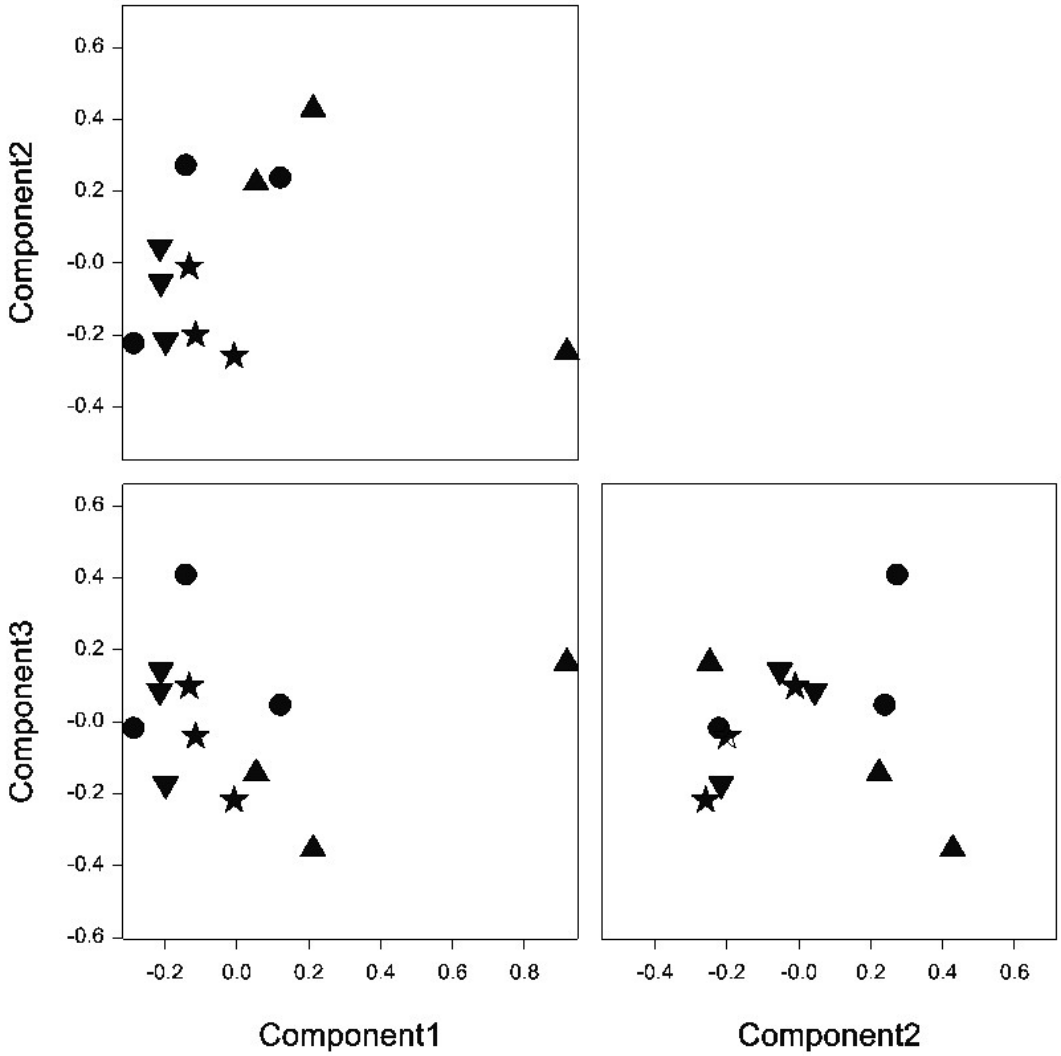

Figure 34. Principle coordinate analysis (PCO) of DGGE profiles obtained from steers receiving pangola hay supplemented with various RDP sources during Experiment 2. Symbols indicate supplement treatments (● = Control, ▲ = Urea, ▼= BCAA, ★ = Casein).

Bacterial diversity indices. A number of diversity indices were calculated for all profiles examined, the results of which are presented for steers fed Mitchell grass (Table 29) and pangola grass (Table 30). The number of bands (species richness), Simpson and Shannon diversity indices and species evenness were determined for both basal hays and supplements. Steers fed Mitchell grass-based diets demonstrated very similar predicted values for diversity and evenness indices across all treatments. Interestingly, the mean number of bands in each treatment as detected by BioNumerics software decreased with higher-order N supplementation, with control diets displaying the most bands and casein the least. ANOVA analysis and pairwise tests between means demonstrated no significant difference between any of the calculated indices with regards to dietary treatment. A number of changes were seen in profile attributes when basal dietary pasture type was changed to

pangola. The values obtained for the Shannon diversity index and species evenness remained similar regardless of diet, however the average number of bands for each diet increased significantly ($P < 0.05$) as higher order N supplements were added to the diet. Likewise, the values calculated for the Simpson diversity index also showed an increasing trend, but the differences observed were not significant.

Table 29. Diversity measures (bacterial species richness estimates, Simpson and Shannon diversity indices and evenness values) obtained from denaturing gradient gel electrophoresis profiles of the rumen bacterial community in *Bos indicus* steers fed Mitchell grass hay alone, or Mitchell grass hay supplemented with urea-ammonium sulphate (US), US plus branch chain amino acids (USAA) and casein.

Steer number	Diet	Number of bands	Simpson Index	Shannon Index	Evenness
686	Control	57	26.39	3.57	0.8821
852	Control	61	36.38	3.78	0.9190
491	Control	61	34.44	3.76	0.9137
Control Mean		59.67	32.40	3.7	0.9037
809	US	55	29.42	3.66	0.9121
686	US	57	33.75	3.70	0.9155
541	US	50	29.03	3.60	0.9195
US Mean		54.00	30.73	3.65	0.9157
524	USAA	59	32.67	3.70	0.9073
871	USAA	57	28.59	3.60	0.8908
519	USAA	46	28.10	3.50	0.9130
BCAA Mean		54.00	29.79	3.60	0.9037
541	Casein	55	35.61	3.75	0.9359
851	Casein	46	23.18	3.44	0.8997
871	Casein	50	30.10	3.62	0.9268
Casein Mean		50.33	29.63	3.61	0.9208

Table 30. Diversity measures (bacterial species richness estimates, Simpson and Shannon diversity indices and evenness values) obtained from denaturing gradient gel electrophoresis profiles of the rumen bacterial community in *Bos indicus* steers fed pangola grass hay alone, or pangola grass hay supplemented with urea-ammonium sulphate (US), US plus branch chain amino acids (USAA) and casein. Different alphabetical superscripts within a column indicate significant differences between diet means within a diversity measure ($P < 0.05$).

Steer number	Diet	Number of bands	Simpson Index	Shannon Index	Evenness
524	Control	53	24.07	3.54	0.8905
574	Control	57	29.73	3.65	0.9032
871	Control	54	28.07	3.64	0.9129
Control Mean		54.67[a]	27.29	3.61	0.9022
871	US	58	26.28	3.59	0.8844
686	US	58	33.89	3.74	0.9219
574	US	57	31.36	3.74	0.9260
US Mean		57.67[a]	30.51	3.69	0.9167
686	USAA	56	26.86	3.60	0.8954
541	USAA	60	34.06	3.72	0.9097
851	USAA	58	33.81	3.73	0.9186
USAA Mean		58.00[a]	31.58	3.69	0.9079
574	Casein	60	32.39	3.71	0.9052
851	Casein	62	38.86	3.85	0.9337
686	Casein	65	36.58	3.80	0.9113
Casein Mean		62.33[b]	35.94	3.79	0.9167

The rumen bacterial community has been shown to change in response to an alteration in dietary intake by the host animal, especially with introduction of supplementation where the magnitude of change in dietary composition available to the rumen microbes is considerable (Kocherginskaya *et al*. 2001; Tajima *et al*. 2001; Klieve *et al*. 2007). Assessing bacterial community changes through the use of molecular profiling techniques allows the detection and identification of species dynamics resulting from these dietary modifications. Previous work completed in experiment 1 demonstrated that an increase in protein content and a decrease in NDF content of pasture hays had some effect upon the rumen bacterial community, but dominant members of these communities appeared to remain unchanged. In experiment 2, high levels of three forms of N supplementation were fed to provide a significant increase in the amount of available RDN within the rumen when fed in conjunction with tropical pasture hays.

The dendrograms resulting from the analysis of DGGE profiles of the rumen bacterial community in steers fed Mitchell grass hay based diet demonstrates a limited degree of similarity between both animals and diets. Despite this, no clustering of profiles occurred at a level greater than the 80% similarity threshold set previously. This finding was reinforced by the statistical analysis of the data used to generate the dendrograms, which concluded that there were no significant differences between steers receiving different sources of RDN-supplementation with this hay type. In some instances, there appeared to be a weak run effect observed, however this was neither significant nor consistent. A visual comparison of profiles shows that a number of common and dominant bands are present in all profiles. Interestingly, these bands persist regardless of steer or diet. Three steers (steer number 686, 541 and 871) participated in more than one experimental run, but the associated

profiles did not cluster together and insufficient data was available to complete further analysis of bacterial community dynamics between diets within individuals.

Similar to DGGE profiles produced from animals consuming Mitchell grass based diets, those receiving pangola also displayed some degree of similarity between profiles. Once again, however, no clustering of profiles took place at a level greater than the 80% similarity threshold. Additionally, the analysis of data produced from profile information also resolved no significant differences between dietary profiles from supplementation of pangola hay with the different RDN sources. The dendrogram displayed a weak degree of clustering relating to diet, with small clusters of two steers appearing (574 and 686 supplemented with both urea and casein), however again none of these were significant. One profile produced by steer 871 when supplemented with urea displayed a markedly different bacterial population profile (approximately 35% similarity). Interestingly, this unique pattern disappears when the same steer was fed only the pangola hay control diet. Due to the lack of samples available for this steer receiving other RDN supplements because of treatment design, it is impossible to ascertain whether the unique banding pattern was attributed to the introduction of supplementation or another confounding factor such as non-specific PCR amplification or template contamination. Four steers participated in more than one experimental run, with steers 574 and 686 consuming three separate diets; again there was no animal effect observed in clustering patterns. Visual inspection showed the presence of a number of dominant bands, similar to those seen on Mitchell grass diets, which remained constant throughout the experimental period and were not affected by animal or diet.

The results obtained from the above DGGE profile analysis of RDN supplementation of tropical hays were further supported by the analysis of diversity data calculated from band enumeration and intensity attributes within profiles. No significant differences in rumen bacterial profiles with regards to band number, Simpson or Shannon diversity indices, or evenness measures were observed between any RDN supplementation of Mitchell grass hay. However, a trend in the reduction of the number of bands and diversity indices was noted. This may have been caused by the low levels of readily available carbohydrates within the diet, leading to reduced breakdown of higher order RDP and availability of component amino acids and peptides for incorporation into microbial protein (Bach *et al*., 2005). In examining the same supplements of pangola hay, a significant increase in the number of bands within profiles was seen as the complexity of RDN supplement increased, in direct contradiction to that observed on Mitchell hay. As a result, an increase in both Simpson and Shannon diversity was also observed, but due to the large amount of residual variation due to steer effects, these differences could not be regarded as significant. These results indicate that the growth of a number of sub-dominant bacteria was enhanced with the introduction of a more digestible hay with a higher CP content. Interestingly, the average values obtained for both diversity and evenness are comparable to those reported in other DGGE profile analyses from ruminants, where energy concentrates (corn) were incorporated into hay-based study diets (Kocherginskaya *et al*., 2001; Larue *et al*., 2005).

The results obtained from this study appear to suggest that the nutritive value of the basal diet has some impact upon the effects of RDN supplementation. This has been previously shown to be one of the major factors affecting the degradation of protein within the rumen (Bach *et al*., 2005). The Mitchell grass hay was lower in digestibility and thus was most likely to be more limiting to bacterial growth. This finding agrees with the results obtained by Cruz Soto *et al*. (1994) who demonstrated increased digestion of NDF when both NPN and dietary protein was supplied and that the potential stimulatory effects of the addition of amino acids was most likely reliant upon the provision of sufficient energy within the basal diet. Further to this, amino acids and peptides can only be incorporated into microbial protein when energy is available in addition to providing an energy

source available to microbes through deamination (Bach *et al*., 2005). This has the flow-on effect of reducing the amount of RDN available for incorporation in new amino acids synthesised by the rumen microbes.

The dominance of bands within a profile can be correlated to the proportion of a given species within a population (Milinovich *et al*., 2008). Interestingly, as in earlier work, these common and dominant bands appear across all profiles regardless of animal or diet on each hay type and may represent species that contribute significantly to microbial protein received by these animals. Despite the continuity of these bands across steers and diets, the clustering patterns within the resulting dendrograms failed to reach a level of significance and thus, the variability between profiles must be the result of species variation at a sub-dominant level within rumen microbiota between diets. As whole rumen contents were examined in this study, the resolution of these changes may be enhanced by the inclusion of a fractionation method, as carried out in Experiment 1, to identify whether these changes occurred within the liquid fraction or appeared in populations associated with fibre within the rumen. Higher order RDN supplements, such as casein and BCAA, have been demonstrated to stimulate growth rates in a number of fibrolytic bacterial species allowing for an increase in the amount of nutrients released downstream from structural carbohydrate breakdown (Wallace *et al*., 1997; Bowen 2003). Due to the likely simultaneous response of all rumen microbes to increased nutrient release, the response to supplementation is difficult to demonstrate using 16S rDNA-based DGGE assays. Additionally, the effects of supplementation on a given rumen bacterial community may have been diluted by the non-continuous nature of the animals used for the study. If the design had allowed for the continuous use of all animals receiving all treatments, microbial community dynamics between supplementation types may have been more clearly resolved.

In conclusion, the results obtained in this study demonstrate that the provision of various sources of supplemental RDN affects the rumen bacterial community, dependent upon the inherent nutritional value of the basal diet upon which supplementation is applied. Analysis of DGGE patterns demonstrated no significant effect of RDN supplementation on structural changes of the rumen bacterial community across both tropical hay types, regardless of RDN complexity. A number of dominant bacteria were detected across a number of animals on all dietary treatments, but alterations in the banding patterns of sub-dominant community members were also evident, with the supplementation of higher quality pangola hay resulting in a significant increase in the number of bands detected within profiles.

4.6.3 Experiment 3. The effect of rumen degradable protein or single cell organism on microbial genetic profiles

Denaturing gradient gel electrophoresis profiles of the rumen liquid phase of each steer over three runs were obtained and analysed by cluster analysis. A threshold of 80% similarity was used as a criterion to evaluate the DGGE clusters.

High supplementation and controls over three runs. Rumen bacterial (liquid phase) DGGE profiles of control cattle and those supplemented with high algae (S290) or high urea-ammonia sulphate (US250) over the three runs showed that each steer harboured very diverse microbial communities (Figure 35). The DGGE profiles from two of the three control animals had well defined dominant bands that were unusual in that they were so heavily pronounced indicating that a high proportion of this bacterial group was present. Interestingly, these pronounced bands were present only in runs 2 and 3 in samples from cattle fed solely on a Mitchell grass hay diet but who had previously been fed, in the feeding period immediately prior to this, a high level of N. One band belonged to an uncultured Spirochaete, the other was an uncultured rumen bacterium from the order Bacteroidales.

Interestingly, these bacteria were not dominant in the Control group in run 1. In this run, treatments were implemented at the commencement of the experiment where there had been no N supplementation in the period immediately prior to experimentation. To explain this anomaly, we propose that the explosive population of Spirochaetes, and possibly also the Bacteroidales, in the control animals of run 2 and 3 resulted from these bacteria advantageously scavenging microbial protein from starved and dying bacteria following the marked reduction in dietary N for microbial growth and survival when previously high dietary N was reduced to very low levels (control). Spirochaetes and members of the Bacteroidales species are known to be proteolytic. It is also of interest that Spirochaetes have been reported to grow in close proximity to cell wall degrading microbes and often contaminate isolations of cellulolytic bacteria (Cheng *et al*., 1984). A DGGE gel of these same animals but of the solid phase digesta (Figure 36) showed very similar bacterial community profiles regardless of treatment. The striking bands found in the control 2 and 3 animals were not present in the solid phase profiles indicating that these particular Spirochaetes and Bacteroidales were entirely planktonic with no association to plant material.

Microbial genetic profiling (Figures 35 and 36) revealed that there were fewer dominant species present in the digesta of cattle supplemented with a high level of Spirulina, indicating a shift in the microbial population possibly related to growth rate of specific species. There appear to be minor changes in the banding pattern between cattle supplemented with Spirulina and US, and those cattle not supplemented. These changes may be related to bacterial retention time in the rumen, bacterial lysis or predation by protozoa, or due to direct effects of the nutrient composition of the Spirulina preferentially favouring one species of microbe over others. Dominant DGGE bands detected in previous experiments were the same as those detected in this experiment. Identification of the six dominant and differential bacterial species, within and between treatments, is under investigation. Of particular interest is a unique band (Figure 35) found in the solid phase of animals supplied high levels of Spirulina algae. Due to the limitation of harvesting a large number of clones needed to represent the diverse rumen profiles of the various treatments, next generation sequencing technologies (454 sequencing) is currently being applied to the control animals and high algae supplemented animals. This new technology will allow us to describe the entire microbial profiles of these differing supplemental treatments in the future.

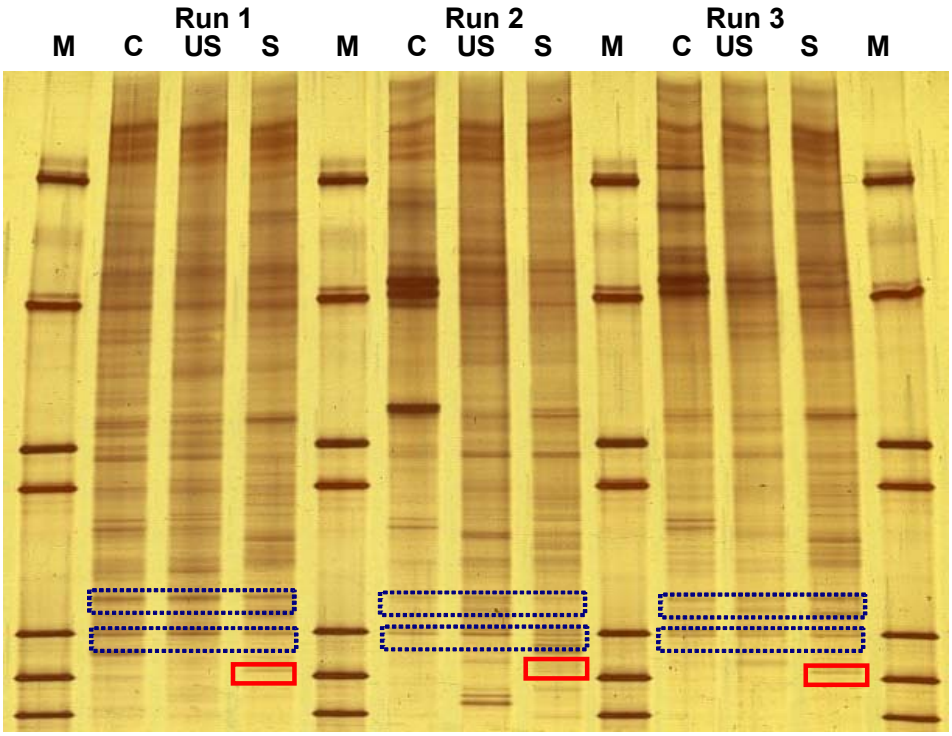

Figure 35. Microbial population profiles associated with the liquid fraction of the rumen contents of animals fed Mitchell grass hay alone (C; 62.3g RDP/kg DOMI) and Mitchell grass hay supplemented with urea-ammonium sulphate (US; 245g RDP/kg DOMI) and Spirulina (S; 359g RDP/kg DOMI). Profiles are from different animals over three consecutive runs. Bands in dotted (blue) outline boxes are dominant species (A and B) reported in Figure 25; bands in solid (red) outline boxes that demonstrate differential banding patterns between Spirulina and US supplement treatments. M is fractionated size marker.

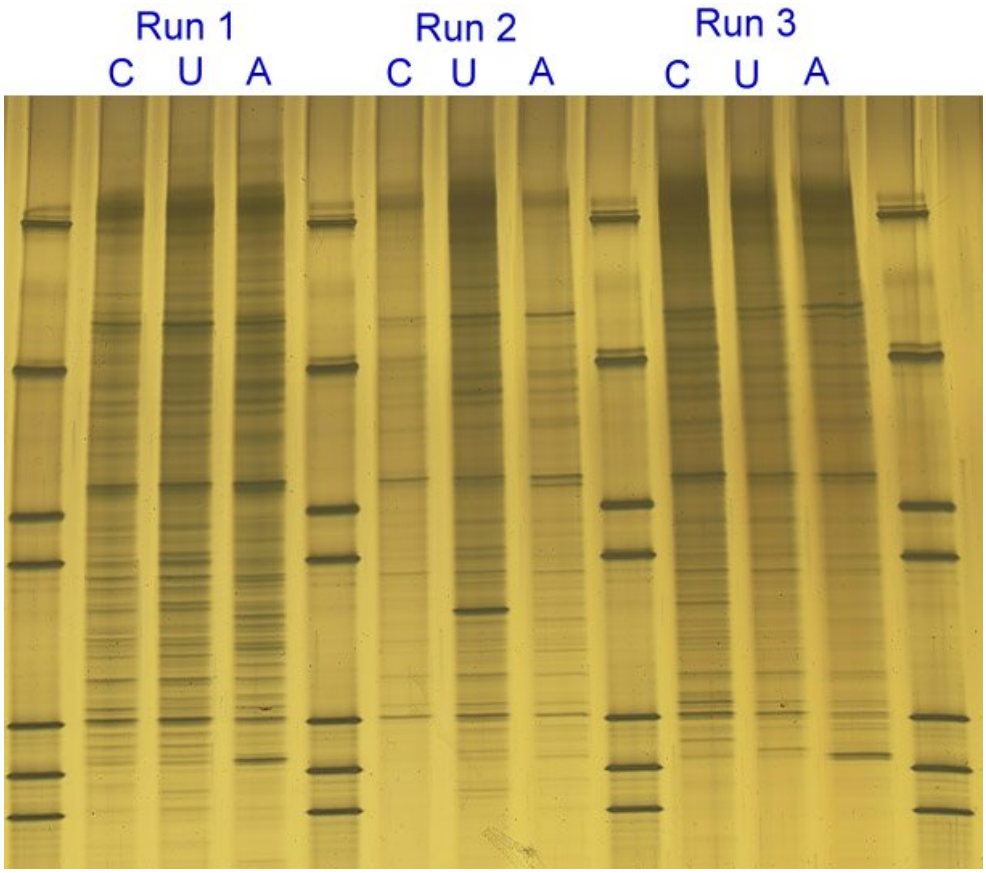

Figure 36. Microbial population profiles associated with the solid fraction of the rumen contents of animals fed Mitchell grass hay alone (C; 62 g RDP/kg DOMI) and Mitchell grass hay supplemented with urea-ammonium sulphate (US; 245 g RDP/kg DOMI) and Spirulina (A; 359 g RDP/kg DOMI). Profiles are from different animals over three consecutive runs.

The clustering analysis dendrogram of the controls and high supplementation liquid phase profiles over the three runs is shown in Figure 37. Clustering of these profiles showed differences between treatments, with S290 (run 3) clustering separately from the rest of the treatments. S290 (run 2 and 3) clustered together but their similarity was just under 80% (i.e. not regarded as identical). The treatments that had higher than 80% similarity were the control and US250 (run 3) and control and US250 (run 1). This data reveal that between animal variability may be greater than treatment variability and there may be an animal effect or a run effect. The dendrogram from solid phase profiles over the three runs from controls and the highest supplement treatments is shown in Figure 38. This dendrogram shows much greater similarity between treatments indicating that the solid phase associated bacteria are more stable regardless of dietary changes.

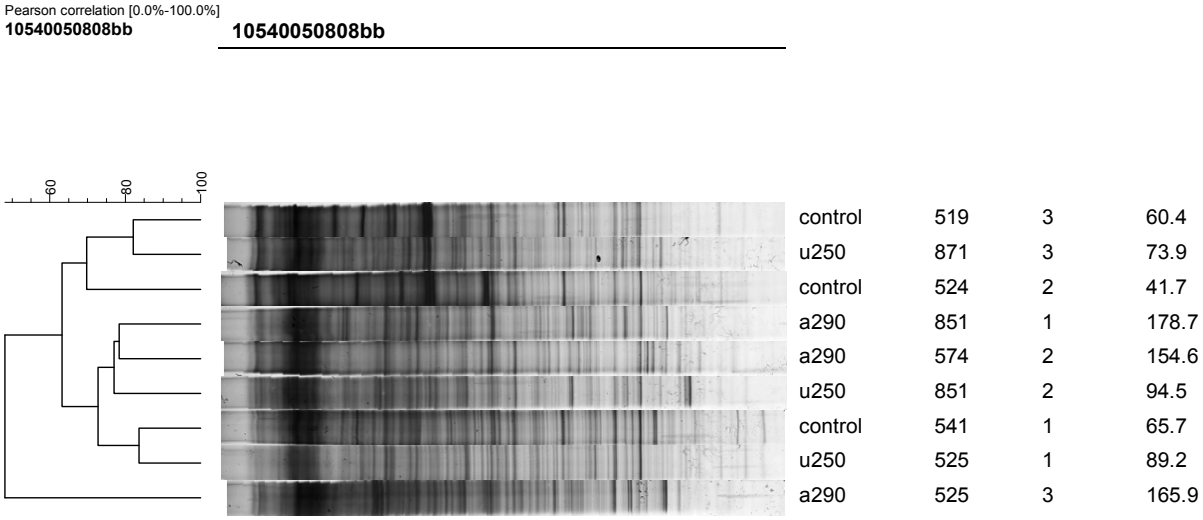

Figure 37. Dendrogram from profiles (liquid phase) from controls and the highest supplement treatments of experiment 3 over runs 1, 2 and 3. The information columns included are the treatment, animal number, run number and EMCP values (g MCP/kg DOM) respectively.

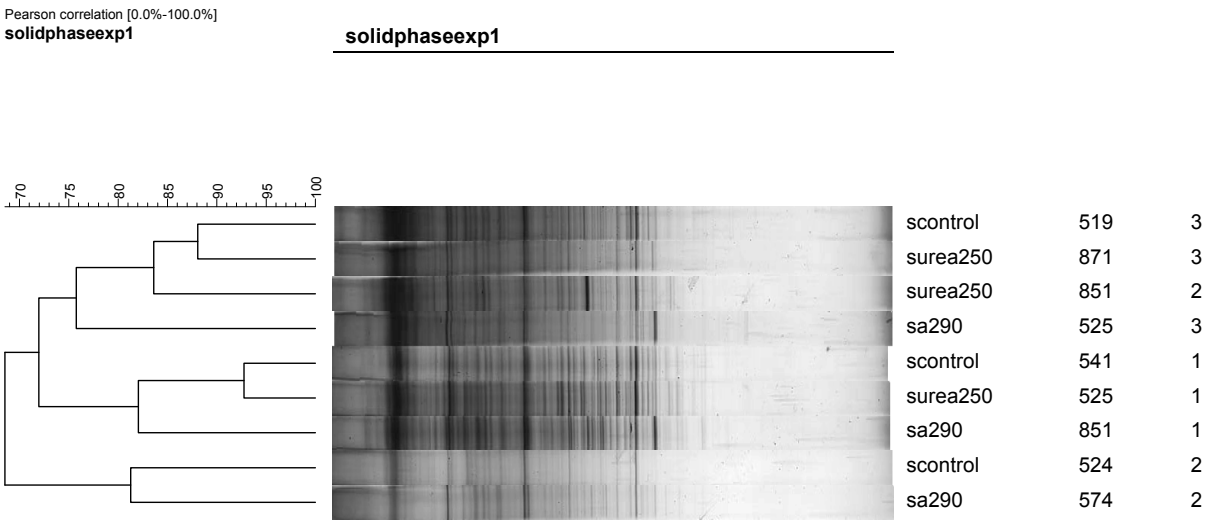

Figure 38. Dendrogram from profiles (solid phase) from controls and the highest supplement treatments of experiment 3 over runs 1, 2 and 3. The information columns included are the treatment, animal number, run number and EMCP values (g MCP/kg DOM) respectively.

Animal comparisons over the three runs. The DGGE profiles of all animals across the three runs were compared over three DGGE gels, so as to determine any carry over treatment effect and to

determine any animal effect. It was of particular interest to observe if the striking and unusual bands found in control run 2 and 3 animals (524 and 519 respectively) were found in these animals given other treatments. These bands only appeared in the controls of which the diet was of poor quality, but where the animals had previously been fed a diet of adequate N (either US130 or S130) on the preceding run. These bands disappeared when the animal was supplemented with N in the next experimental treatment run within the incomplete latin square design. Dendrograms (Figure 39) of the animal comparison DGGE gels illustrate a tendency for individual animals to cluster over the three runs regardless of treatments. This is evident in animal 541, 442 and 553 where their similarity was greater than 80% (effectively identical profiles). Animal 851 also clustered over the three runs but only at 70% similarity. Exceptions of within animal clustering were animals 524 and 519 when fed the control basal diet (run 2 and 3 respectively) and animals 574 and 525 when supplemented with high algae (run 2 and 3 respectively). It appears that at very high EMCP (S290 treatment) or very low EMCP (control), profiles tended to cluster as a treatment and separately but were not similar within treatments (<70%). These results suggest in cattle a genetic, or animal, effect on rumen microbial profiles. These profiles stay similar for each animal across treatments if they are supplemented with medium levels of either US or Spirulina algae. However the genetic or animal effect on profiles is overridden when animals are fed either a very low quality diet (control) or high levels of supplements (S290 and US250).

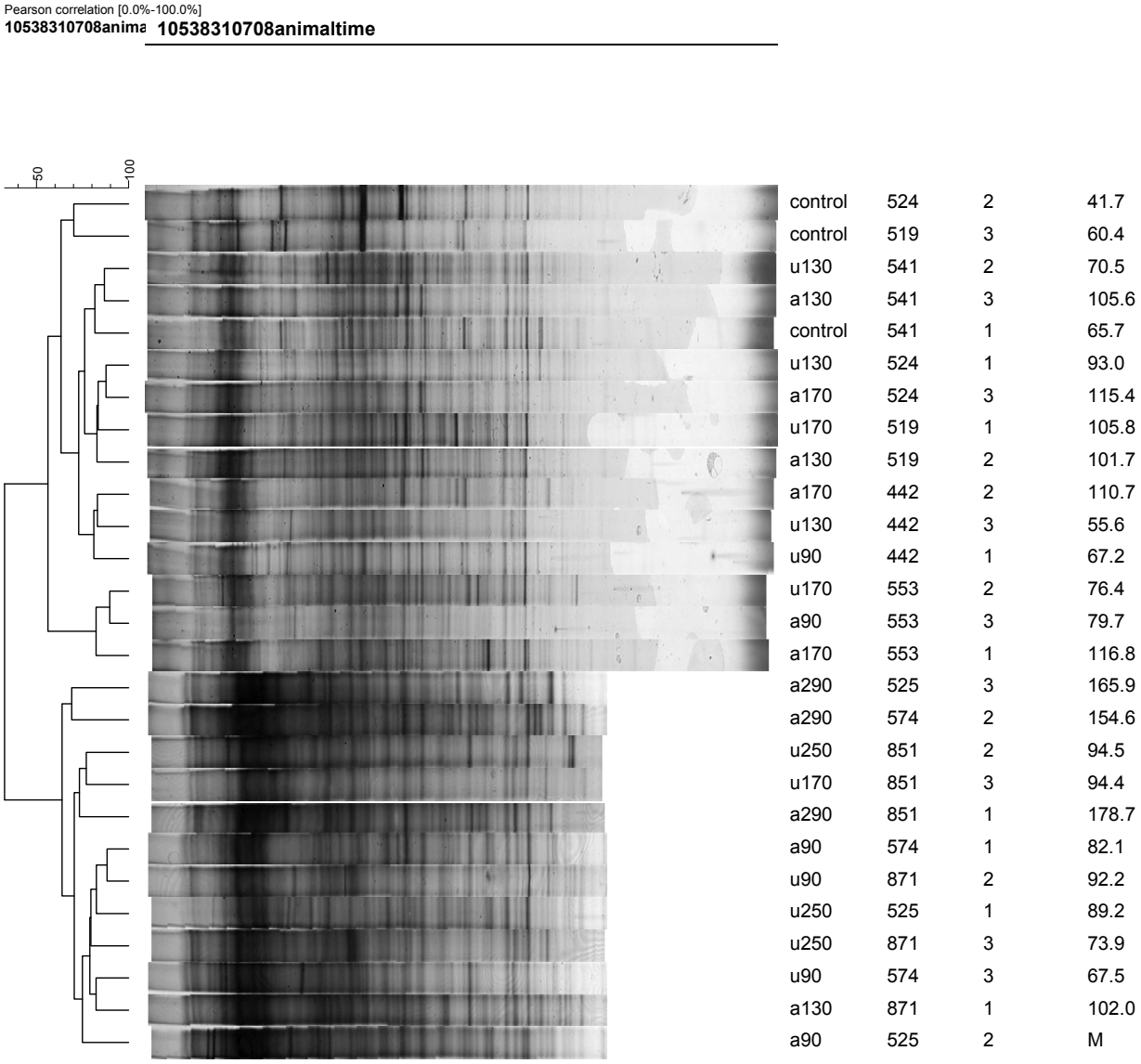

Figure 39. Dendrogram of controls and all treatments of experiment 3 over runs 1, 2 and 3. The information columns included are the treatment, animal number, run number and EMCP values (g MCP/kg DOM) respectively.

Urea supplementation. Figure 40 illustrates the effects of urea supplementation at all levels. Cluster analysis separated control run 2 and control run 3 from all other treatments at a 70% similarity. Control run 1 was separate from the controls of other runs and separate from urea treatments. Profiles for all levels of urea supplementation were very similar but higher urea treatments (US170 and US250) formed 2 sub-clusters. This similarity is also reflected in similar EMCP values. There did not appear to be any time/run effect.

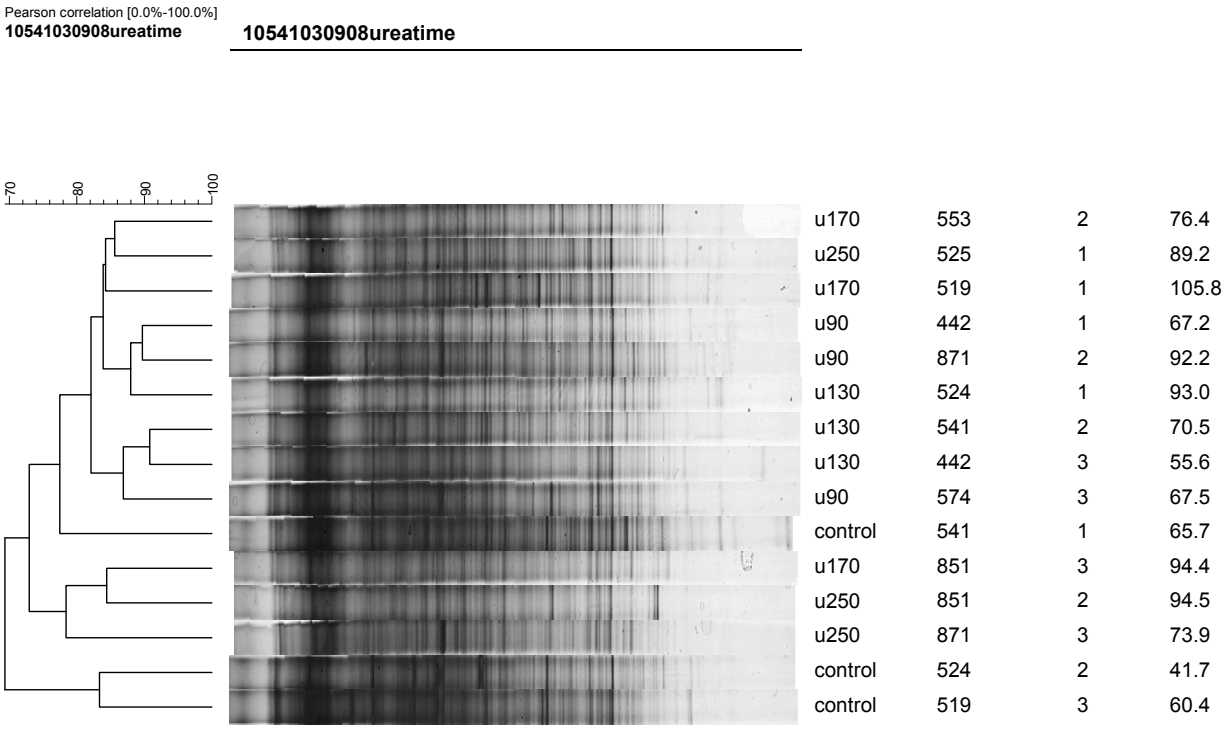

Figure 40. Dendrogram of controls and urea treatments of experiment 3 over runs 1, 2 and 3. The information columns included are the treatment, animal number, run number and EMCP values (g MCP/kg DOM) respectively.

Algae supplementation. Figure 41 illustrates the effect of Spirulina algae supplementation. Cluster analysis separated the profiles of S290 treatment in run 2 and 3 from the controls and other levels of algae supplementation. The S290 treatment from run 3 demonstrated less than 50% similarity to the other treatments and S290 from run 2 was of only 60% similarity to the other treatments. Controls from run 2 and 3 clustered together. These controls had the lowest EMCP values. Control in run 1 and the lower levels of Spirulina algae supplementation (S90 and S130) formed a separate sub-cluster with a similarity of over 82%. This corresponded to EMCP values of between 65.7 to 115.4 g MCP/kg DOM). There did not appear to be any time/run effects.

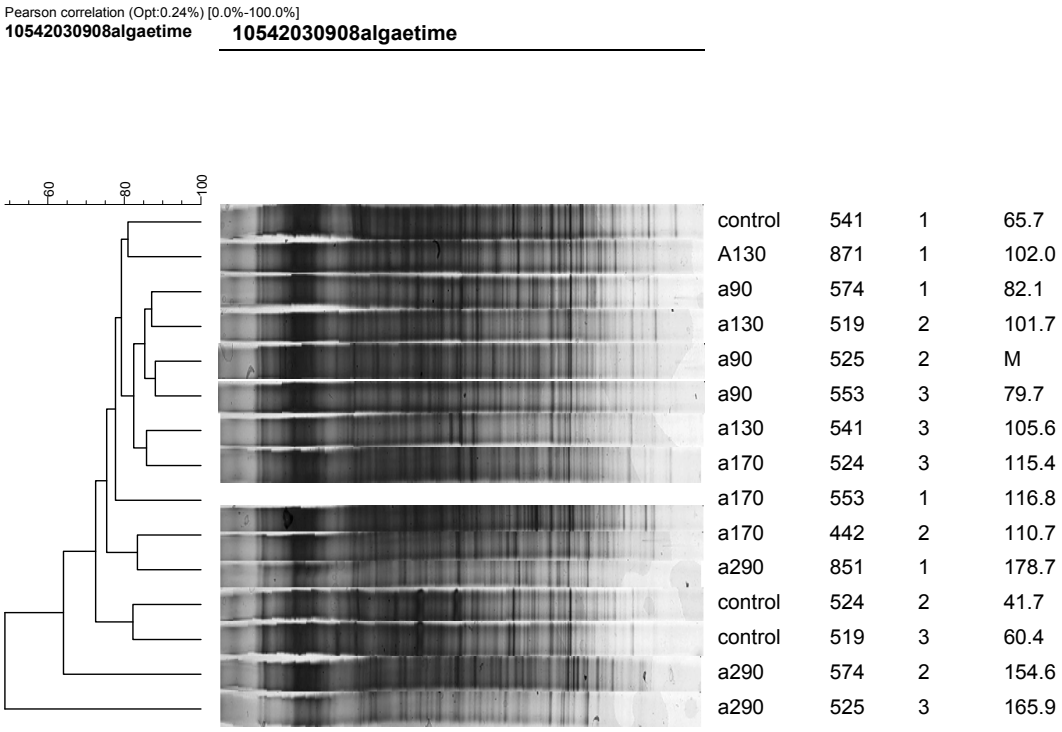

Figure 41. Dendrogram of controls and Spirulina algae treatments of experiment 3 over runs 1, 2 and 3. The information columns included are the treatment, animal number, run number and EMCP values (g MCP/kg DOM) respectively.

In conclusion, it would appear that there is an overall core of dominant bacteria in the rumen that, while generally similar between animals, are impacted on by an animal effect suggesting that the genetics/physiology of individual animals exert an influence on the rumen ecosystem. However, this influence is over-ridden at either high or low levels of N (as urea or Spirulina algae) in the diet. Most interestingly, the profiles at high and low planes of N supplementation appear to cluster relative to high and low EMCP values and this may suggest a correlation between bacterial community structure and EMCP at the extremities of the range. The species compositions of these bacterial communities will be defined using new next generation sequencing technologies (454 sequencing).

4.6.4 Experiment 4. Microbial genetic profiles in animals selected for growth rate on low quality diets

Dominant bands identified in previous studies are apparent in this experiment (Figure 42). Common dominant bands exist between individual animals, regardless of treatment or post-weaning growth rate. Differences in band intensity are apparent in steers divergent in post-weaning growth. However, the trends are inconsistent between nutritional treatments and experimental runs. Cluster analysis is still to be undertaken in this experiment and further analysis of the microbial genetic profiling in this experiment will be completed by a PhD student using new next generation sequencing technologies (454 sequencing). This is expected to be completed in 2012. The DGGE

pattern suggest that the results are similar to the previous experiments in that a core band of species are common but that some bands (species) are distinct for individual animals.

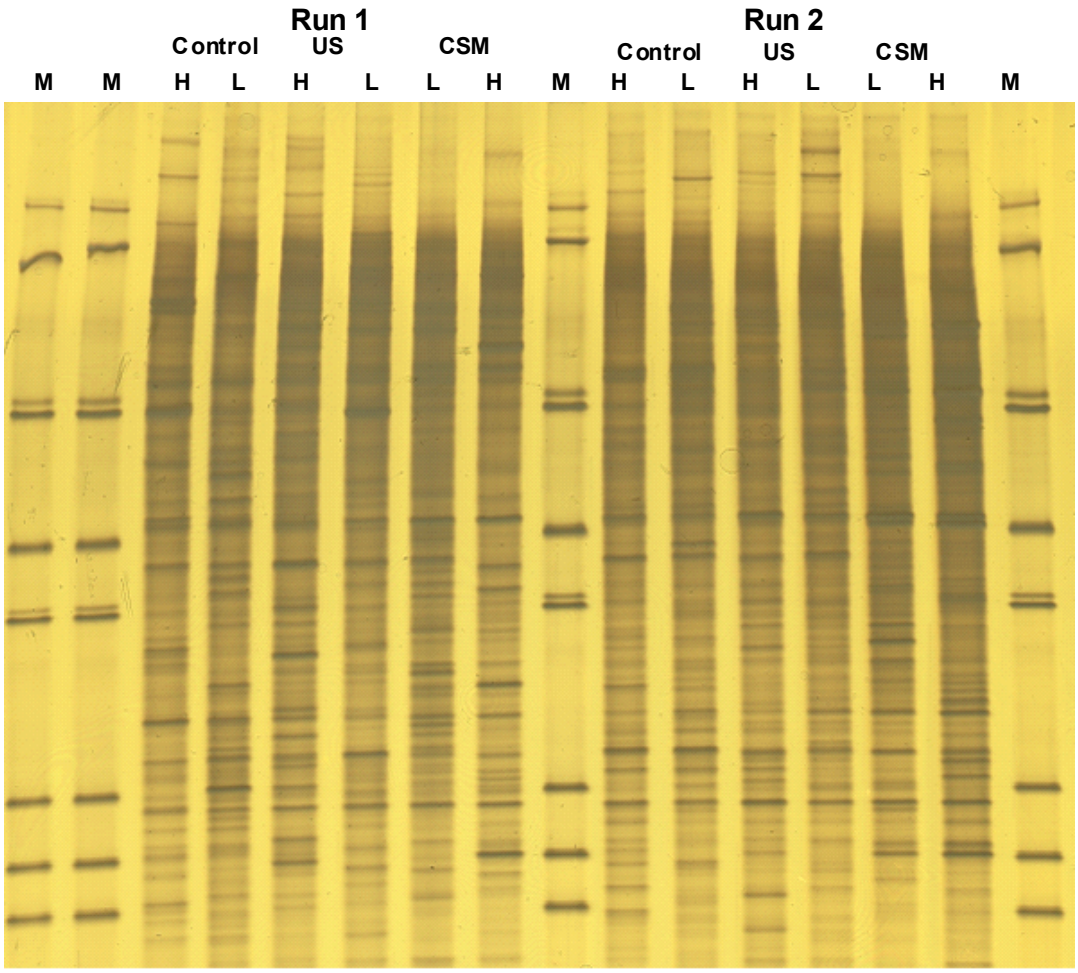

Figure 42. Microbial genetic profile within the rumen of steers divergent in post-weaning growth rates (Moderate (H) or low (L) relative growth rates) fed Mitchell grass hay (Control) or Mitchell grass hay supplemented with urea-ammonium sulphate (US) or cottonseed meal (CSM). Each lane is the microbial genetic profile of a different individual animal over two consecutive runs, offered the highest level of each of the two supplements.

4.6.5 Experiment 5. Microbial genetic profiles of animals consuming different forms of N supplement (including algae)

Common dominant bands exist between individual animals, regardless of treatment (Figure 43). Dominant bands are relatively stable and do not appear to be influenced by treatment. The dendrogram (Figure 44) shows that the results are consistent with previous experiments which describe a relatively stable microbial genetic profile of the dominant species within the rumen of cattle fed different forages or supplements. These dominant bands appear to be consistent between experiments.

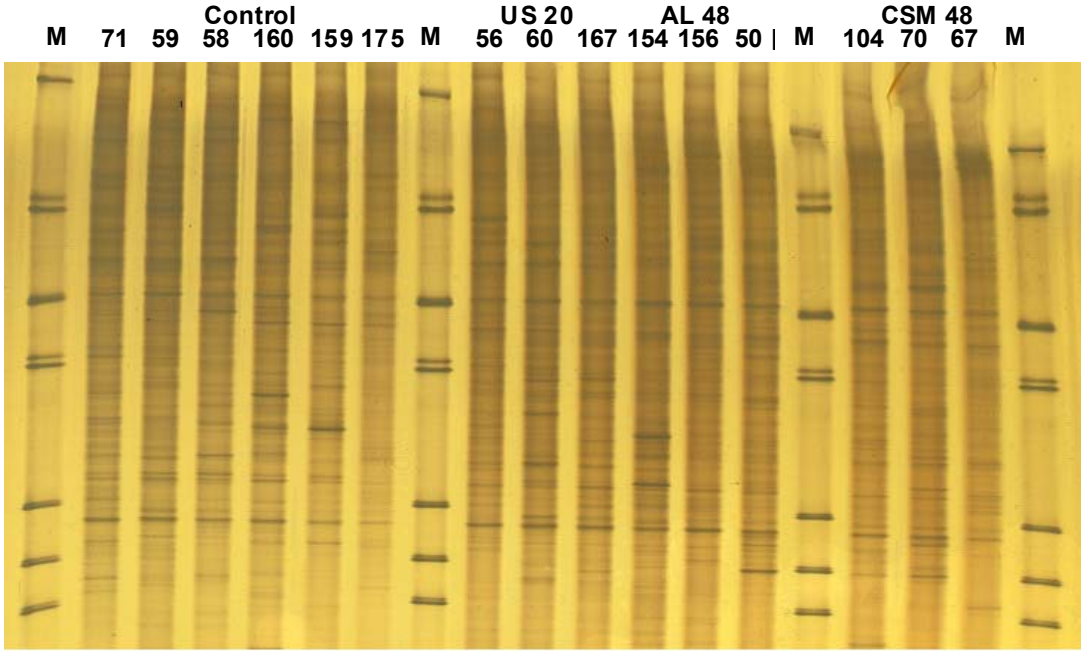

Figure 43. Microbial genetic profile within the rumen of steers fed speargrass hay (Control), or speargrass hay supplemented with urea-ammonium sulphate (US20; 0.2 g N/kg W/d), Spirulina (AL48; 0.48 g N/kg W/d) and cottonseed meal (CSM48; 0.48 g N/kg W/d). Each lane is the microbial genetic profile of an individual animal with animal identification number given on each lane.

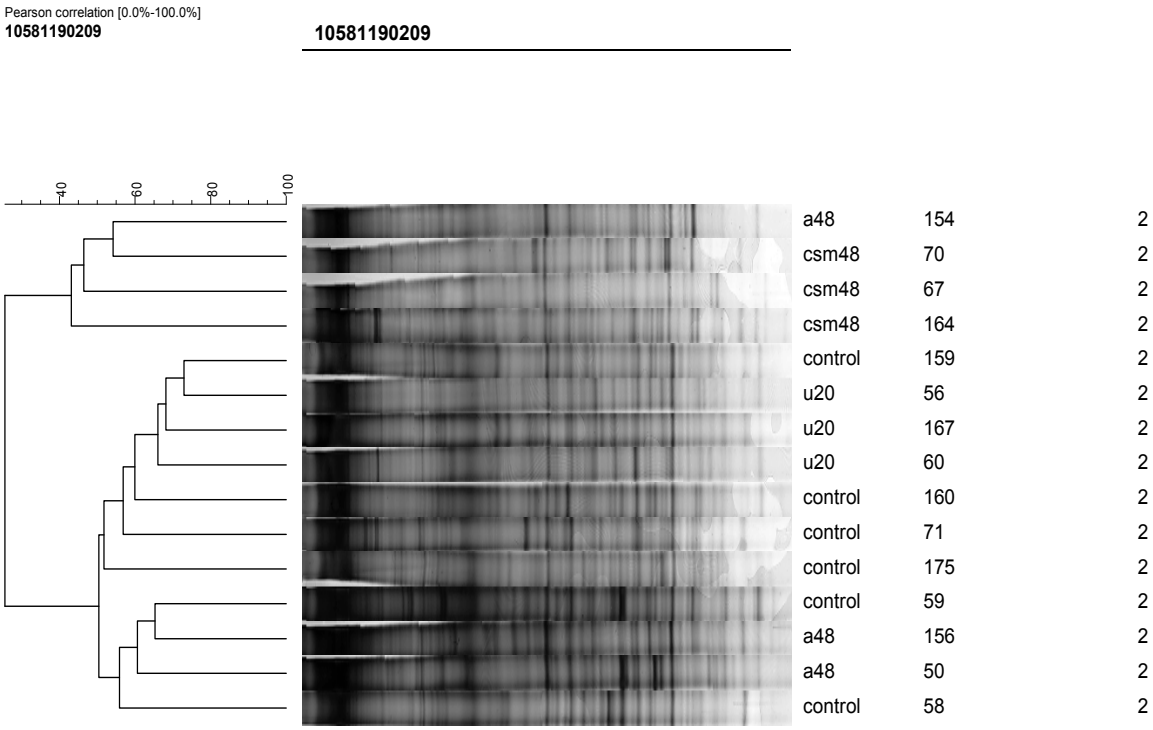

Figure 44. Dendrogram of controls and high treatments of experiment 5. The information columns included are the treatment, animal number respectively.

4.6.6 General conclusions and future directions of the microbial genetic profiling work within this project

Across all experiments it appears that the dominant species within the rumen microbe population are relatively stable under different feeding strategies. It is, at this stage, unknown what changes have occurred amongst the less dominant species and the impacts this may have on the entire rumen microbe population and MCP production. It does appear that variation in the MGP between individuals within a treatment may be greater than variation between treatments. This is somewhat reflected in the results from experiment 4, where animals growing differently but fed the same diets presented different MGP. The reasons for this individual to individual animal variation in MGP and the implications for production warrant further investigation.

Work on the bacterial community profiles using new procedures will continue so as to identify the bacterial species from samples collected from animals in this experiment and it is expected that from all of the experiments within the project a core of dominant bacteria will be identified as will those that are in communities that apparently correlate with the higher EMCP values. This more detailed analysis by PhD students will be completed within 2 years. This analysis is in addition to that expected from the milestones of this project but which will add considerably to the interpretation of the current results. These will be reported separately to MLA.

5 Success in achieving objectives

5.1 Objective 1. Determine the effects on animal production and microbial protein production of key nutritional manipulations and correlate these with changes in rumen microbial populations

This objective was achieved. Five experiments used various nutritional manipulations to examine the response of intake and microbial protein production. A feature of the experiments was the very high levels of supplement added in Experiment 2 where very high levels of casein (RDP) and branch chain amino acids (BCAA) plus phenylalanine were used. These treatments had been used before but not at these levels. There were no significant responses to these supplements at these high levels which could be applied practically. A novel single cell protein source, Spirulina algae was used in Experiments 3 and 5 with significant increases in MCP production and liveweight gain. Four forages varying markedly in CP were used in Experiment 1 with marked differences in MCP production. Two groups of animals selected for divergent liveweight gain were used in Experiment 4 but there were no differences in rumen function to explain this difference in liveweight gain. The differences appeared to be related to metabolic reasons based on IGF-1 concentration. The rumen bacterial population was studied in all these experiments and, while there were some differences in specific species present due to supplement and individual animals, the bulk of the dominant bacterial species appeared relatively constant across all experiments. When animals shifted from high N supplements to low dietary N there was a marked increase in scavenger species of microbes. The population profile, examined by dendogram analysis, indicated that within an individual animal the profile remained relatively constant irrespective of treatments but that there were marked differences between individuals or clusters of individual animals. Thus MCP production and EMCP appeared to be related more to the factors which affect growth of microbes rather than the species present within the dominant bacterial profile.

5.2 Objective 2. From a knowledge of these changes in rumen ecology, target those bacterial species associated with high efficiencies by using specific nutrient manipulations and quantify these effects in terms of MCP production and animal production.

This objective could not be achieved as the species changes were not marked in Experiment 1 and so no single species could be targeted. The alternative approach taken was to target particular types of bacteria with nutrients that from the literature were hypothesized to affect their growth and presumably presence and dominance within the rumen bacterial profile of species. The cellulolytic bacteria were targeted in Experiment 2 and although we were successful in changing the BCFA concentration in the rumen with high RDP or BCAA supplements, there were no significant effects on MCP production. There were some minor shifts in bacterial species and novel bacterial species were identified as being among the dominant bacterial species present in the rumen. The objective of providing a package of nutrients through an algal supplement was examined in Experiment 3 with Mitchell grass and this was very successful in increasing MCP production. In Experiment 5, this was extended to examine the liveweight gain response of weaner steers consuming speargrass hay and at levels of supplementation of approximately 4 g algal DM/kg W/d there was a significant increase in liveweight gain of approximately 0.95 kg/d (from -0.16 kg/d to + 0.79 kg/d) similar to a CSM supplement. Cottonseed meal gave a better liveweight gain response than algae at lower levels of supplementation. Supplementation with Spirulina algae was the most successful of all the new strategies studied.

The dominant bacterial populations appeared similar across this wide diversity of experiments and feed types. There were some specific changes and novel bacteria identified in response to treatments. The most successful treatments (Spirulina algae and CSM) did not differ in bacterial populations nor was there any one or more group of bacterial species which markedly changed (increased or decreased) in response to these treatments especially when there were marked changes in rumen dilution rate. Only at very high levels of N from urea or algae were there some shifts in bacterial populations. This indicates that the increase in MCP production and EMCP was more related to factors which affect growth of bacteria (total nutrient supply and rumen dilution rate) rather than major shifts in the population of bacteria. The marked shifts in the population of bacteria that occurred appeared to be related to large dietary changes when animals moved from high N supplements to low dietary N and scavenger species of bacteria increased in the population profile. Individual animal differences in specific bacterial population profiles were at least as great as the changes seen with dietary manipulation except at very high N levels of supplementation. The bulk of the bacterial species appeared relatively constant across all dietary manipulations.

5.3 Objective 3. Develop nutritional strategies based on these findings to increase microbial protein production, intake and hence liveweight gain of cattle at pasture

This objective could not be achieved given that there were no major changes in the bulk of the bacterial species present. Instead, as part of the process of generating treatment differences in specific nutrient supply by which to examine changes in bacterial species, the effect of these specific nutrients or package of nutrients on MCP production, EMCP and liveweight gain was examined. The development of response curves of liveweight gain to low levels of Spirulina supplementation was very successful. The response curves to both Spirulina algae and CSM was done over a very low range (0 to approximately 5 g/kg W/d). It is not known what would happen with supplementation at higher levels or at pasture. These levels were chosen to have application for algal inclusion within algal ponds associated with watering points and so algae could be supplemented via the drinking water. However at levels of algal bloom in ponds (maximum of 1.2 g DM/L) this would only provide approximately 0.2 g algal DM/kg W/d, and from the response curves which we have developed, this level is far too low to give a significant liveweight gain response. It would be possible to supplement algae in a similar arrangement to a urea water medicator but the source of the algae would need to be external to that grown within an algal bloom pond. The results of Experiment 2 indicated that amino acid supplementation in the drinking water would not result in extra liveweight gain even in the presence of adequate N from urea.

6 Impact on meat and livestock industry – now & in five years time

6.1 Impact on meat and livestock industry – now & in five years time

This project has shown that algae (Spirulina was used here) is a valuable supplement for cattle at pasture. It appears highly degradable in the rumen and at low levels is inferior to cottonseed meal and similar to urea. However, algae can be fed at high levels, unlike urea, and so much higher responses can be obtained. At these levels (approximately 4-5 g algal DM/kg W/d) the response is similar to cottonseed meal. The use of algal ponds to supplement cattle on the rangelands is not feasible to deliver supplement as the highest concentration of algae in such ponds is too low to deliver modest amounts through the drinking water. Nevertheless, algae might be a safer way than

urea to deliver high amounts of N and avoid urea toxicity through water medicators and algal ponds could achieve that. At present the technology for on farm use of such ponds is too difficult for routine use and the potential for inappropriate algal species to bloom with toxicity issues would need to be fully researched and addressed. However, there is potential to develop algal ponds especially on the rangelands as a way to produce protein as a supplement. The current cost of algae is based on their use as a human food with quality assurance requirements. This means the cost is prohibitive with no immediate application to the beef industry. Other sources of algae are becoming available from C sequestration and biofuel industries and their cost structure will be much more competitive with traditional feed protein sources. The results of this project indicate that these algal sources will have a valuable role and the response curves developed here can have immediate application in using the algal sources as a supplement. Algal species differ in their CP and lipid content and fatty acid composition. There will be a need to evaluate the different species used by these industries.

Rumen degradable protein at high levels increased microbial protein production but the levels required and the potential sources prohibit this approach as a means of increasing liveweight gain. The use of traditional protein meal (e.g. cottonseed meal used here) and novel sources such as algae provide a practical mechanism to deliver changes in microbial protein production. Branch chain amino acids, even at very high levels, did not change microbial protein production so their use in water medicators to increase microbial protein production does not seem feasible.

The microbial genetic profiling indicated that the bulk of dominant bacterial species in the rumen were relatively constant across a wide range of diets and treatments but interestingly individual animals could be differentiated into groups of animals having similar profiles of bacterial species. Individual animal differences in bacterial population profiles were significant. There were a number of novel bacterial species identified and some specific species appeared or disappeared with various treatments. This work is continuing with new methodology. This is long term work more directed towards understanding exactly what is present within the rumen and how these species respond to forages and supplements. However we now know which are the main species contributing to the microbial protein absorbed by the animal and that the bulk of these are relatively similar across a wide range of forages and supplements of relevance to the cattle industry of northern Australia.

7 Conclusions and recommendations

7.1 Conclusions

1. Algae (Spirulina) appeared an excellent external protein source to increase microbial protein production in the rumen and liveweight gain of weaner steers. It is highly degradable and needs to be fed at levels approximately 4-5 g DM/kg W/d for results to exceed simple N addition through urea.
2. Efficiency of microbial protein production (EMCP) was very difficult to increase from these low CP forages and addition of very high amounts of BCFA or degradable protein had little effect on EMCP. In contrast, a package of nutrients delivered through algae had a large significant effect on microbial protein production.
3. A collation of the data across all experiments and the literature indicated that increasing the supply of rumen degradable protein increased EMCP but the effect was most marked where dilution rate was also increased and or very high dilution rates were in operation. Simple nutrient addition such as casein protein or BCAA had no effect on dilution rate whereas algal supplementation markedly increased dilution rate. It appears that nutrient addition is

important but the conditions in the rumen relating to dilution rate and hence fractional growth rate of microbes and maintenance requirements are much more important. Dilution rate is high with temperate forages but very low with low CP tropical forages.

4. There were no differences between weaners selected post-weaning on liveweight gain in their intake, digestibility, rumen function and dominant microbial species. The only difference was in serum IGF-1 concentration which suggests that the differences in liveweight gain relate to metabolic differences, not differences in rumen function and microbial species. There was no difference between the two groups of weaners in response to a urea based supplement but small differences in response to a cottonseed meal supplement.
5. The dominant bacterial species were relatively stable across this wide range of diets types and supplements and there were many novel species, not previously described, which contributed the bulk of the microbial protein produced by cattle. Some of these species which increased or decreased in response to specific nutrients were identified. Individual animal differences in bacterial profiles within the rumen were apparent and animals could be grouped on the basis of these profiles into animal populations having similar bacterial profiles. High dietary N over-rode the animal effect.
6. Fractionation of samples into liquid and three levels of plant–associated or attached populations appear unnecessary as the three “attached” profiles were effectively identical. The liquid fraction and a single firmly attached fraction are sufficient to adequately cover a description of bacterial diversity.
7. Some bacterial species do appear to be diet specific when high levels of supplements are fed, particularly algae, which may be important contributors to the elevated EMCP values in these cattle. The identity of these species will be determined in due course.
8. It is apparent that extreme populations of protein-scavenging bacteria can proliferate within the rumen when the diet of animals change from a high level of N supplementation to one of a low CP forage without N supplement. These bacteria are Spirochaetes and a member of the *Bacteroidetes* phylum. While they are entirely associated with the liquid fraction, they do not appear to increase EMCP.
9. Profiles at high and low planes of N supplementation appeared to cluster relative to high and low EMCP values and this suggested a correlation between bacterial community structure and EMCP at the extremities of the range.

7.2 Recommendations

1. Algae are a valuable protein source for cattle and sources from C sequestration and biofuel industries should be identified to provide alternative protein meals to the current plant protein meals available to the industry.
2. The development of algal ponds and means of harvesting algae for animal use on farm should be researched. This also has the potential to deliver algae through water medicators and this application should be researched. At present the technology is not robust and also the levels which can be delivered by this route are inadequate for practical increases in liveweight gain.
3. Methane production under algal supplementation is not known. The use of by-products from the C sequestration and biofuel industries with the methane reduction/unit of product associated with increased liveweight gain suggests the overall C footprint of using algae would be beneficial in meeting C emission targets. Species of algae differ in lipid levels and

in fatty acid composition so there may be species specific effects on methane production which should be evaluated.

4. Only *Spirulina platensis* was studied here. In an associated study, *Chlorella* spp. was used with similar effects on rate of digestion and rumen ammonia but microbial protein production and liveweight gain were not studied. These are freshwater species. The effects of marine species, especially those associated with C sequestration and biofuel industries, should be studied. Further response curves need to be developed to confirm the single liveweight gain study reported here.
5. Marine aquaculture and the C sequestration and biofuel industries tend to use marine algal species. These species are generally higher in lipid and have quite different fatty acid profiles. They are usually higher in CLA and the longer chain ω-3 fatty acids (EPA and DHA). In other animal species and dairy cows, these are known to alter fatty acid composition of meat and milk, lamb vigour at birth and alter rate of fat synthesis within intra-muscular, subcutaneous and mammary tissue. All these differences could be exploited for positive effects with rangeland cattle.
6. The fatty acid profile of mixed bacteria from *Bos indicus* are known to differ from *Bos taurus* cattle suggesting different microbial populations. Some of these same fatty acids as appear in algae (see above) are known to depress fat synthesis. The role of these fatty acids and the differences between cattle breed types in fat synthesis and re-alimentation of cattle in the wet season should be investigated.
7. The “core” or fundamental profile of dominant bacteria that appear to be important for digestion of tropical pastures by cattle in northern Australia should be fully defined (probably by higher throughput 454-pyrosequencing) so that future variability of this community as it links to EMCP and nutrition of cattle can be better understood and manipulated where required. An associated study by our group has indicated that there are also differences in the bacterial species which are dominant within the rumen compared to those which flow from the rumen and are absorbed by the animal. To be able to manipulate microbial protein flow from the rumen we need to be able to understand rumen ecology (what species are present and why) and factors which affect their growth and their flow from the rumen.

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9 Appendices

9.1 Appendix 1. Detailed materials and methods

9.1.1 Experiments 1 to 5

Feeding and digestibility. Daily feed intake was determined by measuring the amount of feed offered and refused, at the same time each day. Feed offered and feed residues were bulked over each run, sub-sampled and stored to determine DM content. Faecal output was weighed at the same time each day during the collection period, sub-sampled, bulked and frozen at -20^{o}C before drying to a constant weight in a fan forced oven at 60^{o}C. Urine output was collected in buckets containing 10% H_2SO_4 under the metabolism crates and weighed at the same time each day during the collection period. Sub-samples were bulked over the collection period and frozen at -20^{o}C.

Rumen parameters. Rumen fluid pH was determined immediately upon collection. Duplicate rumen fluid samples were added to 20% metaphosphoric acid (internal standard buffer solution) and stored at -20^{o}C for VFA analysis. Additional duplicate rumen fluid samples were added to HCl to pH <3.0 and stored at -20^{o}C for NH_3-N analysis.

Fractional outflow rate and rumen retention time. Retention time of rumen fluid and digesta was determined using Cr-EDTA and $YbCl_3$ markers, respectively. A single dose of Cr-EDTA solution was administered via the cannula (experiments 1, 2.1, 2,2 and 3) at 55 mL/100 kg W (ca. 154 mg Cr/100 kg W), across four different sites in the rumen of each steer, or mixed with feed (experiment 4; 1.7 g Cr/steer). At the same time, Yb-labelled hay (2 g Yb/steer) was evenly distributed throughout the rumen via the cannula (experiments 1 and 3) or mixed with the feed (experiment 4). Duplicate rumen fluid samples (ca. 10 mL) and single rumen digesta samples (ca. 100 g) were taken prior to dosing (0 h) and at intervals indicated earlier for each experiment and stored at -20^{o}C. The pH of rumen fluid was determined immediately at collection. In some experiments, retention time was also measured by complete evacuation of digesta from the rumen. Total rumen contents were removed by hand, weighed and mixed thoroughly before sub-samples were collected to determine DM content and chemical composition. Retention time of the rumen contents was estimated as described by Minson (1966).

Rate of digestion. Dry matter disappearance of incubated materials in the rumen was determined by measuring residual substrate of the sample and plotting disappearance of DM against time with the disappearance data fitted to the equation

$$Y = a + b (1 – \exp (-ct))$$

Where,

- a = immediately soluble fraction
- b = slowly fermentable fraction
- a + b = potential degradable fraction
- c = degradation rate (%/h)
- t = incubation time (h)

The effective degradability (ED) in the rumen is the function of the size of pools a and b, rate of degradation (c) of pool b and the rate of passage (kp) of pool b from the rumen (Ørskov and McDonald, 1979).

Chemical composition of feeds, residues and faeces. Dry matter was determined by drying samples to a constant weight at 60^{o}C in a forced fan oven. Organic matter content was determined by combusting samples in an electric muffle furnace (Carbolite; England) at 550^{o}C for approximately 4.5 h. The Ankom fibre analyser (ANKOM 220; USA) was used to measure ash free-NDF, ash free-ADF and lignin based on the method of Van Soest *et al*. (1991). Total N content was measured using the Leco system (LECO FP-428; USA) and CP content calculated (N x 6.25).

Analysis of rumen Ammonia-N and volatile fatty acids and plasma urea-N. Ammonia-N concentration in rumen fluid was measured by distillation using a Buchi 321 distillation unit (Buchi Scientific Apparatus Flawil; Switzerland) with saturated sodium tetraborate (>260 g/L) used to adjust pH. The distillate was titrated (TritaLab 840, radiometer; France) with 0.01 M HCl to calculate total N. Volatile fatty acids and branch chain fatty acids (BCFA) were determined by gas chromatography (GC-17 A, Shimadzu; Japan) using a polar capillary column, automatic injector and flame ionization detector. Plasma urea-N concentration was determined spectrophotometrically at 340nm and 37^{o}C, using BUN reagent (Trace Scientific; Australia).

Analysis of Chromium and Ytterbium markers. Chromium in rumen fluid was analysed after centrifugation at 2500 *g* for 5 min, the supernatant was removed and further centrifuged at 2500 *g* for 7 min. Ytterbium was analysed in dried, ground whole digesta by the method of de Vega and Poppi (1997). Background matrix was prepared for standards and all samples were analysed for Cr-EDTA and Yb by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES; USA).

Analysis of purine derivatives in the rumen. Acidified urine samples were thawed, mixed and diluted (1:20) in stock buffer containing 10% $NH_4H_2PO_4$ solution containing an external standard, allopurinol (100 µM). Prior to analysis, diluted urine samples were filtered through a 300 mg C-18 Sep-Pak cartridge. Solutions of known concentrations of allantoin, uric acid, xanthine and hypoxanthine were used to produce standard values, as described by Balcells *et al*. (1992). Purine metabolites (i.e. allantoin, uric acid, xanthine and hypoxanthine) in urine samples were determined by HPLC (Agilent 1100 Series; USA). Separation and quantification were achieved by using a Bondaclone C-18 reversed-phase column (300 mm x 3.9 mm i.d.) (Phenomenex; USA). Data were analysed using Agilent 'Chemstation' software (USA). Estimation of microbial crude protein production was based on the method of Chen and Gomes (1995) and used the endogenous purine excretion for *Bos indicus* cattle of 0.190 $W^{0.75}$, as proposed by Bowen *et al*. (2006). A spectrophotometric method for the analysis of allantoin was also used in some experiments to confirm HPLC analysis.

9.1.2 Microbial genetic profiling across all experiments.

Fractionation of rumen samples. For the liquid phase fraction, a portion of rumen fluid was immediately filtered through a nylon stocking. The filtered portion was well mixed and from each sample 1 mL subsamples were aliquotted into four labelled 1.5 mL microcentrifuge tubes and immediately placed on ice. Samples were centrifuged at 16000 x *g* for 20 min at room temperature. The supernatant was removed and discarded whilst remaining microbial pellets were retained and stored at -20°C prior to gDNA extraction. This fraction was designated the liquid planktonic phase. The solid phase fractions were collected concurrently to liquid samples. Unfiltered rumen contents were placed into two 100 mL containers and immediately frozen at -20°C until further processing in the laboratory. Samples were thawed and squeezed through a nylon stocking and the liquid fraction discarded. The remaining sample was combined with 250 mL of Tris-EDTA (TE) buffer (10mMTris-HCl; pH 8.0; 1mM EDTA) and processed with a blender for 30 sec. Blender walls were scraped, mixed and blended for another 30 sec. This was repeated twice more so that the total homogenisation time was 2 minutes. A portion of this material was then filtered through a nylon

stocking. Four 1 mL aliquots of this filtered liquid were centrifuged at 16000 x *g* for 10 min. Supernatant was removed and the remaining pellets were stored at -20°C. This solid fraction was designated as the loosely attached phase. The remaining solid blended fraction was transferred into a modified coffee plunger with nylon and stainless steel filter. The plunger was depressed and the liquid discarded. This solid fraction was resuspended with 150 mL of 1 x phosphate buffered saline solution and shaken for 30 sec. The suspended digesta was transferred into the plunger and the liquid fraction again discarded. The solid phase was resuspended in 150 mL of 1 x phosphate buffered saline solution with the addition of 0.15% v/v Tween-80 (225µL) and placed on ice for 2.5 h. This was then shaken for 30 sec and transferred to the plunger and the liquid fraction discarded. This washed sample was suspended in TE buffer, mixed and 1 mL samples were extracted and expelled into four 1.5 mL labelled microcentrifuge tubes. Tubes were immediately centrifuged at 16000 x *g* for 20 min, the supernatant discarded and pellet frozen. This solid fraction was the strongly attached phase.

Bacterial DNA extraction. Bacterial pellets from the various phases were thawed and subjected to physical disruption using a bead-beating method based on a procedure described by Whitford *et al*. (1998). Bacterial pellets were resuspended in 1 mL lysis buffer (500 mM NaCl; 50 mM Tris-HCl; pH8.0; 50 mM EDTA; 4% w/v SDS) and added to a 2 mL tube containing 0.5 g of 0.1d mm zirconia beads. Tubes were shaken on a Mini Bead-Beater-8 (Biospec Products Inc.) for 3 min. Tubes were then incubated at 70°C for 15 min then centrifuged at 16000 x *g* at 4°C for 5 min. The supernatant was transferred to a fresh tube and placed on ice. This procedure was repeated with the remaining debris and with the addition of a further 300 µL of lysis buffer. Supernatants were then pooled and proteins and polysaccharides precipitated by adding 260 µL of 10M ammonium acetate and incubating on ice for 5 min. Tubes were then centrifuged in a refrigerated centrifuge at 16000 x *g* for 10 min at 4°C. Supernatants were transferred into fresh tubes and an equal volume of isopropanol added. The resulting suspension was mixed, incubated for 30 min then centrifuged at 16000 x *g* for 15 min at 4°C. The supernatant was again discarded and 1 mL of 70% ethanol added to precipitate nucleic acids. This was centrifuged at 20000 x *g* for 5 min at 4°C and supernatant discarded. The remaining nucleic acid pellet was then dried and resuspended in 100 µL of TE (10 mM Tris-HCl; pH 8.0; 1mM EDTA). Final genomic DNA recovery was then completed using a Qiagen QIAamp DNA kit. To verify the presence of DNA, an aliquot of final flow-through was analysed by electrophoresis at 95 V for 45 min through a 1% TBE agarose gel using a 1 Kb DNA ladder as a standard. After verification, gDNA was diluted 100-fold and stored at -20°C prior to PCR amplification.

PCR amplification of V2V3 region of the 16S rRNA gene. Amplification of the V2-V3 region was performed using primers 341F-GC (5'CCT ACG GGA GGC AGC AG 3') and 543R (5' ATT ACC GCG GCT GCT GG 3'). The diluted gDNA, as described previously, was used as a template for all PCR reactions. The V2-V3 PCR assay using FastStart Taq DNA Polymerase was performed according to the manufacturer's instructions (Roche Applied Science). The thermal profile for the V2-V3 PCR was as follows: an initial denaturation at 95°C, 10 sec annealing at 65°C and 30 sec elongation at 72°C. This is followed by 19 cycles of touchdown PCR of 95°C for 30 sec, 65°C for 10 sec and 72°C for 30 sec with a decrement of 0.5°C per cycle. Following this, 11 cycles of 95°C for 30 sec, 55°C for 10 sec and 72°C for 30 sec was carried out followed by a final extension step of 72°C for 5 min. The presence and size of amplicons was verified using electrophoresis (95 V for 45 min) on 2% TBE agarose gel using a 100 bp DNA ladder as a standard marker. A band of approximately 200 bp was considered to be a positive result. PCR products were stored at -20°C prior to DGGE analysis.

Denaturing gradient gel electrophoresis .The amplified fragments were separated by DGGE based on the protocol of Muyzer *et al*. (2004). This was performed using the Bio-Rad D-Code System (Bio-

Rad, Hercules, CA, USA). The PCR products were separated by electrophoresis through an 8% (w/v) polyacrylamide gel with a denaturing gradient ranging from 30 to 60% (Deplancke *et al*. 2000). A 100% denaturant consisted of 7 M urea and 40% formamide. Gels were polymerised on PAG film for ease of handling (GelBond®, Lonza, MC, USA). The electrophoresis was run for 18 h at 60°C at 100 V (Muyzer *et al*. 2004) in 0.5 x Tris-Acetate-EDTA buffer (20 mM Tris, 10 mM acetate, 0.5 mM disodium EDTA, pH 8). After electrophoresis, the gels were silver stained by immersion in a dehydrating buffer (10% ethanol; 0.5% acetic acid) for 5 min, three times followed by immersion in a silver staining solution (0.1% $AgNO_3$) for 10 min and rinsed twice with distilled water. The gel was then soaked in a developing buffer (1.5% NaOH; 0.01%; 0.015% formaldehyde) until bands reached a desired intensity (between 10 to 20 min). Finally, the gel was washed in a fixation solution (0.75% w/v Na_2CO_3) for 10 min. The exposed side of the stained gel was covered in overhead transparency film for protection and the image scanned using a HP ScanJet 6300C scanner equipped with a transparency adaptor (HP ScanJet XPA).

Analysis of Denaturing gradient gel electrophoresis patterns. Denaturing gradient gel electrophoresis band pattern processing was performed with Bionumerics software version 5.1 (Applied Maths, Saint-Martens Latem, Belgium). Banding profiles were processed by initially defining the lanes, calculating a densitometric curve for each lane, designation of reference standards and definition and quantification of bands. A calibration curve was then calculated to translate all band positions into metrics. Cluster analysis of DGGE patterns was performed using the unweighted pair group method with arithmetic averages (UPGMA) method based on the Pearson correlation coefficient.

DGGE band excision, amplification and purification. Bands of interest were excised from DGGE gels with a sterile 100 µL pipette tip. This tip carrying the associated gel plug was then placed into a PCR tube containing V2-V3 PCR mastermix and the template contained within was amplified. Products were verified on a 2% agarose gel then subjected to DGGE to check for band migration to that of the parent band. Products that showed identical migration to that of the parent band were purified using a QIAquick PCR Purification Kit (QIAGEN Pty. Ltd., Australia) as per the manufacturer's instructions and eluted in 32 µL sdH2O.

Cloning of PCR products. Purified PCR products from excised DGGE bands were cloned into the Eco-RI site of the pCR® 4-TOPO®TA vector (Invitrogen, San Kiego, CA, USA) following the manufacturing instructions. The cloning reaction was 6 µL in total and contained 1 µL of salt solution, 1 µL vector, 2 µL sd H2O and 2 µL of purified PCR product. The reaction was briefly centrifuged and incubated for 15 min at room temperature. One Shot® TOP10 competent cells were transformed according to the manufacturer's instructions (Invitrogen, San Diego, CA, USA). Transformed *E. coli* cells were incubated on ice for 20 min and immediately heat shocked at 42°C for 30 sec. SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to cells and incubated at 37°C for 1 h while rotating at 200 rpm in a Bioline 4500 (Quantum Scientific, Queensland, Australia) incubator-shaker. Transformation dilutions (10 or 20 µL of transformed cells plus 30 µL SOC) were spread on pre-warmed selective LB plates (with 50 µg/mL ampicillin) and incubated at 37°C overnight. Five colonies were picked for each clone using a sterile disposable loop and transferred to a new agar plate whilst residual transformed cells were transferred to microcentrifuge tubes containing 50 µL of sdH2O. The inoculated plate was incubated at 37°C overnight. Microcentrifuge tubes were placed in a 70°C waterbath for 5 min to lyse the host *E. coli* cells then centrifuged at 16000 x *g* for one min to pellet cell debris. Subsequently, 1 µL of supernatant was used as a template to amplify the inserted V2-V3 region of interest. Amplification products were checked using a 2% agarose gel with a 100 bp ladder as a standard marker. PCR products were then analysed on DGGE and compared with original bands of interest. Incubated colonies with the correct insert were picked and placed into 6

mL LB + ampicillin broths in sterile falcon tubes and incubated at 37°C for 12-16 h rotating at 100 rpm. Following incubation, 4 mL of broth was removed and centrifuged at 3000 x *g* for 10 min to obtain a pellet for plasmid DNA extraction. Long-term storage of clones was concurrently achieved by mixing 150 µL of sterile glycerol and 850 µL of remaining broth culture in Nunc cryotubes and frozen at -80°C.

Plasmid DNA extraction. Plasmid DNA extraction of the pelleted cells was performed using a DNA QIAprep SPIN Miniprep kit, as per manufacturer's instructions (QIAGEN). Plasmid DNA was eluted in 50 µL of sdH2O and assessed for purity on a 1% agarose gel with a 1 Kb ladder.

Sequencing. The V2-V3 region of cloned plasmids were sequenced using the T7 primer (5'TAA TAC GAC TCA CTA TAG GG 3") with the ABI PRISMTM BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The PCR sequencing program was programmed to a Corbett Palm-Cycler (Corbett Research, Sydney, Australia) and consisted of denaturation for 1 min at 96°C, then 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Reactions were stopped by chilling at 4°C. Sequencing reactions were cleaned-up by adding 1 µL of 125 mM EDTA, 1 µL 3M sodium acetate (pH 5.2) and 25 µL 100% ethanol. After mixing, the reaction was incubated at room temperature for 15 min then centrifuged at 16000 x *g* for 15 min. The supernatant was removed, 35 µL of 70% ethanol added and centrifuged at 16000 x *g* for 15 min. This was repeated and the supernatant removed. Pellets were finally covered in foil and air dried in the dark before transport to the sequencing facility. The pellet was rehydrated and analysed on an automated, capillary sequencer (ABI PRISM 3130xl Genetic Analyser, Applied Biosystems) at the Griffith University DNA Sequencing Facility (Queensland).

9.2 Appendix 2. Acknowledgements

9.2.1 The research team

This work was conducted collaboratively between the University of Queensland (UQ) and Queensland Primary Industries and Fisheries (QPIF). The work was successfully conducted through the contributions of scientists and technicians from both organisations and post-graduate students, enrolled at UQ but based at either UQ or QPIF. We acknowledge the contributions to the project of the following people:

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