final report

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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 (MCP) production in cattle and the response of bacterial species within the rumen of cattle fed various pastures and supplements. Supplementation with Spirulina and Chlorella algae markedly increased MCP production and the efficiency of MCP (EMCP) and liveweight gain of weaner steers supplemented with Spirulina was similar to that of steers supplemented with cottonseed meal at the highest intakes tested in the current project. 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 intake and between individual animals and these changes were associated with differences in liveweight gain. However, weaner steers selected for differences in post-weaning liveweight gain had a similar profile of dominant bacterial species in the rumen when grazing as one mob or when fed the same basal diet and supplements in pens. Microbial protein production appears 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 live-weight 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.

The main findings of this project have been reported earlier (NBP0350, Final Report 30.09.2009), this addendum includes 454-pyrosequencing results from Experiments 3, 4 and 5 (of which some data was reported in the original final report) and two additional experiments conducted on the nutritive value of a range of algae species (Experiment 6) and cattle responses to algae species (Experiment 7),

1. Microbial genetic profile (MGP) in the rumen of steers offered algae or urea supplements determined by 454-pyrosequencing (Experiment 3).

2. Microbial genetic profile in the rumen of steers that were of divergent liveweight gain during the post-weaning period, determined by 454-pyrosequencing (Experiment 4).

3. Microbial genetic profile in the rumen of steers offered algae, cottonseed meal or urea supplements and the relationship with liveweight gain, determined by 454-pyrosequencing (Experiment 5).

4. Chemical composition and relative degradability of a range of algae species and commonly used protein supplements (Experiment 6).

5. Rumen function and MCP production of steers fed a low CP hay supplemented with various algae species and cottonseed meal (Experiment 7).

The main findings provided in this addendum include:

- Supplements of algae (*Spirulina platensis*) significantly increased MCP production and EMCP. No particular bacterial species (or groups of bacteria) were associated with the increased EMCP that was measured but greater diversity in the population of bacteria in the rumen was apparent in steers that received the Spirulina supplement (Experiment 3).
- There was no association between the rumen microbe genetic profile and post-weaning liveweight gain of steers. There was steer to steer variation in the rumen bacterial community but this was not related to post-weaning liveweight gain (Experiment 4).

- Liveweight gain was positively associated with the diversity of the bacterial population within the rumen of steers, as indicated by the number of operational taxonomic units present (Experiment 5).
- Diversity within the rumen bacterial population was associated with N intake but did not vary between supplements *per se* (Experiment 5).
- Increased rumen N supply increased the diversity of the rumen bacteria population but a large number of core bacteria remained relatively stable in response to increasing rumen N supply (Experiments 3, 4 and 5).
- The composition and nutritive value of algae species is highly variable. Algae species may be useful supplements for animal production when used to meet specific nutrient deficiencies (Experiment 6).
- Algae species with a high N content, such as *Spirulina* and *Chlorella*, can be supplied to steers on low CP basal diets with comparable results to traditionally used protein supplements, such as cottonseed meal (CSM) (Experiment 7).

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1 Experiments

1.1 The effect of rumen degradable protein or single cell organism on microbial genetic profiles in the rumen of steers fed a low protein hay (Experiment 3)

Background

This experiment measured the intake, MCP production, rumen function and the MGP of steers fed a low CP hay and supplied with increasing amounts of non-protein nitrogen (NPN) or the single cell micro-algae *Spirulina platensis*.

Experimental design and materials and methods

Details of the experimental design, materials and methods, rumen function and preliminary MGP results are provided in the final report. Briefly, nine Brahman crossbred steers with rumen fistula were used. The steers were approximately 18 months of age and 271 ± 17 kg in liveweight (W) at the commencement of the experiment. An incomplete Latin square design consisting of nine steers, two supplement types over three experimental runs was used. The basal diet was Mitchell grass (*Astrebla* spp.) hay (5.1 g N/kg DM; 766 g NDF/kg DM) supplemented with increasing amounts of urea and ammonium sulphate (US) or Spirulina (114 g N/kg DM; 35 g NDF/kg DM). Feed intake, digestibility, MCP production, rumen function and the MGP were determined.

Results and discussion

Briefly, MCP production and the EMCP increased in response to increasing RDP intake from both supplements, with a higher response measured for the Spirulina supplement. Hay and total DM intake and fractional outflow rate of liquid from the rumen were greater in response to increasing RDP intake from Spirulina than US. Initial denaturing gradient gel electrophoresis (DGGE) screening of the MGP demonstrated that there was an overall core of dominant bacteria in the rumen that, while generally similar between animals, were impacted on by an animal effect suggesting that the genetics/physiology of individual animals exert an influence on the rumen ecosystem. However, this influence was over-ridden at either high or low levels of N (as US or Spirulina) in the diet. Most interestingly, the profiles at high and low planes of N supplementation appeared 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 composition of these bacterial communities was then defined using next generation sequencing technologies (454-pyrosequencing). All indices for richness and diversity were measured at 1000 sequences per sample. Richness and diversity indexes increased with increasing N supplementation but peaked at treatment level 170 g RDP/kg DOM for both Spirulina and US. Higher richness and diversity values were found in the Spirulina treatments (Table 1).

A total of 14 known phyla were identified, with Firmicutes and Bacteroidetes accounting for over 90% of the population. The third largest phylum present in all treatments was the unclassified grouping representing sequences that could not be classified into any known phylum, which accounted for approximately 4% of the population. The remaining

bacteria consisted of low abundance phyla which together represented <3% of the total bacterial community and were not present consistently in all of the treatments. There were 63 core operational taxonomic units (OTUs) identified as being present in 100% of the samples. Forty-one of these core bacteria were Bacteroidetes, nineteen were Firmicutes, one was a Fibrobacter, one was a Proteobacter and the remaining one an unclassified bacteria (Figure 1). Analysis of the core OTUs at the genus level showed they belonged to *Prevotella* (12 OTUs), *Fibrobacter* (OTU 5704), *Oscillibacter* (OTU 4390), *Coprococcus* (OTU 4948), *Pseudobutyrivibrio* (OTU 9864) and *Butyrivibrio* (OTU 11339). A number of core OTUs could only be classified to the phylum Bacteroidetes (17 OTUs), the class Deltaproteobacteria (OTU 267), the orders Clostridiales (OTUs 481 and 5537) and Bacteroidales (OTU 5194), and the families Lachnospiraceae (5 OTUs), Ruminococcaceae (8 OTUs), Prevotellaceae (4 OTUs), Porphyromonadaceae (OTU 4931) and Flavobacteriaceae (OTU 10981). The OTU 5537, in the order Clostridiales, was present as a very large proportion (3-5%) of the bacterial community across all animals. Non-core OTU network graphs (Figure 2) and associated PCA analyses were conducted to investigate the relationship between non-core OTUs in rumen samples and treatment and EMCP. These data sets were each colour coded and show a very high proportion of shared non-core OTUs. The samples clustering closely together in the middle of the network (large white diamonds) are a function of the large number of shared OTUs. There was no clear clustering with respect to treatment or EMCP.

The rumen bacterial community structure of steers at both a phyla and genera level were largely stable in spite of different supplements and large changes in rumen function associated with increases in MCP production and EMCP. The similarity of bacterial community structure between treatments indicates that improvements in MCP production were related more to better growth conditions for all bacteria as a whole. It appears that supplementation with Spirulina removed a limitation to microbial growth observed when inorganic N alone was supplied. The stability of the bacterial community is likely linked to the amount of genetic diversity it contains. The high bacterial diversity within the rumen of steers fed tropical pastures, regardless of treatment, provides a number of potential metabolic pathways, creating a greater ability for the system to absorb the effects of dietary changes (Edwards *et al.*, 2009). The addition of a N supplement, particularly Spirulina, did increase the diversity of bacteria in the rumen. However, regardless of supplementation a large core of bacteria remained constant for every steer. Minor species in the rumen community that make up the extra diversity may still possess important and yet unrecognized ecological functions. However no particular species or group of bacteria appeared to be associated with the high EMCP values.

In conclusion, no particular group or species of bacteria was associated with high EMCP. While the addition of increasing amounts of N supplement did increase the diversity of bacteria in the rumen, a large core of bacteria remained relatively stable for each animal. Increased EMCP is likely to be a result of a relatively similar increase in the major bacteria species rather than a change in proportion of any group of or single bacterial species in the rumen.

Table 1. Biodiversity indices of the liquid phase rumen bacterial populations of steers fed Mitchell grass hay (control) and Mitchell grass hay supplemented with increasing amounts of Spirulina (AL) or urea and ammonium-sulphate (US).

Treatment	chao1	PD_whole_tree	observed_species	shannon	equitability
Control	788.5	23.9	367.0	7.6	0.9
US90	865.6	24.7	383.3	7.8	0.9
US130	866.4	25.4	410.0	8.0	0.9
US170	919.2	26.0	411.0	8.0	0.9
US210	863.7	25.0	394.0	7.8	0.9
AL90	942.9	26.8	416.5	8.1	0.9
AL130	1279.3	26.9	420.0	8.0	0.9
AL170	963.1	27.8	445.5	8.2	0.9
AL290	1002.2	26.9	426.5	8.0	0.9

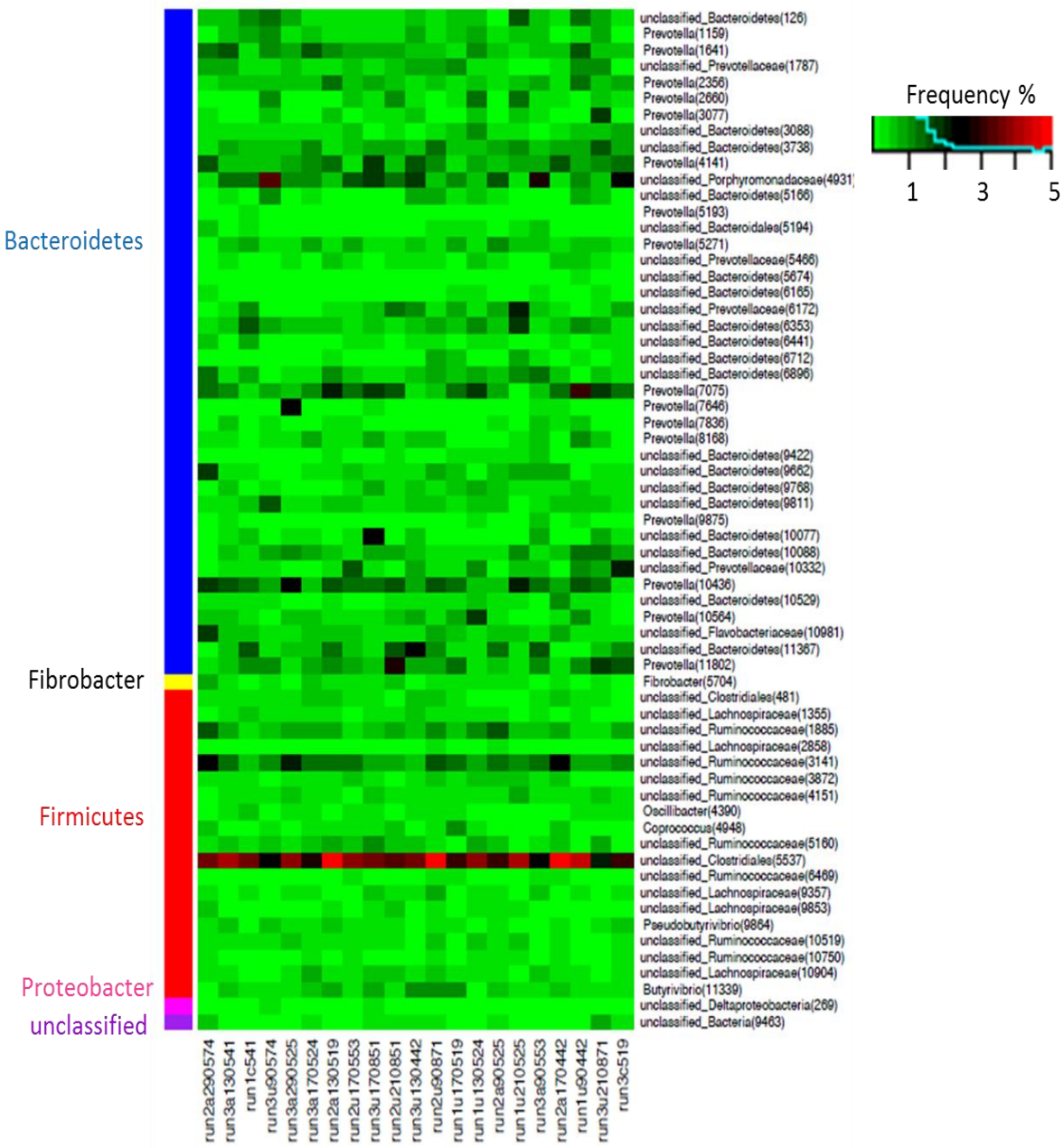
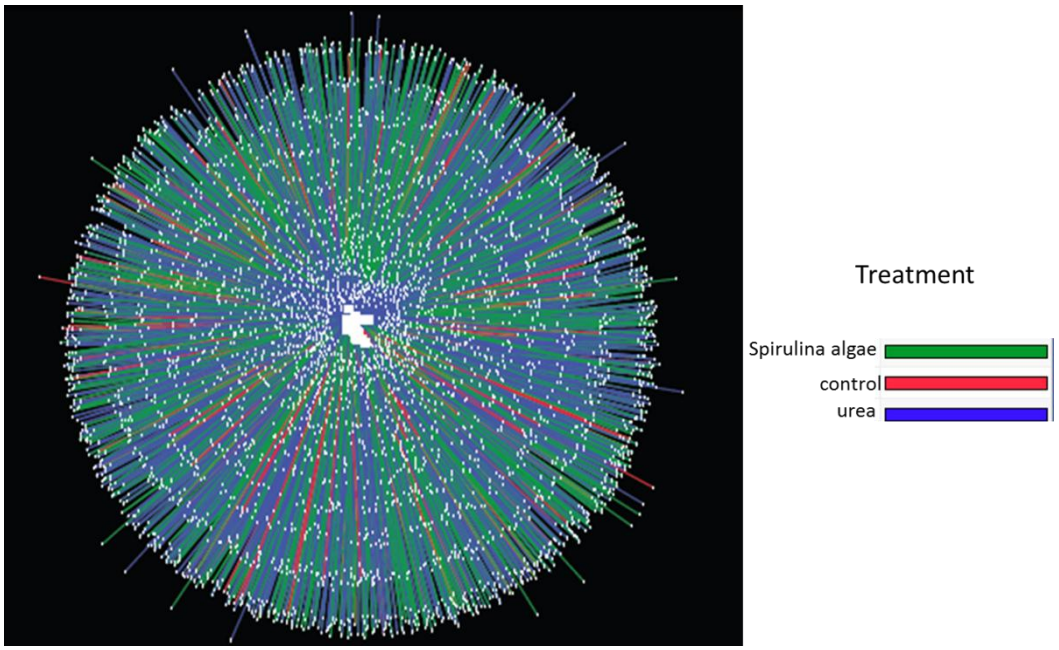


Figure 1. Heatmap of common operational taxonomic units (OTUs) present in 100% of the liquid phase rumen samples of steers fed Mitchell grass hay and Mitchell grass hay supplemented with increasing amounts of Spirulina or urea and ammonium-sulphate at increasing levels. *The relative abundance of sequences per OTU increases with colour intensity from green to red. Animal number and treatment are displayed along the x-axis and OTU identification number and ribosomal database project classification are along the right hand side.*



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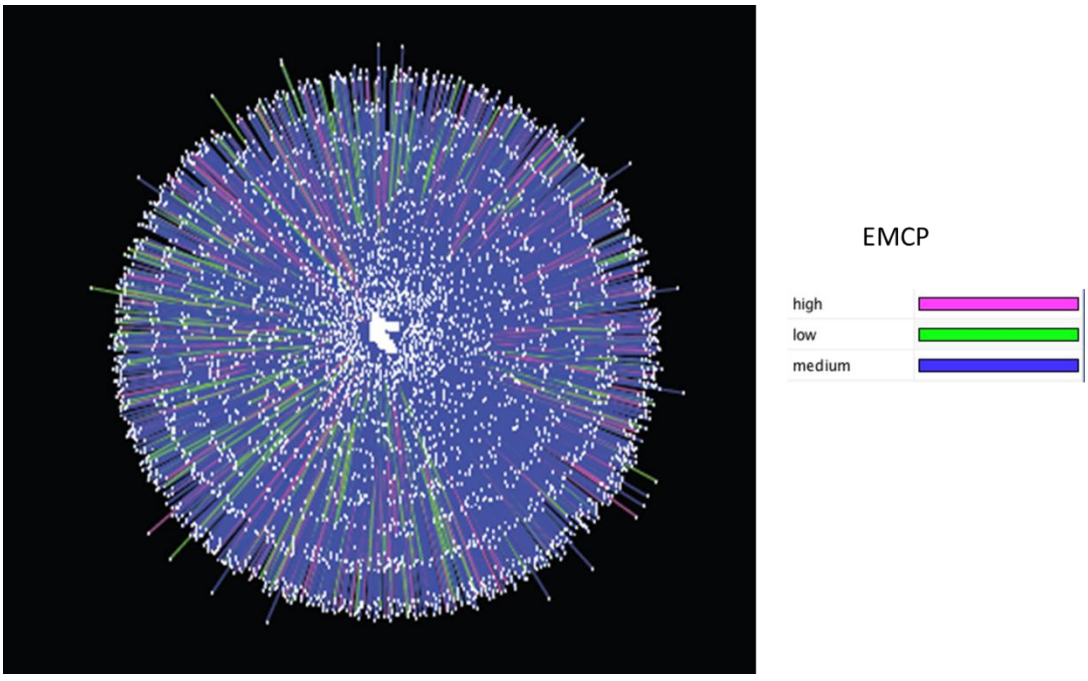


b.

Figure 2. Association between rumen bacterial non-core operational taxonomic units (OTUs) and treatment (a.) and efficiency of microbial protein production (EMCP) (b.) of steers receiving Mitchell grass hay and Mitchell grass hay supplemented with Spirulina or urea and ammonia sulphate. *Each of the large white nodes represents a sample, and the smaller white nodes represent an individual OTU. Coloured lines represent the treatments (a.) and EMCP levels (b.; low <130 g MCP/kg DOMI; medium =130 to 170 g MCP/kg DOMI; high >170 g MCP/kg DOMI). Interconnecting lines are shared OTUs between several samples.*

1.2 Rumen function and microbial genetic profile in steers selected for divergent post-weaning growth rate on low quality diets (Experiment 4)

Background

This experiment measured the intake, rumen function and MGP of steers selected for moderate and low post-weaning liveweight gain after grazing dry season speargrass (*Heteropogon contortus*) based pastures after weaning and the response of these parameters to supplements of US or CSM.

Experimental design and materials and methods

Details of the experimental design, materials and methods, rumen function and preliminary MGP results are provided in the final report. Briefly, steers grazed speargrass based pastures for 100 days after weaning during the dry season. Steers that were of similar liveweight at weaning (182 $\pm$ 2.3 kg) but different liveweight 100 days later (low or moderate post-weaning liveweight gain) were selected for the experiment (8 pairs of steers with one low (172 $\pm$ 3.5 kg) and one moderate (197 $\pm$ 4.3 kg) liveweight gain steer/pair). All steers were fed Mitchell grass hay (6.4 g N, 610 g NDF/kg DM) without supplementation, followed by Mitchell grass hay supplemented with increasing amounts of US or CSM. Feed intake, digestibility, MCP production, rumen function and the MGP were determined.

Results and discussion

Briefly, there were no differences in hay or total DM intake, EMCP or rumen function of low or moderate liveweight gain steers. Steers that had low post-weaning liveweight gain had lower MCP production than moderate post-weaning liveweight gain steers, when offered increasing amounts of CSM but there was no difference in response to increasing US intake.

Denaturing gradient gel electrophoresis data, cluster analysis and principal componenet analysis (PCA) (Figure 3) and biodiversity indexes (Table 2) of rumen bacterial diversity based on Pearson similarity co-efficients, indicated no association between rumen MGP with highest and lowest post-weaning liveweight gain steers. These results did indicate variation between steers in the rumen bacterial communities, however, there was no evident pattern or clustering of steers into liveweight gain groups. Thus, there was no evident relationship between DGGE banding patterns and steers with low or moderate liveweight gain. In addition, there were no associations between MGP and intake or rumen parameters in response to increasing supplementary N intake.

The beta diversity of the rumen fluid bacterial community between samples was estimated by phylogenetic distance between pyrosequencing OTUs using weighted UniFrac. Visualisation of the pattern of beta diversity by PCA showed clustering associated with N source (CSM or urea-ammonium sulphate) (Figure 4 A). The first and second components of the PCA explained 58.7% of the variation in the data. The second component (PC2) showed the separation of the sampled bacterial communities of steers fed CSM and US. However, the rumen bacteria community was not influenced by the amount of N intake regardless of the supplement offered (Figure 4 B-C).

The microbial community resolution and phylogenetic information obtained from the 454-pyrosequencing approach examined the effects of N supplementation on specific taxonomically classified rumen bacteria. The major phlya within the rumen of steers fed the control, CSM and US supplemented diets were *Firmicutes* and *Bacteroidetes*. Samples collected from the rumen of steers fed the control diet (Mitchell grass hay) had similar relative proportions of *Firmicutes* (49%) and *Bacteroidetes* (45%). The variation in the ratio of *Firmicutes* and *Bacteroidetes* was affected by type and amount of N supplement. With increasing intake of CSM resulting in a proportional increase in *Firmicutes* sequences relative to *Bacteroidetes* In contrast, increasing intake of US resulted in a proportional decrease in the relative abundance of *Firmicutes* sequences relative to *Bacteroidetes*. Other phyla such as *Proteobacteria, Fibrobacteres, Spirochaetes,* SR1*, Tenericutes,* TM7, *Actinobacteria* and *Fusobacteria* accounted for less than 1 % of total sequences*.* Moreover, a small proportion of sequences were identified as unclassified Bacteria across the treatments. In general, there were similar taxonomical groups between the control and N supplements (CSM and US). Within the phylum *Firmicutes*, the predominant taxonomical groups were unclassified *Clostridiales* and the genera Ruminococcaceae and Lachnospiraceae on each diet. The abundance of unclassified *Clostridiales* increased significantly with increasing N intake of CSM supplement. However, the relative abundance of unclassified *Clostridiales* group was lower (P<0.05) compared with the control group. In the phylum *Bacteroidetes*, the predominant genus was *Prevotella* and unclassified *Bacteroidetes* accounted for approximately 40% of the sequences on all the treatments. Although the relative abundance of *Prevotella* was similar among the treatments, the proportion of this group was greater for the lowest N intake of CSM. A lower abundance of OTUs and sequences of unclassified *Bacteroidales* and *Prevotellaceae* accounted for approximately less than 5% of the sequences detected.

The dominant bacterial OTUs and their relative abundance across all treatments are presented on a heatmap (Figure 5). The dendrogram analysis based on these dominant OTUs showed that steers fed high amounts of CSM (0.72-0.75 and 1.0-1.05 g N/kg W.d) cluster together and were different to the others steers. The second cluster consisted of steers consuming the Mitchell grass hay only (control) and a low amount of US (0.12-0.17 and 0.18-0.22 g N/kg W.d). The third cluster consisted of steers consuming the highest US (0.23-0.29 g N/kg W.d) and the low and intermediate amount of CSM (0.26-0.29 and 0.41-0.44 g N/kg W.d). All the sequences of these dominant OTUs were over 98% similar to sequences associated rumen bacterial species identified on Genbank (data not showed). In the microbial community within the rumen of steers fed the two highest CSM amounts, the predominant *Firmicutes* OTUs were *Ruminococcaceae* (OTU_2039) and unclassified Clostridiales (OTU_7859 and OTU_6645). *Pseudobutyrivibrio* (OTU_2696) was the most abundant OTU in the rumen of control and US supplemented steers. Within the phylum *Bacteroidetes*, unclassified *Bacteroidetes* (OTU_5424 and OTU_349) and *Provotella* OTUs (OTU_2165 and OTU_3605) were the most abundant in the rumen of steers supplemented with US and the lowest amount of CSM.

In conclusion, the analysis of genetic profiles of the most abundant groups of bacteria within the rumen of steers of divergent post-weaning liveweight gain suggests that microbial populations were not associated with the differences observed in post-weaning liveweight gain. The response of steers to increasing N intake from different supplement sources may be associated with changes in the rumen bacteria community structure.

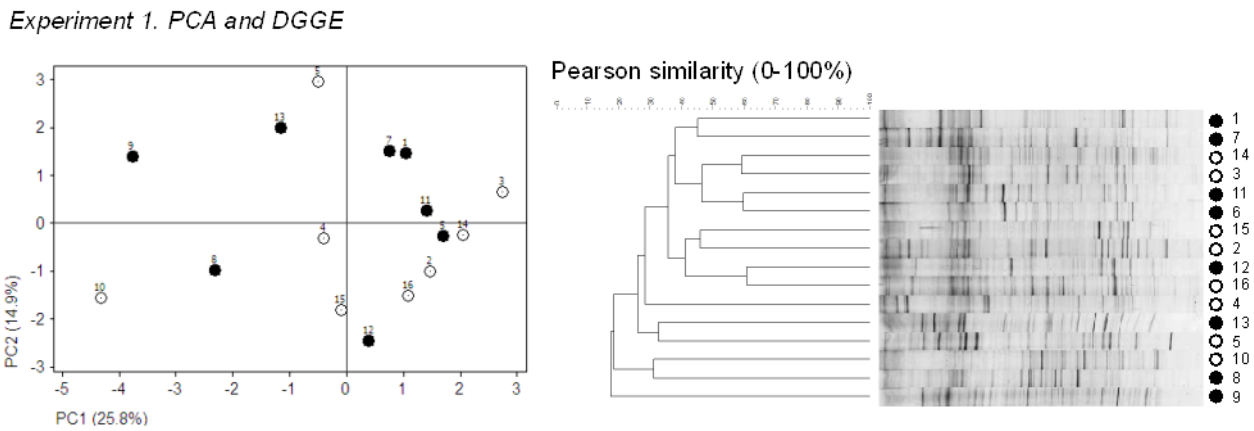


Figure 3. Cluster analysis and principal component analysis (PCA) of ruminal bacterial liquid-phase denaturing gradient gel electrophoresis (DGGE) profiles using the Pearson similarity matrix for steers of the highest (●) and lowest (○) post-weaning live weight gain (LWG) grazing dry season native pastures. *Sixteen steers run on a single DGGE gel.*

Table 2. Ruminal bacterial community diversity indexes (number of bands, Shannon-Wiener and evenness) calculated using ruminal bacterial liquid-phase denaturing gradient gel electrophoresis (DGGE) data for steers divergent in post-weaning liveweight gain (LWG) grazing Speargrass in the dry season. *Values are means with pooled standard error of the mean (SEM)*.

	Low LWG	Moderate LWG	SEM
n	8	8	
Number of bands	27.0	24.8	1.4
Shannon–Wiener	2.96	2.87	0.07
Evenness	0.898	0.895	0.008

All means within rows are not significantly different (P = 0.05)

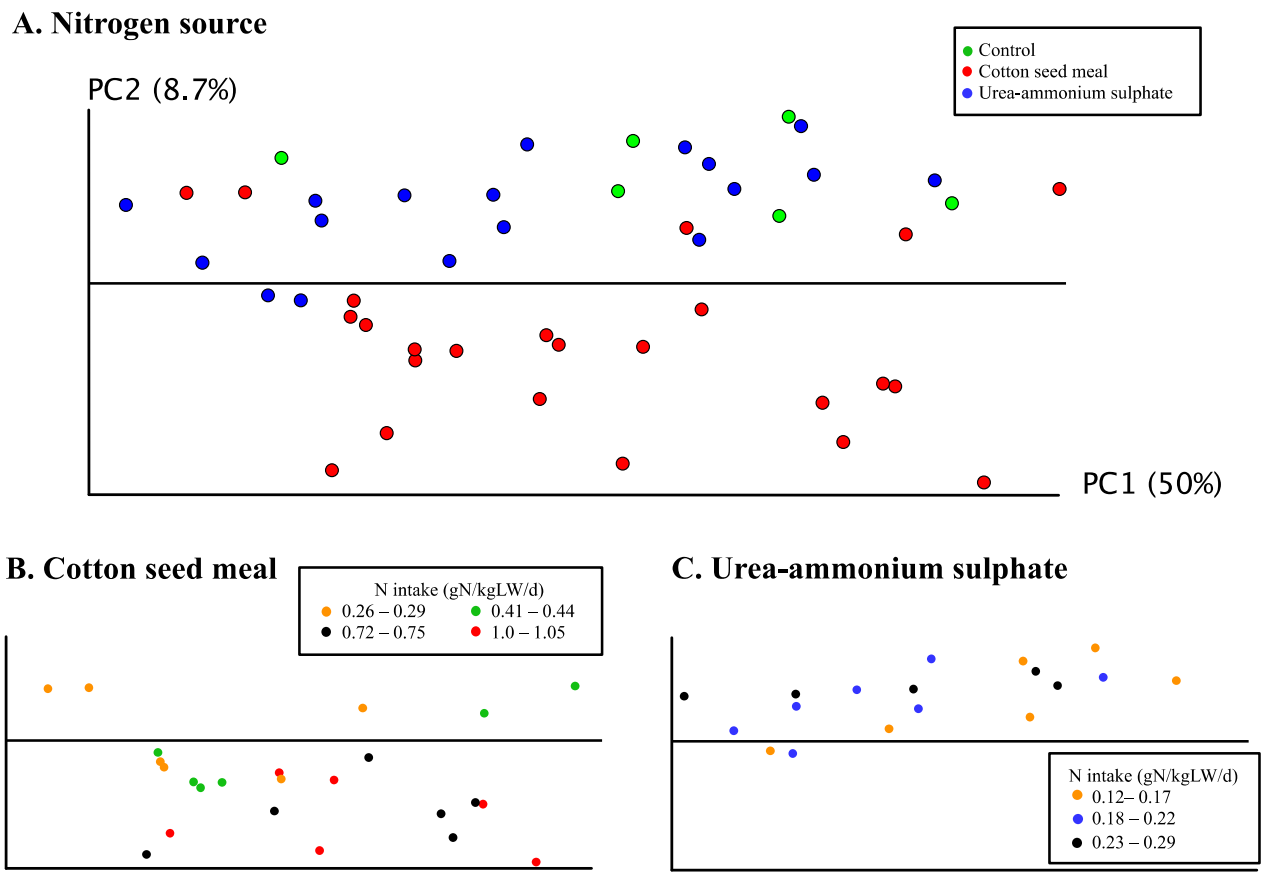


Figure 4. Principal component analysis (PCA) of rumen bacterial liquid-phase pyrosequencing using the weighted UNIFRAC for steers grouped on supplement (cotton seed meal and urea-ammonium sulphate) (A.) and the amount of N intake of cottonseed meal (B.) and urea-ammonium sulphate (C.).

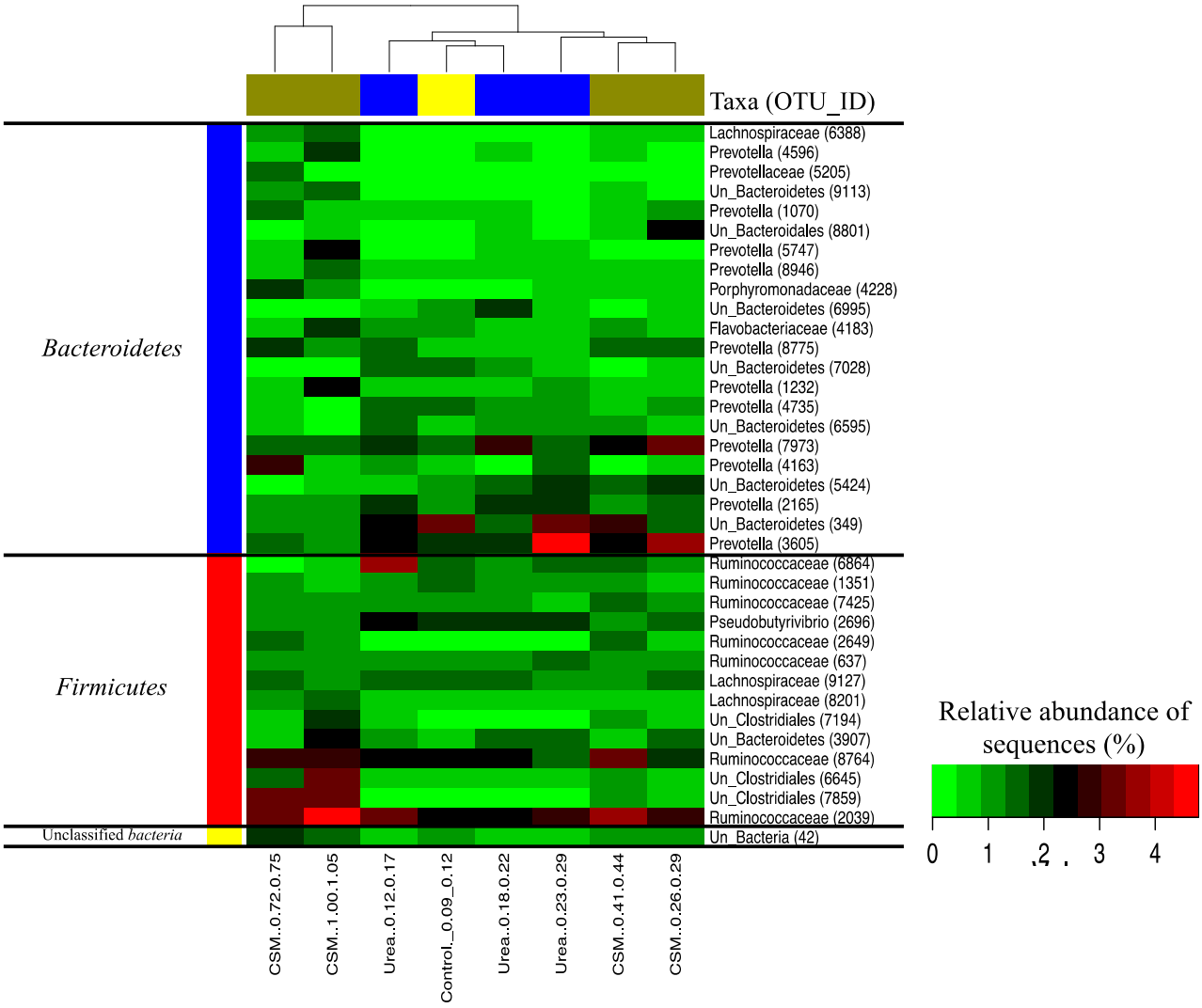


Figure 5. Heatmap and taxonomical classification of the dominant rumen fluid associated bacterial operational taxonomic units (OTUs) obtained with 454-pyrosequencing of steers fed Mitchell grass hay (control, C) and increasing amounts of cottonseed meal (CSM) and urea and ammonium sulphate (US) classified using the Ribosomal Database Project (version 2.0). ***Treatments are on the x-axis and OTU identification and ribosomal database project classification are on the y-axis. The relative abundance of sequences per OTU increases with colour intensity from green to red. Taxonomical groups that are not classified with a cultured species are denoted as unclassified (Un).***

1.3 Liveweight gain of animals consuming different forms of N supplements (Experiment 5)

Background

This experiment examined the liveweight gain response of weaner steers fed a basal diet of speargrass hay to increasing intake of US, Spirulina and CSM supplements. Intake, digestibility, liveweight gain and rumen MGP were measured.

Experimental design and materials and methods

Details of the experimental design, materials and methods, rumen function and preliminary MGP results are provided in the final report. Briefly, 42 steers were fed a basal diet of speargrass hay *ad libitum* and offered increasing amounts of US (0.04, 0.08, 0.16 and 0.20 g N/kg W.d) and Spirulina and CSM (0.08, 0.16, 0.32 and 0.48 g N/kg W.d) supplements (n=3/amount/supplement) or no supplement (control; n=6) for 70 days. Intake and liveweight were measured weekly and digestibility was determined during week 7 of the experiment. Rumen samples were collected for MGP work on day 50 of the experiment.

Results and discussion

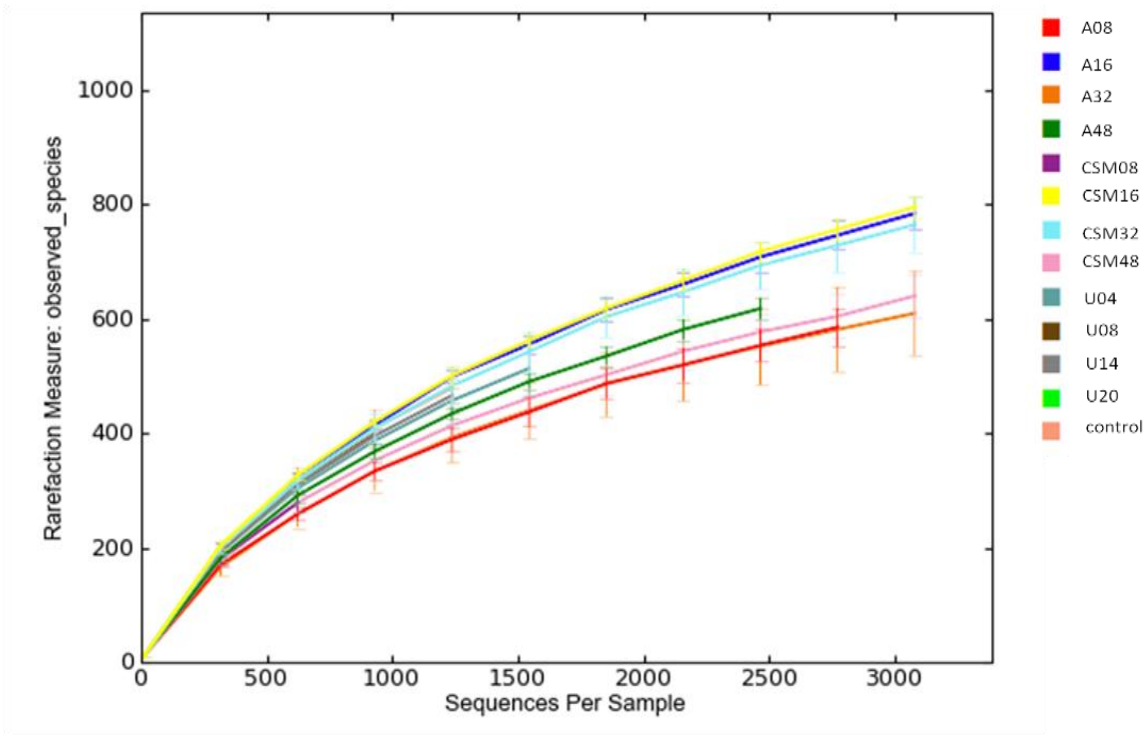
Briefly, liveweight gain increased in response to increasing supplement N intake from all three supplements. Cottonseed meal had a quadratic response curve for liveweight gain with better responses than Spirulina at low levels of supplementation. At higher levels of supplementation there was little difference between Spirulina and CSM. Hay intake increased in a quadratic fashion in response to all supplements with no difference between supplements. Initial DGGE analysis of rumen samples demonstrated that common dominant bands exist between individual animals, regardless of treatment and the dominant bands are relatively stable and do not appear to be influenced by treatment.

Results from 454-pyrosequencing demonstrated that the number of OTUs initially increased in response to increasing N intake from each of the three supplementary N sources and then decreased at higher N intakes (Table 3). Rarefaction curve analysis, which represents total community diversity of bacteria within a rumen sample, did not show any relationship between OTU number and treatment (Figure 6 a.). However, at high N intakes there was a decrease in OTUs compared with low N intakes, across all supplements (Figure 6 b. and Figure 7). Steers that had the highest increase in liveweight over the experiment had the highest number of OTUs and hence the greatest amount of diversity of bacteria in the rumen (Figure 8); steers that had liveweight loss or low liveweight gain had fewer OTUs and hence less diversity of bacteria in the rumen. This result initially appears to contradict the findings of Experiment 4, in which no differences in MGP were associated with differences in post-weaning liveweight gain, however the liveweight gain of the two groups of animals in Experiment 4 (low and moderate liveweight gain) were comparable to the negative and low liveweight gain animals in Experiment 5. Therefore it is likely that differences in the diversity of the MGP are only evident at more extreme differences in liveweight gain than those measured in Experiment 4. The medium amounts of all supplements offered to steers (US14, AL16 and CSM16) resulted in the greatest richness of species (Chao01), the number of OTUs

at an observed sequence (observed species index) and the bacterial community diversity (Shannon and Simpson indices), however these treatments were not significantly different to control for any of the measured indices (Table 4).

Table 3. Average number of operational taxonomic units (OTUs) found in the liquid phase of rumen samples in steers fed Speargrass hay (control) or speargrass hay supplemented with Spirulina (AL), urea and ammonium-sulphate (US) or cottonseed meal (CSM) at increasing amounts. *SEM, standard error of the mean*.

Treatment	OTU count	SEM
Control	711	119
US04	538	33
US08	802	384
US14	609	64
US20	417	75
CSM08	333	22
CSM16	1754	49
CSM32	1297	92
CSM48	837	47
AL08	639	3
AL16	1021	156
AL32	783	11
AL48	963	241
Average	821	



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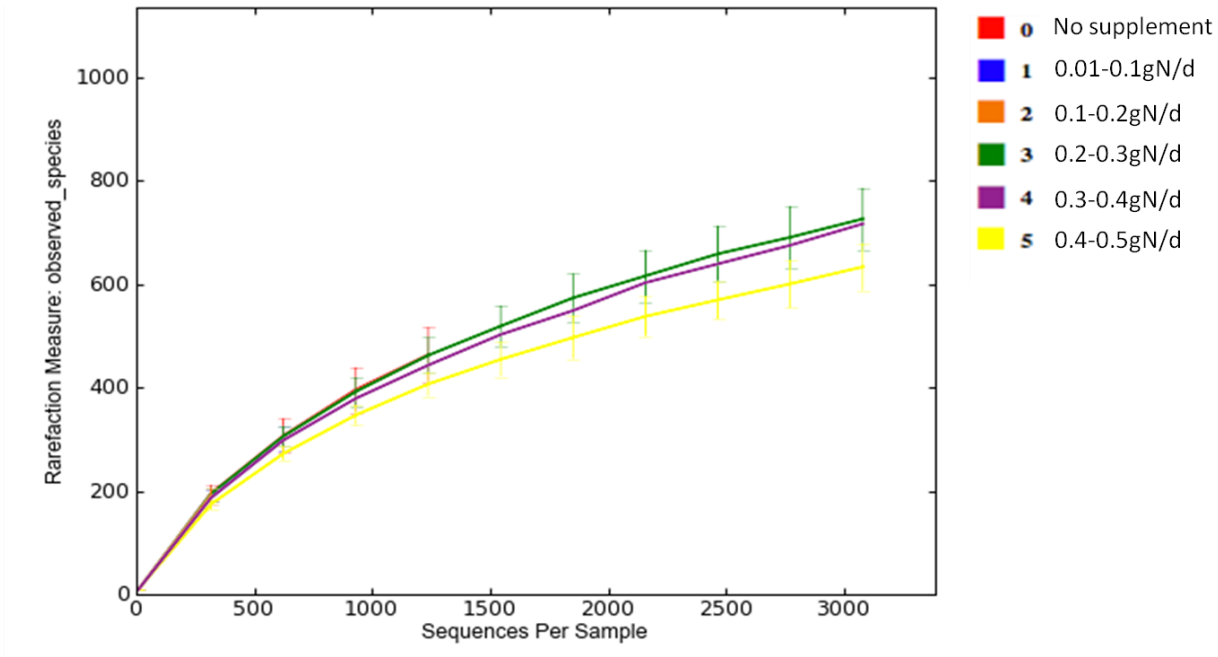

b.

Figure 6. Rarefaction plots of liquid phase rumen samples from steers fed Speargrass hay (control) and Speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM) across treatment (a.) and N intake (b.). *The x-axis represents the sequences per sample (sampling depth) and the y-axis is the measure of diversity (number of observed species).*

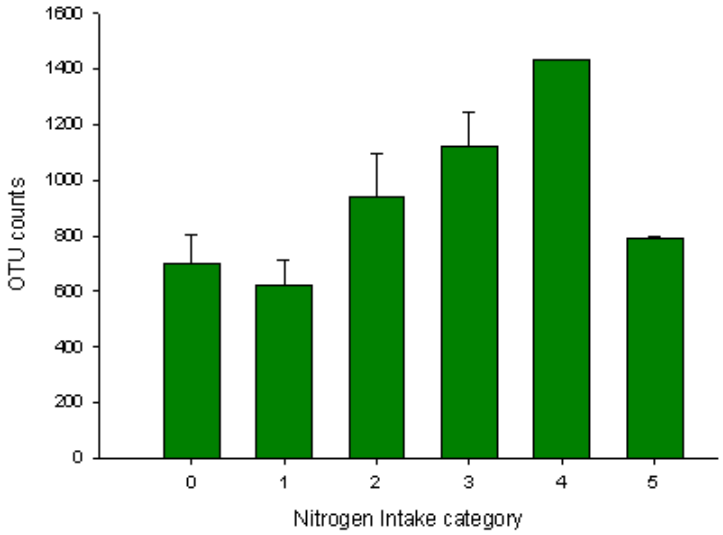

Figure 7. Relationship between operational taxonomic unit (OTU) count and N intake category of steers fed Speargrass hay (control) and Speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). *N intake categories are, 0=no supplementary N, 1=0.01-0.1 g N/d, 2=0.1-0.2 g N/d, 3=0.2-0.3 g N/d, 4=0.3-0.4 g N/d and 5=0.4-0.5 g N/d; OTU count is average OTUs in each category; error bars represent the standard error of the mean*.

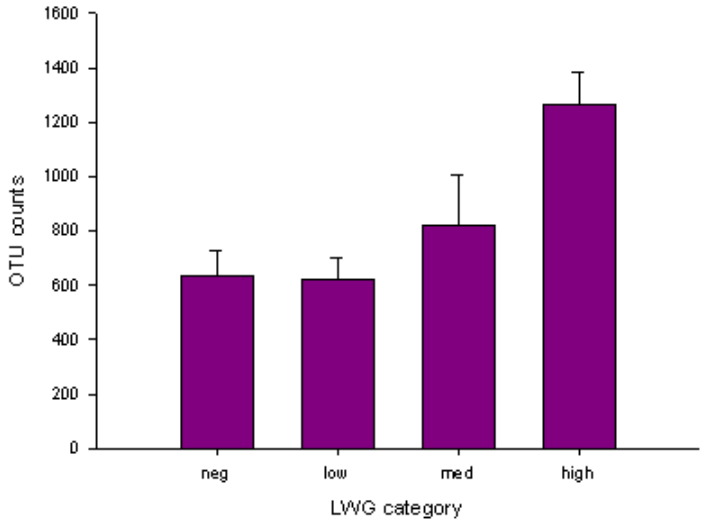

Figure 8. Relationship between the operational taxonomic unit (OTU) count and liveweight gain (LWG) category of steers fed speargrass hay (control) and speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). *LWG categories are neg<0 kg LWG, low=0-15 kg LWG, medium=15-30 kg LWG and high>30 kg LWG, across the entire experiment; error bars represent the standard error of the mean.*

Table 4. Biodiversity indices of the liquid phase rumen bacterial populations of steers fed speargrass hay (control) and speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). *Alphabetical superscripts indicate significance at P≤0.05 level for each index.*

		Observed	Diversity Indices	
	Chao1	species	Shannon	Simpson
Control	705.3^{ab}	316.6^{ab}	7.69^{ab}	0.990^{ab}
US04	646.4^{abcd}	303.9^{abc}	7.49^{abcde}	0.984^{d}
US08	651.8^{abcd}	313.6^{ab}	7.71^{abc}	0.991^{ab}
US14	662.4^{abc}	310.0^{abc}	7.56^{abcd}	0.986^{cd}
US20	610.0^{bcd}	297.1^{abcd}	7.51^{abcde}	0.987^{bcd}
CSM08	519.3^{d}	278.7^{cd}	7.40^{cde}	0.987^{bcd}
CSM16	750.5^{a}	326.8^{a}	7.77^{ab}	0.991^{ab}
CSM32	716.2^{ab}	317.8^{ab}	7.73^{abc}	0.991^{ab}
CSM48	574.8^{cd}	278.6^{cd}	7.44^{bcde}	0.990^{abc}
AL08	544.2^{cd}	259.0^{d}	7.20^{de}	0.985^{cd}
AL16	733.4^{ab}	325.3^{ab}	7.80^{a}	0.992^{a}
AL32	543.6^{abcd}	258.8^{d}	7.15^{e}	0.984^{d}
AL48	648.6^{abcd}	291.5^{bcd}	7.48^{abcde}	0.989^{abc}

A total of 15 phyla were identified across all the steers in the experiment, with 95% of these being Firmicutes and Bacteroidetes. There appears to be a trend towards a higher proportion of the population belonging to the phylum Bacteroidetes in response to increasing CSM and AL supplement intake. The third largest phylum, present in all treatments at approximately 4%, was the unclassified grouping which consists of sequences that could not be classified into any known phylum. The remaining bacteria were comprised of low abundance phyla which together represent <3% of the total bacterial community and were not present consistently in all of the treatments. *Prevotella* was the most common genus, comprising 22% across all samples, with other genera found at less than 1%. These includes *Butyrivibrio* (0.63%) and *Acetivibrio* (0.17%). Approximately 76% of the population could not be classified to a genera level.

There were 20 core OTUs, identified as being present in 100% of the samples, consisting of 15 Firmicutes and five Bacteroidetes (Figure 9; Table 5). Analysis of the core OTUs at genus level showed they belonged to *Ruminococcus* (OTU 1316), *Prevotella* (OTUs 3026, 4083, 4234, 4539) and Lachnospiraceae *Incertae sedis* (OTU 1922). A number of core OTUs could only be classified to the phylum Bacteroidetes (OTU 1195), the order Clostridiales (OTUs 277, 1427, 2742, 2921, 3728, 4666), and the families Lachnospiraceae (OTUs 281 and 1568) and Ruminococcaceae (OTUs 462, 681, 1952, 3542, 4111). These core bacteria represent around 25% of the total sequences in the rumen samples from control and US supplemented steers. When either AL or CSM supplements were included in the diet these core sequences represented about 15% of the total sequences. The OTU 2921, is in the order Clostridiales, and was present as a very large proportion of the bacterial community across all animals. In the controls and US supplemented steers this OTU accounted for

approximately 10% of all sequences. When AL or CSM supplements were included in the diet the proportion of the OTU 2921 reduced to approximately 4% of total sequences, while the proportion of the OTU 462 (family Ruminococcaceae) and OTU 4539 (*Prevotella*) increased 10-fold. Sequences of the 20 core OTUs were subject to GenBank for identification and were all shown to be between 95 to 100% similar (≈450 bp) to sequences from uncultured rumen bacteria. In addition these sequences were subject to a Greengenes search to find the closest cultured relative. Only OTU 1316 (*Ruminococcus flavefaciens*) was found to be closely related (99%) to a cultured organism. Two OTUs (4083 and 1922) were found to be 95% similar to *Prevotella ruminocola* and *Ruminococcus gauvreaui* respectively.

Twenty OTUs were statistically specific to the AL and CSM supplement diets, two OTUs were statistically specific to the CSM supplement diets and three OTUs statistically specific to US supplement diets (Table 6). These supplement specific OTUs constituted only a small proportion of total OTUs in each sample (<1%). These sequences were all shown to be between 95 to 100% similar to sequences from uncultured rumen bacteria. These supplement specific OTUs were phylogenetically diverse across each treatment group, with five of the OTUs belonging to the phylum Bacteroidetes, 17 to Firmicutes, one to Actinobacteria and the SR1 a candidate division.

The PCA and OTU network analysis illustrates the interrelationships of diet and bacterial OTUs. These results showed two distinct clusters corresponding with protein supplementation and urea supplementation (Figure 10) but there was no clustering with regard to the level of supplemental N intake (data not presented). These results suggest that although many OTUs were shared between these two supplemental groups, US supplementation and AL and CSM supplementation supported distinct bacterial communities, as seen by OTUs present only in these samples. The OTU network relating to liveweight gain categories revealed two distinct clusters corresponding to low and high liveweight gain (Figure 11). A low liveweight gain cluster was associated with US supplementation, while the high liveweight gain cluster was associated with AL and CSM supplementation. Operational taxonomic units from the rumen of control steers, which correlated with the liveweight loss category, were associated with both the major clusters of high and low liveweight gain but had fewer unique OTUs, indicating lower bacterial diversity.

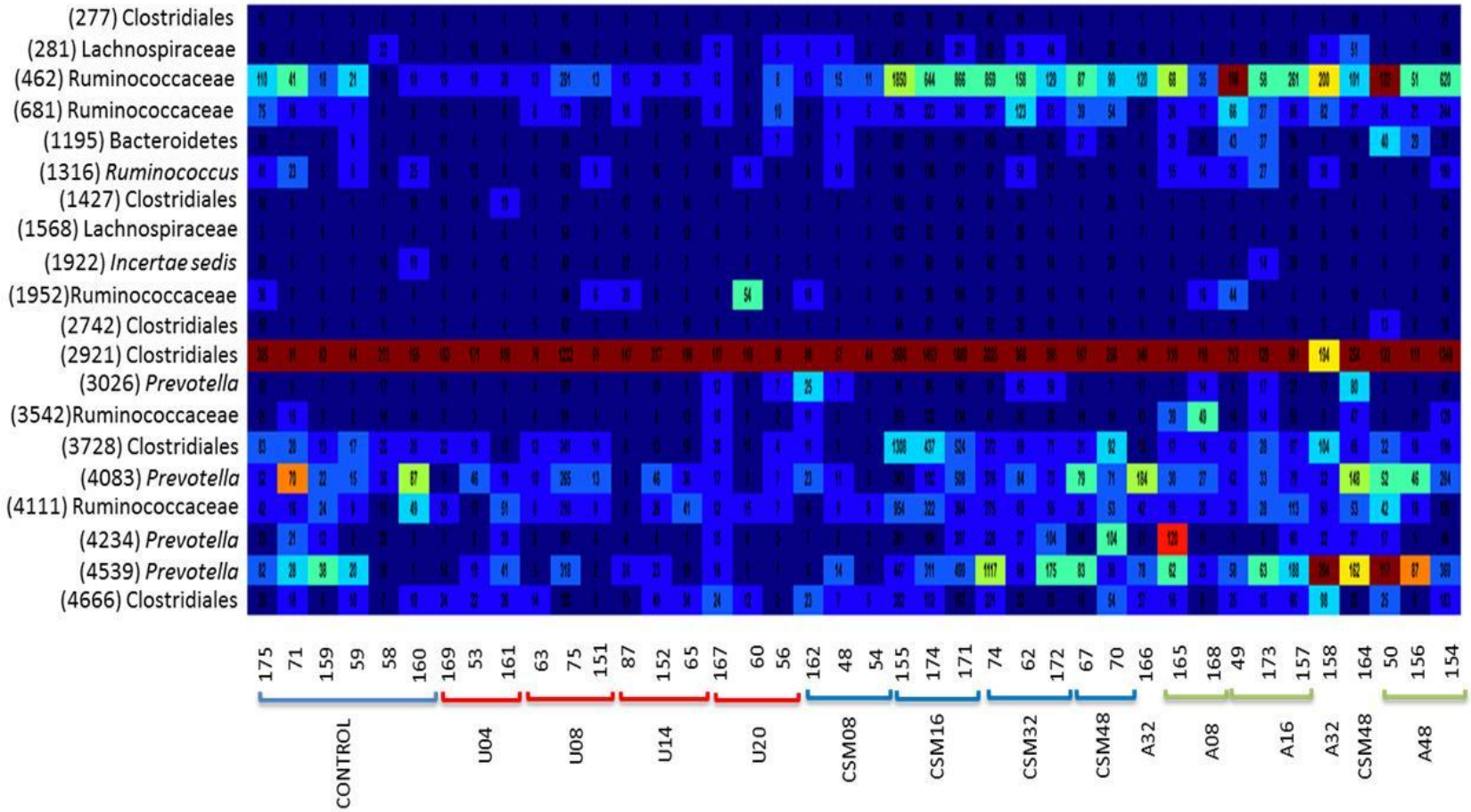

Figure 9. Heatmap of the dominant rumen fluid associated bacterial operational taxonomic units (OTUs) obtained with 454-pyrosequencing of steers fed speargrass hay (control) and speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). ***Animal number and treatment are on the x-axis and OTU identification and ribosomal database project classification are on the y-axis. The relative abundance of sequences per OTU increases with colour intensity from blue to maroon.***

Table 5. Core bacterial operational taxonomic units (OTUs) in rumen samples collected from steers fed speargrass hay (control) and speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). *OTUs were classified using data from the Ribosomal Database Project (RDP) and referenced data from the GenBank database. Closest relative and reference according to GenBank is listed along with its accession number and percent identity. Closest cultured relative along with accession number is listed second. Cultured species are not included if the percentage similarity is less than 90%. *Indicates that sequences submitted to GenBank remain unpublished.*

				Classification	
OTU No.	**RDP classification**	**Accession No.**	**Similarity**	**Bacterium**	**Author**
277	Clostridiales	HM104859	97%	uncultured Firmicute- rumen	Fernando *et al*. (2010)
281	Lachnospiraceae	EF686576	100%	uncultured rumen-bacterium	Mao *et al*. (2008)
		(AB008552	93%	*Eubacterium ruminantium*)	Nagamoto *et al*. (1997)*
462	Ruminococcaceae	GU324404	100%	rumen bacterium NK4A214	Kenters *et al.* (2011)
		(HM626173	91%	*Oscillibacter sp*.)	Park (2010) unpub.
681	Ruminococcaceae	HM104905	100%	uncultured Firmicutes-rumen	Fernando *et al.* (2010)
		(L35515	90%	*Acetivibrio cellulolyticus*)	Lin *et al.* (1994)*
1195	Bacteroidetes	EF436405	99%	uncultured rumen bacterium	Yang *et al.* (2010)
1316	*Ruminococcus*	AB185779	100%	uncultured rumen bacterium	Ozutsumi *et al.* (2005b)
		(AM748742	99%	*Ruminococcus flavefaciens*)	Jindou *et al.* (2008)
1427	Clostridiales	AY854292	99%	uncultured bacterium cattle	Nelson *et al.* (2003)
1568	Lachnospiraceae	EU843314	99%	uncultured rumen bacterium	Brulc *et al*. (2009)
		(D14148	92%	*Coprococcus eutactus*)	Ezaki *et al.* (1994)
1922	*Incertae sedis*	HM104971	100%	uncultured Firmicute-rumen	Fernando *et al.* (2010)
		(EF529620	95%	*Ruminococcus gauvreaui*)	Domingo *et al.* (2008)
1952	Ruminococcaceae	HM104781	96%	uncultured Firmicute- rumen	Fernando *et al.* 2010
		(Z49863	92%	*Sporobacter termitidis*)	Grech-Mora *et al.* (1996)
2742	Clostridiales	EU844768	99%	uncultured rumen bacterium	Brulc *et al*. (2009)
2921	Clostridiales	GQ327565	99%	uncultured rumen bacterium	Kong *et al.* (2010)
3026	*Prevotella*	AB501155	98%	*Prevotella sp.*	Kobayashi *et al.* (2009)*
3542	Ruminococcaceae	EU259444	99%	uncultured rumen YRC68	Yang *et al*. (2010)
		(AY949857	93%	*Clostridium sp.*)	Chen *et al.* (2010)
3728	Clostridiales	GU324386	100%	rumen bacterium NK4A237	Kenters *et al.* (2011)
		(AY949857	92%	*Clostridium sp.*)	Chen *et al.* (2010)
4083	*Prevotella*	HM104872	98%	uncultured Bacteroidete-rumen	Fernando *et al.* (2010)
		(AJ009933	95%	*Prevotella ruminocola*)	Avgustin *et al.* (1994)
4111	Ruminococcaceae	AY854277	100%	uncultured cattle bacterium	Nelson *et al.* (2003)
		(AY136666	91%	*Bacteroides capillosus*)	Bernard&Munro (2002)*
4234	*Prevotella*	AF018500	95%	uncultured rumen bacterium	Whitford *et al.* (1998)
		(AJ009933	94%	*Prevotella ruminocola*)	Avgustin *et al.* (1994)
4539	*Prevotella*	AB185583	99%	uncultured rumen bacterium	Ozutsumi *et al*. (2005)
		(AJ009933	94%	*Prevotella ruminocola*)	Avgustin *et al.* (1994)
4666	Clostridiales	HM104845	99%	uncultured Firmicute- rumen	Fernando *et al.* (2010)

Table 6. Supplement specific operational taxonomic units (OTUs) in rumen samples collected from steers fed speargrass hay (control) and speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). *OTUs were classified using data from the Ribosomal Database Project and referenced data from the GenBank database. Closest cultured relative along with accession number and percentage similarity found using Greengenes is included in brackets. Cultured species are not included if the percentage similarity is less than 90%. *Indicates that sequences submitted to GenBank remain unpublished.*

OTU No.	RDP classification	Accession No.	Classification		
			Similarity	Bacterium	Author
OTUs specific for AL and CSM supplements					
1083	Clostridiales	AB034007	99%	Uncultured - rumen	Tajima *et al*. (2000)
		(GQ461729	92%	*Eubacterium sp.*)	Tong *et al*. (2009)*
1318	Clostridiales	HM04945	99%	Uncultured Firmicute	Fernando *et al*. (2010)
3216	Clostridiales	GQ327762	99%	Uncultured - rumen	Kong *et al.* (2010)
		(X76161	93%	*C. aminobutyrium*)	Collins *et al.* (1994)
1670	Clostridiales	GQ327245	96%	Uncultured - rumen	Kong *et al* (2010)
		(L34621	95%	*Eubacterium hallii*)	Ludwig&Woese (1995)*
3714	Prevotellaceae	AM884044	97%	Uncultured - rumen	Sadet-Bourgeteau *et al.* (2010)
		(FJ794074	94%	*Butyrivibrio hungatei*)	Li *et al*. (2009)*
72	Prevotellaceae	EU844794	96%	Uncultured - rumen	Brulc *et al* (2009)
4664	Bacteroidales	EU719269	96%	Uncultured - rumen	He *et al.* (2008)*
3417	Ruminococcaceae	EU845249	99%	Uncultured - rumen	Brulc *et al*. (2009)
		(NR026104	91%	*Clostridium cellobioparum*)	Rainey&Stackebrandt (1993)
997	Ruminococcaceae	GQ327248	99%	Uncultured - rumen	Kong *et al.* (2010)
		(NR025670	94%	*Bacteroides capillosus*)	Bernard and Munro (2009)*
2617	Ruminococcaceae	EU842234	100%	Uncultured - rumen	Brulc *et al*. (2009)
		(HM037995	91%	*E. coprostanoligenes*)	Crouch *et al*. (2010)*
408	Firmicutes	EU844497	99%	Uncultured - rumen	Brulc *et al*. (2009)
1736	Butyrivibrio	AY699273	98%	*Butryrivibrio fibrisolvens*	Al Jassim *et al.* (2004)*
3473	Bacteroidetes	DQ673480	99%	Uncultured - rumen	Yang *et al*. (2010)
2927	Bacteroidetes	HM104986	100%	Uncultured Bacteroidetes	Fernando *et al*. (2010)
2602,	*Incertae sedis*	AY858413	99%	Uncultured	Nelson *et al*. (2003)
		(L76597	95%	*Ruminococcus gnavus*)	Wilson and Duncan (1996)*
2141	*Incertae sedis*	GQ327486	99%	Uncultured - rumen	Kong *et al*. (2010)
		(NR027579	95%	*Ruminococcus lactaris*)	Wilson and Duncan (1996)*
754	Clostridia	EU472033	100%	Uncultured – rumen	Ley *et al*. (2008)
5368	Clostridia	AB270336	99%	Uncultured - rumen	Tajima *et al*. (2007)
495	Coriobacteriaceae	EU843364	99%	Uncultured - rumen	Brulc *et al*. (2009)
		(AB558168	95%	*Atopobium parvulum*)	Sakamoto (2010)*
4549	SR1*Incertae sedis*	FJ480094	99%	Uncultured environ. SR1	(Davis *et al.* (2009)
OTUs specific for CSM supplement					
4753	Clostridiales	GQ448104	98%	Uncultured - rumen	Paustian *et al*. (2009)*
1293	Ruminococcaceae	HM105399	100%	Uncultured rumen Firmicute	Fernando *et al*. (2010)
		(NR025670	91%	*Bacteroides capillosus*)	Bernard and Munro (2009)*
OTUs specific for US supplement					
2814	Lachnospiraceae	AB270112	99%	Uncultured - rumen	Tajima *et al*. (2007)
		(EF529620	96%	*Ruminococcus gauvreaui*)	(Domingo *et al.* (2008)
2628	Clostridiales	HM104871	97%	Uncultured rumen Firmicute	Fernando *et al*. (2010)

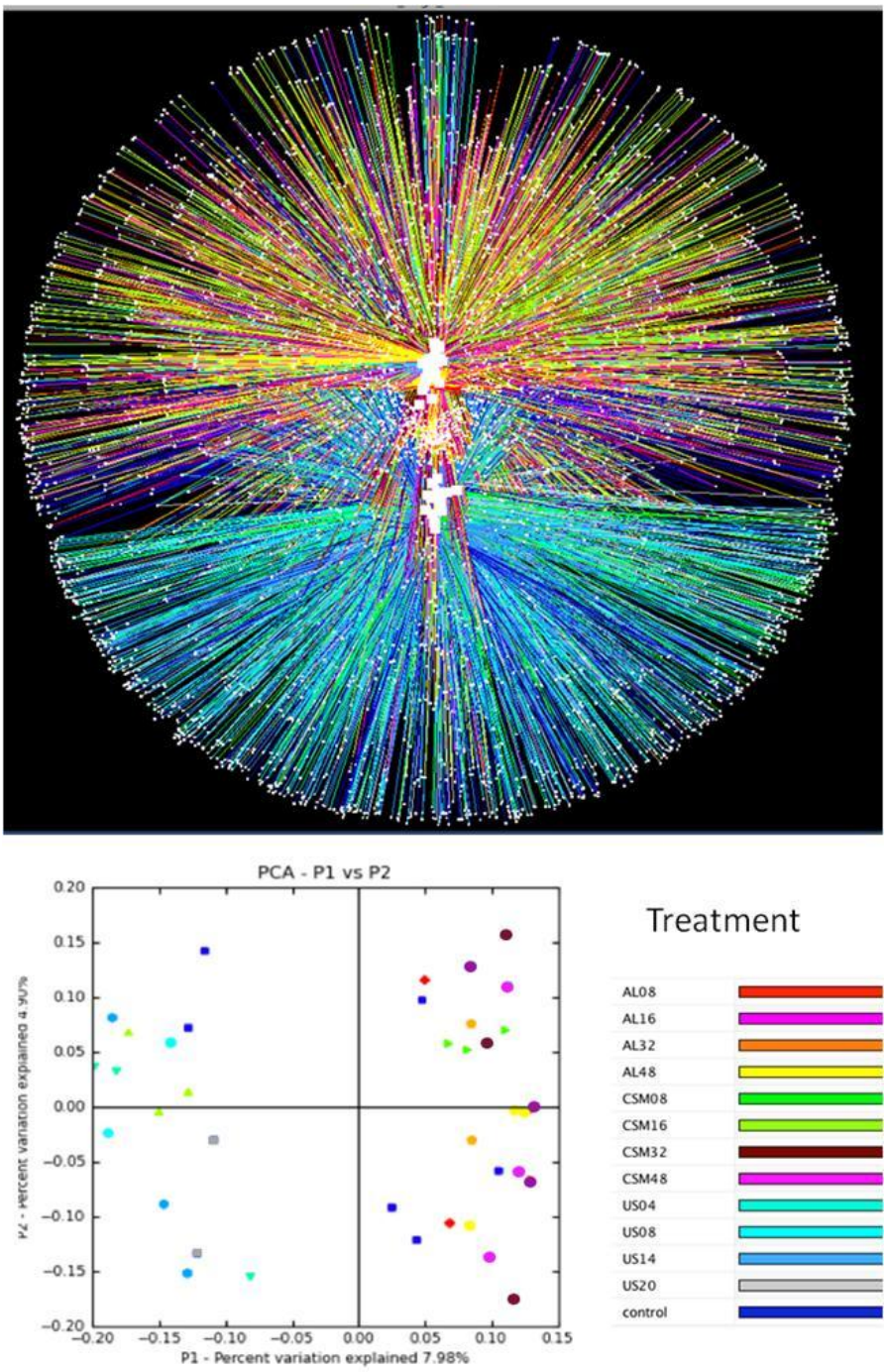

Figure 10. Rumen bacterial operational taxonomic unit (OTU) network and associated principal co-ordinate analysis (PCA) of steers fed speargrass hay (control) and speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). *Each of the large white nodes represents a sample and the smaller white nodes represent an individual OTU. Coloured lines represent the treatments. Interconnecting lines are shared OTUs between several samples.*

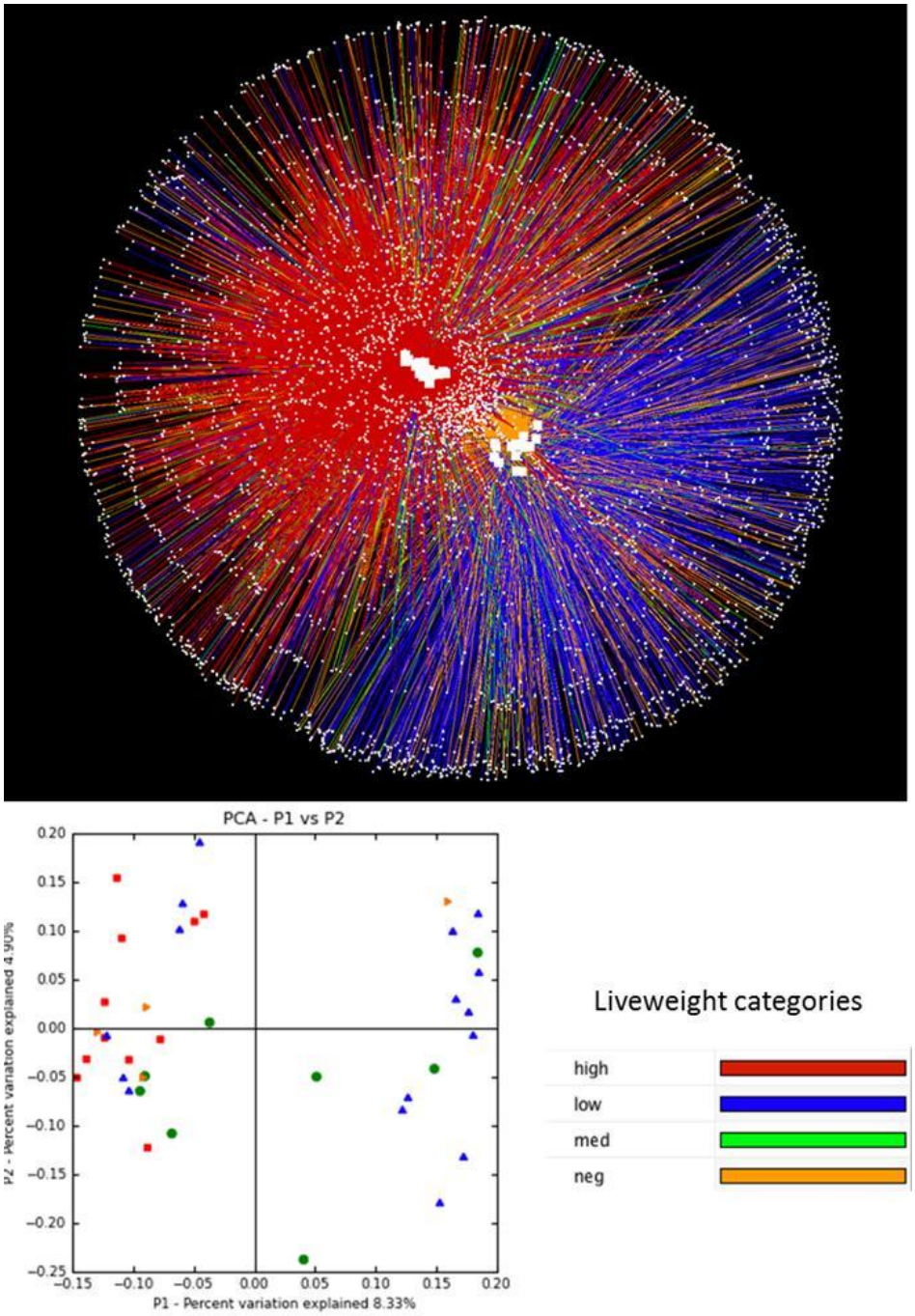

Figure 11. Rumen bacterial operational taxonomic unit (OTU) network and associated principal co-ordinate analysis (PCA) of steers fed speargrass hay (control) and speargrass hay supplemented with increasing amounts of urea and ammonium sulphate (US), Spirulina (AL) or cottonseed meal (CSM). ***Each of the large white nodes represents a sample and the smaller white nodes represent an individual OTU. Coloured lines represent the liveweight gain (LWG) categories where neg<0 kg LWG, low=0-15 kg LWG, med=15-30 kg LWG and high>30 kg LWG, across the entire experiment. Interconnecting lines are shared OTUs between several samples.***

1.4 Chemical composition and relative degradability of a range of algae species (Experiment 6)

Background

This experiment was conducted to evaluate the nutritive value of a range of algae species and compare them with a range of commonly used protein supplements. The algae species and other feeds analysed in this experiment are provided in Table 7.

Table 7. Source, supplier and classification of the various algae species, protein sources and hays examined within the present experiment.

Sample	Supplier	Classification
Chlorella pyrenoidosa	Phytofoods[1]	Micro-algae
Dunaliella salina	Nutrakol[2]	Micro-algae
Nannochloropsis sp.	JCU[3]	Micro-algae
Nannochloropsis oculata	JCU[3]	Micro-algae
Schizochytrium sp.	Aquafauna[4]	Micro-algae
Spirulina platensis - A	Phytofoods[1]	Micro-algae
Spirulina platensis - B	Aquafauna[4]	Micro-algae
Chaetomorpha linum	JCU[5]	Macro-algae
Cladophora patentiramea	JCU[5]	Macro-algae
Enteromorpha sp.	JCU[5]	Macro-algae
Casein	Murray-Goulburn[6]	True protein
Zein	Sigma-Aldrich[7]	True protein
Canola meal	Brisbane Export Corp.[8]	Supplement
Copra meal	Mi-Feed[9]	Supplement
Cottonseed meal	Riverina Stockfeeds[10]	Supplement
Palm kernel meal	Brisbane Export Corp.	Supplement
Soybean meal	Riverina Stockfeeds	Supplement
Mitchell grass	Grown in Longreach district, QLD	Hay
Pangola grass	Grown in Mt. Cotton district, QLD	Hay

[1]Commercial production source for human consumption, Phytofoods, Labrador, QLD.; [2]Commercial production source for aquaculture, Nutrakol, Perth, WA.; [3]Grown under experimental conditions in a bioreactor, James Cook University (JCU), Townsville, QLD.; [4]Commercial production source for aquaculture, Aquafauna, Hawthorne, California, USA.; [5]Collected from the Pacific reef and GFB inlet, James Cook University, Townsville, QLD.; [6]Sodium caseinate, Murray-Goulburn, Brunswick, VIC.; [7]Zein (maize prolamine protein), Sigma-Aldrich, Castle Hill, NSW.; [8]Brisbane Export Corp., Brisbane, QLD.; [9]Mi-Feed, Yandina, QLD.; [10]Riverina Stockfeeds, Brisbane, QLD.

Experimental design and materials and methods

Samples were analysed for organic matter (OM), N, ash-free neutral detergent fibre (NDF) and ash-free acid detergent fibre (ADF), crude lipids (CL), water soluble carbohydrates (WSC), minerals, amino acids and relative degradability *in vitro*.

Results and discussion

The micro-algae *Spirulina* and *Chlorella* had the highest CP content of all feed sources evaluated, and were much higher than feeds typically used as feed supplements (e.g. CSM) (Table 8). *Schizochytrium* spp. had the highest CL content of all feeds analysed. Fibre content of all the algae species, including the macro-algae, were lower than traditionally used protein supplements. The mineral profile of the algae species evaluated was highly variable (Table 9). Sodium content was greatest in the marine algae species and *Spirulina* and *Chlorella* had relatively high P content compared to the other algae species, and this was comparable to the P content of CSM and canola meal. There was little difference in the amino acid profile of the algae species evaluated in this study (Table 10). The *in vitro* degradability, relative to casein, was highest for *Spirulina* and *Dunaliella* algae and lowest for copra meal (Table 11). With the exception of *Chlorella* and *Schizochytrium*, the algae spp. investigated had higher relative degradability than the traditionally used protein supplements.

In conclusion, the variability in composition and degradability of the algae species investigated here suggests they may be useful supplements for ruminants when specific nutrient (e.g. protein or mineral) deficiencies exist or when specific production goals are targeted. Some algae species displayed characteristics that suggest they may be beneficial if offered as supplements or included in supplement mixes, with results comparable or higher than those of traditionally protein supplements. *Spirulina* sp. and *Chlorella* sp. may have a role as supplements under low CP grazing scenarios. *Dunaliella* sp. and *Nannochloropsis* sp. may be included in supplements to regulate acceptance and intake. *Schizochytrium* sp. will increase lipid intake, and supply polyunsaturated fatty acids, when included in diets. .

Table 8. Chemical composition of algae species, protein sources and Mitchell and pangola grass hays.

	Composition (g/kg DM)					
Source	**OM[1]**	**CP[2]**	**CL[3]**	**NDF[4]**	**ADF[5]**	**WSC[6]**
Chlorella pyrenoidosa	936	580	136	4	0	22
Dunaliella salina	280	78	101	0	0	10
Nannochloropsis oculata	688	312	36	4	14	38
Nannochloropsis sp.	235	67	23	4	0	22
Schizochytrium sp.	904	120	198	0	3	143
Spirulina platensis - A	906	675	114	63	0	19
Spirulina platensis - B	861	668	109	1	0	132
Chaetomorpha linum	584	195	34	166	104	7
Cladophora patentiramea	323	110	20	100	97	13
Enteromorpha sp.	607	303	46	170	90	20
Casein	960	933	na[7]	0	na[7]	0
Zein	981	931	na[7]	1	na[7]	7
Canola meal	919	412	52	270	165	131
Copra meal	935	211	91	552	297	125
Cottonseed meal	927	465	42	174	128	113
Palm kernel meal	962	144	155	700	357	33
Soybean meal	931	515	29	83	43	162
Mitchell grass hay	888	39	na[7]	701	390	48
Pangola grass hay	925	89	15	752	414	36

[1]Organic matter (OM); [2]crude protein (CP); [3]crude lipid (CL); [4]ash-free neutral detergent fibre (NDF); [5]ash-free acid detergent fibre (ADF); [6]water soluble carbohydrates (WSC); [7]not analysed (na).

Table 9. The mineral composition of algae species and protein supplements.

Name	P	Ca	Mg	Na	K	S	Fe	Cu	Co	I	Zn	Mo	Mn
	(g/kg DM)						(mg/kg DM)						
Chlorella pyrenoidosa	11.1	2.0	2.3	0.6	10.3	7.2	86	13	1	16.1	56	2	17
Dunaliella salina	0.8	1.5	20.9	177.0	9.5	26.6	296	21	1	2.8	36	2	99
Nannochloropsis oculata	9.6	45.7	6.8	42.0	12.6	16.0	114	17	1	2.1	53	2	42
Nannochloropsis sp.	1.7	10.9	20.1	194.3	11.9	26.3	196	7	1	1.7	58	2	50
Schizochytrium sp.	1.6	0.3	1.1	22.4	5.8	24.7	138	28	1	1.6	54	1	35
Spirulina platensis - A	8.6	3.8	3.1	9.4	13.7	8.9	7	4	1	1.5	3	1	0
Spirulina platensis - B	6.8	0.9	2.0	18.7	15.0	8.7	6	3	1	1.3	40	1	0
Chaetomorpha linum	1.4	24.1	8.0	45.0	67.2	28.9	629	7	1	29.2	21	1	51
Cladophora patentiramea	0.8	14.6	7.1	34.9	40.2	30.0	681	13	1	65.3	55	1	41
Enteromorpha sp.	3.6	6.9	10.9	57.7	43.5	31.4	734	4	1	53.0	8	1	29
Casein	6.2	0.3	0.0	11.5	0.2	7.8	10031	19	7	16.1	53	6	573
Zein	0.1	0.0	0.0	7.0	1.1	6.9	619	22	3	na[1]	74	3	224
Canola meal	9.3	5.0	4.5	1.8	11.2	8.3	90	13	0	na[1]	236	1	5
Copra meal	4.8	0.8	2.5	0.7	18.9	3.3	538	76	1	na[1]	1880	2	57
Cottonseed meal	9.4	1.8	5.2	1.6	13.4	5.4	180	13	2	na[1]	260	3	17
Palm kernel meal	3.8	1.9	1.7	0.1	5.3	2.1	125	19	3	na[1]	69	1	112
Soybean meal	5.6	2.3	2.4	0.0	21.2	4.8	31	6	3	na[1]	6	1	4

[1]**not analysed**

Table 10. The amino acid (AA) profile of various algae species

Name	Asp[1]	Thr[2]	Ser[3]	Glu[4]	Gly[5]	Ala[6]	Val[7]	Met[8]	Ile[9]	Leu[10]	Tyr[11]	Phe[12]	His[13]	Lys[14]	Arg[15]
	(% of total AA)														
Chlorella pyrenoidosa	9.6	5.4	5.0	13.3	6.3	8.9	6.4	2.6	4.5	9.8	3.9	5.3	2.7	9.3	7.1
Dunaliella salina	12.7	7.0	5.7	14.9	6.3	8.2	7.9	2.2	5.7	10.3	2.9	5.7	2.0	3.3	5.3
Nannochloropsis oculata	10.4	5.8	5.7	13.7	6.9	10.7	6.8	2.3	4.6	9.4	3.2	5.3	2.7	6.4	6.1
Nannochloropsis sp.	12.4	6.7	6.4	14.2	6.7	9.9	7.0	1.7	5.2	8.6	3.1	6.7	2.0	4.2	5.1
Schizochytrium sp.	10.5	6.2	6.1	20.1	5.5	7.9	6.0	3.3	4.3	8.1	3.2	4.5	2.8	3.6	7.8
Spirulina platensis - A	10.7	5.8	5.8	16.5	5.4	8.1	6.7	2.1	6.1	9.5	4.3	4.7	2.2	4.6	7.6
Spirulina platensis - B	9.9	5.7	6.0	16.3	5.2	8.2	7.0	2.5	6.3	9.7	4.6	4.6	2.2	4.7	7.0
Chaetomorpha linum	16.6	4.9	5.7	15.1	6.1	5.4	5.5	2.0	4.0	7.0	4.7	5.2	2.3	8.0	7.4
Cladophora patentiramea	16.3	5.7	5.1	14.2	6.7	6.3	6.8	2.5	4.4	7.7	3.5	4.9	2.5	6.4	6.9
Enteromorpha sp.	13.1	6.2	6.2	13.8	6.8	9.4	6.7	2.4	5.2	8.5	3.4	6.0	2.2	4.2	5.9

[1]Aspartic acid (Asp); [2]Threonine (Thr); [3]Serine (Ser); [4]Glutamic acid (Glu); [5]Glycine (Gly); [6]Alanine (Ala); [7]Valine (Val); [8]Metionine (Met); [9]Isoleucine (Ile); [10]Leucine (Leu); [11]Tyrosine (Tyr); [12]Phenylalanine (Phe); [13]Histidine (His); [14]Lysine (Lys); [15]Arginine (Arg).

Table 11. *In vitro* degradability of various algae species and protein supplements

Source	N (%)	Ammonia-N[1] (mg NH_3N /g DM)	Degradability (%)	Degradability relative to casein (%)
Chlorella pyrenoidosa	9.3	56.9	10	16
Dunaliella salina	1.3	55.1	43	69
Nannochloropsis oculata	5.0	65.8	38	60
Nannochloropsis sp.	1.1	51.0	25	39
Schizochytrium sp.	1.9	49.8	21	33
Spirulina platensis - A	10.8	95.4	42	67
Spirulina platensis - B	10.7	97.7	46	73
Chaetomorpha linum	3.1	58.6	34	53
Cladophora patentiramea	1.8	51.7	26	41
Enteromorpha sp.	4.8	65.2	30	48
Casein	14.9	162.7	63	100
Zein	14.9	52.1	2	3
Canola meal	6.6	65.8	23	36
Copra meal	3.4	48.0	3	5
Cottonseed meal	7.4	61.5	15	24
Palm kernel meal	2.3	53.0	19	30
Soybean meal	8.2	77.0	33	52
Mitchell grass	na[2]	na[2]	na[2]	na[2]
Pangola grass	1.4	50.1	9	19

[1]AmmoniaN released
[2]not analysed (na).

1.5 Rumen function and microbial protein production of steers fed speargrass hay supplemented with various algae species or cottonseed meal (Experiment 7)

Background

Experiment 3 of the project demonstrated that the provision of increasing amounts of *Spirulina* to cattle increased feed intake, MCP production and the fractional outflow rate of liquid from the rumen compared to a NPN source. Experiment 6 of the project demonstrated that other algae species have properties which may also stimulate feed intake, MCP production and the fractional outflow rate of material from the rumen. This objective of this experiment was to examine the effect of *Spirulina*, *Chlorella* and *Dunaleill*a algae species on feed intake, MCP production and rumen function in cattle and compare these results with CSM.

Experimental design and materials and methods

Five Brahman-cross steers (187 ± 4 kg) were offered five treatments diets in a 5x5 latin-square design, consisting of five replicates (steers) and five treatments (diets).
The five treatments were:

1. Speargrass (*Heteropogon contortus*) hay (SG) *ad libitum* (Control),
2. *Spirulina platensis* supplement (4 g Spirulina DM/kg W.d) plus SG *ad libitum* (Spirulina),
3. *Chlorella pyrenoidosa* supplement (4.7 g Chlorella DM/kg W.d) with SG *ad libitum* (Chlorella),
4. *Dunaliella salina* supplement (4 g Dunaliella DM/kg W.d) with SG *ad libitum* (Dunaliella), and
5. CSM supplement (6.0 g CSM DM/kg W.d) with SG *ad libitum* (CSM).

Feed intake, digestibility, MCP production and rumen function were measured.

Results and discussion

Steers offered *Spirulina* had a higher intake of SG hay (approximately 30%) than both unsupplemented or *Dunaliella* supplemented steers (Table 12). Supplementation with CSM or *Chlorella* did not significantly increase SG hay intake above unsupplemented steers. Intake of *Dunaliella* was much lower (0.7 g DM/kg W.d) than the target intake of 4.0 g DM/kg W.d. Steers supplemented with *Spirulina* and CSM had higher total dry matter intake than Control and *Chlorella* and *Dunaliella* supplemented steers. Nitrogen intake was similar between steers supplemented with *Spirulina*, *Chlorella* and CSM, as planned. Digestibility of OM was highest for steers supplemented with *Chlorella* and CSM and lowest for steers supplemented with *Dunaliella*. Microbial protein production and EMCP was higher in steers supplemented with *Spirulina*, *Chlorella* and CSM compared with unsupplemented or *Dunaliella* supplemented steers with no difference between the three supplementary sources of N (Table 13). Rumen ammonia-N concentration was significantly higher in the rumen fluid of steers supplemented with *Spirulina* compared with unsupplemented or *Dunaliella* supplemented steers. The concentration of total VFA in the rumen fluid of steers did not differ between treatments. Steers supplemented with *Spirulina* had the highest percentage of valeric, isovaleric and isobutyric acids. Retention time of Cr-EDTA was shorter in the rumen of steers

supplemented with *Spirulina* and CSM, with no difference between these two supplements, resulting in an approximately 1.9-fold increase in fractional outflow rate of steers fed *Spirulina* compared with unsupplemented steers. Steers supplemented with *Chlorella* had higher plasma glucose concentrations than the other steers with no significant difference between the other treatments. Steers supplemented with *Spirulina*, *Chlorella* and CSM had approximately 3 to 6-fold higher plasma urea concentration than unsupplemented steers or steers supplemented with *Dunaliella*, respectively, with no difference between these three treatments.

In conclusion, this experiment demonstrates that *Spirulina* and *Chlorella* have the potential to be used as protein supplements for cattle grazing low CP pastures in the future, with results comparable to CSM likely when fed to supply equivalent amounts of N. Intake of *Dunaliella* was low in the present experiment. However, given its high mineral content it may have a role as a supplement for ruminants to address specific mineral deficiencies, at lower intakes than employed in the present study, or as an intake regulator.

Table 12. Hay, supplement, total dry matter (DM), N and digestible organic matter (DOM) intake and DM and organic matter (OM) digestibility of steers fed speargrass hay alone (SG) or supplemented with Spirulina, Chlorella, Dunaliella and cottonseed meal (CSM)[1]

Parameter	SG	Spirulina	Chlorella	Dunaliella	CSM	SEM
Hay intake (g DM/kg W.d)	11.4^{a}	16.0^{b}	12.1^{ab}	11.6^{a}	14.3^{ab}	1.3
Supplement intake (g DM/kg W.d)	na[2]	4.0^{b}	4.7^{c}	0.7^{a}	6.0^{d}	0.3
DM intake (g DM/kg W.d)[3]	12.6^{a}	21.2^{c}	18.2^{b}	13.4^{a}	21.5^{c}	1.5
DOM intake (g OM/kg W.d)[3]	5.4^{a}	9.0^{c}	8.1^{b}	5.6^{a}	9.6^{c}	1.3
N intake (g DM/kg W.d)[3]	0.06^{a}	0.53^{b}	0.50^{b}	0.09^{a}	0.53^{b}	0.02
DM digestibility (%)	41.8^{ab}	45.5^{ab}	47.9^{b}	41.2^{a}	47.6^{b}	2.7
OM digestibility (%)	46.5^{b}	48.9^{bc}	51.0^{c}	44.4^{a}	51.4^{c}	2.5

[1]Values are means with standard error of the mean (SEM). Different alphabetical superscripts across the rows indicate significant difference between treatments (P<0.05); [2]na = not applicable; [3]Includes 300 g molasses/d.

Table 13. Microbial production (MCP), efficiency of MCP (EMCP), concentration of ammonia N, molar percentage of volatile fatty acids (VFAs), retention time (RT) and fractional outflow rate (FOR) in the rumen and glucose and urea concentration in the plasma of steers fed speargrass hay alone (SG) or supplemented with Spirulina, Chlorella, Dunaliella and cottonseed meal (CSM)[1]

Parameter	SG	Spirulina	Chlorella	Dunaliella	CSM	SEM
MCP (g/d)	57.8^{a}	167.7^{b}	170.8^{b}	58.3^{a}	185.7^{b}	38.0
MCP (g/kg W.d)	0.28^{a}	0.82^{b}	0.87^{b}	0.29^{a}	0.89^{b}	0.16
EMCP (g MCP/kg DOMI)	52.1^{a}	90.7^{b}	105.9^{b}	56.5^{a}	90.5^{b}	16.2
Ammonia N (mg/L)	26.9^{a}	187.7^{b}	82.9^{ab}	38.6^{a}	136.9^{ab}	75.7
Total VFA (mM)	54.9	52.3	57.1	51.3	61.5	14.7
Acetic (% total VFAs)	74.5^{bc}	71.7^{ab}	75.1^{c}	76.5^{c}	70.2^{a}	2.22
Propionic (% total VFAs)	15.4^{ab}	15.5^{b}	14.5^{ab}	14.2^{a}	14.9^{ab}	0.79
Isobutyric (% total VFAs)	0.48^{a}	1.51^{b}	0.60^{a}	0.56^{a}	0.64^{a}	0.19
Butyric (% total VFAs)	8.95^{a}	7.95^{a}	8.71^{a}	7.88^{a}	13.10^{b}	1.58
Isovaleric (% total VFAs)	0.37^{a}	1.09^{b}	0.54^{a}	0.40^{a}	0.60^{a}	0.22
Valeric (% total VFAs)	0.28^{a}	2.27^{b}	0.52^{a}	0.44^{a}	0.58^{a}	0.32
RT (h)	24.6^{a}	13.7^{b}	21.9^{a}	23.0^{a}	14.7^{b}	2.92
FOR (%/h)	4.1^{a}	7.7^{b}	4.9^{a}	4.6^{a}	6.9^{b}	0.96
Glucose (mmol/L)	4.24^{a}	4.12^{a}	5.54^{b}	4.94^{a}	4.32^{a}	0.83
Urea (mmol/L)	2.20^{a}	6.02^{b}	6.67^{b}	1.06^{a}	5.88^{b}	1.79

[1]Values are means with standard error of the mean (SEM). Different alphabetical superscripts across the rows indicate significant difference between treatments (P<0.05).

2 Conclusions and Recommendations

2.1 Conclusions

1. The dominant bacterial species in the rumen 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.
2. Bacterial diversity in the rumen was associated with liveweight gain in response to increasing N supply from different sources of N.
3. Divergence in liveweight gain post-weaning was not associated with any particular bacteria, or grouping of bacteria, within the rumen of steers.
4. Algae species are variable in composition and nutritive value and may be used as supplements to alleviate specific nutrient deficiencies in animal production, including protein deficiency in ruminants.

5. Growing steers fed low crude protein diets will respond to Spirulina and Chlorella supplementation in a comparable fashion to traditionally used protein supplements, such as cottonseed meal suggesting these protein sources may potentially be used by industry in the future.

2.2 Recommendations

1. 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 so that future variability of this community, as it links to EMCP and nutrition of cattle, can be better understood and manipulated where required. At present these species have not been described nor studied for function.
2. Further work be conducted to evaluate and develop the relationship between liveweight gain and bacterial diversity within the rumen of cattle fed a range of diets.
3. The development of algal ponds and means of harvesting algae for animal use on-farm should be researched.
4. Further work be conducted to assess other reported benefits of various algae species on the composition of meat and milk and on neonatal vigour, in a range of cattle classes (e.g. growing weaners, finishing steers, lactating females) under different dietary regimes (e.g. roughage, grain)
5. The potential use of algae by-products from biofuel production for livestock production should be evaluated (thus far the algae species evaluated have not undergone any post-harvest processing, apart from drying).

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4 Appendices

4.1 Appendix 2. Acknowledgements

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- Dr Karen Harper – Experiments 3 and 5
- Mr Emilio Martinez – Experiment 4
- Mr Diogo Costa – Experiments 6 and 7

4.2 Appendix 1. Description of the 454-pyrosequencing methodology and data analysis

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 Yu and Forster (2005). Genomic DNA (gDNA) extraction was then completed using commercial column extraction (Qiagen QIAamp DNA kit, QIAGEN Pty. Ltd., Doncaster, Australia) based upon a method published by Yu and Forster (2005).Extracted samples were visualised by gel electrophoresis with 1% agarose and ethidium bromide and DNA concentration was calculated using the Nanodrop 8000 Spectrophotometer (ThermoFisher scientific, Australia) as per manufacturer's instructions. The purity of the DNA was determined by measuring the absorbance ratio at 260 nm/280 nm. Samples with a purity ranging from 1.7 – 2.0 were diluted with sdH2O to a final concentration of 10 ng/µL and used for subsequent analysis.

Polymerase chain reaction

PCR was performed using fusion primers manufactured by Integrated DNA technologies (IDT) based on 16S rRNA gene primers 341f (Watanabe *et al.*, 2001) and 787r (Allen *et al.*, 2001). For each sample the forward primer (341f) also contained a TAG (4bp of TCAG) and individual multiplex identifiers (MIDs) (Roche, Australia). The PCR reaction mixture was made to a final volume of 50 µL with the following reagents; 1.25 µL of 10 µM of Primer 341f-TAG-MID and of Primer 787r-TAG; 1.0 µL dNTP mix (10 µM) (Roche, Australia); 10 µL high fidelity (HF) Phusion buffer; 0.50 µL HF Phusion polymerase (Finnzymes, Australia); and 2 µL of 10 ng/µL (c. 20 ng) of genomic DNA. Samples were vortexed and centrifuged briefly before placing into the thermocycler.

Thermocycling was performed in an Eppendorf Mastercycler using Eppendorf Cycle Manager 1.1.3 software (Eppendorf, North Ryde, Australia). Optimised conditions were: - Lid preheated to 101^{o}C; initial denaturation 98^{o}C for 30 sec; followed by 30 cycles of: 98^{o}C for 10 sec; 65^{o}C for 20 sec; 72^{o}C for 15 sec; followed by a final extension step at 72^{o}C for 10 min and a 'cooling' step at 12^{o}C for 5 min. PCR products were excised from agarose gels after electrophoresis and purified using QIAGEN gel purification kit (QIAquick PCR Purification Kit, QIAGEN, Pty Ltd, Doncaster, Australia) according to manufacturer's instructions, and eluted in 30µL of Elution Buffer.

454-pyrosequencing

Approximately 300 ng of purified PCR product, in a final volume of 30 µL was sent to the Australian Genomic Research Facility (AGRF) at the University of Queensland for pyrosequencing where amplicons were pooled in equimolar amounts and emulsion PCR performed as per manufacturer's instructions. Pyrosequencing was undertaken on a Roche 454 Genome Sequencer FLX (Roche Diagnostics Corporation, Mannheim, Germany). Data was returned in two formats; firstly as raw data unaltered as standard flowgram files (sample.sff), and secondly as deconvoluted data. The deconvoluted data was sorted into original samples based on barcodes and included individual samples in FastA format (sample.fna), combined sample data in FastA format (contig.fna) and quality files (contig.qual and contig.ace).

Data analysis

Data was analysed using the QIIMEs (Quantitative Insights Into Microbial Ecology) pipeline of software (Caporaso *et al.*, 2010b). There are a number of steps involved in data analysis. Firstly the conversion of raw data in the standard flowgram files (.sff files) into sff.txt files (required for denoising). Data was denoised using a python script (denoise.py) and fast denoise process within QIIMEs using default settings. Denoised data was converted into FastA files (.fna or. fasta) and quality score files (.qual files) for the assessment of DNA sequence quality. As samples had been pooled together, the following step involved the de-convolution of data back into individual samples by barcodes and also filtered sequences based on sequence length (minimum of 430 bp and maximum of 470 bp), low quality score, incorrect barcodes and forward priming sites. Sequences were then grouped based on similarity using operational taxonomic units (OTUs) with a threshold of 97% sequence similarity which has been suggested as equating to the species level when examining full length 16S rRNA sequence (Stackebrandt and Goebel, 1994). However, as sequences in this experiment were partial length (approximately 450bp), the 97% similarity was more likely to represent the genus level.

A representative sequence for each of the OTUs was selected using the 'most abundant' method and then aligned with a nominated core set with the 16S rRNA Greengenes core set (http://greengenes.lbl.gov/) (DeSantis *et al.*, 2006) and PyNAST (Caporaso *et al.*, 2010) was used as the alignment method. The alignment was then used to check for chimeric sequences using ChimerSlayer. Chimeric representative sequences were then removed from the alignment and subsequently, any members belonging to that OTU, were also removed. The representative sequences (minus chimeras) were then assigned a taxonomic classification using the Ribosomal Database Project (Cole *et al.*, 2009) as the reference database. To remove non-informative data (e.g. comprised of gaps only) within the alignment it was filtered using a file obtained from the Greengenes website (http://greengenes.lbl.gov/). A phylogenetic tree was then constructed from the filtered alignment using the fasttree method. An OTU table was constructed which consisted of a matrix of OTUs, the number of individual sequences within each OTU and the taxonomic classification of OTUs for each sample.

A graphical display showing only the OTUs that were found in all of the samples (designated as core OTUs) as well as the number of sequences per OTU was generated using a heatmap. A network of the OTUs was also constructed using a QIIMEs script and visualized using the open source platform for complex network visualisation

Cytoscape© (Smoot *et al.*, 2011). Large nodes (diamonds) depict the sample, smaller nodes represent OTUs. Edges (lines) connect OTUs from samples. Interconnecting lines depict shared OTUs between samples.

Taxonomic classifications were also summarized for each sample as a percentage composition of the total community. Both Genus and Phylum levels were summarized for each sample and graphically illustrated as stacked bar graphs. Various alpha diversity indices measuring the diversity within a sample were calculated within QIIMEs for each sample. These include Chao1 (Chao, 1984), Observed Species and the Shannon and Simpson (Hayek and Buza, 1996). Statistical analyses of these indices were performed using ANOVA with orthogonal terms in GenStat. Beta diversity, measures the diversity between samples. These indices were calculated within QIIMEs using the beta diversity metrics of weighted and unweighted UniFrac which take into account the evolutionary relationships between sequences. This was then related to metadata (such as food intake, N intake and LW gain) available to generate both 2D and 3D Principal Coordinate analysis (PCA) plots. Statistical analysis, using ANOVA, was also performed which pulled out the OTUs of significance ($P \leq 0.05$) based on treatment so that unique OTUs were revealed.