



Report for AGRIndustries

“Responses in Rumen Cellulose Digestion and Microbial Metabolism to Different Forms of Nitrogenous Supplementation *InVitro*”

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September, 2009

Summary

Three nitrogenous feed supplements supplied by AGR Industries in liquid form and identified as “Sample A”, “Sample B”, and “Sample C”¹ were evaluated in an *in vitro* rumen system.¹

A study was made to determine whether the supplements enhanced rumen digestion of fibre (cellulose). The rates of release of ammonia from the three nitrogenous supplements *in vitro* were determined, as well as the effects of the supplements on pH, volatile fatty acid production and gas production by rumen microorganisms.

Supplement C generated a higher level of cellulose digestion ($P < 0.05$) and tended to promote higher VFA than Supplements A and B. With all supplements, pH was relatively stable and always above the minimum value that is considered likely to restrict digestion of feed materials in the rumen. Microbial metabolic activity was higher with Supplement C than with the other supplements.

The nitrogen (N) concentrations of Supplements A, B and C (supplied as solutions) were 4.93, 3.91 and 4.02 g/100 g, respectively. There was rapid release of ammonia from all supplements after they were mixed with rumen contents, with the rate of release being fastest for Supplement A, intermediate for B and slowest for C. Recovery of the N from supplements in ammonia that was not assimilated by microorganisms was 58-69%.

Because the ammonia concentrations *in vitro* were well above the concentrations considered likely to restrict rumen microbial digestion or cell growth *in vivo*, it is

¹ For the convenience of those readers who do not wish to read this report in its entirety we advise that this was a “double blind” investigation in which UNE were completely unaware of the identity and preparation method of each nitrogenous feed sample. In September 2009, after completion of all field and laboratory work and also the submission of draft version of this report AGR Industries formally advised UNE of the constituent ingredients and method of preparation used to prepare each of the three nitrogenous feed samples. A copy of this letter has been included as Appendix 6. AGR provided this advice for purposes of discussion regarding the possible mode of action and explanation regarding the observed results of these *in vitro* trials. Please refer to the “Postscript” section for more details on the identity of Samples A, B and C and hypotheses that may explain the higher level of cellulose digestion and microbial metabolic activity as observed with Supplement C.

The AGR Industries letter revealed that Sample A consisted of urea thermally reacted with molasses under acidic conditions. Sample B was a simple solution of urea and salts in molasses. Sample C was AGR Industries own proprietary product “AGRiliq” which was made according to an AGR proprietary alkaline pH-controlled process using molasses, urea and alkalis and acids as ingredients.

unlikely that the higher level of digestion and microbial metabolic activity observed with Supplement C was due only to the ammonia released from this supplement.

Introduction

The *in vitro* method that uses rumen contents from donor animals is commonly used to evaluate the nutritive value of feedstuffs and the effects of feed supplements on microbial digestion and cell growth in the rumen of sheep and cattle. Although not capable of completely simulating *in vivo* digestion processes, *in vitro* methods are a rapid and cost-effective alternative to animal studies (Getachew *et al.* 1998).

The aim of this experiment was to assess *in vitro* the effect of three nitrogenous supplements on feed cellulose digestion by a rumen microbial population, and to determine the production of ammonia, VFA, gases and microbial cells over a 24-hour period.

Materials and Methods

Experimental Design

The experiment consisted of 4 treatments (Supplements A, B and C plus an un-supplemented control) arranged according to a stratified block design. There were 3 replicate flasks for each of the 4 treatments.

Animals and rumen contents

Inoculum for the *in vitro* studies was obtained from donor sheep (cross-bred Merino wethers with rumen fistulas) maintained on pasture and supplemented with chaffed lucerne (*Medicago sativa*) hay. Feed and water were freely available to the sheep prior to sampling. Ruminant ingesta were collected 3-4 h after the sheep were offered their morning feed. Rumen fluid was withdrawn via the ruminal fistulas using a syringe

connected to 1 cm silicon tubing, ending in a blind brass plug, perforated with 1 mm holes. The sample was drawn under vacuum into a 2-litre Winchester bottle, previously flushed with CO₂ and pre-warmed to 39°C and carried in the insulated container. The rumen ingesta were taken immediately to the laboratory in an insulated container.

Materials

Prior to sampling, 2 L of buffer formulated to mimic sheep's saliva was prepared according to the method of McDougall (1948). The buffer was sparged with CO₂ for approximately 30 min to reduce pH to 6.8. Cellstar® 250 ml tissue culture flasks (Greiner-bio-one, GDR) were used as incubation vessels. These were modified by replacing the lids with silicon stoppers (Cole Palmer Instr. Co., Japan) through which 2 14 g, 38 mm indwelling sampling tubes had been introduced. The plastic sampling tubes were in turn sealed by 3-way stopcocks (Discofix®Braun,GDR) that enabled fluid to be withdrawn without allowing atmospheric gases to enter the flask (to maintain strict anaerobic conditions). Fluid samples were extracted from the flask liquid via a 5ml disposable syringe (Terumo Corp., Tokyo, Japan) connected to the sampling tubes. Gas samples were obtained using a 50 ml syringe (Terumo Corp., Tokyo, Japan), connected to the other sampling port. Prior to addition of inoculum and sealing, a glass marble was inserted in each flask to aid agitation. An 11 cm diameter filter paper (0.445-0.462g, Whatman No. 2, England – a source of N-free cellulose) was inserted into each treatment flask, to act as an additional source of energy substrate.

To start the incubations, 100 ml each of rumen fluid and 100 mL McDougall's buffer were dispensed into each flask under CO₂. About 1.1 g of Supplements A, B and C were each accurately weighed (see Appendix 1) and added to 9 flasks (3/supplement) and another three flasks were incubated with no added supplement solution (controls). A 5 ml subsample of the rumen fluid/buffer mixture was taken from the control flasks prior to commencement of incubation to represent the zero-time concentrations of ammonia and VFA. The flasks were sealed and placed in an agitating water bath at 39° C.

All flasks were sampled for gas production and liquid during the incubation period at 1, 3, 5, 7, 11, 19 and 24 h. pH was measured within 2 min of sampling, and samples were then frozen (-18°C) pending analysis. At the conclusion of the trial, samples of the incubation solution were removed from all flasks and stored at -20°C. The filter papers were removed from the incubation flasks, washed with distilled water and dried at 50°C for 48 h, prior to weighing, so that loss in cellulose dry weight could be estimated.

Analysis

Nitrogen content of the feed supplements and the filter papers after incubation was determined by Dumas combustion (FP 2000, Leco Corp.). Ammonia concentrations in the incubation fluid were determined by the salicylate-cyanurate method on a segmented flow analyzer (Skalar San⁺⁺ System, Netherlands). Ammonia present in the flasks at different times during the incubation period (the net effect of ammonia production and ammonia utilization for microbial growth) was determined by multiplying the fluid ammonia concentration (mgN/mL) by the volume of fluid in the flask (mL). The ammonia in each flask arising specifically from the added N supplements was determined by subtracting the ammonia present in the control flasks at each sampling time, i.e. the ammonia produced from the rumen fluid inoculum alone was subtracted from the total amount produced from the inoculum and the added supplement.

The ammonia concentrations versus time data for different incubation solutions were fitted by non-linear least squares regression (WinSAAM; Stefanovski *et al.*, 2003) to an equation with two exponentials of the form:

$$Y_t = -P \cdot \exp(k_1 \cdot t) + P \cdot \exp(-k_2 \cdot t)$$

where t = time (min), P represents the intercept of the second exponential function at time zero, and k_1 and k_2 are the respective rate constants for the buildup and decay components of the curve.

Microbial dry matter (DM) colonization of the filter paper (a pure, N-free cellulose source) was estimated by assuming that N retained on the filter paper at the end of the incubation period was present in colonizing microorganisms containing 8 gN/100 g DM. The net DM disappearance of cellulose was calculated as the apparent DM loss of filter paper corrected for microbial DM present.

Volatile fatty acid (VFA) concentrations were determined by Gas Liquid Chromatography (Varian CP 3800) using iso-caproate as an internal standard to provide a reference for the volume of sample injected.

Gases were collected at atmospheric pressure into new disposable plastic syringes connected to outlets in the incubation flasks. Carbon dioxide was removed from the total gases collected by dissolving it in 0.5 mL 1M NaOH in the syringes and the remaining gas was assumed to be methane.

Calculations

Because the rumen fluid inoculums used to create the in vitro rumen system was already metabolic active, control flasks with no additional added materials were used to determine baseline values that were subtracted from totals to give net values for ammonia and VFA production in the in vitro flasks.

Results

The total N concentrations of the three unknown supplements A, B and C (supplied as solutions) were 4.93, 3.91 and 4.02 g/100 g, respectively. These differences in N concentration meant that 45-57 mg of N was added to each of the replicate flasks; however there was no relationship ($P < 0.10$) between the amount of N added to any flask and DM disappearance in the flask (DM loss) during the incubation period. Both apparent DM loss (%) (start weight-final weight of filter paper) and true filter paper DM loss (%) (apparent DM loss corrected for microbial DM) were greatest in flasks containing Supplement C (Table 1).

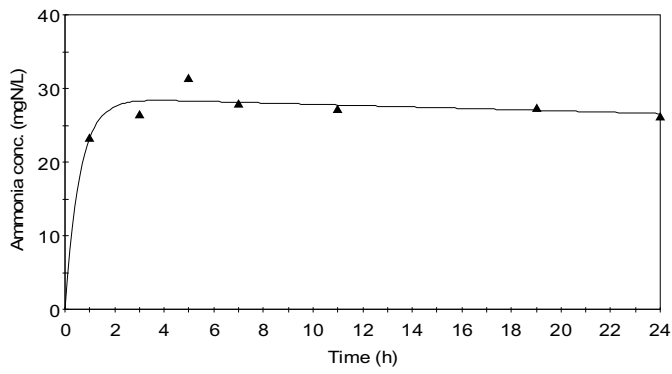
Table 1. Cellulose digestion and microbial colonization in an in vitro rumen after addition of N supplements (A, B and C)

<i>Parameter</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>S.E.</i>	<i>P-value</i>
Apparent cellulose DM loss (%)	13.4 ^{ab}	10.7 ^a	16.9 ^b	0.99	0.012
Estimated microbial DM (g) ¹	0.018	0.019	0.023	0.00199	0.292
Net DMD (%)	17.5 ^a	15.0 ^a	22.0 ^b	1.02	0.007

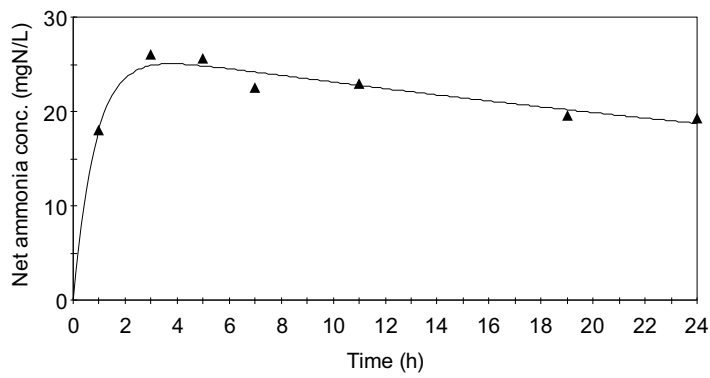
¹Estimated microbial attachment to filter paper at the end of incubation

Ammonia concentrations in the flasks at the start of the incubations were about 34 mgN/L and increased in all flasks during the incubations, indicating that more ammonia was produced than was utilised by the microorganisms present. Ammonia production was considerably greater in all flasks with added supplementary N. The ammonia released due to degradation of N in the supplements (or possibly the presence of ammonia itself) caused the ammonia concentrations to increase rapidly during the early part of the incubation.

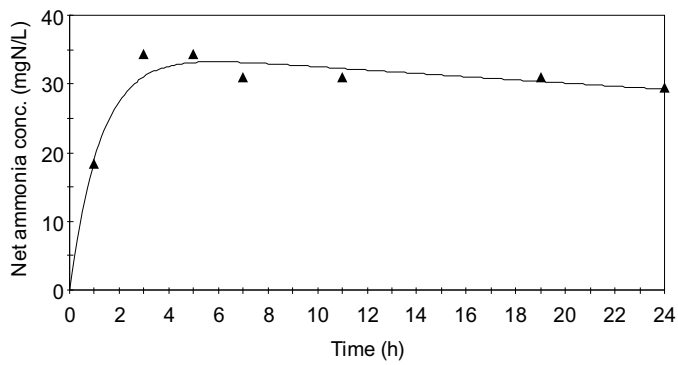
Of particular interest was the amount of ammonia produced in the supplemented flasks after subtracting the ammonia produced in the control flasks. Ammonia derived from the supplements caused concentrations to rise quite rapidly and reach maximum values at 3.8-5.9 h, after which concentrations declined (Figure 2). Details of curve fitting are and summarized in Table 2 (see also Appendix 4). The rate constants for the rising part of the ammonia concentration *v.* time curves (representing the initial release of ammonia from the supplements) were greatest for Supplement A (1.65/min), intermediate for Supplement B (1.2/min), and slowest for Supplement C (0.80/min) (Table 2). On the other hand, ammonia utilization (represented by the rate constant of the second exponential function defining the declining part of the curve) was slowest for Supplement A fastest for Supplement B (Table 2).



A



B



C

Fig. 2. Net ammonia concentrations resulting from degradation of supplements A, B and C during the *in vitro* incubations

Table 2. Parameters of equations describing the buildup and decay of net ammonia production after addition of supplements A, B and C to the *in vitro* rumen system

Parameter	A	B	C
Intercept (mgN/L)	28.8	26.9	35.1
Rate constant k_1 (min^{-1})	1.65	1.18	0.797
Rate constant k_2 (min^{-1})	0.00331	0.0150	0.00762
Time to reach maximum (h)	3.8	3.8	5.9
% of added N recovered in ammonia at the time of maximum ammonia concentration	65.5	58.0	68.5

The results in Table 2 confirm that there was rapid release of ammonia from all supplements after they were mixed with rumen contents. The buildup rate for ammonia concentration (rate constant k_1) was fastest for supplement A, intermediate for B and slowest for C. The time taken for ammonia to reach its peak value was also longest for supplement C (5.9 h). Some ammonia-N was assimilated for microbial growth; however, recovery of the supplementary N in ammonia that was not assimilated by microorganisms was 58-69%.

Volatile fatty acid production

Volatile fatty acid (VFA) concentrations increased in all flasks during the period of incubation due to fermentation of both feed residues and energy containing materials in the supplements. Mean values for total and individual VFA productions in the control flasks (3 replicates of each supplement) and each of the treatments A, B and C (3 replicates) are shown in Appendix 2. The VFA production in response to addition of supplements A, B and C (corrected for VFA production in the control flasks) are shown in Figs 3-6. There appeared to be a short lag in VFA production and possibly some utilisation of VFA in the 2 - 3 h period after the start of the incubation.

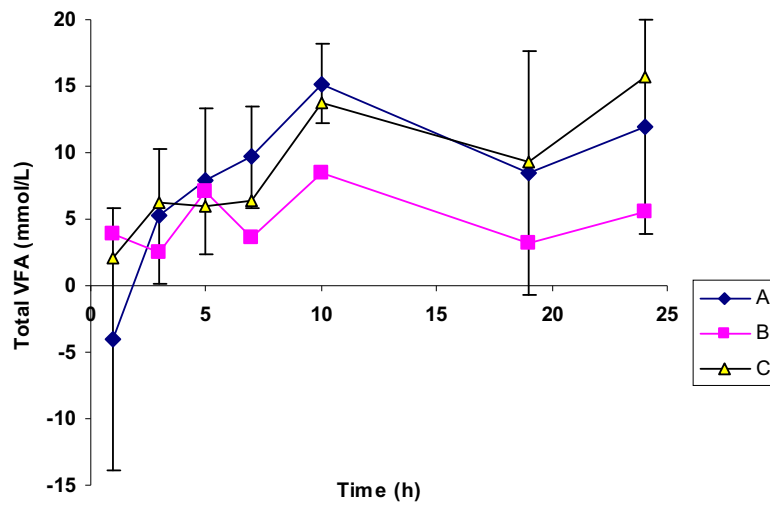


Figure 3. Net production (\pm SD) of total volatile fatty acids (VFA) in flasks containing Supplements A, B and C (corrected for VFA production in control flasks)

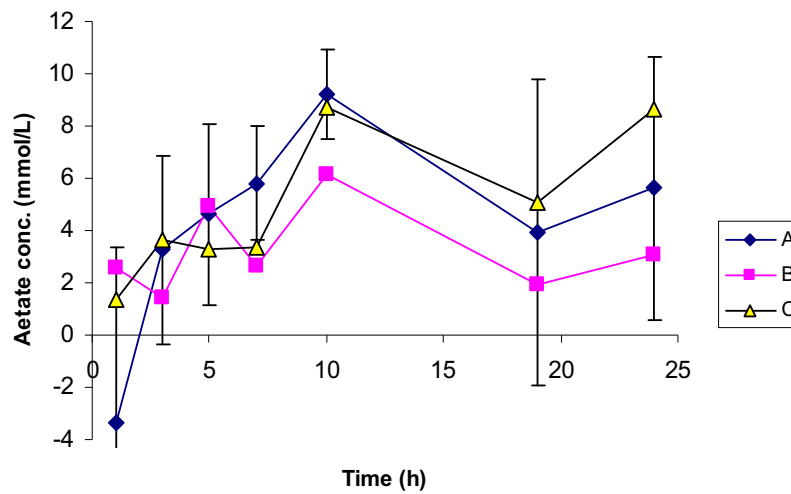


Figure 4. Net production (\pm SD) of acetate in flasks containing Supplements A, B and C (corrected for acetate production in control flasks)

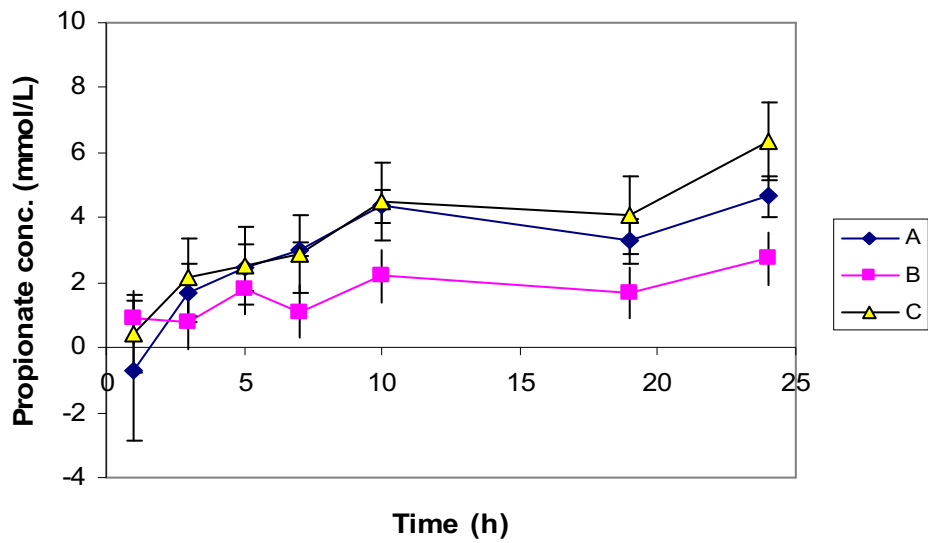


Figure 5. Net production (\pm SD) of propionate in flasks containing Supplements A, B and C (corrected for propionate production in control flasks)

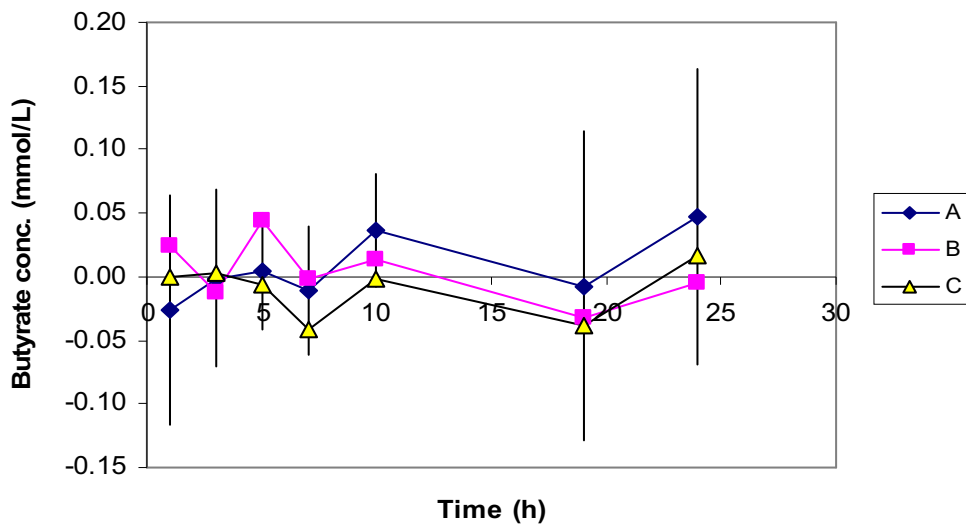


Figure 6. Net production (\pm SD) of butyrate in flasks containing Supplements A, B and C (corrected for butyrate production in control flasks)

Production of acetate and propionate was clearly detectable during the incubations (Figs 4 and 5) whereas butyrate production, which is always produced in lesser amounts, was at the limits of detection.

pH and gas production

The pH was relatively stable in all flasks (treatments and controls), varying in a small range between 6.75 and 6.95. Gas production in control flasks and supplemented flasks are shown in Appendix 5 and the *net* productions of total gases and methane are given in Fig. 8.

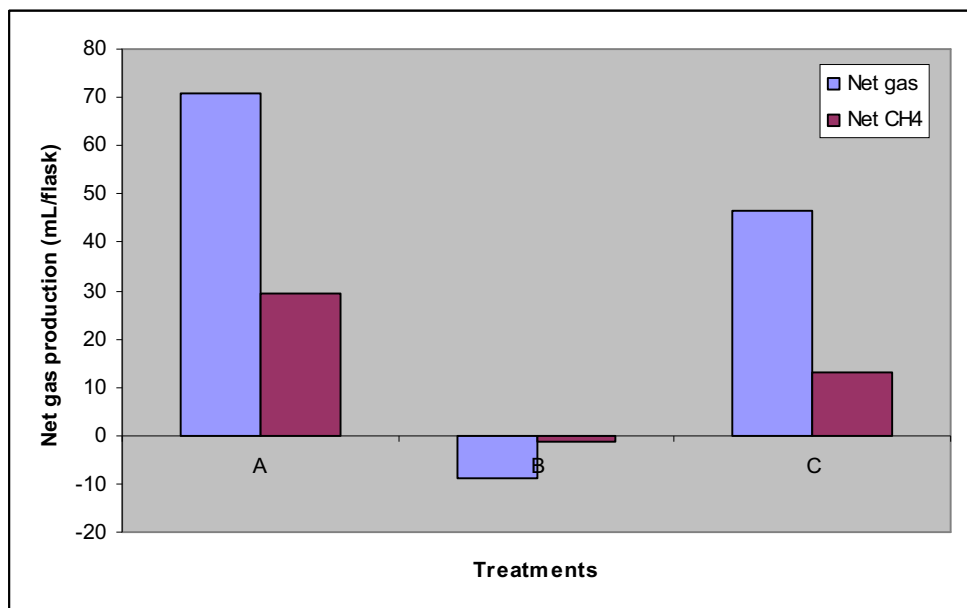


Figure 8. Net production of total gases and methane in flasks containing Supplements A, B and C (corrected for gas production in control flasks)

Amounts of methane produced in flasks to which Supplements A and C were added were greater than in those containing Supplement B for which the amounts of gas produced were not detectably greater than amounts generated in the control flasks.

Discussion

General comments

About half of all solar energy trapped by plant photosynthesis on the planet is fixed into the chemical structure of cellulose and ruminant animals are the only mammals that can liberate this energy. Ruminants can do this because they harbour anaerobic microbes in the rumen which produce enzymes (cellulases) that can degrade the polymeric structure of cellulose to glucose. Cellulases are not synthesized by mammalian tissues. Additionally, ruminants have another ability acquired from the rumen microorganisms – an ability to use ammonia as a source of N for protein synthesis (Nolan and Leng, 1984). This protein is produced by microorganisms as they grow in the rumen, and the protein is ingested by the host animal when the microbes leave the rumen and pass into the intestines where the protein is digested, absorbed and made available for tissue growth in the ruminant animal itself. These proteins contain the so-called essential amino acids that mammalian tissues cannot synthesise.

Feed supplements which provide ammonia or NAN to rumen microbes in situations where these are limiting nutrients to microbial metabolism, are likely to have positive effects on the growth/production of the host animal, because this will result in the greater and more complete breakdown of cellulosic materials and creation of greater amounts of microbial biomass.

Fibre/cellulose digestion

Filter paper apparent DM loss, true DM loss and microbial colonization of filter paper indicate that Supplement C was most effective in promoting cellulose digestion. This suggests that Supplement C either supplied N or energy more effectively to the microbial population, or that it supplied more of other limiting nutrients (e.g. intermediate-length or branched chain fatty acids, haem-like materials or minerals such as sulphur) that were

limiting to microbial metabolism than the other treatments. The pH of the inoculum was in the ideal range for cellulolytic rumen microorganisms.

It could also be postulated that the relatively low level of activity reflected in microbial DM production may have been because the surface of the filter paper was fully colonized in all treatments. It may aid differentiation between treatments to increase the amount of cellulose substrate supplied if similar techniques are used in the future.

Gas production and pH of incubation fluids

Gas production in the various treatments was generally consistent with DM disappearance, insofar as Supplement A produced the greatest amount of gas, although the treatment differences were not statistically significant. Care should be taken when relating these results to the likely situation *in vivo*, as the incubations were conducted using highly buffered media, which would have contributed to the relatively high and stable pH as well as contributing some of the released CO₂.

VFA production

VFA productions (total and acetate, and propionate,) were likewise consistent in trend to DMD, with Treatment C having greatest production and B the least.

Although there were statistically significant differences between supplemented flasks and control flasks, again there were no statistically detectable differences in VFA production between the supplements. This is probably not surprising as the supplements provided relatively small amounts of fermentable energy, and most likely had even smaller between-supplement differences in fermentable energy to support microbial growth.

Ammonia release and utilisation

Ammonia released into the flasks was derived from the continuing degradation of nitrogenous materials in the rumen fluid inoculum, augmented by that released from the digestion of the N supplements. The ammonia produced from the inoculum was determined in control flasks and subtracted from the treatment flasks to determine the N release from the supplements themselves. Some of the latter will have been used during growth of micro-organisms in the medium during the incubation period. The amount used for this purpose will have been small relative to the ammonia produced, but because of this assimilation, the maximum recovery of added N as ammonia in the flasks (58-69%) will have been under-estimated.

The ammonia concentration in all treatments increased rapidly (see Table 2) indicating that ammonia production in the *in vitro* rumen greatly exceeded ammonia assimilation rate by the microorganisms for growth (and their consequent protein synthesis during growth). The k_1 parameter of the fitted curves is a rate constant, i.e. the fractional rate of buildup per minute in net ammonia concentration in the flasks during the early part of the incubation period.). The k_1 values indicate that all the supplements were rapidly degraded by the action of microbial enzymes present in the rumen inoculum. It is therefore highly likely that all of the supplements will also be degraded rapidly in the rumen of sheep in commercial production systems. However, Supplement C was degraded more slowly than Supplement A and B and this may be related to the form in which the N was included in the supplements.

If it is considered important to rank rapidly degraded N supplements in the future, more frequent sampling during the early part of the *in vitro* incubation period would be needed to provide additional statistical 'power' to this type of study, and to enable small differences in ammonia release rates to be determined. In addition, studies of the rate of release of N from these supplements in the rumen of sheep given widely different types of diets would provide additional information on the efficacy of these supplements as sources of N for microbial growth in the rumen of animals on, for example mature temperate pastures versus mature tropical pastures. The importance of achieving slower

ammonia release rates from these types of supplements assumes greater importance when animals are ingesting their basal diet in discrete meals. Also it is important to have ammonia released and available in synchrony with the release of the fermentable energy in the diet (Trevaskis *et al.* 2001).

The k_2 parameter is a rate constant that mainly represents the utilization of ammonia in the latter part of the incubation. The declining concentration of ammonia in this period indicates that more ammonia was being assimilated by microorganisms than was being produced. It also confirms that an actively growing population of rumen microorganisms was present throughout the incubation period (i.e. microbial growth was still present in the later stages of the incubation).

Conclusions

Active fermentations were maintained throughout the period of the incubations. Values for pH were stable throughout the incubations, suitable for cellulolytic rumen microorganisms, and unaffected by the type of supplement. Treatment C generated higher (statistically significant) levels of cellulose DM disappearance, and tended ($P>0.05$) to produce more VFA during the incubation period than Supplements A and B. This indicates that greater levels of microbial metabolic activity were promoted by materials present in Supplement C although, given the rapid release of ammonia at the onset of incubation in all treatments and the high concentrations of ammonia present in all the flasks throughout the incubations, it is unlikely that the higher levels of metabolic activity were due only to provision of ammonia from Supplement C.

Postscript

Upon completion by UNE of the in vitro trials and the draft report, AGR were invited by UNE to provide comment. This subsequently led to discussion between AGR and UNE regarding the possible modes of action and explanation regarding the observed results of these in vitro trials. During these discussions AGR quickly formed the view that maximum benefit would be derived from these post trial discussions if they were conducted with both parties being fully aware of true identities of the nitrogenous Samples A, B, & C. Consequently, AGR informed UNE regarding the key ingredients and methods of preparation that were employed in the preparation of samples A, B & C. Please refer to AGR letter dated 28th September, 2009 which has been included as Appendix 6 in this report.

In summary, these three supplements were closely matched in their nitrogen, salt and carbon levels. The three supplements have been identified by AGR as:

1. Supplement A, an acidic thermally reacted molasses-urea blend, which was then neutralized, using a procedure similar to that described by Malik and Chopra (1979).
2. Supplement B, a simple solution of urea and salts in molasses.
3. Supplement C, 'AGRiliq' made according to an AGR proprietary alkaline pH-controlled process using molasses, urea and alkalis and acids as the ingredients. As a result of this process, the supplement contains a range of nitrogenous compounds including heterocycles such as pyrroles.

Arising from these post trial discussions, AGR Industries have put forward two hypotheses to explain the higher level of cellulose digestion and microbial metabolic activity as observed with Supplement C (AGRiliq). These hypotheses are:

1. the pyrrolic compounds within Supplement C ('AGRiliq') are metabolized to produce caproic acid, which is vital for efficient cellulolytic activity.

2. the alkali treatment which forms a stage of the 'AGRiliq' production possibly degrades anti-nutritive or inhibitory phenolic compounds which were originally present in the molasses ingredients, and so enhance microbial function.

Detailed discussions of Hypothesis #1 including further observations on the in vitro trial results and relevant/supporting scientific references

Fibre/cellulose digestion

Besides pH control, cellulose digestion is promoted by intermediate length fatty acids, such as valeric and caproic acids (Bentley et al, 1954; Bentley et al, 1955; Dehority et al, 1967).

The minority VFA caproic acid, which is important for cellulolytic activity, can be provided by the metabolism of the pyrrolic compounds within Supplement C ('AGRiliq'). For instance, El-Shazly (1952) has shown that pyrrole derivatives such as proline are enzymatically cleaved to δ -amino acids, which are further broken down to ammonia and intermediate-length VFAs. Please find possible evidence of an observed increase levels of Caproate in Sample C within the following section.

Also, the pyrrole rings of indole derivatives are susceptible to cleavage by anaerobes (Gu et al, 2002), including rumen microbes (Mohammed et al, 2003). Thus a number of pathways exist for the microbial utilization of the pyrrole groups in Supplement C.

Furthermore, Lacoste (1960) has also demonstrated that the δ -amino acids which result from pyrrole ring cleavage serve as precursors for intermediate-length and branched-chain fatty acids, again yielding ammonia as a byproduct. The ammonia then becomes available for the normal microbial anabolic processes. Normally, the production of these longer chain fatty acids is understood to proceed via the condensation of shorter chain units (Gray et al, 1952). However, this ring-cleavage mechanism provides an alternative synthetic route.

Minority VFA production

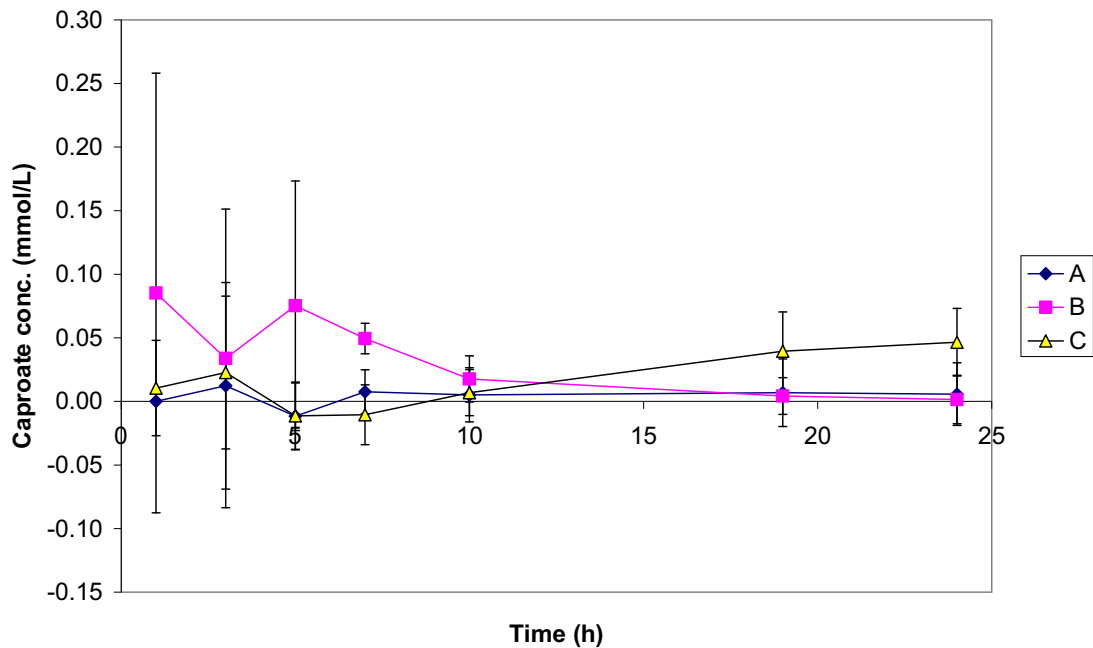


Figure 7. Net production (\pm SD) of caproate in flasks containing Supplements A, B and C (corrected for caproate production in control flasks)

Production of most of the minority VFAs was not significantly different from the control. However, caproate production showed a possible significant increase in sample C compared to samples A and B during the incubations (Figs 7). Further trials, which employ a more accurate analytical method, would be required to definitively determine whether or not there is a significant increase in Caproate levels in the Sample C beyond the control and Samples A and B.

It should also be noted that a limiting compound such as Caproate, which is essential for efficient cellulolytic activity, would therefore not be expected to be present in high levels.

Detailed discussions of Hypotheses #2 including further observations on the in vitro trial results and relevant/supporting scientific references

Another possible reason for the improved cellulolytic activity observed with Supplements A and C is the removal of antinutritive factors. Molasses contains a range of phenolic compounds, including ferulic, coumaric, and syringic acid derivatives (Yokota and Fagerson, 1971; Palla, 1982; Guimarães et al., 2006; Payet et al, 2006) which are inhibitory towards cellulolytic activity (Chesson et al, 1982).

It is possible that the alkali treatment which forms a stage of the 'AGRiliq' production (sample C) degrades these anti-nutritive or inhibitory compounds much like the process described by Hartley and Jones (1978) for reduction of phenolic compounds in straw in order to improve its digestibility. Indeed, both the acidified molasses ('Thermal' – Supplement A) and the alkali-treated molasses ('AGRiliq' – Supplement C) show improved cellulose digestion rates, since these phenols are degraded under acidic or alkali conditions (Krygier et al, 1982). Similarly, Borja et al (1993) have shown that removal of these phenolic compounds significantly improves the anaerobic digestion of molasses.

Conclusions arising from post-investigation discussions between UNE and AGR Industries

1. It is possible that additional factors which promote cellulolytic activity are present in Supplement C ('AGRiliq'). Additional limiting nutrients, especially intermediate-length fatty acids such as caproic acid which are required by cellulolytic organisms may be produced by the metabolism of pyrrolic compounds within this supplement.
2. Furthermore, microbial function is possibly enhanced by the degradation of inhibitory phenolic compounds during the AGRiliq production process.

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Appendices

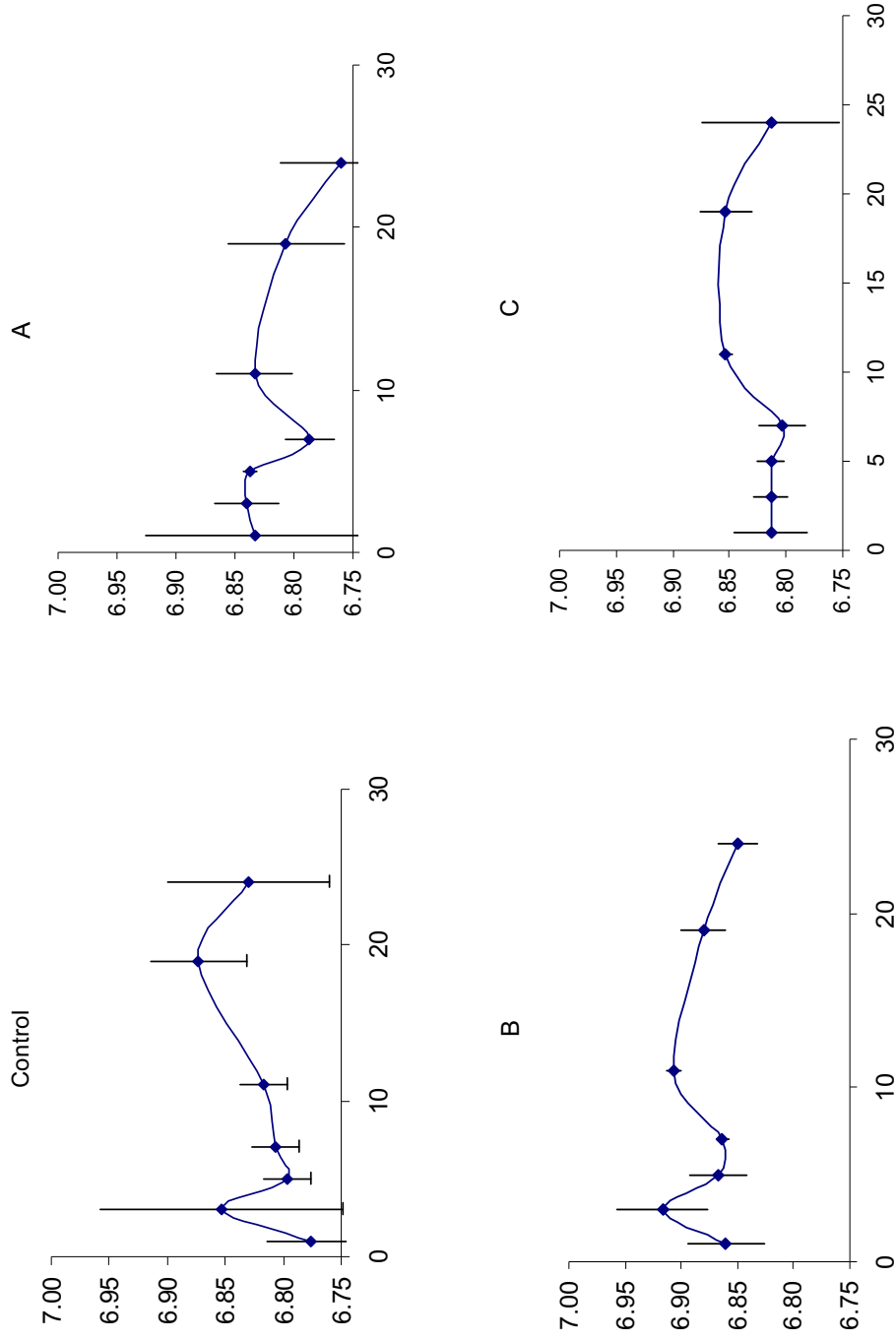
Appendix 1.

Set-up of 12 flasks at start of incubation

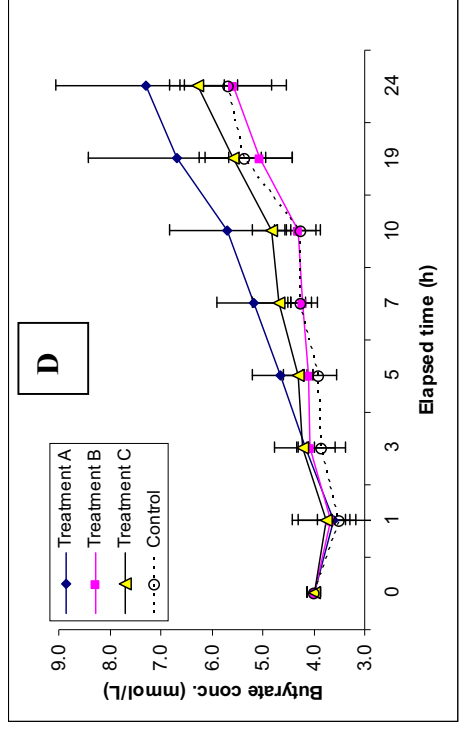
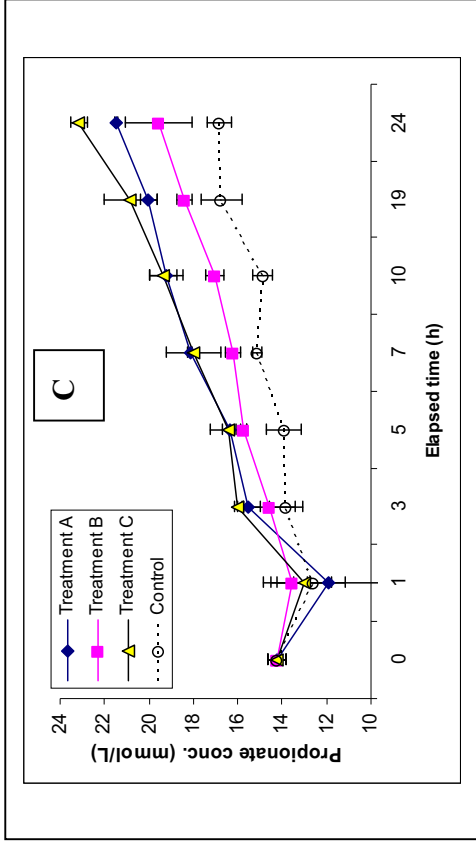
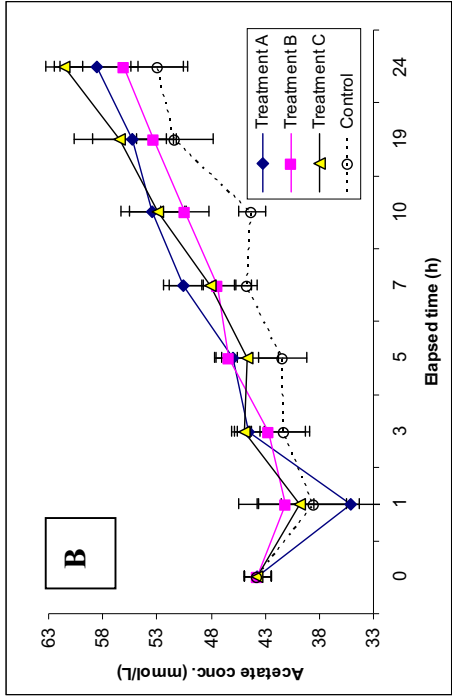
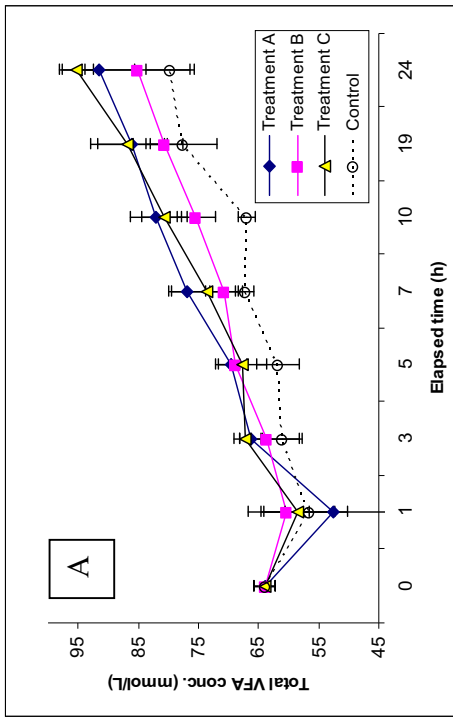
Flask	Supplement (g)	Filter		Rumen	Buffer
		paper g	fluid mL	fluid mL	mL
1	A	1.08	0.457	100	100
2	A	1.12	0.447	100	100
3	A	1.10	0.461	100	100
4	B	1.10	0.446	100	100
5	B	1.15	0.459	100	100
6	B	1.10	0.462	100	100
7	C	1.10	0.455	100	100
8	C	1.15	0.454	100	100
9	C	1.16	0.445	100	100
10	Control	0.00	-	100	100
11	Control	0.00	-	100	100
12	Control	0.00	-	100	100

Appendix 2.

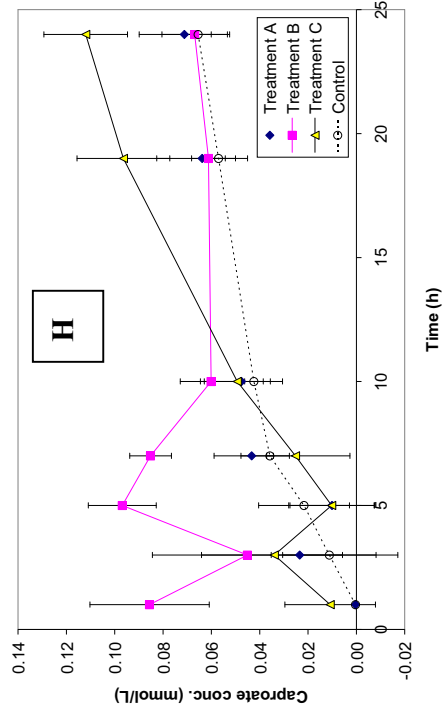
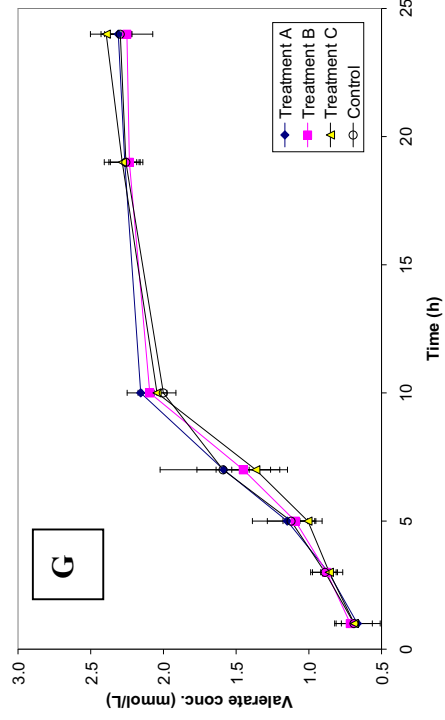
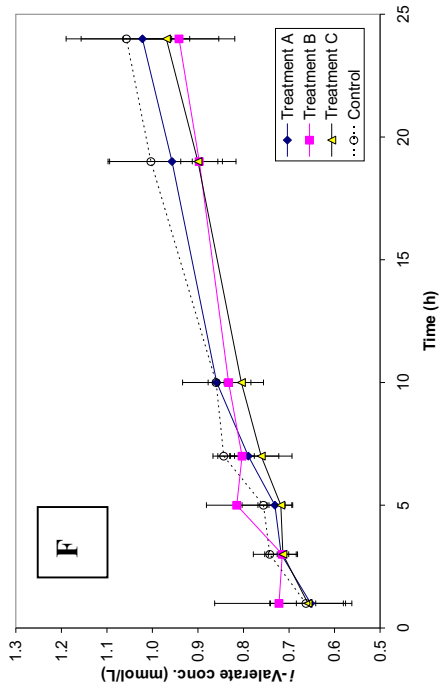
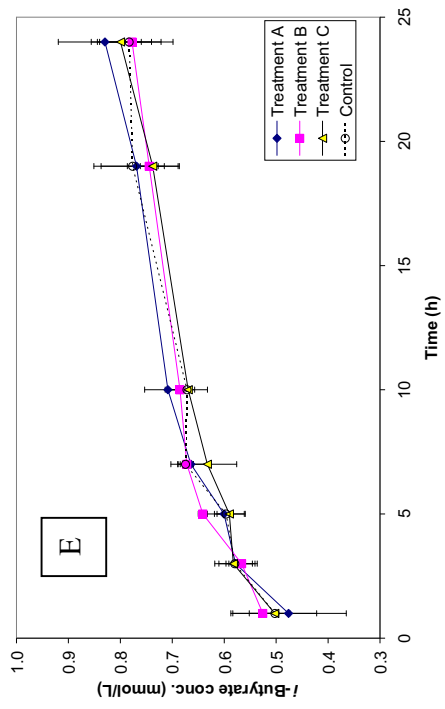
Mean pH values (\pm SD, n=3) over time (h) in the incubation media



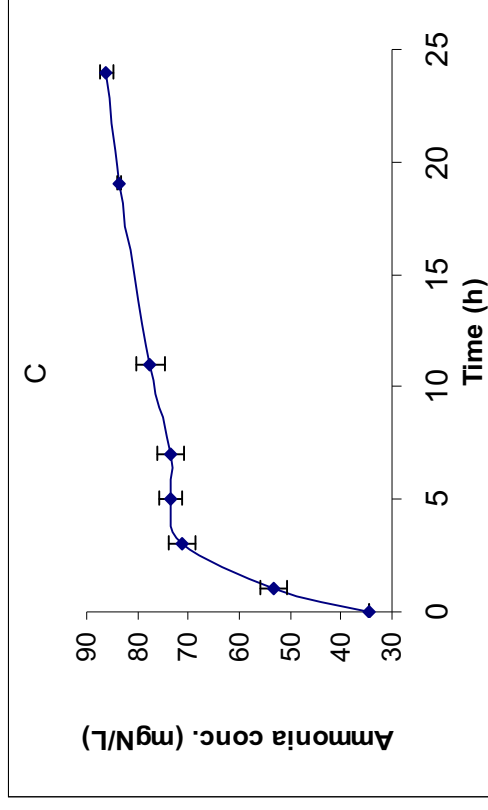
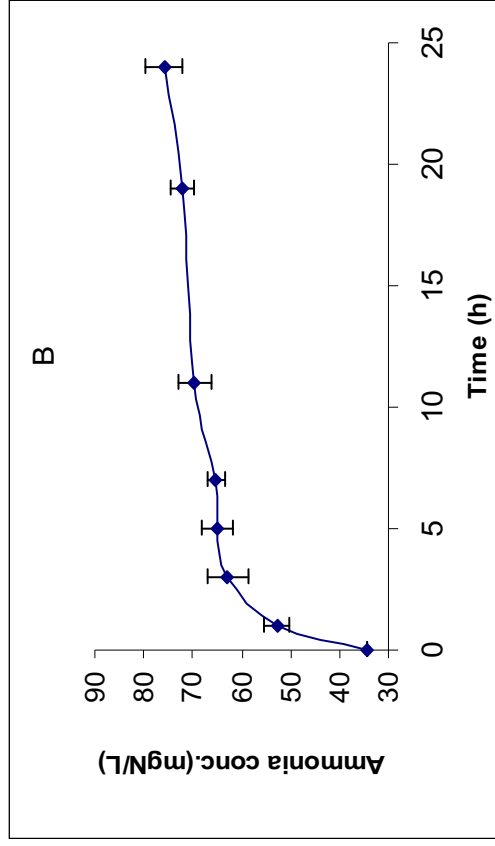
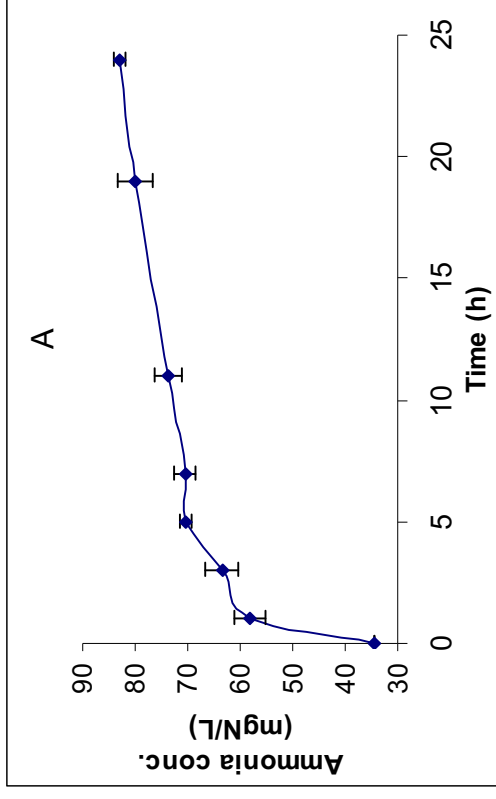
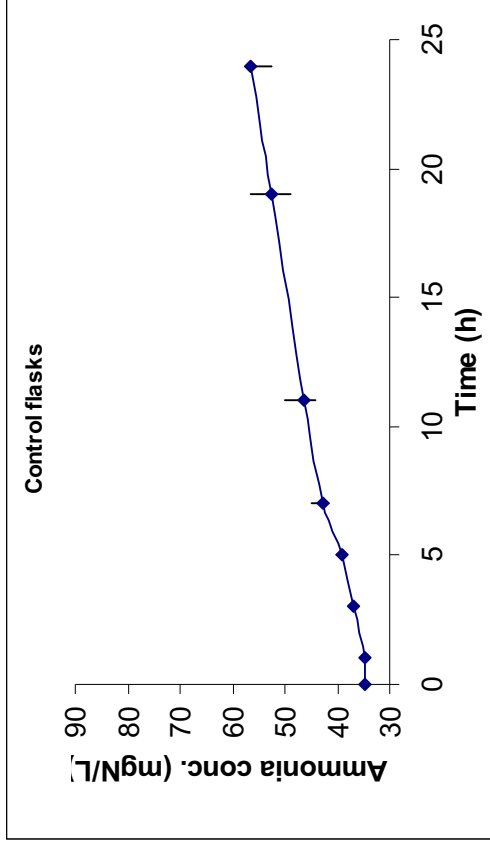
Appendix 2. (cont.) Mean (\pm SD) total volatile fatty acid concentrations (A), and concentrations of acetate (B), propionate (C) and butyrate (D) (mmol/L of flask contents, n=3) over time in the incubation media



Appendix 2 (cont). Mean (\pm SD) concentrations of *iso*-butyrate (E), *iso*-valerate (F), valerate (G) and caproate (H) (mmol/L) of flask contents, $n=3$ over time in the incubation media.

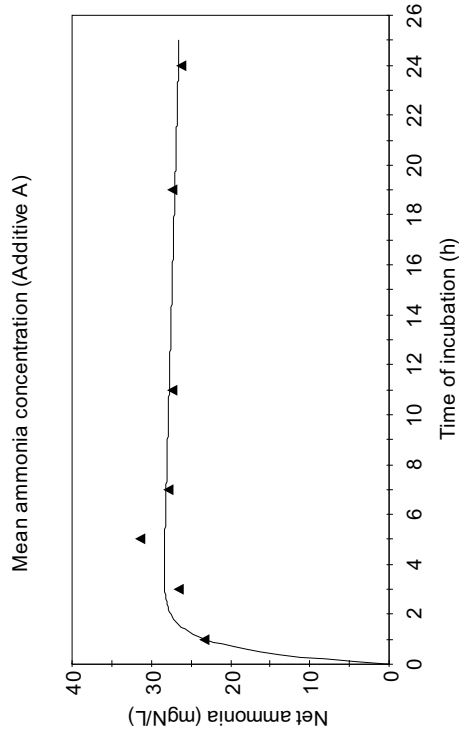


Appendix 3. Mean ammonia concentrations (\pm SD, n=3) over time in the incubation media



Appendix 4A. (Ammonia curve fitting)

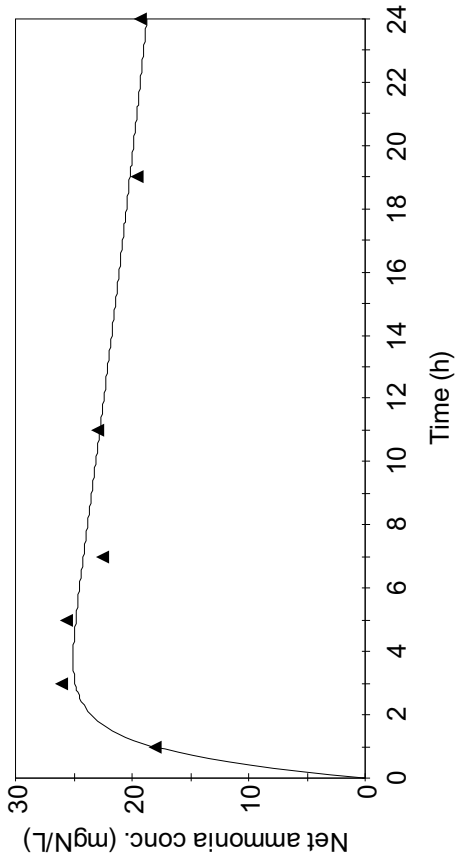
Two-exponential curve fitted to average net ammonia concentrations in flasks containing Supplement A over time



Sum of squares = 10.8

A-Category	Form	CurrentValue	Minimum	Maximum	FSD
K(51)	A	28.78	0	100	0.03198
P(1)	A	1.65162	0.001	10	0.09745
P(2)	A	0.003306	0	1	0.49735

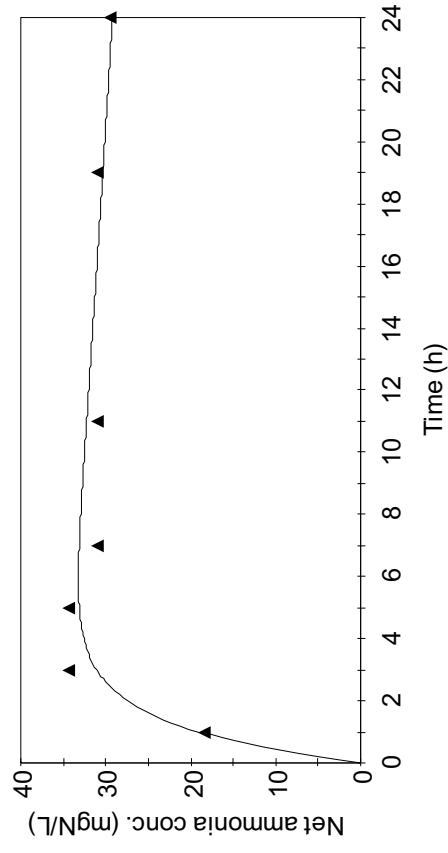
Appendix 4B. Two-exponential curve fitted to mean net ammonia concentrations (mgN/L) in flasks containing Supplement B over time (h)



Sum of squares = 4.49

B-Category	Form	CurrentValue	Minimum	Maximum	FSD
K(51)	A	26.85	0	100	0.0290
P(1)	A	1.1758	0.001	10	0.0757
P(2)	A	0.0150	0	1	0.1155

Appendix 4C. Two-exponential curve fitted to mean net ammonia concentrations (mgN/L) in flasks containing Supplement C over time (h)

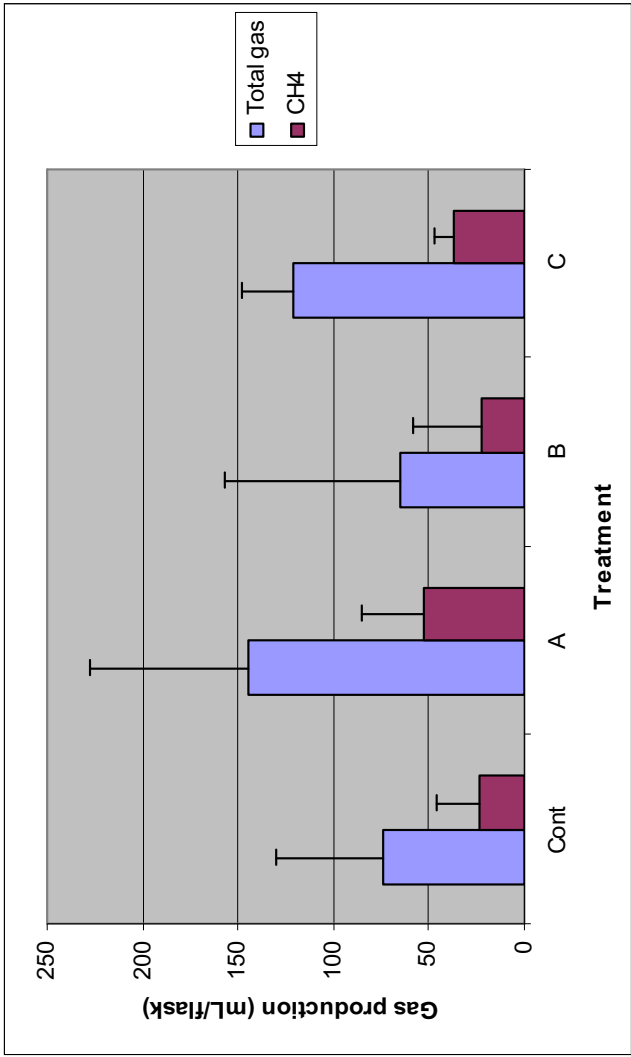


Sum of squares = 14.9

C-Category	Form	CurrentValue	Minimum	Maximum	FSD
K(51)	A	35.08	0	100	0.0385
P(1)	A	0.797	0.001	10	0.0624
P(2)	A	0.00762	0	1	0.2476

Appendix 5.

Total gas and methane gas production (mL/flask \pm SD, n=3) over 24 h



Appendix 6. – AGR Letter to UNE

28 September 2009

John Nolan
Professor, Animal Nutrition
School of Environmental and Rural Science
University of New England, NSW 2351, Australia

Subject: Nitrogenous Supplements – In vitro AGRiliq Trial

Dear Professor Nolan,

You would already be aware that the three nitrogenous supplements, "Sample A", "Sample B" and "Sample C" (prepared for purposes of the in vitro AGRiliq trials) were labeled by a third party so that neither AGR's Principal Scientist nor UNE were aware of the true identity of the said samples until after completion of the in vitro trials and UNE draft report.

Subsequent to the completion of the UNE draft report into these trials, AGR formed the opinion that maximum benefit would be derived from any post trial discussions between AGR and UNE if both parties were made fully aware of the of true identities of the nitrogenous Samples A, B, & C and, in particular, the key ingredients and methods of preparation that were employed in the preparation of Samples A, B & C.

Consequently, just prior to the telephone conference (held 5th June) between yourself, Dr. John Goopy, Dr. Calos and Antony Sachs, I revealed to Dr. Calos the identity of each of the three nitrogenous samples and I am aware that Dr. Calos shortly thereafter also informed yourself and Dr. Goopy.

AGR believe that it is appropriate that I also formally advise you in relation to the identities of the three nitrogenous samples as prepared by our company so that this information can be noted within your final report.

I therefore confirm the key ingredients and method of preparation for each of Sample A, Sample B and Sample C as follows:

1. Supplement A, an acidic thermally reacted molasses-urea blend, which was then neutralized, using a procedure similar to that described by Malik and Chopra (1979)ⁱ.
2. Supplement B, a simple solution of urea and salts in molasses.
3. Supplement C, 'AGRiliq' made according to an AGR proprietary alkaline pH-controlled process using molasses, urea and alkalis and acids as the ingredients. As a result of this process, the supplement contains a range of nitrogenous compounds including heterocycles such as pyrroles.

I also confirm that care was taken by AGR to ensure each of the three samples were closely matched in their nitrogen, salt and carbon levels.

Yours faithfully



Gregg Chapple

General Manager

AGR Industries

ⁱ Malik N.S., Chopra, A.K. (1979). A note on uromol prepared with narrow urea-molasses ratio. *Indian J. Anim. Sci.* **49**, 386 – 388.