In vitro digestion of ryegrasses harvested in the morning and afternoon to manipulate water soluble carbohydrate concentration

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ABSTRACT

The concentration of water soluble carbohydrate (WSC) increases during the day and the difference between morning and afternoon is greater and more consistent than the difference between cultivars. An *in vitro* batch culture system was used to compare the fermentation of ryegrass cultivars that differed in WSC, after harvesting samples in the morning (08:00 h) and afternoon (16:00 h). Bottles were placed in a shaking incubator at 39°C and removed at 0, 2, 4, 6, 8, 12 and 24 hours to measure pH and concentrations of ammonia and volatile fatty acids (VFA). Ryegrasses harvested in the afternoon had 81-114 g/kg dry matter more WSC, 3-12 g/kg dry matter less neutral detergent fibre and 14-25 g/kg DM less crude protein than morning samples. Compared to morning harvested ryegrass cultivars, afternoon harvested ryegrass cultivars had the lowest yields of VFA. Afternoon-harvested ryegrass cultivars, especially high sugar ryegrass, were rapidly fermented and after four hours the pH of high sugar ryegrass decreased to less than 5.8, which has been reported to cause sub-optimal fibre digestion. It may be possible to manipulate the WSC concentration of a pasture diet by altering grazing management practices. Further research is required to characterise the optimum concentration of WSC for efficient rumen fermentation.

Keywords: water soluble carbohydrate; high sugar ryegrass; *in vitro* digestion; pH ammonia, volatile fatty acids.

INTRODUCTION

Ryegrasses have been selected in the United Kingdom (UK) for higher water soluble carbohydrate (WSC) concentration in their dry matter (DM) as a means for improving the balance of fermentable energy and nitrogen (N) in the rumen. Some evaluations have shown up to 20% increase in milk yield from "high sugar" grasses (HSG) relative to control ryegrasses, in the UK environment (Miller *et al*., 2001), while others have not (Miller *et al*., 2000; Moorby *et al*., 2006). Where positive responses were measured there was also an increase in digestible DM intake and in the efficiency of dietary N utilisation for milk production (Moorby *et al*., 2006). In New Zealand (NZ), a HSG cultivar from the UK has not resulted in the same accumulation of WSC above controls when evaluated in studies with grazing dairy cows over several years and seasons (Cosgrove *et al*., 2010). In the NZ studies the WSC concentration differed from Controls by between 9 and 41 g/kg DM, compared with a difference of 39 to 81 g/kg DM between a HSG and a Control at eight European sites (Halling *et al*., 2005). Water soluble carbohydrate concentration in forages can also be manipulated by altering the time of the day the grass is harvested or grazed, or varying nitrogen supply. Concentrations of WSC are lower in the morning and higher in the afternoon (Abrahamse *et al*., 2009). This difference is greater and more consistent than differences obtained from cultivar selection (Tas *et al*., 2006; Cosgrove *et al*., 2007a; 2007b). Understanding the effects of these changes in chemical composition on fermentation in the rumen will help determine whether further increases in the concentration of WSC will have both productive and environmental benefits.

The objective of this experiment was to compare the fermentation characteristics of ryegrass cultivars differing in the concentration of WSC, by harvesting in the morning and in the afternoon, using an *in vitro* rumen batch fermentation technique.

MATERIALS AND METHODS

Digestion kinetics using *in vitro* techniques were measured in three ryegrass cultivars that were harvested in the morning and afternoon to manipulate WSC content. Fresh leaf samples of a diploid ryegrass selected for increased WSC (a "high sugar ryegrass"; cv. Aberdart; HSG), a diploid perennial ryegrass (*Lolium perenne*; cv. Impact; STG) and a tetraploid Italian (annual) (*Lolium multiflorum*; cv. Moata; IRG) ryegrass were harvested at 08:00 h (AM) and 16:00 h (PM) in October 2005 (Spring) from Massey University's No. 4 dairy farm and frozen immediately with liquid nitrogen and stored at -20^{o}C until being prepared for the experiment (April 2007). Samples were collected on a single day from one paddock of each

TABLE 1: Dry matter (DM) concentration and chemical composition (g/kg DM) of High sugar, Italian and Standard ryegrass cultivars harvested in the morning (AM) and afternoon (PM).

Ryegrass	High sugar		Italian		Standard	
Harvest time	AM	PM	AM	PM	AM	PM
Dry matter	157	244	141	208	151	245
Soluble sugars and starches	204	285	161	275	164	243
Crude protein	212	208	274	249	240	218
Acid detergent fibre	244	231	212	209	233	227
Neutral detergent fibre	459	447	379	382	438	432
Lipid	31	28	35	26	34	31
Ash	106	102	111	104	113	107

ryegrass cultivar prior to being grazed by dairy cows in the Spring 2005 experiment to measure the differences in milk production from cows grazing HSG, STG or IRG (Cosgrove *et al*., 2007a, b).

The ryegrass was prepared for *in vitro* batch culture incubation using the method described by Burke *et al*. (2000). Briefly, frozen grasses were maintained frozen at all times and minced with a Kreft Compact meat mincer, so that the grasses had a particle size distribution similar to herbage material chewed by ruminants (Burke *et al*., 2000). The mincing component was encased with a polystyrene container with dry ice to ensure the grass did not thaw during mincing to avoid plant cell deterioration.

Sub samples of minced forage were taken, oven-dried at 60°C for 48 hours, to determine DM concentration. Nutrient composition was predicted using near infrared reflectance spectroscopy (NIRS: feedTECH, Palmerston North). Estimates of WSC were derived from a NIRS calibration previously developed from wet chemistry analysis of fresh ryegrass herbage harvested in New Zealand. All other components were estimated from the standard calibration for pasture (feedTECH, Palmerston North).

In vitro incubation

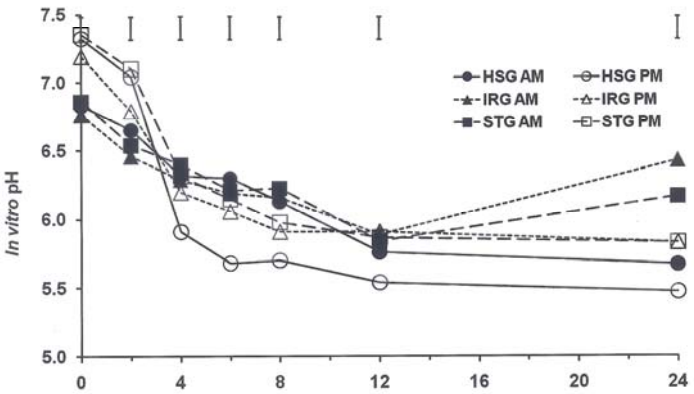
About 3.0 g of freshly minced forage was weighed into 50 mL vented bottles and warmed to 39°C with 12 mL of McDougall's buffer, 0.5 mL of reducing agent and 3 mL of strained rumen liquor as described by Burke *et al*. (2000). Rumen inocula for all incubations was pooled from two rumen cannulated non-lactating Holstein-Friesian cows that were fed good-quality ryegrass, that was not any of the grasses being evaluated *in vitro*. The experiment was conducted as one run using a Gallenkamp orbital incubator (Watson Victor Ltd, Wellington) set at 90 oscillations/minute.

Triplicate bottles of each ryegrass were removed after 0, 2, 4, 6, 8, 12 and 24 hours of incubation, pH of the fluid recorded and a 1 mL aliquot was taken from each bottle, acidified with 0.15 µL of hydrochloric acid, mixed and centrifuged (14,000 *g*) for 10 minutes. The supernatant was kept frozen and subsequently analysed for ammonia (NH_3) concentration (Neeley & Phillipson, 1988). Ammonia concentrations were corrected using the background concentrations in rumen inocula and expressed as mmol NH_3 per mol feed nitrogen (N) incubated. An additional 1.5 mL aliquot was taken from triplicate bottles removed at 2, 6, 12 and 24 hours, centrifuged (14,000 *g*) for 10 minutes and the supernatant bulked within grasses, kept frozen until analysis to determine concentrations of volatile fatty acids (VFA). Volatile fatty acid concentrations were determined using gas liquid chromatography (Attwood *et al*., 1998), corrected using background concentrations in rumen inocula and expressed as mg VFA/g DM incubated (Burke *et al*., 2006).

Statistical analysis

All data were analysed using the GLM procedure of SAS (2008) with ryegrass cultivar (HSG, IRG, STG), harvest time (AM, PM), sample time (0 to 24 hours) and their interactions considered as fixed effects. Due to differences in DM concentration in the AM and PM samples, the analysis of pH data included the DM incubated in each bottle as a covariate. Ammonia and VFA values were corrected for the amount of plant N and DM incubated in each bottle, respectively. The DM in each bottle was not used as a covariate for these variables. Differences between the least squares

FIGURE 1: Least-square means of *in vitro* pH, adjusted for dry matter incubated by covariate analysis, of grasses harvested in the morning (AM) or afternoon (PM). Standard error of the means are presented as bars.



means calculated for ryegrass cultivar, harvest time, sample time and their interactions were determined and were significant at $P \leq 0.05$.

RESULTS

Chemical composition

The DM concentration of grasses ranged from 141 to 245 g/kg, with AM grasses having lower DM concentration than PM grasses (Table 1). As a result an average of 0.47g DM was incubated in all AM bottles and 0.73g DM was incubated in all PM bottles. The WSC concentration of grasses ranged from 161 to 204 g/kg DM in the AM, and from 243 to 285 g/kg DM in the PM. The WSC concentrations for all grasses harvested in the PM were greater by 81–114 g/kg DM, than those harvested in the AM. The higher WSC concentration resulted in lower crude protein (CP) (4-25 g/kg DM) and neutral detergent fibre (3-12 g/kg DM) concentrations.

In vitro batch culture

Although incubations were buffered, changes in pH indicated extent of digestion, and decreased at different rates for the three cultivars and harvest times over the 24 hour incubation period (Figure 1). After adjusting for the amount of DM incubated in each *in vitro* bottle, the pH for PM harvested ryegrass decreased more rapidly (HSG > STG = IRG) than AM ryegrasses for the first four hours of the incubation. The pH value for PM harvested HSG was 5.9 at 4 hours, whereas for all other ryegrasses (AM and PM) the pH value was between 6.2 and 6.4 ($P < 0.05$). The pH nadir for PM ryegrasses occurred between 6 and 12 hours of the incubation, whereas for AM ryegrasses it occurred at 12 hours ($P < 0.05$).

TABLE 2: Molar percentage of acetate, propionate and butyrate and (acetate+butyrate)/propionate (A+B:P) ratio after 24 hours of incubation (%) for High sugar, Italian and Standard ryegrass cultivars harvested in the morning (AM) or afternoon (PM). No statistical analysis was carried out on these values as they have been determined from sub-samples taken from the bulking of triplicate bottles at each time.

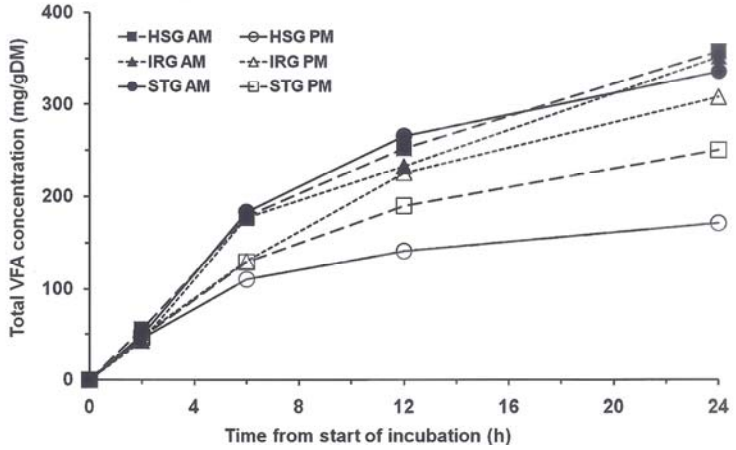
Ryegrass cultivar	Harvest time	Molar percentage of volatile fatty acids after 24 hours			
		Acetate	Propionate	Butyrate	A+B:P ratio
High sugar	AM	61.3	22.2	12.8	3.3
	PM	56.8	31.2	10.8	2.2
Italian	AM	61.5	21.5	12.0	3.4
	PM	51.7	26.3	18.4	2.7
Standard	AM	61.2	21.0	13.0	3.5
	PM	48.0	29.1	20.5	2.4

Concentrations of individual VFAs showed differences between AM and PM ryegrasses, but not between ryegrass cultivars. At six hours of incubation, total VFA concentrations for all AM ryegrasses were greater than those of all PM ryegrasses (Figure 2). After 12 hours the VFA concentration from AM and PM harvested IRG was similar, but concentration remained lower for PM, than AM harvested cultivars.

For the three ryegrasses, the AM samples produced more acetate and less propionate after 24 hours of the incubation (Table 2). After 24 hours AM samples contained 61.2-61.5% acetate and 21.0-22.2% propionate compared to PM samples which contained 48.8-56.8% acetate and 26.3-31.2% propionate. Consequently the mean (Acetate+Butyrate)/Propionate ratio for all ryegrass cultivars after 24 hours was greatest for AM harvested (3.3-3.5) than PM harvested (2.2-2.7) ryegrasses.

Mean NH_3 concentrations over the 24 hours are illustrated in Figure 3. The Ryegrass cultivar x Harvest time x Sample time interaction was not statistically significant ($P \geq 0.05$). However, because of the difference in CP content between all ryegrass cultivars and harvest times there was a clear difference after 24 hours of incubation, with PM harvested ryegrass cultivars having lesser NH_3 concentrations (HSG, 2.1; STG, 3.2; IRG, 8.7 mmol/mol plant N) than AM harvested ryegrass cultivars (HSG, 12.7; STG, 18.2; IRG, 24.0 mmol/plant N).

FIGURE 2: Net yield of volatile fatty acids (VFA; mg/g DM) produced when High sugar (HSG), Italian (IRG) and Standard (STG) ryegrass harvested in the morning (AM) or afternoon (PM) were incubated *in vitro* for 24 hours. No statistical analysis was carried out on these values as they have been determined from sub-samples taken from the bulking of triplicate bottles at each time point.

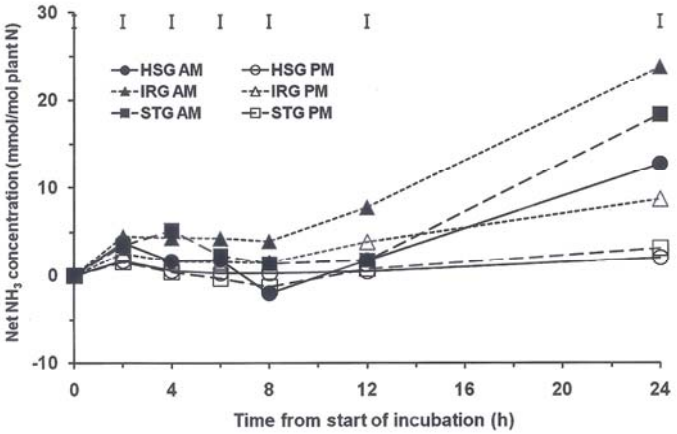


DISCUSSION

Concentrations of WSC were 81-114 g/kg DM higher for each ryegrass cultivar harvested in the PM than when harvested in the AM. The magnitude of the difference was greater than the means reported by Cosgrove *et al*. (2007b) of 52, 59 and 61 g/kg DM in Spring 2004, Spring 2005 and Autumn 2006, respectively. The larger differences in WSC concentration between AM and PM samples in this experiment may be a result of sub-sampling variation and/or the method of preparation of ryegrass samples for NIRS analysis. In both studies ryegrass samples were collected and frozen immediately with liquid nitrogen. However, the reported values in Cosgrove *et al*. (2007b) referred to freeze-dried samples, while in this study the samples were frozen for 18 months, then minced and oven dried at 60°C for 48 hours and then ground. Recent reports suggest that differences in drying method affect the measurement of WSC in forage samples (Pelletier *et al*., 2010).

Volatile fatty acids represent about 70-80% of the energy absorbed by ruminants. Rates of production and proportions of individual VFAs have important implications for nutrient supply. Overall, VFA yields after 24 hours of incubation represented 17 to 36% of DM incubated, in agreement with Burke *et al*. (2006) for *in vitro* VFA yields and (Acetate + Butyrate)/Propionate ratios for forages with diverse composition. In the current study, the differences in chemical composition of ryegrasses, especially between AM and PM harvest were sufficient to affect both VFA and NH_3 concentrations and the proportions of individual VFAs, but not in the direction that was expected. Surprisingly, the ryegrasses harvested in the AM produced the greatest amount of VFA consistently over the entire 24 hour incubation, and the PM harvested ryegrasses the least. The biggest difference was for the HSG cultivar where PM harvested HSG produced half as much VFA after 24 hours of incubation as that harvested in the AM (171 versus 356 mg/g DM). The differences between the other ryegrasses were smaller (IRG, 43 mg/g DM and STG, 101 mg/g DM). It is not clear why the VFA concentration was lower for PM harvested ryegrass cultivars, however Abrahamse *et al*. (2009) did report higher rumen VFA concentrations (125-155 mmol/L versus 103-111 mmol/L) after cows grazed pastures with 12 to 44g/kg DM more WSC. In the study reported here it is hypothesized that the lower VFA concentrations in PM harvested ryegrasses, especially HSG, could be related to the rate at which the pH declined in the first four hours of the incubation, and low pH, especially for the PM harvested HSG. This may have inhibited fibre degradation after 4 to 6 hours of incubation. An *in vitro* pH threshold value of 5.8 has been suggested for fresh forages; below this value, fermentation may be affected (de Veth & Kolver, 2001b; Wales *et al*., 2004). This occurred after four hours for PM harvested HSG.

FIGURE 3: Net yield of ammonia (NH_3; mmol/mol plant nitrogen; N) produced when High sugar (HSG), Italian (IRG) and Standard (STG) ryegrass harvested in the morning (AM) or afternoon (PM) were incubated *in vitro* for 24 hours. Standard error of the means are presented as bars.



The high proportion of propionate relative to acetate associated with forages containing high concentrations of WSC can be attributed to two factors. The production of lactic acid can increase the proportion of propionate relative to acetate. Also, low pH (<5.8) can inhibit fibre digestion, resulting in less acetate being produced relative to propionate (de Veth and Kolver, 2001a; Wales *et al*., 2004). It is possible in this *in vitro* experiment that both factors were causing the results observed.

In vitro production of NH_3 indicates the balance between wasteful production and lack of utilisation of NH_3 that occurs when protein is degraded during digestion (De Visser *et al*., 2007). Ryegrasses selected for higher WSC are expected to improve efficiency of N utilisation (Nocek & Russell, 1988; Kingston-Smith & Theodorou, 2000) and reduce the environmental cost of excess N, providing absorbed amino acids are utilised for production. However, the effect of WSC concentration on NH_3 concentrations were small, compared to concentrations and proportions of VFA. Lower NH_3 concentration from PM harvested material may be a consequence of lower CP concentration, rather than the change in WSC concentration, *per se*.

Together the pH and VFA data indicates that there may be an optimum concentration (<240

g/kgDM) of WSC that will benefit rumen fermentation and subsequently animal production. Even though WSC concentration between ryegrass cultivars has only differed a small amount in animal experiments, the data presented here may help explain why some animal experiments have produced positive responses when grazing HSG (Autumn), whereas there has been no effect in others (Spring; Cosgrove *et al*., 2007a; 2010). It will also help determine whether further increases in WSC concentration will be beneficial for animal production.

CONCLUSION

The concentration of WSC in ryegrass increases throughout the day. This experiment suggested major effects on fermentation characteristics such as pH and VFA production which could affect animal performance. Before modifying grazing management practices or embarking on further breeding programmes that increase the concentration of WSC in ryegrass, more research is required to establish the optimal concentration of WSC for pastoral diets.

ACKNOWLEDGEMENTS

The field study comparing ryegrass cultivars was funded by DairyNZ for the benefit of dairy farmers. The *in vitro* study reported here was funded by Massey University. The authors would like to acknowledge Mike Hickey, Francisco Dias and Jason Peters for their assistance with this experiment.

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