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***In vitro* fermentation of [¹⁵N-] ryegrass and ruminal digesta of sheep grazing a ryegrass-based pasture in the morning or in the afternoon**

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Abstract

Ruminal digesta contents of eight fistulated sheep grazing morning- or afternoon-allocated herbage were used to examine *in vitro* fermentation patterns, nitrogen (N) partitioning and microbial N use using (¹⁵N-) perennial ryegrass (*Lolium perenne* L.) as a substrate. Samples of digesta contents were collected two hours after a new allocation of a ryegrass-based pasture. Atom percent excess (APE) of vegetative plant material in the substrate was 4.5% (44.9 mg ¹⁵N/g plant N). An *in vitro* batch culture system was used to examine fermentation characteristics at predetermined times after ingestion (0, 2, 6, 12, and 24 h). Despite similar concentrations of total volatile fatty acids, concentrations of acetate and yields of ammonia were greater for ruminal digesta contents of afternoon-allocated herbage. *In vitro* true dry matter disappearance was similar among digesta treatments, but microbial N (MN; 50.4 vs. 44.0 mg) and microbial efficiency (MN/available N; 42.1 vs. 33.3%) were greater for digesta contents of afternoon-allocated herbage. Under the current conditions, enhancing water soluble carbohydrate in afternoon herbage was not at the expense of N concentration, and promoted a lipogenic type of fermentation. Increased microbial efficiency, rather than increased ammonia uptake, suggested improvements in N utilisation in digesta contents collected from sheep grazing afternoon herbage.

Keywords: grazing; *in vitro* fermentation; labelled ryegrass; ammonia; volatile fatty acids

Introduction

In temperate grasses, the concentration of total non-structural carbohydrates, particularly the water soluble carbohydrate (WSC) fraction, increases throughout the day as products of photosynthesis accumulate (Delagarde et al. 2000). During daylight, the process of carbon (C) fixation occurs at a greater rate than that of C losses to the atmosphere. In addition to increased dry matter (DM) and WSC concentrations, grazing management strategies that exploit these temporal benefits often lead to temperate herbage with reduced crude protein (CP) concentrations (Burke et al. 2011) and neutral detergent fibre (NDF) concentrations (Gregorini et al. 2006; Abrahamse et al. 2009) compared with morning-allocated herbage.

Ruminal fermentation processes from afternoon-allocated herbage often result in reduced ammonia loads (Lee et al. 2002; Trevaskis et al. 2004; Tavendale et al. 2006). These reductions have been associated with reductions in urinary N excretion (Kingston-Smith & Theodorou 2000). Proportions of individual volatile fatty acids (VFA), rather than total VFA concentrations, are often altered by these temporal changes in chemical composition. However, these alterations in individual VFA have been inconsistent. Afternoon-allocated herbage has led to a more glucogenic (propionate relative to acetate and butyrate) type of fermentation in most (Lee et al. 2002; Tavendale et al. 2006; Gregorini et al. 2008; Burke et al. 2011) but not all instances (Abrahamse et al. 2009; Vibart et al. 2011).

Microbial incorporation of N compounds from plant CP degradation can be determined by means of tracer substances such as ¹⁵N. Furthermore, the use of stable isotopes such as ¹⁵N, as a measure of N incorporation into ruminal microorganisms has been extensive (Salter et al. 1979; Wang et al. 2000; Berthiaume et al. 2010). Fewer studies, however, address the direct incorporation of labelled plant N into microbial N (Hristov et al. 2001; Kamoun et al. 2007). The use of this internal N marker allows for a measure of direct contribution of plant N to the different pools of N in the rumen as opposed to the contribution of an external marker such as ammonium sulphate, ammonium bicarbonate or urea added to the incubation medium as a source of supplemental N. Therefore, the objective of this study was to examine *in vitro* fermentation patterns and N partitioning of (¹⁵N-) perennial ryegrass as a substrate incubated with either ruminal digesta contents of sheep grazing morning (AM)- or afternoon (PM)-allocated herbage.

Materials and methods

The current experiment was conducted during the spring of 2009 between late October and November at AgResearch, Grasslands Research Centre, Palmerston North. The experiment was conducted as one run in a completely randomized design with two ruminal digesta treatments (AM and PM) and three replications per treatment at each of five predetermined times (0, 2, 6, 12, and 24 h of incubation).

Preparation of labelled ryegrass

A hydroponic nutrient solution containing ($^{15}\text{NH}_4$) $_2\text{SO}_4$ was used to label plant N for a measure of direct contribution of plant N to the different pools of N in the rumen. Labelled ryegrass was obtained by growing perennial ryegrass in a hydroponics system with a supplemental substrate solution. Concentrations of each ingredient are expressed in g/100 L of solution: KNO_3 , 10.1; $(\text{NH}_4)_2\text{SO}_4$, 6.3 [labelled ^{15}N source, enriched at 10 atom percent excess (APE)]; KH_2PO_4 , 3.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.7; K_2SO_4 , 3.1; Fe-EDTA, 1.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.1; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.20; H_3BO_3 , 0.057; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.022; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.016; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.013; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.008. The concentration of total N and ^{15}N -enrichment of the plants was assessed at three-week intervals. Fertilisation was adjusted accordingly.

Herbage was harvested, frozen in liquid N and subsequently stored at -20°C . Representative subsamples were freeze-dried and ground through a 1-mm screen for chemical analysis using near infrared reflectance spectroscopy (FeedTECH, AgResearch Grasslands, Palmerston North, New Zealand) and for total N and ^{15}N -enrichment determination (Waikato stable isotope unit, University of Waikato, Hamilton, New Zealand) using a Dumas elemental analyser (Europa Scientific ANCA-SL, Europa Scientific Ltd., Crewe, UK) interfaced to an isotope mass spectrometer (Europa Scientific 20-20, Europa Scientific Ltd., Crewe, UK).

Ruminal digesta treatments

Eight Romney wethers with a mean live weight \pm standard error of 52.3 ± 1.8 kg LW and previously fitted with a ruminal cannula, were used to examine ruminal fermentation characteristics from morning- vs. afternoon-allocated spring herbage (Vibart et al. 2011). Briefly, wethers grazed for 24 hours on the same strip and were allocated a new strip of pasture at 08:00 h (AM; $n = 4$ wethers) or at 16:00 h (PM;

$n = 4$ wethers). Samples of ruminal digesta contents were collected two hours after a new allocation of the ryegrass-based pasture at 1000 h (AM), and 1800 h (PM), to be incubated *in vitro* with (^{15}N -) perennial ryegrass as the sole protein source.

In vitro fermentation

An *in vitro* batch culture system, similar to that described by Burke et al. (2011), was used to examine fermentation characteristics at predetermined times. Approximately 2.5 g DM equivalent of freshly minced (^{15}N -) ryegrass was weighed and added to 250 mL vented conical flasks. Forage samples were warmed to 39°C in an incubation chamber (Gallenkamp orbital incubator, Watson Victor Ltd., Wellington, New Zealand) for 45 min, and flushed with CO_2 before the addition of a buffer solution (McDougall, 1948; 60.0 mL), a reducing agent (cysteine sulphide; 2.5 mL), and strained rumen liquor (15.0 mL). Rumen liquor obtained from pooled ruminal digesta contents of AM ($n = 4$ donors) or PM ($n = 4$ donors) sheep was strained through four layers of cheesecloth. McDougall's buffer was modified to contain 0.25 g/L of ammonium sulphate. Subsamples of pooled ruminal digesta were collected for the determination of background ^{15}N .

Flasks in triplicate were removed after 0, 2, 6, 12, and 24 hours of incubation. *In vitro* pH was measured at each sampling using a calibrated pH meter (MeterLab, PHM210, Radiometer Pacific Limited, Copenhagen, Denmark). At each time point, subsamples were withdrawn for ammonia and VFA determinations. Ammonia concentrations were determined according to a colorimetric procedure (Weatherburn 1967). VFA concentrations were determined by gas liquid chromatography (Attwood et al. 1998). Bacterial cells were separated by ultracentrifugation (Wang et al. 2000); residual (microbial plus residual feed; ^{15}N -RN) and microbial (liquid-associated bacteria; ^{15}N -MN) N fractions

Table 1 Dry matter (DM) and chemical composition of the herbage offered to sheep prior to incubations and of the labelled ryegrass incubated *in vitro*. P values in italic indicate values approaching significance between $P = 0.05$ and $P = 0.10$.

Variable	Herbage allocation				Substrate ^{15}N - ryegrass
	AM	PM	Standard error of mean	P value	
Dry matter (g/100 g)	14.7	16.0	0.7	0.20	21.5
Water soluble carbohydrates (g/100 g DM)	14.0	15.7	0.6	<i>0.08</i>	14.5
Crude protein (g/100 g DM)	17.6	18.0	1.0	0.80	21.3
Neutral detergent fibre (g/100 g DM)	50.5	49.9	0.8	0.64	47.9
Acid detergent fibre (g/100 g DM)	25.1	25.1	0.5	0.97	23.9
Lipid (g/100 g DM)	3.1	2.8	0.1	<i>0.09</i>	3.2
Ash (g/100 g DM)	9.0	8.7	0.2	0.41	9.6
Metabolisable energy (MJ/kg DM)	12.2	12.5	0.1	<i>0.08</i>	13.0
Organic matter digestibility (%)	81.7	83.0	0.7	0.21	87.0
Atom percent excess (%)					4.5

Table 2 *In vitro* pH, ammonia and volatile fatty acids (VFA) from *in vitro* fermentation of a labelled substrate [^{15}N -] ryegrass] incubated with ruminal digesta contents of sheep grazing a ryegrass-based pasture in the morning (AM) or in the afternoon (PM). Bold text indicates significant at $P < 0.05$. Italic text indicates approaching significance with P values between 0.05 and 0.10.

Measurement	Treatment	Time of incubation (hours)					Standard error of mean	P value		
		0	2	6	12	24		Treatment effect (T_r)	Time effect (T_i)	$T_r \times T_i$
<i>In vitro</i> pH							0.04	0.32	<0.001	0.27
	AM	6.96	6.56	6.46	6.03	5.55				
	PM	7.01	6.61	6.44	5.97	5.66				
Ammonia ($\mu\text{g/mL}$)							6.6	<0.001	<0.001	0.01
	AM	87.7 ^a	101.9 ^a	25.6	6.1	93.9 ^a				
	PM	127.3 ^b	150.6 ^b	52.5	6.9	140.9 ^b				
Total VFA (mmol/L)							2.6	0.05	<0.001	<0.001
	AM	19.6	29.2	63.2	124.1	128.1				
	PM	28.1	38.3	66.8	107.5	140.6				
Acetate (molar percentage)							0.7	<0.001	<0.001	0.003
	AM	64.0	67.5	58.2	54.2	54.9 ^a				
	PM	62.8	68.9	58.8	56.4	59.7 ^b				
Propionate (molar percentage)							0.6	0.004	<0.001	<0.001
	AM	22.0	20.7	33.0	34.9	32.8 ^a				
	PM	23.4	20.7	33.0	31.7	27.7 ^b				
Butyrate (molar percentage)							0.3	0.10	<0.001	0.02
	AM	11.5	9.9	7.9	9.3	10.1				
	PM	11.1	8.5	7.1	10.1	10.2				
Valerate (molar percentage)							0.03	0.80	<0.001	<i>0.06</i>
	AM	0.96	0.73	0.65	1.15	1.45				
	PM	1.04	0.73	0.71	1.12	1.31				
Isoacids (isobutyrate + isovalerate; molar percentage)							0.04	<0.001	<0.001	0.04
	AM	1.63	1.17	0.34	0.53	0.81 ^a				
	PM	1.71	1.17	0.43	0.68	1.13 ^b				
Acetate / Propionate ratio							0.06	0.009	<0.001	<0.001
	AM	2.91	3.26	1.77	1.56	1.68 ^a				
	PM	2.68	3.33	1.78	1.78	2.16 ^b				

Different superscripts for time of incubation, between time of sampling rumen liquor for each variable, indicate values that differ significantly ($P < 0.05$).

were analysed for total N and ^{15}N (Waikato Stable Isotope Unit, University of Waikato, Hamilton, New Zealand).

Calculations and statistics

The isotopic composition of samples (RN and MN) was originally expressed in delta units ($\delta^{15}\text{N}$, ‰), and converted to atom percent excess (APE) using the $^{15}\text{N}/^{14}\text{N}$ of the atmospheric N_2 as a standard. Enrichment of ^{15}N in RN and in MN was defined as ^{15}N APE above the natural abundance of ^{15}N measured in background ruminal digesta samples. The ^{15}N APE in the substrate was calculated assuming a natural abundance of 0.3663 atom % in plant material (3.663 mg $^{15}\text{N}/\text{g}$ N). Yield of microbial N (MN; mg) was estimated according to the following equation (Wang et al. 2000):

$$\text{MN} = (\text{APE in RN}/\text{APE in MN}) \times \text{RN}$$

where APE in RN and in MN are the percent excess of ^{15}N in the residual and microbial fractions, and RN is the total amount of N in the residues (microbial plus residual feed, mg). Chemical composition and *in vitro* fermentation data were analyzed using the GLM procedure of SAS (2002). Time of allocation for chemical composition data, and ruminal digesta, sampling time and interactions for *in vitro* fermentation data, were considered as fixed effects. Differences between the least squares means were calculated. Significance and trends were established at $P \leq 0.05$ and $P \leq 0.10$, respectively.

It is important to note that, unlike other experiments where *in vitro* pH, ammonia and VFA concentrations are corrected, often based on Time 0 values (Burke et al. 2011) before different dietary treatments are reported, in the current experiment these values remained uncorrected. The treatments examined in this study were the ruminal digesta contents from AM or PM allotted sheep.

Table 3 Dry matter (DM) degradation and efficiency of microbial N use from *in vitro* fermentation of a labelled substrate [^{15}N -] ryegrass] incubated with ruminal digesta contents of sheep grazing a ryegrass-based pasture in the morning (AM) or in the afternoon (PM). EMPS = Efficiency of microbial protein synthesis (mg of microbial N per g of DM apparently digested). Treatment x time interactions were deemed non-significant as all P values were greater than 0.5. Bold text indicates significant at $P < 0.05$. Italic text indicates approaching significance with P values between 0.05 and 0.10.

Measurement	Treatment		Standard error of mean	P value	
	AM	PM		Treatment	Time
<i>In vitro</i> apparent DM digestibility (%) after 24 h incubation	78.6	75.2	1.9	0.21	<0.001
<i>In vitro</i> true DM digestibility (%) after 24 h incubation	80.0	77.2	1.7	0.26	<0.001
Microbial N (mg)	44.0	50.4	1.9	0.04	<0.001
EMPS (mg of microbial N/g of DM apparently digested)	36.3	41.9	2.0	<i>0.07</i>	<0.001
Microbial N (% of available N)	33.3	42.1	2.7	0.03	<0.001

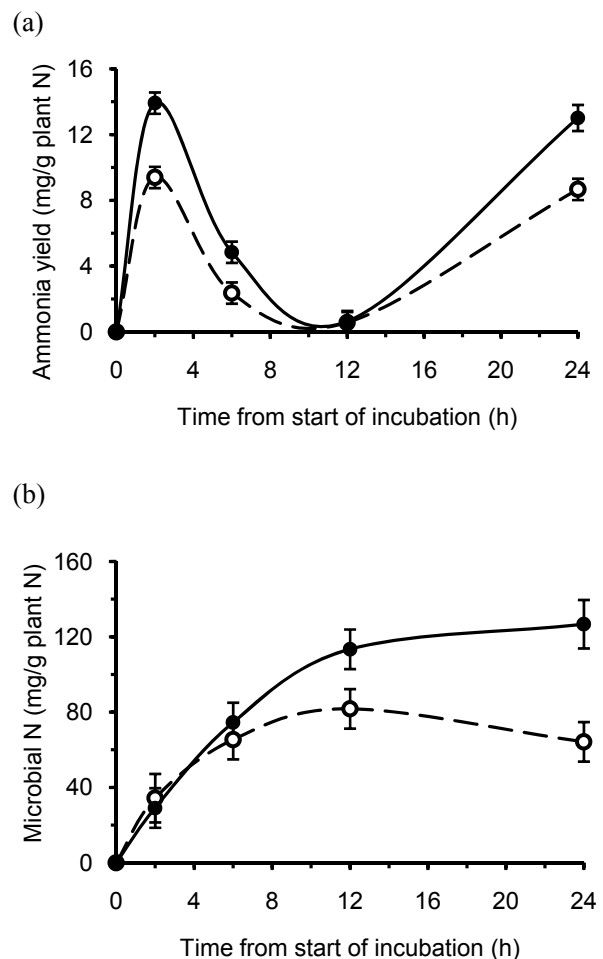
Results

Water soluble carbohydrates (WSC), lipids, and metabolisable energy (ME) concentrations tended ($P < 0.10$) to be greater for the PM-allocated herbage (Table 1). Atom percent excess (APE) of the (^{15}N -) ryegrass was 4.5% (44.9 mg ^{15}N /g plant N).

In vitro pH values did not differ among digesta treatments (Table 2), but ammonia concentrations and ammonia yields (mg/g plant N) (Fig. 1a) were greater ($P < 0.001$) for ruminal digesta contents of PM-allocated herbage. Concentrations of total VFA tended ($P = 0.05$) to be greater for ruminal digesta contents of PM-allocated herbage; this trend was obtained by incorporating Time 0 in the analysis. The molar percentages of acetate and the isoacids isobutyrate and isovalerate were greater ($P < 0.05$) for ruminal digesta contents of PM-allocated herbage, whereas the molar percentage of propionate was greater ($P = 0.004$) for ruminal digesta contents of AM-allocated herbage. Consequently, the acetate to propionate ratio was greater ($P < 0.05$) for ruminal digesta contents of PM-allocated herbage.

In vitro apparent and true (corrected for microbial DM) dry matter disappearance (DMD) were similar among digesta treatments ($P > 0.10$) (Table 3). Mean values of *in vitro* true DMD were 42.1, 46.0, 68.1 and 78.6% after 2, 6, 12 and 24 hours of incubation, respectively. Yield of microbial N (MN), calculated from APE in RN and in MN ($P = 0.04$), and microbial N use (MN/available N, %, $P = 0.03$; and mg MN/g plant N, $P = 0.008$) (Fig. 1b) were greater for digesta contents of PM-allocated herbage. None of the above degradation and microbial N measures exhibited treatment by time interaction effects, except for a trend ($P = 0.05$) in microbial N use (mg MN/g plant N) (Fig. 1b).

Figure 1 (a) Patterns of ammonia yield (mg/g plant N) and (b) microbial N (mg/g plant N) from *in vitro* fermentation of a labelled substrate [^{15}N -] ryegrass] incubated with ruminal digesta contents of sheep grazing a ryegrass-based pasture in the morning (AM; O) or in the afternoon (PM; ●). The error bars represent the standard error of the mean.



Discussion

The underlying hypothesis was that afternoon-allocated herbage would lead to reduced *in vitro* ammonia loads, a more glucogenic type of fermentation and enhanced microbial growth compared with morning-allocated herbage. Under the field conditions leading to this study (Vibart et al. 2011), allocating a 24 hour strip of fresh pasture during the afternoon resulted in only small increases in WSC concentration (17 g/kg DM of additional WSC). This marginal response was attributed to unseasonal weather conditions occurring during the field stage prior to the *in vitro* study. In the present study, enhancing WSC in afternoon herbage was not at the expense of N concentration, and tended to be greater in ME concentration (Table 1). Lipid, and to a lesser extent ash, rather than N and NDF concentrations, were offset by the marginal increase in WSC concentrations of the afternoon-allocated herbage.

Ammonia concentrations and ammonia yields (Table 2 and Fig. 1a) were greater for the ruminal digesta contents of afternoon-allocated herbage. These differences were particularly noticeable during the early (up to six hours) and late (24 hours) phases of incubation similar to that of Burke et al. (2011). Ammonia N for PM digesta contents accounted for 11.4, 4.0, 0.5 and 10.7% of plant N after 2, 6, 12 and 24 hours of incubation. Corresponding values for AM digesta were 7.7, 1.9, 0.5 and 7.1%. These results were attributed to differing ingestive behaviour taking place at the time of ruminal contents sampling, rather than the divergent chemical composition imposed by time allocation of herbage (Vibart et al. 2011). Grazing behaviour of ruminants grazing temperate pastures changes considerably throughout the day; the longest and most intense grazing events take place at, or near, dusk (Orr et al. 1997). These events have been associated with a greater intake and fermentation rate occurring at this time of the day (Penning et al. 1991; Orr et al. 1997). In our study, grazing in the afternoon, particularly around sampling time, presumably equated to a greater proportion of herbage DM intake at a slightly greater WSC and similar CP concentration, leading to greater ammonia concentration. Consistent with these findings, greater concentrations of the protein-derived isobutyric and isovaleric fatty acids, from the metabolism of valine and leucine respectively, also support greater fermentation occurring during the first four to six hours following afternoon allocation of herbage (Vibart et al. 2011).

The digestion of (¹⁵N-) ryegrass with ruminal digesta contents from differing time allocation had a notable effect on individual VFA. Consistent with *in vivo* findings (Vibart et al. 2011), acetate concentrations and molar percentages were greater for the afternoon-allocated herbage, and promoted a lipogenic type of fermentation. The greater molar percentage of propionate of ruminal digesta contents

of morning-allocated herbage was somewhat unexpected. However, similar findings were reported by Abrahamse et al. (2009) for a ryegrass-based pasture that was allocated to cows either after the morning or afternoon milking. These findings, along with a greater lipogenic type of fermentation, led to greater milk fat concentration of afternoon-allocated dairy cows (Abrahamse et al. 2009). Overall, VFA yields accounted for ~18% of DM incubated; these results are consistent with those reported by Burke et al. (2011) for perennial ryegrasses.

The efficiency of microbial protein synthesis, expressed as mg MN per g of apparent DMD, tended to be greater for digesta contents of afternoon-allocated herbage. Expressed as MN per available N (%), microbial efficiency was greater for digesta contents of afternoon-allocated herbage. Hypothetically, the rate of substrate fermentation is roughly proportional to the rate of microbial growth in that more rapidly fermented substrates yield more microbial mass, but there is no certainty that substrate disappearance will necessarily reflect enhanced microbial protein synthesis. Effectively, increased microbial protein synthesis has resulted from the use of high-sugar grasses (Merry et al. 2003), from alfalfas selected for high WSC concentrations (Berthiaume et al. 2010), from using afternoon- vs. morning-harvested alfalfas (Brito et al. 2009) and from grazing afternoon- vs. morning-allocated wheat (Gregorini et al. 2008).

Under the current conditions, enhancing WSC in afternoon herbage was not at the expense of N concentration, and promoted a lipogenic type of fermentation. Increased microbial efficiency, rather than increased ammonia uptake suggested an improvement in N utilisation in digesta contents collected from sheep grazing afternoon herbage.

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