In vitro determination of the characteristics of fresh and frozen-thawed alfalfa and ryegrass

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Abstract Current ruminant feeding systems depend on knowledge of the composition of feeds and the rate and extent of degradation of feed organic matter (OM) and crude protein (CP) in the rumen. The effect of storage and preparation of samples on *in vitro* gas production and fermentation characteristics of two common forage species, namely alfalfa and ryegrass were studied. Samples were prepared as fresh (F), frozen-thawed (FT) and FT + starch (FT + S) before *in vitro* evaluation. The fractional rate of loss of organic matter (OM) and the total N and total VFA production during 12h of incubation were significantly faster for alfalfa than for ryegrass. Model parameters describing changes in OM loss and total N appearance differed significantly between F samples and FT and FT + S samples; there was a significant interaction between forage species and preparation method for fractional degradation rate of total N. A significant interaction between forage species and preparation method at 6 h incubation changed the rankings. The propionate:acetate ratios after 12 h incubation were similar for alfalfa and ryegrass but were lower for F and FT samples than for FT + S samples. After 12 h of incubation, alfalfa produced more gas, total VFA (mmol/g OM) and microbial crude protein (mg/g OM) than ryegrass, whereas F samples produced more fermentation products than FT and FT + S samples. *In vitro* degradation characteristics of forage samples were influenced by forage species, but also by sample preparation method; therefore, consistent use of one sample preparation method is recommended when comparing degradation characteristics of forage species *in vitro*.

Keywords: *in vitro* fermentation, forages, preparation method, fresh, frozen-thawed

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Introduction

Alfalfa (*Medicago sativa* L.) and ryegrass (*Lolium perenne* L.) are common pasture species grazed by Australian livestock. Current ruminant feeding systems depend on knowledge of the composition of feeds being ingested and the rate and extent of degradation of feed organic matter (OM) and crude protein (CP) in the rumen. There is, however, little information on the rumen fermentation characteristics of fresh forages for use in ruminant feeding systems (Gosselink et al.,2004). Determination of *in vivo* forages intake and digestibility is time-consuming, laborious and expensive, where; feeding trials require large quantities of feed and are unsuitable for large-scale feed evaluation (Coelho et al., 1988; Carro et al., 1994). *In situ* and *in vitro* techniques are simpler and less expensive for predicting digestibility and potential feed intake.

In vitro techniques have been extensively used to determine fermentation characteristics of the ruminant feeds (Krishnamoorthy et al., 2005), but the procedure used for preparation of fresh forage samples before *in vitro* (or *in situ*) evaluation can affect the results (AFRC 1992; Huntington and Givens, 1995). For example, fresh forage produced less gas and VFA than dried forage (Lowman et al., 2002). Drying that involves heat is known to change the composition, physical state or solubility of the feed. Fermented feeds such as silage, when dried, will have increased losses of volatile compounds (Snyman and Joubert, 1992). It has also been suggested that fermentation characteristics of feed will be over-

estimated when dried samples are assessed by *in vitro* methods (Rymer et al., 2005). Hydrolysis of disaccharide sugars to monosaccharides occurs when fresh herbage is thawed after freezing but is considered to be insignificant (McRae, 1970). However, a reduction in soluble nitrogen (N) content was also observed and McRae et al. (1975) suggested this was mainly due to rupturing of the vacuolar membrane of the plant cell during freezing, thus resulting in precipitation of proteins. Similar effects of freezing were noted by Kohn and Allen (1992) who reported that freezing forage samples affected neutral detergent fiber, acid detergent fiber, lignin, and ash contents in different ways depending on forage type and duration of freezing.

Forage samples are usually either dried (oven- or freeze- dried) or frozen and thawed before being analyzed. These preparation methods offer convenience and flexibility when many samples are to be evaluated. However, *in vitro* gas production and fermentation characteristics of fresh forage have not been compared with the same material after freezing and thawing and it is possible that different storage and preparation methods used after collection of samples could lead to errors when the intention is to determine the nutritive value of fresh forages as ingested by grazing animals.The process of freezing fresh forage is time-consuming and during this period cellular respiration continues, leading to degradation of sugars. Thawing also takes time, and cell damage after freezing can result in loss of cell contents, and compositional changes in the forage. Frozen-thawed grass can be used for feeding animals for up to 12 h after removal from the freezer to avoid the risk of loss of soluble carbohydrate components during thawing (McRae, 1970).

There is limited information on the differences between preparation methods of forages for determination of *in vitro* gas production and fermentation characteristics. Therefore, the objective of this study was to compare the *in vitro* gas production and fermentation characteristics of fresh and frozen-thawed alfalfa and ryegrass. A third treatment was included in which starch was added to the *in vitro* substrates to compensate for possible losses of soluble carbohydrates during the freezing and thawing processes.

Materials and methods

Freezing the forages

Alfalfa (*Medicago sativa* L.) and perennial ryegrass (*Lolium perenne* L.) were grown in 4-L pots in a glasshouse for greater control over the growing conditions. The potting mix was almost free of N; the required N for plant growth was provided from fertilizer (8 g Multigro fertilizer N = 10.1%, P = 3.5%, K = 5.5%, S = 16.3%, Ca = 7.8% added monthly). The potting mixtures were constituted from sand (50%), soil (25%) and peat moss (25%).

Second cut alfalfa (pre-blooming, 17% DM, 26% CP) and ryegrass (4th leaf stage, 13% DM, 18% CP) were incubated using the *in vitro* gas production technique. Samples of each forage species were cut 5 cm above the soil surface, thoroughly mixed, and sub-sampled. Forage samples were cut 24 h before *in vitro* incubation was started and placed in sealed plastic bags and stored at -20 ºC. Next morning, frozen forages were removed from plastic bags and the contents were spread out and left in a foil tray on the laboratory bench at room temperature for about 30 min, while a second sample was cut to be used as the fresh sample. Fresh (F) and frozen-thawed (FT) samples were macerated for 30 sec in a blender before being placed in 1-L incubation bottles.

Rumen fluid collection

Rumen inoculum for *in vitro* incubation was prepared from four rumen-cannulated Merino wethers, housed indoors in individual metabolic cages in an animal house. The sheep had continuous access to fresh drinking water and were offered a basal diet of alfalfa (600 g/d, 92% DM, 19% CP) and oaten chaff (400 g/d, 91.5% DM, 7.5% CP) once a day at about 1000h. Approximately 600 mL of rumen fluid was collected from at about 0900h. Rumen fluid was dispensed into a 5-L Schott bottle (filled with CO_2) and transferred to the laboratory in a thermos flask containing water at 40ºC.

In vitro procedure

In vitro incubation was carried out in duplicate. The rubber stopper of the incubation bottle was equipped with a Bunsen valve that was connected through a silastic tube to a glass measuring cylinder that was inverted with its mouth under water so that gas production during incubation could be determined.

Weighed samples (approximately 5 g DM per incubation) of F and FT alfalfa or ryegrass were

added to the bottle; for FT + S samples, 4g DM of FT alfalfa and ryegrass plus 1 g starch (85652 Fluka, Biochemica) were added to replicate bottles. Each sample was mixed with 300 mL McDougall's buffer solution (pH: 7 ± 0.1) and macerated for 30 sec using a household blender set at high speed. The slurry was transferred directly to the incubation bottle. Rumen fluid inoculum (200 mL) was strained through cheese cloth and gassed again with CO_2 and then added to the bottle containing the sample. The tubes were finally re-gassed with CO_2, sealed and incubated.

Bottles containing rumen fluid and buffer but no sample (blanks) were also prepared in the same manner. Blanks and samples in the glass bottles were incubated in a shaking water bath at 39°C for 12 h. Samples of fluid were taken, without opening the bottles, at 0, 3, 6, 9 and 12 h using a sampling syringe. The samples taken at zero time were immediately placed into a container of crushed ice to reduce the rate of fermentation. These samples were assumed to represent fermentation in the first 30 min of incubation. To obtain each sample, fluid was removed from an incubation vessel using the sampling syringe and returned to the vessel at least three times before 20 mL was finally removed, transferred to a 30-mL centrifuge tube and placed on crushed ice. The sample pH was recorded immediately (Eco scan pH 5/6, Eutech instruments) and the remaining fluid was then centrifuged (20,000 x ***g***, 15 min, 4°C). The supernatant fraction was acidified with 0.25 mL 18M H_2SO_4 and stored at -18°C for analysis of ammonia-N. Bacteria were separated from the solid fraction but the amounts separated were later found to be too small to enable determination of the dry matter (DM) mass or N concentration.

Total VFA concentration and molar proportions of acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acids were determined in the rumen fluid supernatant (Erwin et al., 1961) using a gas liquid chromatograph (Model 427, Packard Instrument Co., USA). Iso-caproic acid was used as an internal standard. The gas chromatograph was connected to a recording data processor (Model 604, Packard Instrument Co., USA).

Total-N content of feed samples was determined on finely ground feed DM (1-mm sieve) using an N analyzer (Leco FP 2000) or, in the rumen fluid supernatant, by micro-Kjeldhal digestion followed by steam-distillation and ammonia titration

Calculations

Production of VFA and gas during incubation (mmol/g OM) was adjusted for the corresponding blanks. Additional information about fermentation and MCP was predicted from the stoichiometry of acetate, propionate and butyrate synthesis (Czerkawski, 1986). It was assumed that growth of cells was not limited by an insufficiency of carbon intermediates or ammonia, minerals or other essential nutrients. As well as being measured directly, gas production was also calculated according to Cone and van Gelder (1999) as follows:

$$Gas(ml) = V_m\left(H_A + 2H_B + 0.87H_T \right)$$

where V_m is the molar gas volume (24 L mol/at 20°C), H_A is acetic acid, H_B butyric acid and H_T is the total amount of VFA produced (mmol/g OM).

The percentages of total N and OM removal from the incubation mixture were determined using the model:

$$y = a(1 - e^{-bt})$$

where *a* is the potentially degradable fraction and *b* is the rate of degradation of fraction *a*.

Statistical analysis

Data were analyzed as a 2 × 3 factorial design using repeated measures ANOVA (SAS 2003). There were two forage species (S): alfalfa (L) and ryegrass (R) and three preparation methods (PM) viz: fresh (F), frozen-thawed (FT) and frozen-thawed + starch (FTS). The statistical model was:

$$Y_{ijk} = \mu + S_i + PM_j + (S.PM)_{ij} + T_k + \varepsilon_{ijkl}$$

where *i* is species (L and R); *j* is method of sample preparation (F, FT and FTS); Y_{ijk} is observation *k* in level of *i* of factor *S* and level *j* of factor *PM;* S_i is the effect of level of *i* of factor *S* (species); PM_j is the linear effect of level of *j* of factor *PM*; $(S.PM)_{ij}$ is the interaction between level of *i* of factor *S* with level *j* of factor *PM;* T_k is time of incubation (h); ε_{ijkl} is the random error with mean 0 and variance σ^2.

Mean comparison was performed using Fisher's least significant difference (LSD) if the F-ratio was significant.

Results

In vitro gas production

Cumulative gas production (mmol/g OM) for alfalfa (L) and ryegrass (R) prepared as F, FT and FT + S during 12 h *in vitro* incubation is shown in Figure 1.

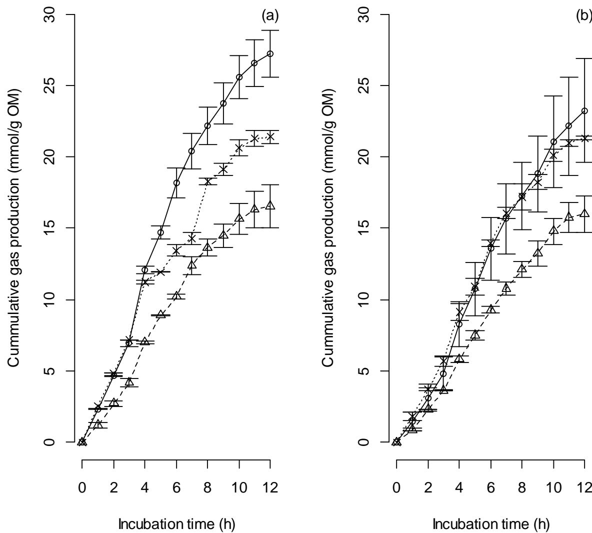

Figure 1. *In vitro* (mean ± SE) cumulative total gas production (mmol/g OM) for (a) alfalfa and (b) ryegrass prepared as fresh (—○—), frozen-thawed (--Δ--) and frozen-thawed + starch (···x···) at different incubation times (h).

Cumulative gas production was higher ($P = 0.0009$) for fresh samples of alfalfa than FT and FT + S samples. There were also significant ($P = 0.02$) interactions between PM and time but not within ryegrass samples on cumulative gas production. However, fresh and FT + S ryegrass samples had similar mean cumulative gas production compared with FT + S samples. There was no significant effect due to either S or PM on cumulative gas production ($P > 0.05$) and there were also no significant two-way and three-way interactions (see Figure 1).

Percentage N as ammonia and pH

In vitro incubation of alfalfa produced significantly ($P < 0.0001$) less NH_3 as a percentage of total N than ryegrass (47.2 ± 3.61 vs. 55.9 ± 3.97). Preparation method also significantly ($P = 0.009$) affected percentage N as NH_3 such that values for samples prepared as FT were lower than F and FT + S samples (48.2 ± 4.63 vs. 53.9 ± 4.51 vs. 52.6 ± 5.11). Significant interactions were also found between S and PM ($P = 0.0009$), S and incubation time ($P = 0.003$) and PM and incubation time ($P = 0.04$). A significant ($P = 0.01$) 3-way interaction was also present.

There was no significant ($P > 0.05$) effect on pH of either S or PM. The overall mean ± SE of the pH of the incubation solution was 6.8 ± 0.02. However, there was a significant ($P = 0.04$) interaction between S and PM, suggesting that the effects were not additive.

Measured and calculated production of products

Mean (± SE) quantities of products formed after 12 h *in vitro* incubation for alfalfa (L) and ryegrass (R) prepared as F, FT and FT + S are shown in Table 1.

In vitro TVFA production for ryegrass was consistently less than for alfalfa, irrespective of PM treatment. Alfalfa prepared as FT produced less gas (16.5 mmol/g OM) than any other S and PM combination, but their differences were not significant. The lack of interaction between S and PM for measured and calculated characteristics suggested that their effects were additive (see Table 1)

Acetate: propionate ratio

Mean (± SE) propionate: acetate (C3:C2) ratios at 3, 6, 9 and 12 h *in vitro* incubation for alfalfa (L) and ryegrass (R) prepared as F, FT and FT + SFT + S is shown in Table 2.

The propionate: acetate ratio increased with duration of incubation. There was no difference between S and PM on this ratio during the 12 h incubation period, but there were differences ($P < 0.05$) due to PM at all incubation times. An interaction ($P = 0.03$) between S and PM was present after 6 incubation but not after 3, 9 and 12 incubation.

In vitro fermentation characteristics

Percentages of total N and OM remaining after 12 h *in vitro* incubation for alfalfa and ryegrass prepared as F, FT and FT + S are given in Table 3.

The fractional rate of degradation (b) of TVFA, total N and OM was at all times slower for ryegrass than for alfalfa ($P < 0.05$). Ryegrass soluble fraction (a) for total N was higher than alfalfa ($P = 0.002$) which might be due to higher values for all PM samples of ryegrass (see Table 3).

Table 1. Amount of fermentation products formed after 12 h *in vitro* incubation (mean ± SE).

S	PM	Measured		Calculated	
		TVFA (mmol/g OM)	Gas (mmol/g OM)	MCP (mg/g OM)	Gas (mmol/g OM)
L	F	5.3^{a} ± 0.03	27.2^{a} ± 1.63	201.6^{a} ± 1.10	28.3^{a} ± 1.63
	FT	4.2^{bc} ± 0.03	16.5^{c} ± 1.51	160.8^{bc} ± 1.21	17.6^{b} ± 1.51
	FT + S	4.4^{b} ± 0.05	21.4^{ab} ± 0.45	168.4^{b} ± 2.24	22.4^{ab} ± 0.45
R	F	4.3^{b} ± 0.004	20.7^{ab} ± 1.07	162.8^{b} ± 0.33	21.7^{b} ± 1.07
	FT	3.8^{cd} ± 0.23	19.0^{b} ± 4.26	145.2^{cd} ± 8.53	20.0^{b} ± 4.26
	FT + S	3.7^{d} ± 0.20	18.6^{b} ± 2.59	143.2^{d} ± 7.75	19.6^{b} ± 2.59
	SED	0.18	3.2	6.9	3.2
Main effects					
L		4.6^{a} ± 0.21	21.7^{a} ± 2.04	176.9^{a} ± 7.96	22.7^{a} ± 2.04
R		3.9^{b} ± 0.13	19.4^{a} ± 1.38	150.4^{b} ± 4.95	20.5^{a} ± 1.38
	SED	0.10	1.9	4.0	1.9
	F	4.8^{a} ± 0.29	23.9^{a} ± 2.06	182.2^{a} ± 11.21	25.0^{a} ± 2.06
	FT	4.0^{b} ± 0.15	17.8^{b} ± 1.98	153.0^{b} ± 5.72	18.8^{b} ± 1.98
	FT + S	4.1^{b} ± 0.21	20.0^{ab} ± 1.35	155.8^{b} ± 7.99	21.0^{ab} ± 1.35
	SED	0.13	2.3	4.8	2.3
P	S	0.0005	0.26	0.0005	0.26
	PM	0.002	0.09	0.0017	0.09
	S.PM	0.14	0.22	0.13	0.22

Forage species (S) alfalfa (L) and ryegrass (R) prepared (PM) as fresh (F), frozen-thawed (FT) and frozen-thawed + starch (FT + S).
Means within a column without a common superscript differ (P < 0.05).
a is the soluble fraction or easily degradable and *b* is the fractional rate of degradation.
SED: standard error of the difference.

Table 2. Acetate: propionate concentration ratio after 12 h *in vitro* incubation (mean ± SE).

S	PM	Incubation time (h)			
		3	6	9	12
L	F	2.3^{b} ± 0.05	2.6^{b} ± 0.02	2.7^{ab} ± 0.09	2.9^{c} ± 0.01
	FT	2.5^{b} ± 0.04	2.6^{b} ± 0.06	2.6^{abc} ± 0.13	3.0^{ab} ± 0.05
	FT + S	2.1^{b} ± 0.03	2.4^{c} ± 0.05	2.5^{bc} ± 0.02	2.7^{d} ± 0.10
R	F	2.2^{b} ± 0.04	2.4^{c} ± 0.01	2.6^{bc} ± 0.05	2.9^{bc} ± 0.02
	FT	3.4^{a} ± 0.53	2.8^{a} ± 0.02	2.9^{a} ± 0.05	3.1^{a} ± 0.01
	FT + S	2.2^{b} ± 0.11	2.3^{c} ± 0.08	2.3^{c} ± 0.15	2.6^{d} ± 0.02
	SED	0.31	0.06	0.13	0.06
Main effects					
L		2.6^{a} ± 0.08	2.5^{a} ± 0.05	2.6^{a} ± 0.05	2.9^{a} ± 0.07
R		2.3^{a} ± 0.29	2.5^{a} ± 0.09	2.6^{a} ± 0.11	2.9^{a} ± 0.09
	SED	0.18	0.04	0.07	0.04
	F	2.2^{b} ± 0.05	2.5^{b} ± 0.05	2.6^{ab} ± 0.05	2.9^{b} ± 0.01
	FT	2.9^{a} ± 0.34	2.7^{a} ± 0.05	2.7^{a} ± 0.09	3.1^{a} ± 0.04
	FT + S	2.1^{b} ± 0.06	2.3^{c} ± 0.04	2.4^{b} ± 0.08	2.7^{c} ± 0.04
	SED	0.22	0.05	0.09	0.05
P	S	0.14	0.70	0.83	0.36
	PM	0.02	0.0007	0.03	0.0002
	S.PM	0.12	0.03	0.11	0.35

Forage species (S), alfalfa (L) and ryegrass (R) prepared (PM) as fresh (F), frozen-thawed (FT) and frozen-thawed + starch (FT + S).
Means within a column without a common superscript differ ($P < 0.05$).
SED: Standard error of the difference.

Discussion

Two forage species, alfalfa and ryegrass, were incubated using the *in vitro* gas production technique in three different forms, *viz.* F, FT and FT + S. Both S and PM influenced the degradation characteristics of the two forages.

True OM digestion rate of legumes was faster than for grasses *in vitro* (Niderkorn et al., 2011), while TVFA production rate *in vitro* from fermentation of Persian clover was higher than perennial ryegrass (Williams et al., 2005). Preparation methods in the current study did alter the rankings such that, samples prepared as FT + S had faster fractional rates of degradation (b) for total N and OM and higher TVFA production than F and FT prepared samples (see Table 1). In contrast, Lowman et al. (2002) found that other methods of drying (microwave or oven-drying) had no affect on DM loss and VFA production rate from perennial ryegrass samples. However, oven drying of browse species investigated by Parissi et al. (2005) depressed OM digestibility. When S and PM were considered together in this study, forage species prepared as FT + S had the highest ranking for OM digestibility (Table 1), presumably because the added starch would have been completely digestible.

No difference between legumes and grasses in C3:C2 ratio at 3.5 and 24 h incubation times was found by Niderkorn et al. (2011). However, Williams et al. (2005) found the glucogenic:lipogenic ratio of VFAs was lower for ryegrass than clover. Also, the C3:C2 ratio was higher when the rate of fermentation was higher and the TVFA produced corresponded to the amount of fermentable OM ingested (Boss and Bowman, 1996). Our results indicated that rate of fermentation of OM was higher when fresh material was fermented (Table 3). By adding starch to the incubation vessels containing alfalfa prepared as FT the C3:C2 ratio increased, suggesting that starch was more rapidly fermented than the fermentable OM in FT alfalfa, thus promoting propionate and inhibiting acetate production. However, the fractional rate of degradation of TVFA and the C3:C2 ratios was still generally higher than when the same amount of fresh sample was incubated suggesting soluble carbohydrate concentrations were higher in the F than in FT samples. This hypothesis warrants further investigation.

Table 3. Percentage of N (Total N) and organic matter (OM) *in vitro* incubation after 12 h *in vitro* incubation (mean ± SE).

S	PM	Total N		OM	
		a	*b*	*a*	*b*
L	F	94.7^{bcd} ± 2.75	0.30^{b} ± 0.014	54.3^{a} ± 1.40	0.18^{ab} ± 0.016
	FT	72.0^{d}NA	0.57^{a}NA	43.3^{c} ± 1.41	0.18^{b} ± 0.032
	FT + S	85.0^{cd} ± 5.03	0.32^{b} ± 0.016	43.2^{c} ± 0.08	0.24^{a} ± 0.007
R	F	132.8^{a} ± 17.15	0.14^{c} ± 0.034	48.8^{c} ± 0.99	0.13^{bc} ± 0.008
	FT	111.9^{ab} ± 0.67	0.17^{c} ± 0.008	55.0^{a} ± 1.70	0.08^{c} ± 0.002
	FT + S	106.9^{bc} ± 2.43	0.34^{b} ± 0.035	38.5^{b} ± 2.06	0.18^{b} ± 0.010
SED		10.5	0.03	2.0	0.02
Main effects					
L		86.3^{b} ± 4.56	0.36^{a} ± 0.052	46.9^{a} ± 2.39	0.20^{a} ± 0.015
R		117.2^{a} ± 6.72	0.22^{b} ± 0.041	47.4^{a} ± 3.13	0.13^{b} ± 0.017
	SED	6.1	0.02	1.2	0.01
	F	113.7^{a} ± 13.08	0.22^{b} ± 0.049	51.5^{a} ± 1.75	0.16^{b} ± 0.016
	FT	92.0^{b} ± 13.30	0.30^{a} ± 0.131	49.2^{a} ± 3.48	0.13^{b} ± 0.030
	FT + S	96.0^{ab} ± 6.73	0.33^{a} ± 0.017	40.8^{b} ± 1.60	0.21^{a} ± 0.018
	SED	7.5	0.02	1.4	0.02
P	S	0.002	<0.0001	0.71	0.002
	PM	0.06	0.01	0.0007	0.009
	S.PM	0.46	0.0003	0.002	0.42

Forage species (S) alfalfa (L) and ryegrass (R) prepared (PM) as fresh (F), frozen-thawed (FT) and frozen-thawed + starch (FT + S).
Means within a column without a common superscript differ (P<0.05).
a is the soluble or easily degradable fraction and *b* is the fractional rate of degradation of *a*.
SED: Standard error of the difference.

The VFA production at 3.5 h of incubation was greater for legumes than for grasses but after 24 h, no differences were found (Niderkorn et al., 2011). In the current study, gas production was higher for alfalfa than ryegrass and this was consistent across the three preparation methods as indicated by the

absence of a significant interaction between S and PM (see Figure 1). However, FT + S samples produced more TVFA than FT prepared samples for both forage species. The calculated MCP, based on production rates of VFA, was also higher for alfalfa than ryegrass and higher for F samples than FT and FT + S samples (Table 1). This suggested that frozen samples contained less fermentable substrates, perhaps because of respiration after cutting or losses of soluble substrates during thawing. If frozen-thawed grass is ingested by feeding animals within 12 h after removal from the deep freezer, the risk of loss of soluble carbohydrate components during thawing is low (MacRae, 1970). The effect of added starch was also negligible (FT versus FT + S) in the current study. Therefore, with this range of possibilities, and the demonstration that the fermentation of the FT samples increased when starch was added. It is therefore difficult to explain what changes occurred in the fermentable carbohydrate concentrations in FT samples. This increases uncertainty about evaluations of fresh forages by the *in vitro* gas production methods using frozen-thawed samples. Freezing can reduce N solubility because of vacuolar membrane breakdown which in turn can markedly boost the potential microbial utilization of the dietary protein by ruminants (MacRae et al., 1975). Further investigation as to why freezing and thawing of ryegrass changed the rankings in gas and MCP production is required. In the current study, the characteristics of VFA production and the differences in C3:C2 ratios, suggested that alfalfa was degraded more quickly than ryegrass (see Figure 1). Samples prepared as FT had the lowest *in vitro* VFA production for either forage species, while F prepared samples produced the highest TVFA. Samples prepared as FT had the lowest cumulative gas production but patterns of degradation were similar for alfalfa and ryegrass (Figure 1).

In the current study, samples of forages prepared as F, FT and FT + S were macerated for 30 sec in a blender before incubation, therefore forage particle size may have affected the rate of forage DM fermentation. Forage rankings for digestibility have been shown to be influenced by sample preparation method with mincing (which simulates chewing), being more suitable than chopping and freeze-drying followed by grinding (Barrell et al., 2000). These workers also found that chopping tended to reduce VFA production rates relative to mincing and freeze-drying. The effect of sample preparation on 'high sugar' *Lolium perenne* varieties compared to a control was investigated by Lee et al. (2002). They found that freeze-dried and ground samples had a significantly lower lag and a faster degradation rate than FT samples. Freeze-dried and ground samples also produced less ammonia-N concentrations than FT samples. Total VFA production did not differ between preparation methods, but higher C3:C2 ratios were recorded from the freeze-dried and ground samples than from the FT samples.

Implications

The *in vitro* gas production method used in the current study demonstrated differences in the fermentation patterns and degradability parameters for fresh alfalfa and ryegrass, and provided useful comparative information about their potential nutritive value. Our study confirmed the higher nutritive value of legumes over grasses. However, the significant interaction between species and storage and preparation methods for some characteristics indicated that rankings may change within and between a forage species when different storage and preparation methods are used. In particular, freezing and storing samples before *in vitro* evaluation could lead to false conclusions about the nutritive value of fresh forage ingested by grazing animals. In short, this study highlighted the need for standardization of sample storage and preparation methods before *in vitro* evaluation of forage samples is conducted.

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