Anim. Res. 51 (2002) 269–277

DOI: 10.1051/animres:2002023

Original article

In vitro investigation into the nutritive value of *Lolium perenne* bred for an elevated concentration of water-soluble carbohydrate and the added effect of sample processing: freeze-dried and ground vs. frozen and thawed

Michael R.F. Lee[a]*, Alison E. Brooks[a], Jonathan M. Moorby[a], Mervyn O. Humphreys[a], Michael K. Theodorou[a], John C. MacRae[b], Nigel D. Scollan[a]

[a] Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23 3EB, UK
[b] Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK

(Received 6 September 2001; accepted 18 July 2002)

Abstract — A novel *Lolium perenne* variety bred for an elevated concentration of water-soluble carbohydrate (WSC) Ba11353 (high WSC; HWSC), which when grazed exhibited higher liveweight gains in lambs compared with a control (AberElan), was analysed in vitro to determine its relative nutritive value. The effect of sample preparation was also investigated: frozen and thawed (FT) vs. freeze-dried and ground (FDG). The nutritive value of the grasses was determined by in vitro measurements of gas production, which allowed assessment of both the rate and extent of fermentation of the grasses. Five FT and FDG samples of the two grasses (HWSC and control; n = 20) were suspended in an anaerobic medium, inoculated with rumen digesta and incubated at 39 °C for 120 h, with readings of gas pressure and volume made at regular intervals. At the end of the experimental period samples of liquid phase were taken to determine ammonia nitrogen and volatile fatty acid (VFA) concentrations. The WSC concentration of the HWSC was greater than the control ($P = 0.03$) whereas fibre concentrations were higher in the control ($P = 0.002$ and $P = 0.01$ for acid and neutral detergent fibres, respectively). The gas produced from the HWSC forages (both FT and FDG) had lower half-lives (the time at which half the total gas pool was produced), lag-times ($P < 0.001$) and a greater predicted asymptote gas yield ($P = 0.003$) when compared to the corresponding control forage. For both grass varieties, the FDG treatment significantly reduced gas production half-life and lag-time and increased the gas yield and degradability compared to the FT treatment. Liquid phase ammonia-N concentrations were lower with HWSC ($P = 0.04$) compared to control. The FDG treatment of the grass led to a further reduction in ammonia-N concentration compared to the FT samples ($P < 0.001$). Total VFA production was greater ($P < 0.001$) for both HWSC treatments compared to

* Correspondence and reprints
Tel.: +44 (0)1970 823084; fax: +44 (0)1970 823245; e-mail: michael.lee@bbsrc.ac.uk

the control, but no significant differences were noted between FT and FDG treatments. The glucogenic/lipogenic acid ratios (propionate/(acetate + butyrate)) were greater with HWSC ($P < 0.001$). The FDG treatment further increased this ratio compared to the FT treatment ($P = 0.002$). This study helped to determine the nutritive value of high WSC grass through an evaluation of its fermentation properties when incubated in vitro with inoculated anaerobic media; it also provided evidence of how preparation of the samples can influence the results.

water soluble carbohydrate / gas production / nutritive value / grass / fermentation rate

Résumé — Détermination in vitro de la valeur nutritive de *Lolium perenne* sélectionnée pour sa concentration élevée en glucides hydrosolubles et effet de la préparation de l'échantillon: lyophilisé et broyé vs. congelé et décongelé. Une nouvelle variété de *Lolium perenne*, sélectionnée pour sa concentration élevée en glucides hydrosolubles (Ba11353, HWSC) et qui permet des gains de poids vifs plus importants chez les agneaux lorsqu'elle est comparée à un témoin (AberElan), a été étudiée in vitro pour estimer sa valeur nutritive. L'effet de la préparation des échantillons a été également examiné : congelés et décongelés (FT) vs. lyophilisés et broyés (FDG). La valeur nutritive de l'herbe a été déterminée par des mesures in vitro de la production de gaz, qui ont permis d'évaluer la vitesse et le potentiel de fermentation du fourrage. Cinq échantillons FT et FDG de deux variétés de ray-grass anglais (HWSC et témoin ; n = 20) ont été mis en suspension dans un milieu anaérobie, inoculé avec des digesta de rumen, et incubés à 39 °C pendant 120 h. La lecture de la pression et du volume de gaz s'est faite à intervalles réguliers. À la fin de la période expérimentale, des échantillons de phase liquide ont été prélevés pour déterminer les concentrations en azote ammoniacal et en acides gras volatils (VFA). La concentration en WSC de HWSC était plus importante que celle du témoin ($P = 0,03$) tandis que les concentrations en fibres étaient plus élevées chez le témoin (ADF, $P = 0,002$ et NDF, $P = 0,01$). Le gaz produit à partir des fourrages HWSC (FT et FDG) a eu un T50 inférieur (temps correspondant à la moitié de la production totale de gaz), un temps de latence inférieur ($P < 0,001$) et une plus grande production de gaz à l'asymptote ($P = 0,003$) comparativement au fourrage témoin correspondant. Pour les deux variétés de ray-grass, le traitement FDG a diminué significativement le T50 et le temps de latence et a augmenté la production de gaz et la dégradabilité des fourrages comparés au traitement FT. Les concentrations en azote ammoniacal dans la phase liquide ont été inférieures avec HWSC ($P = 0,04$) comparées au témoin. Le traitement FDG de l'herbe a conduit à une réduction supplémentaire de la concentration en azote ammoniacal comparativement aux échantillons FT ($P < 0,001$). La production d'acides gras volatils totaux a été plus importante ($P < 0,001$) pour les deux traitements HWSC comparativement au témoin, mais aucune différence significative n'a été notée entre les traitements FT et FDG. Les ratios acides glucogéniques/acides lipogéniques (propionate/(acétate + butyrate)) étaient plus élevés avec HWSC ($P < 0,001$). Le traitement FDG a encore augmenté ce rapport comparé au traitement FT ($P = 0,002$). Cette étude a permis de déterminer la valeur nutritive du fourrage HWSC par une évaluation de ses propriétés de fermentation lors de l'incubation in vitro dans des milieux anaérobies inoculés ; elle a également mis en évidence la façon dont la préparation des échantillons peut influer sur les résultats.

glucide hydrosoluble / production de gaz / valeur nutritive / herbe / taux de fermentation

1. INTRODUCTION

In ruminants fed on fresh forage up to 80% of protein entering the duodenum maybe of microbial origin [1]. For the production of protein, the micro-organisms need access to a non-protein nitrogen source (ammonia, amino acids, peptides) and a readily available energy source (water-soluble carbohydrate; WSC). In most traditional forages there is a large proportion of soluble nitrogen, however, the levels of WSC are low (< 10%) which may reduce

the efficiency of microbial protein synthesis and vice-versa there maybe potential for a forage exhibiting elevated levels of WSC to increase microbial protein synthesis.

Biological procedures that assess the initial rate of digestion of a feed can be used to gain insights into the nutritive value of high WSC grass for ruminants, and more particularly the rumen microbial population. In vitro measurement of gas production [14] allows assessment of both the rate and extent of fermentation of feeds, which is an indirect measure of microbial growth on a given feed and thus an indicator of the nutritive value of that feed in the ruminant animal. Many in vitro experiments use freeze-dried and ground samples [2], however, in this experiment frozen and thawed grass was also used, as this is more stable in terms of chemical composition than fresh grass, to determine whether sample processing had an effect on the fermentation rate.

The objectives of this study were to determine the nutritive value of grass samples which were taken from a previous experiment [8] which demonstrated a significant increase in liveweight gain of suckling lambs on a high WSC *Lolium perenne* variety Ba11353 (HWSC) versus a control variety AberElan. The study also examined if the mode of preparation (freeze-dried and ground vs. frozen and thawed) induced any effect on their rate of fermentation in vitro.

2. MATERIALS AND METHODS

2.1. Experimental design

Five replicate samples of two grasses (*Lolium perenne*) var. Ba11353 high WSC (HWSC) and control (AberElan) either freeze-dried and ground (FDG) or frozen and thawed (FT) were incubated in anaerobic medium inoculated with rumen fluid for 120 h. Regular measurements of gas production and volume were taken to assess rate of fermentation via the France et al. [5] gas production model.

2.2. Preparation of digestion media and samples

The digestion medium was made up as described by Theodorou et al. [14]. The grasses used were bulked sub-samples taken from a previous experiment (Lee et al. [8]; which also describes the management of the grasses before cutting). Grass samples were cut at 3 cm above ground level with herbage shears at 10:00 h at weekly intervals for 9 weeks from five random locations in three paddocks for each variety. The samples were then stored at –20 ^{o}C prior to the start of the experiment. Half of the whole sample was removed, freeze-dried and ground using a Cyclotec 1093 grinder fixed with a 1 mm mesh screen (Foss Tecator, Hoganas, Sweden) FDG and the other half defrosted and cut into 1cm length strips. Five replicate 1.00 g samples of the two FDG grasses and five replicate 5.00 g samples of the two FT grass samples (var. HWSC and control) were weighed out into 160 mL serum bottles, to give approximately equal masses on a dry matter basis. Five extra bottles were used, as controls with no added grass. Grass samples were analysed for dry matter content and total WSC concentration. The bottles were gassed with carbon dioxide and 85 mL of the digestion medium was added before the bottles were stoppered, crimped with an aluminium seal and incubated at 39 ^{o}C prior to inoculation.

2.3. Inoculation

Two litres of rumen digesta were taken two hours after feeding from two sheep fistulated at the rumen, fed on standard grass silage ad libitum, thoroughly mixed and transported to the laboratory, within 1 h, in a pre-heated vacuum flask. The rumen fluid was strained through double layer of

muslin into a CO_2-filled beaker, squeezing the muslin to obtain maximum liquid. The serum bottles were inoculated with 10 ml of strained fluid using a 10 mL syringe, which was then attached to a gas pressure transducer to adjust the headspace gas pressure in each bottle to zero. The bottles were incubated in a water bath maintained at 39 °C.

2.4. Sampling

Gas pressure and volume was recorded at intervals of 3, 6, 9, 12, 15, 18, 21, 24, 28, 33, 38, 45, 60, 70, 94, 140 h post inoculation. After each reading, the bottles were shaken and returned to the incubator. At the end of the fermentation period, the supernatant was separated from the particulate substrate and adherent microbial biomass by vacuum filtration through pre-weighed sintered disc filters (100 µm porosity 1; Gallenkamp; Fisher Scientific UK, Loughborough, Leicestershire). These residues were washed with 100 mL of deionised water and freeze dried to constant weight for determination of residual dry matter. The pH of the supernatant was measured using a Hydrus 400 pH probe (Fisher Scientific UK, Loughborough, Leicestershire), 1 mL was taken and acidified with 100 µL of 2M HCl and used for ammonia-N (NH_3N) analysis and another 1 mL was acidified with 100 µL of orthophosphoric acid and analysed for volatile fatty acid (VFA) concentration.

2.5. Chemical and statistical analysis

Water-soluble carbohydrate concentration was determined spectrophotometrically, after extraction in water, using anthrone in sulphuric acid on a Technicon Autoanalyser attached to a peak height analyser calibrated with fructose working standards [15]. Ash and by mass difference organic matter (OM) was analysed by igniting at 550 °C for 6 h in a muffle furnace. Volatile fatty acids in the supernatant were determined by gas chromatography using Chrompack CP 9002 (CP-Sil 5CB column 10 m × 0.25 mm ID; Chrompack, UK) and following the method of Zhu et al. [20]. In vitro dry matter digestibility was determined following the method of Jones and Hayward [6]. Ammonia nitrogen was assessed enzymatically using glutamate dehydrogenase on a discrete analyser (FP-901M Chemistry Analyzer, Labsystems Oy, Helsinki, Finland; Test kit No. 66-50, Sigma-Aldrich Co. Ltd., Poole, Dorset). Total nitrogen was determined by micro-Kjeldahl technique using 'Kjeltec equipment' (Perstorp Analytical Ltd., Maidenhead, Berkshire). Neutral detergent fibre was determined as described by Van Soest et al. [17] and acid detergent fibre was analysed according to Van Soest and Wine [18] using the Tecator Fibretec System equipment (Tecator Ltd., Thornbury, Bristol).

In vitro dry matter (DM) disappearance was calculated as the difference between the DM of the initial sample and incubated washed residue DM. A ratio (final/initial) was calculated and subjected to a general analysis of variance with treatment (FDG vs. FT) × variety (HWSC vs. control) as the treatment using Genstat 5: Lawes Agricultural Trust, [7]. Gas production data obtained from the fermentation of each forage, corrected for negative controls, were fitted to the model described by France et al. [5] using the Maximum Likelihood Programme (MLP; [11]). The equation was:

$$Y = A\{1 - e^{[-b(t-T) - c(\sqrt{t} - \sqrt{T})]}\}$$

where Y is cumulative gas production (mL), A is the asymptote (i.e. gas pool size), T is lag time, b (h^{-1}) and c ($h^{-0.5}$) are fractional rate constants. These calculated parameters, along with measured effluent parameters, VFA, pH and ammonia-N, were subjected to a general analysis of variance using the same model as for in vitro DM disappearance.

3. RESULTS

The chemical composition of the forages used is given in Table I. The WSC concentration and in vitro digestibility were higher in HWSC ($P = 0.03$ and $P = 0.007$, respectively), whilst fibre was lower ($P = 0.002$ and $P = 0.01$ for ADF and NDF respectively) than the control grass. The total nitrogen concentration of the grasses was not significantly different.

The gas production curves are shown in Figure 1. The mathematical estimation of half life and lag time, calculated according to the model described by France et al. [5] is presented in Table II. The curves for all grass samples showed a short lag phase of between 0.28 and 1.43 h and the derived asymptotes for gas produced ranged from about 269 to 321 mL. The gas produced from the incubated HWSC forages (FT and FDG) had lower half-lives (the time at which half the total gas pool was produced),

Table I. Chemical composition ($g \cdot kg^{-1}$ DM) of the two *Lolium perenne* grasses Ba11353 high WSC (HWSC) and the control (AberElan) (n = 5).

	HWSC	Control	s.e.d.	Significance
Dry matter ($g\ DM \cdot kg^{-1}$)	173.1	172.6	12.5	NS
Organic matter	923.6	923.2	8.4	NS
Water-soluble carbohydrate	179	95	6.7	0.03
Total nitrogen	24.9	31.1	2.6	NS
Acid detergent fibre	246.1	286.1	14.0	0.002
Neutral detergent fibre	450.9	540.3	24.9	0.01
In vitro dry matter digestibility (%)	0.68	0.64	0.02	0.007

NS = not significant.

Table II. Gas production parameters of the two *Lolium perenne* grasses Ba11353 high WSC (HWSC) and the control (AberElan) freeze-dried and ground (FDG) or frozen and thawed (FT) (n = 5).

	FDG		FT		s.e.d.	Significance		
	HWSC	Control	HWSC	Control		T	V	T × V
Half-life (h)	6.73	10.12	16.98	22.19	0.233	< 0.001	< 0.001	0.001
Lag time (h)	0.28	1.26	1.39	1.43	0.786	< 0.001	< 0.001	0.001
Yield (mL)	320.5	284.7	273.9	268.9	8.74	0.003	0.033	NS
Extent deg.	0.86	0.83	0.89	0.84	0.010	0.044	< 0.001	NS

Half-life = time for half the predicted gas pool to be produced; Lag-time = time for initial fermentation to commence; Yield = predicted asymptote gas yield; Extent deg. = predicted extent of degradation within the rumen; T, V and T × V = treatment, variety, and interaction effects, respectively.

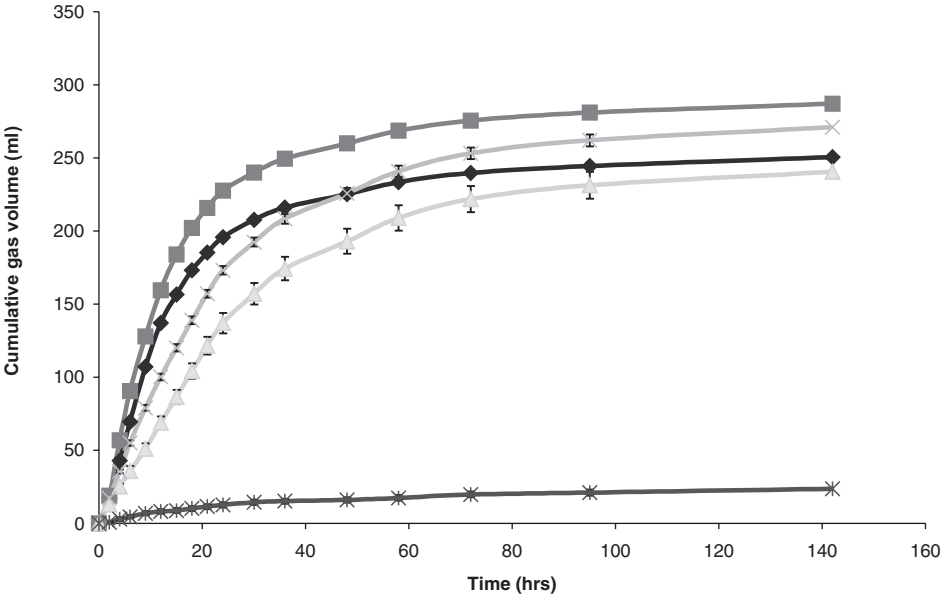

Figure 1. Cumulative gas production of the two grass varieties: Ba11353 high WSC (HWSC) either freeze-dried and ground (■ HWSC FDG) or frozen and thawed (X HWSC FT); control (AberElan) either freeze-dried and ground (◆ control FDG) or frozen and thawed (▲ control FT) and the control bottles with no added grass (✳) when incubated in inoculated digestion media at 39 °C for 120 h. (n = 5 +/– s.e.d).

lag-times ($P < 0.001$) and greater predicted asymptote gas yield ($P = 0.003$) when compared to the corresponding control forage. With both grass varieties the FDG treatment significantly reduced half-life and lag-time and increased the yield and degradability compared to the FT treatment. There were significant ($P = 0.001$) interaction effects between variety and treatment for both half-life and lag time. Mean supernatant VFA and ammonia-N concentrations are given in Table III. Ammonia-N levels were lower in the supernatant with HWSC ($P = 0.04$) compared to control. The FDG treatment of the grass led to a further significant reduction in ammonia-N concentrations compared to the FT treatment ($P < 0.001$). Total VFA production was significantly greater ($P < 0.001$) on HWSC in both treatments compared to control, but no significant differences were noted between FT and FDG treatments. There were significant interaction effects for total and individual VFA production. The glucogenic/lipogenic acid ratios (propionate/(acetate + butyrate)) were greater with the high WSC grass HWSC ($P < 0.001$). The FDG treatment further increased the ratio compared to the FT samples ($P = 0.002$). Molar proportions of acetate were lower ($P < 0.001$) and molar proportions of propionate higher ($P < 0.001$) in the supernatant with the HWSC grass compared to the control. Grass variety or method of preparation did not affect molar proportions of butyrate.

4. DISCUSSION

Davies et al. [2] identified that the initial rate of gas release was proportional to the WSC concentration of the grass ($r^2 = 0.82$), implying an increase in the metabolic activity

Table III. Concentrations of ammonia nitrogen (mmol·L^{-1}) and volatile fatty acids (mmol·L^{-1}) and their corresponding molar proportions in medium after in vitro incubation of the two grasses Ba11353 high WSC (HWSC) and the control (AberElan) freeze-dried and ground (FDG) or frozen and thawed (FT) (n = 5).

	FDG		FT		s.e.d.	Significance		
	HWSC	Control	HWSC	Control		T	V	T × V
				Concentrations (mmol·L^{-1})				
Ammonia-N	19.6	24.0	27.0	29.8	1.63	< 0.001	0.04	NS
Acetate	48.0	34.9	42.9	42.8	1.65	NS	0.001	0.001
Butyrate	6.5	4.6	6.5	6.2	0.54	0.004	< 0.001	0.001
Propionate	22.1	14.2	18.7	17.0	0.82	NS	< 0.001	0.002
Total	79.7	56.1	71.6	69.9	2.76	NS	< 0.001	0.001
Ratio P/(A+B)	0.41	0.36	0.39	0.35	0.006	0.002	< 0.001	NS
				Molar proportions				
Acetate	0.60	0.62	0.60	0.61	0.002	0.016	< 0.001	NS
Butyrate	0.08	0.08	0.09	0.09	0.007	NS	NS	NS
Propionate	0.28	0.25	0.26	0.24	0.003	< 0.001	< 0.001	NS

P/(A+B) = Propionate/(acetate + butyrate).

of the micro-organisms with increasing WSC concentration. The objective of this in vitro study was to determine whether these results could be confirmed when comparing HWSC as the high WSC variety with AberElan as the control where the levels of WSC contrasted greatly, 179 and 95 g·kg^{-1} DM, respectively. Such mechanisms may help to explain the differences in liveweight gain of lambs grazing on these grasses [8]. The comparison of FT and FDG grass was carried out as most in vitro experiments have used dried and ground grass, a treatment which increases surface area and has been reported to increase the rate of digestion [5, 16].

For all the gas production parameters HWSC exceeded control in terms of fermentation rate, yield and degradation. In this respect, the findings support those of Davies et al. [2]. Likewise, all parameters for the FDG treatment significantly exceeded those for the FT treatment and are in agreement with previous observations, that increasing the surface area, by particle size reduction, can increase the rate of fermentation [3, 16]. However, Shain et al. [12] showed that forage particle size had no effect on ruminal metabolism or performance of finishing cattle.

Increasing the concentration of WSC in forage could be expected to increase the initial rate of fermentation in the rumen, as they are released more rapidly than other energy sources and therefore act as the primary energy source for microbial metabolism. In addition, significantly lower amounts of ammonia nitrogen were present in batch cultures where HWSC was the substrate, compared to control, implying that

either bacterial capture of ammonia was greater on the former or that less ammonia was liberated on the HWSC grass or a combination of the two. There were also lower concentrations of ammonia on the FDG treatment compared to the FT treatment. The lower ammonia concentrations encountered with a substrate higher in WSC, indirectly support the findings of Sutoh et al. [13]. They found that when sheep were fed a Lucerne hay diet supplemented with sucrose, a decrease in ruminal ammonia concentration occurred compared to hay alone, accompanied by an increase in nitrogen retention and a decrease in plasma urea and urinary nitrogen excretion. This supports the hypothesis that increasing the readily available energy supply to the rumen increases synchrony between nitrogen and energy supply to the rumen micro-organisms [4, 9]. This may lead to more efficient utilisation of the ammonia by rumen microbes and/or a decrease in the production of ammonia by reducing the need to ferment glucogenic amino acids to provide energy for microbial protein synthesis [10]. However, it is imperative to note that the concentration of ammonia in the vessels was particularly high compared to typical in vivo values, and so may not be comparable. The reduced ammonia levels observed with the FDG treatment may be related to a faster release rate of the soluble carbohydrate from the grass and consequently a greater efficiency of incorporation of the ammonia into microbial protein. Indeed Wadhwa et al. [19] showed that a reduction in the particle size of maize grains increased the rate of release of water-soluble nutrients like WSC. This appeared to be a direct response to the increase in the surface area, making grain starch accessible to the rumen fluid and micro-organisms.

The total VFA produced from FT and FDG grasses did not differ significantly, whereas higher VFA production was noted on the HWSC compared to control with significant interaction effects which may suggest that HWSC may only exhibit this property under certain circumstances i.e. when freeze-dried and ground. In terms of the VFA production patterns, highly significant differences were found between the varieties with similar interaction effects. The molar proportions of acetate were lower, and those of propionate were higher, when HWSC was the substrate compared to control. This trend has been previously reported that with more fibrous and lower WSC content feeds and the inverse with low fibre, high WSC feeds [1]. The differential fibre concentrations of the grasses may also have made an important contribution to the observed differences in the measured gas production parameters and although this was not examined it deserves further investigation.

In conclusion, this study has helped to further the link between WSC concentration and an increased rate of fermentation of grass by a rumen microbial population as an indicator for microbial growth, under controlled conditions. An increase in the WSC appears not only to increase initial rate of fermentation but also the glucogenic/lipogenic VFA ratio and reduces ammonia concentrations. In these respects, the findings give a partial explanation as to why high WSC forages maybe considered to improve the nutritive value of the forage to the grazing ruminant. The study also provided evidence of how preparation of the samples can influence the in vitro rates of fermentation.

ACKNOWLEDGEMENTS

The authors are grateful to Susan Youell for her considerable laboratory expertise during this experiment and Dan Dhanoa for his statistical guidance. This work was funded by a LINK Sustainable Livestock Production programme involving the Ministry of Agricultural Fisheries and Food, Milk Development Council, Meat and Livestock Commission and Germinal Holdings Ltd.

REFERENCES

[1] Beever D.E., Rumen function, in: Forbes J.M., France J. (Eds.), Quantitative aspects of ruminant digestion and metabolism, CAB International, Wallingford, UK, 1993.

[2] Davies R., Brooks A.E., Theodorou M.K., Merry R.J., Farrell M., Humphreys M.O., The relationship between initial rate of fermentation and WSC content of different grasses, Proceeding of the Society of Nutrition and Physiology No. 3 (1994) 193.

[3] Davies Z.S., Brooks A.E., Griffith G.W., Theodorou M.K., Assessment of fermentation characteristics of ruminant feeds using an automated pressure evaluation system, in: Deaville E.R., Owen E., Adesogan A.T., Rymer C., Huntington J.A., Lawrence T.L.J (Eds.), In vitro techniques for measuring nutrient supply to ruminants, Occasional publication, British Society of Animal Science, No. 22, (1998), pp. 190–192.

[4] Dewhurst R.J., Davies D.R., Merry R.J., Microbial protein supply from the rumen, Anim. Feed Sci. Technol. 85 (2000) 1–21.

[5] France J., Dhanoa M.S., Theodorou M.K., Lister S.J., Davies D.R., Isac D., A model to interpret gas accumulation profiles associated with in vitro degradation of ruminant feeds, J. Theor. Biol. 163 (1993) 99–111.

[6] Jones D.I.H., Hayward M.V., The effect of pepsin treatment of herbage on the prediction of dry matter digestibility from solubility in fungal cellulase solutions, J. Sci. Food Agric. 26 (1975) 711–718.

[7] Lawes Agricultural Trust, Genstat 5, Rothamstead Experimental Station, Harpendon, Hertfordshire, UK, 1995.

[8] Lee M.R.F., Jones E.L., Humphreys M.O., Moorby J.M., Theodorou M.K., MacRae J.C., Scollan N.D., Production responses from lambs grazed on *Lolium perenne* selected for an elevated water-soluble carbohydrate concentration, Anim. Res. 50 (2001) 441–449.

[9] MacRae J.C., Reeds P.J., Prediction of protein deposition in ruminants, in: Buttery P.J., Lindsay D.B. (Eds.), Protein deposition in animals, Butterworths, London, 1979, pp. 62–69.

[10] Nocek J.E., Russell J.B., Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production, J. Dairy Sci. 71 (1988) 2070–2107.

[11] Ross G.L.S., MLP: maximum likelihood programme (a manual), Rothamstead Experimental Station, Harpenden, Hertfordshire, UK, 1987.

[12] Shain D.H., Stock R.A., Klopfenstein T.J., Herold D.W., The effect of forage source and particle size on finishing yearling steer performance and ruminal metabolism, J. Anim. Sci. 77 (1999) 1082–1092.

[13] Sutoh M., Obara Y., Miyamoto S., The effect of sucrose supplementation on kinetics of nitrogen, ruminal propionate and plasma glucose in sheep, J. Agric. Sci. 126 (1996) 99–105.

[14] Theodorou M.K., Williams B.A., Dhanoa M.S., McAllan A.B., France J., A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds, Anim. Feed Sci. Technol. 48 (1994) 185–197.

[15] Thomas T.A., An automated procedure for the determination of soluble carbohydrates in herbage, J. Sci. Food Agric. 28 (1977) 639–642.

[16] Thomson D.J., Beever D.E., Coehlo de Silva J.F., Armstrong D.G., The effect in sheep of physical form on the sites of digestion of a dried lucerne diet, Brit. J. Nutr. 28 (1972) 31–41.

[17] Van Soest P.J., Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fibre and lignin, J. Assoc. Official Anal. Chem. 46 (1963) 829–835.

[18] Van Soest P.J., Wine R.H., Use of detergents in the analysis of fibrous feeds. IV. Determination of plant and cell wall constituents, J. Assoc. Official Anal. Chem. 50 (1967) 50–55.

[19] Wadhwa M., Paul D., Kataria P., Bakshi M.P.S., Effect of particle size of corn grains on the release of nutrients and in sacco degradability, Anim. Feed Sci. Technol. 72 (1998) 11–17.

[20] Zhu W.Y., Theodorou M.K., Longlands A.C., Nielson B.B., Dijkstra J., Trinci A.P.J., Growth and survival of anaerobic fungi in batch and continuous culture, Anaerobe 2 (1996) 29–37.