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In vitro protein fractionation methods for ruminant feeds

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ABSTRACT

Estimating protein fractions and their degradation rate are vital to ensure optimum protein supply and degradation in the digestive system of ruminants. This study investigated the possibility of using the ANKOM gas production system and preserved rumen fluid to estimate the protein fractions and *in vitro* degradability of protein-rich feeds. Three *in vitro* methods: (1) gas production method (2) Cornell Net Carbohydrate and Protein System (**CNCPS**), and (3) the unavailable nitrogen assay of Ross (**uN_{Ross}**) were used to quantify protein fractions of four feeds (lupin meal, vetch grain, *Desmanthus* hay, and soybean meal). Rumen fluid mixed with 5% dimethyl sulfoxide and frozen at −20 °C was also compared against fresh rumen fluid in the gas production and uN_{Ross} methods. All three methods ranked the feeds identically in the proportions of available (degradable or 'a + b') protein fractions as vetch grain, soybean meal, lupin meal, and *Desmanthus* hay in decreasing order. The use of fresh rumen fluid produced greater available protein fractions than preserved rumen fluid in all feeds. However, there was no difference between total gas production from lupin meal and vetch grain fermented for 16 h in either rumen fluid source. The *in vitro* degradable CP (**IVDP**) was higher for vetch grain (46 and 70%) at the 4th and 8th hours of incubation than other feeds, whereas soybean meal (85%) exceeded the other feeds after the 16th hour of incubation ($P < 0.001$). The greatest ammonia-N concentration was from soybean meal (1.27 mg/g) and lupin meal (0.87 mg/g) fermented for four hours using fresh rumen fluid. The proportion of fraction 'b' for soybean (82.1% CP) and lupin meals (39.4% CP) from the CNCPS method were not different ($P = 0.001$) from the fraction 'b' estimation of the gas production method for the same feeds ($r = 0.99$). Regardless of the methods, a greater water-soluble protein fraction was found from vetch grain (39.6–46.6% CP), and the proportion of fraction 'c' or unavailable protein in *Desmanthus* hay (39.1–41.5% CP) exceeded other substrates ($P < 0.001$). The strong positive correlation between fractions across different methods and identical ranking of feeds suggests the possibility of using ANKOM gas production apparatus for protein fractionation.

Implications

The tested feeds can be used as a degradable protein source in ruminant feed. The application of the gas production system, along with the procedural modifications to the ammonia-N sampling in ANKOM gas production apparatus, can assist in developing a relatively simple and reliable method as a suitable alternative tool for protein fractionation. Preserving rumen fluid using 5% dimethyl sulfoxide and −20 °C for *in vitro* protein quantification was not as effective as that of fresh rumen fluid.

Introduction

The information on feed quality needs to be precise for the effective mixing of ingredients during feed formulation. Protein is one of the major feed components that affects productivity, as it supports diverse physiological processes in the ruminant body, and could be a limiting factor for an optimized rumen system and for microbial protein synthesis (Roe et al., 1991). Surplus N supply leads to inefficient protein utilization, physiological stress from ammonia toxicity (Patra and Aschenbach, 2018), and a higher N-excretion, whereby more than 70% of the ingested protein can be lost in urine and faeces (Huws et al., 2018; Li et al., 2020), causing adverse effects on the environment (Lebzien et al., 2006; Pacheco and Waghorn, 2008). Furthermore, protein-rich feeds are expensive feed ingredients. Thus, one of the critical factors determining

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the profitability and productivity of ruminant systems is managing their supply through proper feed formulation (Pacheco et al., 2012).

CP is commonly used as a quantification unit for the amount of protein, but it provides inadequate information on the amount of metabolizable protein (ARC, 1980; Gutierrez-Botero et al., 2014). Moreover, chemical composition analysis alone tells us about nutrients within feeds but not about their availability and degradation in the digestive tract of animals (Licitra et al., 1996; Sallam, 2005). Therefore, it is crucial to develop better ways of estimating protein quality, its fractions and their degradation rate to ensure optimum protein supply and digestibility in the digestive system (Ross et al., 2013; Liebe et al., 2018). The *in situ* technique (NRC, 2001), exponential (Ørskov and McDonald, 1979) and Cornell Net Carbohydrate and Protein System (**CNCPS**) (Higgs et al., 2015) mathematical models of protein fractionation divide the CP into three to five fractions based on their degradation in the digestive system.

The *in situ* technique has been extensively used for the determination of protein degradation in ruminants. However, the results are affected by factors such as the bag material, size, insertion, removal and rinsing procedures, sample size, incubation time, number and physiological condition of animals, feed and feeding frequency, microbial contamination, and mathematical models. The degradability of some proteins could be overstated as a result of escaping through the bag pores before degradation (Michalet-Doreau and Ould-Bah, 1992; López, 2005). Moreover, *in situ* technique is costly and more time consuming than *in vitro* techniques (Gosselink et al., 2004). Therefore, *in vitro* procedures can be useful.

Available techniques for the estimation of *in vitro* protein digestibility in the rumen and post-rumen environment have their drawbacks. The enzymatic approach cannot represent the spectrum of proteolytic activities found in the rumen (Stern et al., 1997; Licitra et al., 1998; Hristov et al., 2019). The inhibitor *in vitro* method uses either hydrazine sulphate or chloramphenicol as inhibitors of ammonia-N and amino acid absorption by bacteria (Broderick, 1987; Broderick, 1994). However, this method cannot run beyond 4 hours as the inhibitors become less active, and bacteria become nitrogen-starved (Broderick et al., 1988). Therefore, the inhibitor method is limited to feeds that show rapid degradation within this time and is not applicable to feeds containing high volume of ammonia and soluble nitrogen as these compounds can hinder the estimation of insoluble nitrogen (Cottrill, 1998).

Another alternative is using a modified CNCPS to determine the protein fractions of the feed (Sniffen et al., 1992; Higgs et al., 2015). The CNCPS system fractionates feed protein as ammonia (a_1), soluble true protein (a_2), insoluble true protein (b_1), fibre bound protein (b_2) and indigestible protein (c) (Higgs et al., 2015). The CNCPS model is highly sensitive to variations in chemical composition, environmental factors affecting the composition, and the rate of degradation in predicting the nutritional value of feeds. The unavailable nitrogen assay of Ross (**uN_{Ross}**) is another method for the estimation of degradable, undegradable and intestinal digestibility of rumen undegradable proteins using rumen fluid and enzymes in sequential steps to simulate the digestive system of ruminants (Ross et al., 2013). However, the procedure is lengthy and expensive, which affects applicability. Therefore, there is currently a demand to develop a relatively simple and reliable method for estimating the protein degradation of ruminant feeds.

The gas production method has mostly been used to evaluate *in vitro* fermentation of ruminant feeds for its rapid analysis and low cost. This method is relatively simple to quantify the volume and rate of gas production, and provides opportunity to collect the gas and liquid samples for further analysis (Getachew et al., 1998). Furthermore, it eliminates the confounding effects of *de novo* microbial protein synthesis during fermentation using a mathematical approach (Bueno et al., 2005; Falahatizow et al., 2015). To our knowledge, the automated ANKOM gas production system has not been used to quantify protein fractions; hence, the gas production method of Karlsson et al., 2009 was adapted to the ANKOM equipment in this experiment. The CNCPS and uN_{Ross} assay have not been compared against the gas production method for protein fractionation and the relationship between their results has not yet been established. The protein fractions and *in vitro* protein degradation of *Desmanthus* hay and lupin meal have also not been reported.

The gas production method involves the fermentation of substrates using rumen fluid. Preservation of rumen fluid may be one method to minimize the collection time, cost of handling cannulated animals, avoid seasonal effects on the rumen fluid quality and reduce ethical concerns about cannulation (Getachew et al., 2004; Denek et al., 2010). However, the information on the use of preserved rumen fluid for *in vitro* protein assessment is scarce (Chaudhry and Mohamed, 2012).

The objectives of this study were to compare the gas production method against the CNCPS and uN_{Ross} methods for protein fractionation, to investigate the applicability of using the ANKOM gas production system and preserved rumen fluid to estimate the protein fractions and degradability of protein-rich feeds. This research hypothesized that firstly there would be no difference between the various protein quantification methods in the fraction values of the same feed and secondly that preserved rumen fluid can accurately be used for *in vitro* estimation of protein fractions and degradability.

Material and methods

The study was conducted in animal production, *in vitro* fermentation, and food science laboratories at the University of Melbourne, Australia.

Substrates, rumen fluid, and experimental design

A solvent-extracted soybean meal (*Glycine max* L.), solvent-extracted sweet lupin meal (*Lupinus angustifolius* L.), common vetch grain (*Vicia sativa* L.), and *Desmanthus virgatus* (JCU 2) hay were selected to represent categories of meals, grains, and hay as protein-rich substrates. The meals and grain were purchased from a commercial supplier (Peter Gibbs Stock Feeds, Australia). The hay was collected from Townsville, Queensland and prepared by sun drying the leaf and young stems.

Three litres of rumen fluid were collected twice from four Holstein Friesian dairy cows at Agriculture Victoria Research (Ellinbank, Victoria, Australia) before the morning feeding and transported to the laboratory as described by Gonzalez-Rivas et al. (2016). Cows were grazing perennial ryegrass (*Lolium perenne* L.) pasture, and wheat and barley grain mix (6 kg DM per day per cow) was supplied in the milking parlour. The rumen fluid was filtered using cheesecloth and divided into three bottles. The first and second bottles of rumen fluid were used as fresh for gas production and uN_{Ross} assays, and the remaining rumen fluid was mixed with 5% dimethyl sulfoxide (**DMSO**) (CSA Scientific, 500 ml, Chemsupply) and frozen at −20 °C (**D-20** °C) for 30 days.

A three-way factorial design was used to compare four feeds and three protein fractionation methods (CNCPS, gas production and uN_{Ross} assay) using two kinds of rumen fluid, fresh and preserved. The rumen fluid preservation using D-20 °C was selected following the results of previous studies (Tunkala et al., 2022).

The incubation runs of gas production method were carried out twice on the first and 30th day of post-rumen fluid collection. A total of eight ANKOM modules connected to 250 ml bottles were

incubated for 16 h. The choice of 16 h incubation was based on the procedures of Ross et al., 2013 and other researchers who demonstrated no variation when comparing data from 16 or 24 h incubations in CP disappearance of alfalfa hay (Aghajanzadeh-Golshani et al., 2015) and the *in vitro* degradable CP estimates of fish meal and alfalfa (Falahatizow et al., 2015). The treatments were replicated six times for the CNCPS method and uN_{Ross} assay.

Parameters measured on the substrates

The chemical composition parameters namely DM (g/kg), DM digestibility (**DMD**; % DM), ash (% DM), and ether extract (% DM) were assessed in a commercial laboratory (FeedTest Laboratory, Agrifood Technology, Werribee, Victoria). The ADF (% DM) and NDF (% DM) were determined by an ANKOM^{200} fiber analyzer using the AOAC 2002.4 method (Mertens et al., 2002). The NDF was treated with sodium sulphite (FSS, ANKOM Technology, USA) and thermostable alpha amylase enzyme (FAA, ANKOM Technology, USA). The CP (% DM), acid detergent-insoluble protein (**ADIP**), and neutral detergent-insoluble protein were measured using a Kjeldahl method (method 954.01) (Horwitz, 2010). Ammonia-N concentration was quantified by the colorimetric technique described by Weatherburn, 1967 using a multiscan colorimetric plate reader (Thermo Multiskan Spectrum, Thermo Fisher Scientific, Australia). Soluble protein was determined using a borate-phosphate buffer, and non-protein nitrogen (**NPN**) was analyzed using trichloroacetic acid, as described by Licitra et al., 1996. DM digestibility was measured by pepsin-cellulase method (Dowman and Collins, 1982; AFFIA, 2014).

The protein fractionation methods of the Cornell Net Carbohydrate and protein system

The CNCPS was implemented as originally described by Sniffen et al., 1992 with the modifications reported by Higgs et al., 2015. The CNCPS fractionates CP into five fractions based on solubility: the 'a_1' and 'a_2' fractions are derived from ammonia-N and soluble protein, respectively. The fraction 'b' is a true protein, and 'c' is an unavailable and bound true protein (Van Soest et al., 1981). The fraction C is insoluble in the acid detergent, associated with lignin and Maillard products, resistant to mammalian and microbial enzymes, hence, cannot be degraded in the rumen and post-rumen environments (Sniffen et al., 1992). The fraction 'b' is divided into 'b_1' and 'b_2' based on degradation and passage rates. The 'b_2' fraction was computed from residual proteins of ADF and NDF analysis as indicated in Table 1. The 'b_1' fraction was calculated by subtracting fractions 'a_1', 'a_2', 'b_2', and c from total CP content. The c fraction was estimated from ADIP (% CP) (Higgs et al., 2015) following ADF (% DM) analysis.

Gas production method

Gas production was estimated using an automated ANKOM RF gas production system (ANKOM Technology, Macedon, NY, USA) using the method of Raab et al. (1983), revised by Karlsson et al. (2009), with minor modifications to fit into ANKOM gas production system. A total of 10 g/L of rapidly soluble carbohydrates (3.33 g of maltose, 3.33 g of starch, and 3.33 g of xylose) were added to filtered rumen fluid as described by Aghajanzadeh-Golshani et al. (2015) for pre-incubation conditioning which minimizes the background ammonia-N and stimulates microbial activity. Pre-incubation conditioning was held for three hrs in a 39 °C water bath (20-L Analogue Water bath, WB20; Ratek Instruments Pty Ltd, Boronia, VIC, Australia). Sodium bicarbonate $NaHCO_3$ (3.1 g dissolved in 63 ml of McDougall's buffer per L of rumen fluid) was also added to the rumen fluid under continuous flushing of CO_2 before and during the three h pre-incubation.

The pre-incubated rumen fluid was mixed with McDougall's buffer to obtain a buffered rumen fluid with a 1:2 rumen fluid to buffer ratio. The feed samples were ground into 2 mm particle diameter and sieved to ensure the size. A 500 mg of substrates was weighed in 250 ml ANKOM bottles, mixed with 90 ml buffered rumen fluid, and incubated for 16 h in a 39 °C water bath. Each substrate was replicated in eight ANKOM modules.

Samples were collected during fermentation to estimate ammonia-N concentration at 4 h, 8 h, 12 h and 16 h of gas production and frozen at −20 °C until analysis. Six additional modules were used as a source of ammonia-N samples for up to 12 h for each treatment in two runs, and these modules were removed from the incubation after sampling. The modules from gas production samples were used as a source of ammonia-N samples at 16 h. The use of additional modules as a source of the ammonia-N sample is a modification of the original method to fit into the ANKOM gas production system avoiding the possible change in the gas production after the opening of modules.

The *in vitro* degradable CP (**IVDP**) is a proportion of potentially degradable CP in *in vitro* assay and was calculated for each feed at 4 h, 8 h, 12 h and 16 h of gas production through intercepts of ammonia-N (y, mg/g) and gas production (x, ml/g), as described by Raab et al., 1983 using the equation:

$$\text{IVDP} = \frac{\text{Ammonia} - \text{N at zero gas production } \left(b^0 \text{ intercept}\right) - \text{Ammonia} - \text{N in blank}}{\text{Total N of incubated feed}} \quad (8)$$

The proportion of protein fractions and the effective CP degradation value were estimated by fitting the IVDP to the equations of Ørskov and McDonald (1979) using the exponential regression model of GenStat 21st edition.

$$Y = a + b * (1 - e^{-ct}) \quad (9)$$

where Y is the proportion of CP degraded at time t, a is the proportion of CP degraded at time 0 h, b is the proportion of potentially degradable CP, and c is the degradation rate of fraction b.

The effective CP degradation (**EPD**) value was calculated using the equation of Ørskov and McDonald (1979) as:

$$\text{EPD} = a + \frac{(b * c)}{(k + c)} \quad (10)$$

where a, b, and c are as above, and the passage rate (k) was assumed to be 0.08 h^{-1}.

The unavailable nitrogen assay of Ross

The uN_{Ross} assay is described in detail by Ross et al. (2013) and was conducted as follows; first, 0.5 g of the substrate was weighed and placed into a 125 ml Erlenmeyer flask. Then, 40 ml Van Soest buffer (Van Soest, 2015) with 6.8 pH and 10 ml of rumen fluid was

Table 1
Equations of Cornell Net Carbohydrate and Protein System (CNCPS) used for calculating protein fractions in ruminant feeds as described by Sniffen et al. (1992) and Higgs et al. (2015).

Fraction	Description	Equation[1]	
a_1	Soluble nitrogen	Ammonia × (SP/100) × (CP/100)	[2]
a_2	Soluble true protein	SP × CP/100 − A1	[3]
b_1	Insoluble true protein	CP − (a_1 − a_2 − b_2 − c)	[4]
b_2	Fibre-bound protein	(NDICP − ADICP) × CP/100	[5]
c	Indigestible protein	ADICP × CP/100	[6]

Abbreviations: SP = soluble protein; ADICP = acid detergent-insoluble protein; NDICP = neutral detergent-insoluble protein.
[1] Protein component is expressed in % CP for SP, ADICP, and NDICP.

added to each flask. A total of eight flasks were incubated per treatment in a water bath kept at 39 °C for 16 h. The samples in each bottle were acidified with 2 ml of 3 M HCL and incubated again with 2 ml of 100% pepsin solution for one hour, simulating abomasal digestion, and neutralized by 2 ml of 2 M NaOH to stop the pepsin reaction. The 100% pepsin solution was prepared by dissolving 50 g of pepsin powder (Extra Pure, SLR, Fisher Chemical™, Australia) in 50 ml distilled water.

After the pepsin incubation, a 10 ml enzyme mix containing alpha amylase (FAA, ANKOM Technology, USA), trypsin (EC Number: 232-650-8, model number: MFCD00082094, Sigma-aldrich, Australia), alpha chymotrypsin (EC Number: 232-671-2, model number: MFCD00130481, Sigma-aldrich, Australia), and lipase (Product Code: LL107, CAS No. 9001-62-1, Chemsupply, Australia) in 50, 24, 20, and 4 U per ml, respectively, was added to the ANKOM bottles and incubated for a further 24 h in a water bath at 39 °C. Samples were filtered through a 1.5 µm glass filter (Whatman 934AH) with warm water. The residue was then dried for 48 h in a 60 °C incubator. The N content of the residue was determined using the Kjeldahl method and expressed as a % of the total N in the sample. The entire uN_{Ross} assay procedure was repeated four times using fresh (twice) and preserved (twice) rumen fluids on the first and 30th days of post-rumen fluid collection with a total of eight replicates per treatment. The total N in the residue is unavailable protein and can be compared with ADIP in CNCPS, which is equivalent to fraction c (Sniffen et al., 1992; Ross et al., 2013; Higgs et al., 2015). The amount of available protein to the animal was computed by deducting the unavailable protein from 100.

Statistical analysis

The uN_{Ross} assay uses both rumen fluid and HCl for feed incubation in sequence (Gutierrez-Botero et al., 2014); therefore, the unavailable N fraction of the uN_{Ross} assay is non-utilizable protein in the digestive system. Therefore, the utilizable protein proportion was calculated by subtracting the non-utilizable protein from 100% and compared with 'a + b' of gas production. Fraction 'c' of gas production was computed by subtracting the proportions of fractions 'a' and 'b' from the total CP. Moreover, fractions with similar alphabetic designation in the CNCPS method were combined to use a single value for the correlation analysis with the values from the gas production method as described by Romagnolo et al., 1990.

The mean differences between feeds, sources of rumen fluid and SD were calculated by ANOVA using the Genstat 21st edition statistical package for the CNCPS method using the model:

$Yij = \mu + S_i + R_j + e_{ij}$.where Y_{ij} is the general mean of continuous dependent variables, µ is the mean value of all substrates examined, S_i is the fixed effect of each substrate (i = Soybean meal, Lupin meal, Vetch grain, *Desmanthus* hay or fresh and preserved rumen fluid) on the tested parameter, R_j is the random effect of runs, e_{ij} is the SE term.

The effects of the feed and incubation time on the IVDP and ammonia-N values were evaluated by two-way ANOVA using the Genstat 21st edition statistical package using the model:

$Y_{ij} = \mu + F_i + T_j + FT_{ij} + e_{ij}$.where Y_{ij} is the general mean of continuous dependent variables, µ is the mean value of all substrates examined, S_i is the fixed effect of each substrate (i = Soybean meal, Lupin meal, Vetch grain, *Desmanthus* hay) on the tested parameter, T_j is the fixed effect of time, FT_{ij} is the interaction effect between the independent variables, e_{ij} is the SE term.

The linear correlations among the protein fractions (from the CNCPS and gas production, and uN_{Ross} assay against gas production method using fresh rumen fluid) were determined using Pearson's correlation through correlation's function, and 2D scatter plot of Genstat 21st Edition. The regression equations were derived after regression analysis of protein fraction values of the gas production methods (x) and CNCPS (y) using Genstat.

Results

Chemical composition

Substrates used in this experiment varied in their chemical composition. The percentage of fat content in lupin meal and the ash content in *Desmathus* hay were greater than in the other feeds. Lupin meal and vetch grain had similar proportions of DM contents but were different in fat and DMD, as indicated in Table 2. *Desmanthus* hay had the greatest ADF and NDF contents than other feeds, followed by lupin meal, ($P < 0.001$). The CP content was greater in soybean meal and lowest in *Desmanthus* hay ($P < 0.001$), but the CP and NPN content of lupin meal and vetch grain were not different.

Gas production

The greatest total gas production was yielded from the fermentation of lupin meal using fresh rumen fluid, followed by vetch grain and soybean meal ($P < 0.001$, Fig. 1). There was no difference between total gas production from lupin meal fermented using fresh and preserved rumen fluid for 16 h. The total gas production was lower for *Desmanthus* hay fermented in fresh and preserved rumen fluid compared to other substrates.

The IVDP proportion was affected by the fixed factors and their interaction (feed x rumen fluid x time) ($P < 0.001$, Table 3). The IVDP values increased with the incubation time for all feeds, and the preservation of rumen fluid caused lower IVDP values compared with fresh rumen fluid ($P < 0.001$). The IVDP was greater for vetch grain at the 4th and 8th hours of incubation, whereas soybean meal exceeded the other feeds after the 16th hour of incubation ($P < 0.001$).

Protein fractions

The ammonia-N content was reduced over time for all substrates incubated using fresh and preserved rumen fluid ($P < 0.001$, Table 4). However, the amount of ammonia-N released was greater for feeds fermented using fresh rumen fluid compared to preserved rumen fluid. The greatest ammonia-N concentration was measured from soybean meal and lupin meal fermented for 4 h using fresh rumen fluid. Regardless of the methods, a higher water-soluble protein fraction was found from vetch grain, followed by lupin meal.

The fraction 'a' was slightly overestimated, and fraction 'c' was underestimated in the CNCPS method (Table 5) compared to the observed values of the gas production method for all feeds. The proportion of fraction 'b' for soybean and lupin meals from the CNCPS method were not different ($P = 0.001$) from the fraction 'b' estimation of the gas production method for similar feeds ($r = 0.99$). Soybean meal showed a greater fraction 'b', and the proportion of fraction c or unavailable protein in *Desmanthus* hay exceeded other substrates ($P < 0.001$).

The EPD calculated from protein fractions in the gas production method ranged from 95.1% in lupin meal to 60.8% in vetch grain ($P < 0.001$). The percentage of available protein from uN_{Ross} assay was not different from the 'a' + 'b' proportion of the gas production method for all feeds and rumen fluid types, except for vetch grain and *Desmanthus* hay fermented using the preserved rumen fluid.

The overall estimation of protein fractions between the gas production method and uN_{Ross} assay are strongly correlated ($r \geq 0.90$, $P = 0.001$; Table 4). The protein fraction results from gas production and CNCPS methods were also highly correlated ($r > 0.74$;

Table 2
Chemical composition, fibre and nitrogen contents of ruminant feeds used as substrates in this experiment.

Parameters	Soybean meal	Lupin meal	Vetch grain	*Desmanthus* hay	SED	*P*-value
DM, % sample	82.3	85.0	84.8	91.5		
Fat, % DM	1.20	5.50	1.70	4.90		
Ash, % DM	6.30	3.20	2.60	7.50		
DMD, % DM	93.5	82.2	88.4	61.8		
CP, % DM	46.5	30.4	29.8	16.7	0.83	<0.001
NDF, % DM	11.2	29.7	14.1	35.9	0.69	<0.001
ADF, % DM	12.2	23.8	10.8	29.7	0.35	<0.001
NPN, % N	0.61	1.74	1.89	0.27	0.162	<0.001
ADIP, % CP	0.34	6.43	0.66	40.1	0.441	<0.001
NDIP, % CP	11.9	7.72	37.9	38.5	0.852	<0.001

Abbreviations: DMD = DM digestibility; NPN = non-protein nitrogen; ADIP = acid detergent indigestible protein; NDIP = neutral detergent indigestible protein. The DM, fat, ash, and DMD contents were quantified in a commercial laboratory.

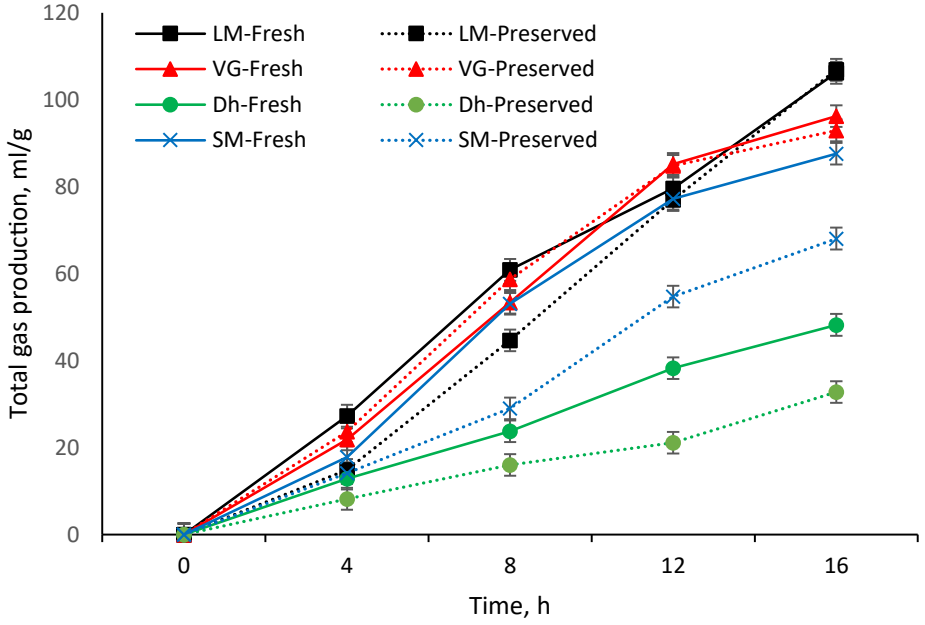

Fig. 1. The total gas production of lupin meal (and), vetch grain (and), *Desmanthus* hay (and), and Soybean meal (and) incubated *in vitro* using fresh and preserved rumen fluid, respectively, in 39 °C water bath for 16 hours. The data were a least mean of eight replications per treatment.

Table 3
The *in vitro* degradable CP (IVDP) of substrates (lupin meal, vetch grain, *Desmanthus* hay, soybean meal) calculated using the intercept of gas production and ammonia-N values at 4, 8, 12, and 16 hours of *in vitro* rumen fermentation.

Time (h)	Lupin Meal		Vetch grain		*Desmanthus* hay		Soybean meal		SED	*P*-value			
	F	P	F	P	F	P	F	P		S	R	T	SxRxT
4	0.35	0.16	0.46	0.30	0.13	0.06	0.34	0.20	0.011	<0.001	<0.001	<0.001	<0.001
8	0.60	0.21	0.70	0.38	0.17	0.04	0.62	0.16					
12	0.77	0.16	0.73	0.40	0.20	0.06	0.77	0.19					
16	0.80	0.12	0.75	0.41	0.25	0.06	0.85	0.13					

Abbreviations: T = time; F = fresh rumen fluid; P = preserved rumen fluid; S = substrates; R = rumen fluid.

$P < 0.001$; Fig. 2). Moreover, all three methods ranked the feeds similarly in the proportions of degradable protein fractions (available or 'a + b') as vetch grain, soybean meal, lupin meal, and *Desmanthus* hay in decreasing order.

Discussion

The protein fractions and fermentation characteristics differed between the methods and the rumen fluid types tested. However, a strong positive correlation was observed between the protein fractions of the feeds using different methods. Moreover, the ranking of feeds based on their protein fractions was identical across all methods. The gas production and uN_{Ross} assay methods behaved similarly in terms of protein fractionation. Therefore, the major finding of this research is that the adaptation of ANKOM gas production apparatus to the gas production technique of Karlsson et al. (2009) has the potential to be an alternative protein quantification tool to CNCPS and uN_{Ross} assay, along with the procedural modifications to the ammonia-N sampling.

The protein fractions of soybean meal fermented using fresh rumen fluid in the gas production and the uN_{Ross} assay methods

Table 4
Effect of protein quantification methods (gas production vs. uNRoss assay) and rumen fluid preservation on ammonia-N (mg/g), protein fractions (a, b, c), effective CP degradation (EPD), and availability of protein after fermentation of protein-rich ruminant feeds (soybean meal, lupin meal, vetch grain, and *Desmanthus* hay).

Parameters	Soybean meal		Lupin meal		Vetch grain		*Desmanthus* hay		SED	*P*-value		
	F	P	F	P	F	P	F	P		S	T	SxT
GP method												
NH_3-N_{4h}, mg/g	1.27	0.53	0.87	0.44	0.66	0.54	0.31	0.16	0.054	<0.001	<0.001	0.12
NH_3-N_{8h}, mg/g	0.97	0.34	0.74	0.20	0.67	0.34	0.26	0.12				
NH_3-N_{12h}, mg/g	0.76	0.28	0.57	0.16	0.41	0.24	0.17	0.05				
NH_3-N_{16h}, mg/g	0.60	0.15	0.37	0.11	0.20	0.16	0.14	0.03				
a, %CP	13.3	13.3	43.5	40.1	48.6	21.5	11.5	22.1	1.46	<0.001		
b, %CP	79.8	51.6	37.6	19.8	47.4	31.1	49.4	24.0	1.12	<0.001		
c, % CP	6.84	35.1	18.9	40.2	4.02	47.4	39.1	53.9	2.836	<0.001		
EPD, %CP	92.2	64.8	80.9	59.8	95.1	52.5	60.8	46.1	3.51	<0.001		
uN_{Ross} assay												
Unavailable, %CP	9.53	41.7	20.9	37.8	6.70	34.3	41.5	79.5	0.383	<0.001		
Available, %CP	90.5	58.4	79.1	62.2	93.3	65.7	58.5	20.5	0.93	<0.001		
Correlation, r	0.93	0.91	0.96	0.92	0.93	0.94	0.90	0.91				

Abbreviations: F = fresh rumen fluid; P = preserved rumen fluid; S = substrates; T = time; GP = gas production; a = soluble protein; b = degradable protein; and c = undegradable protein fraction; uN_{Ross} = the unavailable nitrogen assay of Ross; EPD = effective protein degradation. The degradation rate of 8%/h was used for EPD calculation from Ørskov and McDonald (1979). The correlation values were computed between 'a + b' of gas production with the available fractions of Ross assay.

Table 5
Protein fractions (a_1, a_2, b_1, b_2 and c) of ruminant feeds obtained from the Cornell Net Carbohydrate and Protein System (CNCPS) method.

Protein fractions	Soybean meal	Lupin meal	Vetch grain	*Desmanthus* hay	SED	*P*-value
a_1	8.22	35.6	39.6	10.2	1.012	<0.001
a_2	9.37	23.0	18.2	15.7	0.074	<0.001
b_1	36.8	19.0	33.0	27.7	0.49	<0.001
b_2	45.3	20.4	9.04	41.2	0.078	<0.001
c	0.26	1.95	0.20	5.24	0.018	<0.001

Abbreviations: a_1 = ammonia, a_2 = small peptides and free amino acids, b_1 = insoluble true protein, b_2 = fibre-bound protein, c = indigestible protein.

were within the same range as reported by Ghoorchi and Arbabi (2010) ('a' = 11.3% CP, 'b' = 84.5% CP and 'c' = 4.11% CP), who used the CNCPS method to quantify the protein fractions of soybean meal. Additionally, the protein fractions of soybean meal from the CNCPS method of our study were in the range of the fractions reported by Romagnolo et al. (1990) where fractions 'a', 'b', and 'c' were 13.5, 85.9, and 0.5% of total CP after an *in situ* experiment using cannulated dairy cows. They were also in range with the results of Maxin et al. (2013), who reported 11.8 and 88.3% fractions 'a' and 'b' of soybean meal incubated in rumen fistulated Holstein cows and corrected for particle loss. In our study, the fraction 'a' proportion of vetch grain from gas production and CNCPS methods was slightly higher, and fraction 'b' was lower than the results demonstrated by Ramos-Morales et al. (2010) and Huang et al. (2019) who used in situ method in canulated goat and sheep, respectively. However, they were not different in EPD value compared with five vetch grain varieties, ranging from 73.2 to 92.1% in both studies. The tested feeds showed high EPD values above 80%, except *Desmanthus* hay; hence, they can be used as a degradable protein source in ruminant feed (Ramos-Morales et al., 2010).

The gas production method requires less time, chemicals, and labour than the CNCPS and uN_{Ross} methods. Moreover, the strong positive correlation between fractions, identical ranking of feeds from all three methods examined in this experiment and the equivalent values of fraction 'b' for soybean and lupin meals from the CNCPS and gas production methods using fresh rumen fluid suggests the possibility of using ANKOM gas production system for protein fractionation. However, larger values of protein fraction 'a' and lower proportion of fraction 'c' from CNCPS could imply the advanced efficiency of this method for protein fractionation.

It has been reported that the ammonia-N concentration was reduced when the fermentation time increased as the ammonia-N released from feed was lower than the nitrogen uptake by microorganisms during *in vitro* fermentation (Getachew et al., 2000), causing an inverse relationship between the amount of ammonia-N and IVDP. The increasing IVDP and decreasing ammonia-N values over the incubation period for all feeds resulted from increasing gas production and continuous utilization of degraded protein for microbial protein synthesis. These findings are consistent with previous research that used the gas production method to quantify *in vitro* protein digestion parameters of ruminant feeds (Karlsson et al., 2009; Falahatizow et al., 2015). Generally, the microbial consumption of ammonia-N as input for gas production is a base for estimating protein fractions using a gas production method. Moreover, modification of the original gas production method using additional modules for ammonia-N sampling facilitates the application of ANKOM gas production system for protein quantification. However, the increasing number of modules or runs could affect the time and labour efficiency of the system based on the availability of active modules per run.

The preservation of rumen fluid using D-20 °C negatively affected protein fractionation. The values of IVDP, ammonia-N, and protein fractions from all feeds fermented using preserved rumen fluid were lower compared to fresh rumen fluid. The proteolysis of lysed cells after cold shock and the proliferation of microorganisms in thawed rumen fluid consuming nitrogen from the feeds (Luchini et al., 1996; Fabro et al., 2020) may cause the reduced availability of nitrogen when using preserved rumen fluid. Therefore, testing more options of rumen fluid preservation methods such as liquid nitrogen and −80 °C for *in vitro* protein quantification is still required to find appropriate techniques. It is also possible that an extended fermentation period may assist or even a greater proportion of preserved rumen fluid to buffer may be efficacious.

Prior reports on protein fractions of lupin meal and *Desmanthus* hay are limited. The fraction 'a' of lupin meal in this study was not different from the result of lupin seed fermented using lactating cannulated cows (44.3% CP) (Goelema et al., 1998); however, frac-

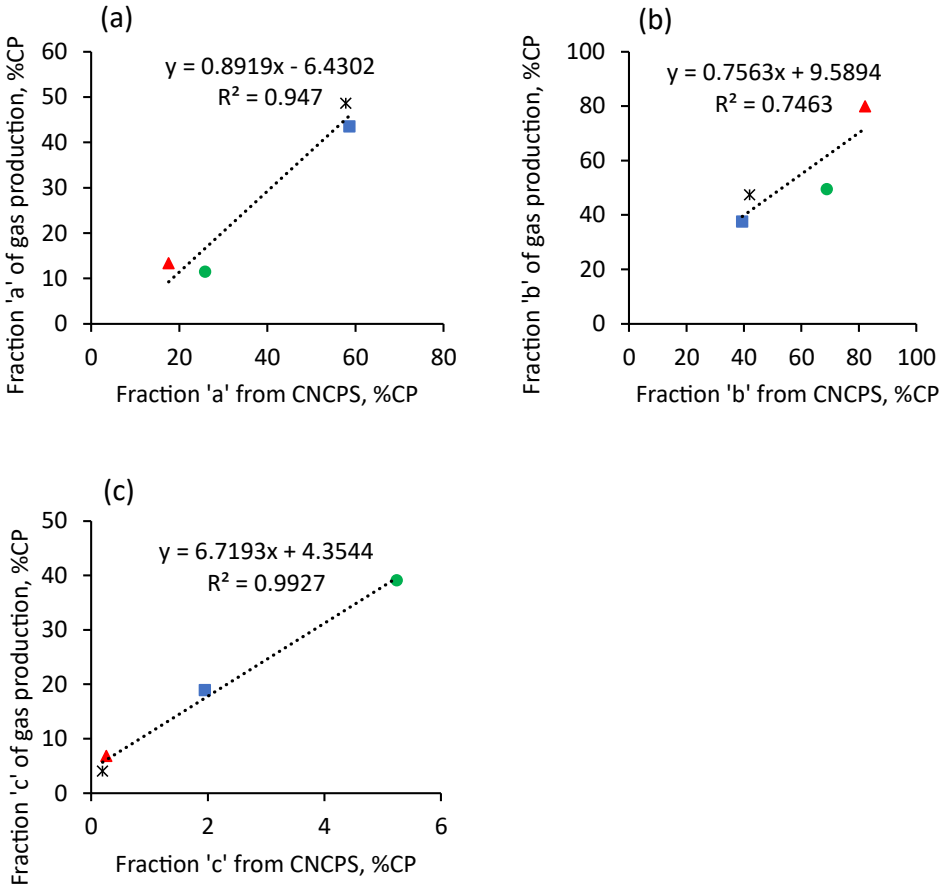

Fig. 2. The linear regression equations, correlation coefficients, and best fit (·····) between the protein fractions 'a' = soluble protein (a), 'b' = degradable protein (b) and 'c' = undegradable protein fraction (c) from the Cornell Net Carbohydrate and Protein System (CNCPS) (X axis) and gas production (Y axis) methods with correlation points for Lupin Meal (■), Vetch grain (✳) *Desmanthus* hay (●) and Soybean meal (▲) samples. The five fractions of the CNCPS method ('a_1', 'a_2', 'b_1', 'b_2' and 'c') were combined to make 'a', 'b' and 'c' fractions (where $a = a_1 + a_2$, $b = b_1 + b_2$) to examine the correlation between similar parameters with the gas production method. The *P*-values for the correlation between protein fractionation methods of ruminant feeds tested were $P < 0.001$.

tion 'b' was lower than the result of these researchers (55.7% DM). The fraction 'a' of lupin meal in the current experiment was lower than fine-sized lupin meal sample (0.8 mm, 74.3% DM) and higher than large-sized lupin meal (4.0 mm, 33.7% DM) fermented using nylon bags in cannulated ewes (Freer and Dove, 1984). Moreover, the fraction 'b' of lupin meal was higher than the fine-sized lupin meal (21.9% DM) and lower than large-sized lupin meal (67.3% DM) reported by Freer and Dove (1984). The change in particle size could increase or decrease the feed surface exposed to the microbial and enzymatic actions, affecting degradability (Goelema et al., 1998; Iommelli et al., 2022). Thus, the differences in feed type and sample preparation could be the source of the variations in the amount of protein fractions.

The findings on *Desmanthus* hay were consistent with the report of Tunkala et al. (2023), who compared three *Desmanthus* species with other five legume hays for their *in vitro* fermentability and protein fractions. The lower gas production and proportion of degradable protein fractions from *Desmanthus* hay resulted from the 4.0–4.6% condensed tannin concentration (Tunkala et al., 2023), and higher amount of ADF and NDF contents. Getachew et al. (2000) demonstrated that the higher concentration of ADF facilitates the formation of a tannin-protein complex, which protects protein digestion. Therefore, the tannin and fibre contents are relevant factors determining *in vitro* protein digestion.

The variations in protein fractions between studies arise mainly from differences in the plant varieties, cultivation environments, and the methods applied for quantification, including the size of feed particles fermented (Bhardwaj and Hamama, 2012; Liebe et al., 2018). The difference between fraction 'a' of CNCPS and gas production methods could be also attributed to the procedural differences used for determination. Moreover, despite the recommendation of Ross et al. (2013), the main difference between the methods is that the higher proportions of fraction 'c' from the gas production and uN_{Ross} assays compared to the CNCPS method could be from a shorter duration of incubation as the gas production curves did not reach a plateau after 16 hours of incubation. The stationary stage of the gas production curve is attained when the degradable substrates are fully depleted, and fermentation of slowly degradable subcomponents is ongoing, which requires prolonged incubation (Groot et al., 1996; Cone et al., 1997). Therefore, extended fermentation periods could change the proportion of fractions for both the fresh and particularly the preserved rumen fluid allowing more time for a microbial breakdown and thus minimizing differences between protein fractionation methods.

Conclusion

All three methods ranked the feeds identically in the proportions of available (degradable or 'a + b') protein fractions as vetch grain, soybean meal, lupin meal, and *Desmanthus* hay in decreasing order. The strong positive correlation between fractions and simi-

lar ranking of feeds indicates that the ANKOM gas production system is suitable for protein fractionation and degradation. However, a comparison of these three methods and two rumen fluid types in a longer duration of incubation may reduce the differences in fraction 'c' and reveal the maximum potential of the methods. The preservation of rumen fluid using D-20 °C negatively affected protein fractionation. Therefore, further studies should be conducted on rumen fluid preservation techniques for *in vitro* protein quantification.

Ethics approval

All procedures were conducted per the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHaMR, 2013). The Department of Jobs, Precincts and Regions Agricultural Research and Extension Animal Ethics Committee approved to conduct this experiment.

Data and model availability statement

None of the data were deposited in an official repository. However, data are available upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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Declaration of interest

The authors declare no conflict of interest.

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