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Methods to study degradation of ruminant feeds

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Ruman degradation is crucial in the supply of dietary nutrients to meet the nutrient demands of the anaerobic microbes and body tissues of ruminant animals. Therefore, it is essential to study the dynamics of rumen degradation of various feeds before their potential use to formulate nutritious diets for ruminant animals. Amongst many methods that have been used in the past, the *in sacco* method has been the most effective method to study rumen degradation. However, this method is undesirable due to its implications for animal welfare and costs. While many *in vitro* methods have been tested as possible alternatives to the *in sacco* method to study rumen degradation of feeds, they were unable to remove the need to use fistulated animals to obtain rumen fluid. Although solubility, enzyme- and faeces-based *in vitro* methods do not require rumen fluid, they still need data from either the *in sacco* method or the rumen fluid-based *in vitro* methods for comparison and validation. Therefore, there is a need to develop *in vitro* methods that do not require the need to surgically modify ruminants to obtain rumen fluid to study rumen degradation. We review the potentials and problems associated with the existing methods to study rumen degradation and their implications for the animal industry in different situations.

Rumen degradation: Animal feed: *In vivo* method: *In sacco* method: *In vitro* method

Introduction

Feeds when ingested by ruminant animals are subjected to microbial degradation in the rumen. The endproducts of the degradation process, i.e. ammonia, amino acids, peptides and volatile fatty acids, are utilised for the synthesis of microbial biomass. The feed escaping rumen degradation, endogenous protein and the microbial biomass entering the duodenum are used to supply energy and protein for the ruminant tissues. Therefore, the nutritional value of a feed depends on its nutrient contents, the extent of rumen degradation and the digestibility of undegraded feed components, especially protein, passing to the small intestine. Ruminants require a dietary supply of protein, sugars, starch and non-structural polysaccharides for the maintenance and synthesis of microbial biomass which is the major protein source needed for their growth and development.

To assess the nutritional status of ruminant animals, rumen degradation of feeds that they receive must be estimated. Here, a good nutritional status is defined as the one where daily nutrient intake matches the daily needs of an animal. For this purpose, a routine method to predict nutrient degradation of feeds in the rumen is needed to formulate animal rations to supply required amounts of ruminally degraded and undegraded nutrients. Therefore, all new feeding systems[1–3] for ruminants emphasise on quantifying the ruminal degradation of feeds more precisely and accurately. However, accurate measurement of degradability is a major problem in the practical implementation of any new feeding system for ruminants. Degradability of various feedstuffs can be determined either from measuring the quantity of nutrients flowing to the duodenum or abomasum of fistulated animals (*in vivo*) or from the measurements of DM or N disappearance from synthetic porous bags suspended in the rumen of fistulated animals (*in sacco* or *in situ*). The *in vivo* method is laborious, expensive, requires large quantities of feed and is largely inappropriate for single feedstuffs, thereby making it unsuitable for routine feed evaluation. The *in vivo* method is also subject to errors associated with the use of digesta flow-rate markers, microbial markers and inherent animal variations[4]. The *in sacco* method, in which the synthetic fibre bags, containing test feeds, are incubated in the rumen for various periods of time has been useful for many years[5,6] to evaluate feedstuffs for DM and N degradation and particle outflow rate. However, the reproducibility among laboratories for this method is poor partly due to the variation in proteolytic activity between animals due to their variable diets and physiological status, etc. Therefore the results obtained for this method may not be equally applicable to all situations unless the method is standardised for a common protocol. As the *in sacco* method requires surgically prepared

Abbreviations: ARF, autoclaved rumen fluid; BMM, Burroughs' mineral mixture; CP, crude protein; ECPD, effective crude protein degradability; NPN, non-protein N; RDP, rumen-degraded protein; UDP, undegradable dietary protein.

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animals, it is undesirable on animal welfare grounds and has associated costs, and so only a limited sample size and numbers can be examined at a time, especially in sheep.

There has been a great interest in developing a cheap and convenient alternative *in vitro* method to obtain estimates for rumen-degraded and -undegraded feeds in order to balance animal rations[7]. These alternatives include solubility in various solvents[8–10] and the gas production technique[11–12], which has been criticised for using the fermentation gas, a nutritional waste product, to evaluate feedstuffs. While gas release is closely related to feed degradation, it does not directly represent the extent of degradation. In fact, feedstuffs with a low rate of gas production may have a higher *in vivo* digestibility than suggested from gas production[11]. This could be due to the added post-rumen activities that only occur during the *in vivo* digestion of a feed. Researchers have also used enzymes for the determination of *in vitro* degradation of feedstuffs[13–15]. However, due to microbial conversions in the rumen, the amount of protein supplied in the diet and its subsequent absorption by the animal (*in vivo*) is less predictable by using commercial enzymes[16]. Consequently, it is not possible to identify any enzyme that will rank the feedstuff in the same order as the *in vivo* method.

The use of rumen fluid for *in vitro* incubation of feeds is well established since its use by Tilley & Terry[17]. However, rumen fluid for such incubations is still usually obtained from rumen-fistulated animals, which is undesirable as described above. Therefore, alternative methods are required for the estimation of rumen degradation of various feeds without involving surgically prepared animals. The present review examines the importance of studying the mechanisms of rumen degradation by involving various methods and their suitability and implications for the systems of feeding ruminants. The present review also explores the potentials and problems that may exist in the development of alternative *in vitro* methods and their ultimate application for the feed and animal industry.

Importance of the reticulo-rumen in degradation of feeds

The rumen (sometimes regarded as the reticulo-rumen) holds about 80 % of the total digesta[18]. It contains micro-organisms that are particularly effective in fibre digestion and so enables ruminants to survive under poor-quality nutritional conditions. The rumen is considered as a continuous anaerobic fermenter that is maintained at constant temperature. Saliva enters continuously and provides appreciable buffering through its contents of phosphate and bicarbonates. The feed is fermented by the action of micro-organisms to yield mainly the volatile fatty acids, carbon dioxide (CO_2) and methane (CH_4). The major energy source of the host animal is provided by volatile fatty acids, while its amino acid supply is derived from the breakdown of microbial and undegraded feed protein passing into the small intestine.

Numerous bacteria and protozoa are present in the rumen (about 1 billion bacteria and 1 million protozoa per ml rumen fluid). Endproducts of fermentation are absorbed continuously, which help to maintain an environment conducive for the microbial growth in the rumen. McDonald *et al.* [19] reported over sixty species of bacteria which are mostly non-spore-forming anaerobes. Protozoa are present in small numbers but, being larger in size than bacteria, they are almost equal in total mass to bacteria. Rumen fungi have also been reported to play an important role in the degradation of ruminant feeds within the rumen ecosystems[20–22]. The ruminant animals can derive various nutrients from ingested feeds with the help of these micro-organisms. However, their ability to degrade proteins and other nutrients to obtain N and energy supply to maintain rumen function and tissue growth is particularly important and so the protein degradation receives special attention in the following sections.

Rumen degradation and nutrient requirements

The nutrient requirements of ruminants are mostly dependent on their stages of growth and production levels. As with non-ruminants, the animal must absorb essential amino acids from the small intestine. However, ruminal micro-organisms are capable of synthesising most of the common amino acids. Therefore, ruminants have the unique ability of their micro-organisms to convert non-protein N (NPN) in association with energy supply to protein, and so these animals can survive by maintaining themselves while consuming only NPN-based diets. Nonetheless, microbial protein synthesis alone is not adequate to maintain the high levels of production of modern ruminants[23]. Therefore, some feed protein that can escape ruminal degradation is required in ruminant diets. This protein is classed as 'undegradable dietary protein' (UDP) in some feeding systems[1] when formulating ruminant diets. The protein that is degraded in the rumen is classed as 'rumen-degraded protein' (RDP). RDP is composed of both quickly degradable protein and slowly degradable protein.

UDP has been demonstrated to be required for maximal growth of beef steers[24]. The amount of amino acid absorbed in the small intestine was greater for cattle fed diets containing a greater portion of UDP relative to RDP. In these diets the rumen ammonia levels were lower for the high-UDP diets. In addition, infusion of amino acid directly to the small intestine also increased DM intake and production, thus demonstrating the importance of UDP entering the small intestine[23,25].

Although provision of dietary protein that escapes ruminal degradation is important for optimising production of high-producing ruminants, feeding to increase microbial protein may also increase the flow of essential amino acids to the small intestine. Microbial growth requires an adequate supply of carbohydrates that are fermented in the rumen, and a rumen environment conducive to microbial growth. Microbial growth may be limited if carbohydrate degradation is too low to supply the energy for microbial activity. Alternatively, on high-starch diets, the pH of the rumen fluid will decrease which will lower microbial growth[26]. The interactions of these factors result in a limit to the maximal amount of protein synthesis that can occur in the rumen.

Microbial protein synthesis is mostly limited by the amount and form of N available to rumen organisms[27].

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Inadequate RDP supply may result in a decrease in protein entering the small intestine due to a depression of microbial protein synthesis. If inadequate N is available to rumen microbes, fibre digestion may also be reduced[28]. When microbes are not available to digest fibre in forages, fibre has a greater filling effect, and hence feed intake can be reduced[29]. Therefore, while it is important to supply an adequate amount of UDP to help meet the needs of the host animal, there is also a requirement for RDP to maintain microbial growth to synthesise microbial protein, degrade dietary fibre, and promote rumen function.

While most of the nutrient needs of rumen micro-organisms are met with ammonia and carbohydrates, microbial growth is maximised by the inclusion of amino acids or peptides in the rumen[30]. *In vitro* studies involving incubation of rumen fluid with amino acid mixtures showed that maximal microbial growth was obtained by supplementation of urea with a mixture of leucine, methionine and histidine[31]. Addition of other amino acids did not result in further increases in microbial growth. Ruminal infusion of casein or supplementation with soyabean meal increased microbial protein synthesis in the rumen above that observed with the urea infusion[26]. Supplementation with soyabean meal or fishmeal gave greater fibre digestion than supplementation with urea[28]. This suggests that amino acid-containing protein meals can improve cellulolytic activity of rumen microbes more that the NPN sources alone.

Accurate formulation of ruminant diets requires attention to the protein and energy interactions. As mentioned earlier, the amount of microbial protein synthesis depends partly on the ruminally available carbohydrate to support microbial growth. Therefore, the amount of RDP required in a ration depends on the level of ruminally available carbohydrate. A study showed faster disappearance of soyabean meal and sunflower-seed meal from nylon bags suspended in the rumen when sheep were fed dried grass instead of a barley-based diet with little fibre[32]. This suggests that nutrient degradation may be affected by the type of carbohydrate and fibre in the ration.

If N available to micro-organisms limits microbial growth, then there may be an advantage in increasing RDP in the rumen. However, once the microbial requirements for RDP have been met, there is no additive effect of additional rumen N[33]. The excess RDP is converted to ammonia in the rumen and diffuses to the blood. In the same way, if the UDP requirement has been met, and no amino acids are limiting, there is no advantage in further increasing the amount of UDP in the diet beyond the effect of providing additional energy. In fact, Chaudhry[34] did not find any positive effect of adding casein, urea and xylose on the *in vitro* digestibility of barley straw when incubated with the rumen fluid from well-nourished sheep. Understanding these possible effects and interactions of rumen protein availability makes it easier to understand why studies often fail to show the positive and combined effects of balancing rations for UDP and RDP. The requirements for each type of protein may depend on other feeds in the diet, and an effect will not be seen unless the level of UDP or RDP (quickly degradable plus slowly degradable proteins) is limiting production.

The only way to be certain to have met the requirements for UDP and RDP when balancing a ration is to supply these protein fractions in excess. However, there are several reasons why feeding excess protein to high-producing ruminants is undesirable, especially due to the higher cost of protein-containing ingredients. Therefore, optimisation of the level of protein ingredients in ruminant diets is important to the profitability of farms. Otherwise, excess dietary protein results in high levels of blood urea and ammonia, which could be toxic to the tissues and may result in an impaired reproductive performance[35], and increased nitrogenous wastes which could harm the natural water supply and the environment[36]. Therefore, excess dietary protein for ruminants is not adequate, so a better understanding of the amounts of different types of nutrients that are required in rations for their utilisation in the rumen is needed. This can be achieved by involving studies that partition dietary proteins into rumen degradable and non-degradable fractions. Researchers have used various methods in the past to study rumen degradation of animal feeds and the following sections summarise the advantages and disadvantages of some of these methods.

Methods for estimating rumen degradation of feeds

Currently, numerous methods involving different procedures are available for estimating degradation of feeds. For example, the *in vivo* methods involve markers and the *in sacco* method requires animals that are surgically modified with rumen cannulae. The *in vitro* methods require the use of rumen fluid, which is obtained from fistulated animals, to estimate either digestibility[17] or gas production[11,12]. There are other *in vitro* methods that involve proteolytic enzymes which are either commercially extracted from non-rumen sources (for example, ficin from fig latex or sap or protease from *Streptomyces griseus*) or extracted from mixed rumen micro-organisms[37,38]. A range of *in vivo*, *in sacco* and *in vitro* methods that are being used are therefore described in the following sections.

In vivo *methods involving internal and external markers*

In vivo methods are the most logical to evaluate degradation of feeds in the rumen. The protocols require animals fitted with cannulae in the reticulo-rumen, the abomasum, or proximal duodenum[4,39]. Also they require suitable methods for determining digesta flow rates and for differentiating microbial protein from dietary protein in the digesta that flows to the small intestine. These procedures are labour intensive and require considerable investment. In addition, increasing concern for animal welfare limits the applicability of such methods. For these reasons, only a few animals can be used in *in vivo* experiments, which could be unreliable due to the large variation observed among animals[4]. The *in vivo* procedure also relies on the accurate estimation of the flow of microbial protein to differentiate it from the feed protein reaching the duodenum. For this purpose several microbial markers have been used. These may be classified as internal markers that are inherently present in micro-organisms and include diaminopimelic acid, aminoethylphosphonic acid

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and nucleic acids (DNA or RNA) or external markers (that are added to the rumen to label the micro-organisms) including ^{35}S, ^{15}N, ^{14}C, ^{3}H and ^{32}P, etc. Despite the use of several markers, there is no single ideal marker to estimate the ruminal microbial protein yield[40].

There is a need for a more practical, repeatable and cheaper method for measuring ruminal degradation of various feeds, particularly for commercial laboratories which do not have access to fistulated animals. In spite of the limitations with the *in vivo* procedure, a routine alternative procedure would only be acceptable following validation against *in vivo* measurements. Different investigators have used several alternative procedures but without reaching a consensus on the suitability of a unified approach.

In sacco *method to estimate feed degradation*

The *in sacco* technique was first suggested by Quin *et al.* [41] and it has since been used by others to estimate utilisation of either forages[42] or concentrates and high-protein feeds[5]. Interest in the technique has intensified since Mehrez & Ørskov[5] critically assessed the factors causing variability in DM and N degradability. They concluded that as long as the bags were large enough to allow free movement of substrate within, the technique could be extremely useful as a rapid guide to determine nutrient disappearance, particularly the rate and extent of nutrient disappearance from the rumen. All modern systems of feeding ruminants[1,2,43] require an estimation of the amount of feed protein escaping ruminal degradation. This estimation is obtained by the *in sacco* technique, which is probably the best-known simple and reliable method to assess the degradability of DM and protein in the rumen[1–3,5,43].

The *in sacco* method requires the use of fistulated animals, which limits its routine use by the commercial laboratories. However, it is widely applied by researchers since it requires fewer measurements, is relatively less labour intensive and so is cheaper as compared with the *in vivo* method. The *in sacco* method involves the sealing of feed samples within nylon, polyester or Dacron bags, which are then suspended in the rumen of sheep or cattle for varying periods of time, followed by determination of the DM and protein in the washed residues. The technique allows the test feed to be incubated in the ruminal environment (i.e. pH, temperature and CO_2), but unlike the normal situation the feed is not subjected to mastication and rumination. Despite its widespread use, the technique has inherent errors that must be taken into account, particularly if comparisons of degradation among different laboratories are to be made. Table 1[44–47] shows possible sources of variations in the use of the *in sacco* method among different laboratories in terms of bag size, sample size, particle size and time (h) of incubation used by different authors. The assumption that the N leaving the bag during washing in water, at 0 h of incubation, is completely degraded may not be true[10,48]. Extensive loss of feed material at 0 time will lead to an overestimation of degradability. Although Table 1 and Table 2[15,49–52] present information in relation to degradable crude protein values only, it is assumed that

Table 1. Some of the variations in the protocols among selected laboratories to obtain *in sacco* data from fistulated sheep

Reference	Dimensions of the bag (cm)	Pore size of the bag (μm)	Weight of incubated feed (g)	Maximum time of incubation (h)	Sheep diets	Tested feeds	DCP (%)	Temperature (°C) and time used for washing
Cottrill & Evans[44]	17 × 9	NA	5	24	*Ad libitum* dried grass	Barley	80·1	30 min
						Soyabean meal	81·5	
						Fishmeal	50·7	
						Maize gluten	33·0	
						00 Rapeseed meal	70·3	
Fonseca *et al.*[45]	6 × 8·5	NA	2	144	*Ad libitum*; 42% rice straw, 42% wheat straw, 16% treated cotton seed, and soyabean as supplement	Rice straw	44–60	Machine wash 40°C for 40 min
						Meadow hay		
						Rye grass		
Rymer & Givens[46]	NA	43	5	72	Hay:maize ratios: 80:20, 50:50 and 20:80	Chopped hay	46, 47 and 30	45 min
						Unground maize	69, 75 and 74	
Verbic *et al.*[47]	10 × 7·5	45–55	3	72	*Ad libitum* direct cut silage and hay	Direct cut silage	67·7	Machine wash for 20 min
						Hay	85·5	

DCP, degradable crude protein; NA, not available.

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Table 2. Some of the variations in the protocols being used by different laboratories to obtain *in sacco* data using fistulated cattle

Reference	Bag dimension (cm)	Bag pore size (μm)	Weight of incubated feed (g)	Maximum incubation time (h)	Cattle diets	Tested feeds	DCP (%)	Washing temperature (°C) and time
Chaudhry[15]	20 × 10	40–50	10	24	Hay + concentrate	Field beans	83	Machine wash for 15 min
						Maize gluten meal	23	
						Distiller's dark grains	74	
						Sunflower-seed meal	85	
De Smet *et al.*[49]	8 × 8	50	2	48	*Ad libitum* 55% maize silage and 45% compound feed	Barley	55·5	Machine wash for 10 min and rinsed 10 min
						Wheat	73·9	
						Maize	52·0	
						Sorghum	20·5	
						Sugarbeet pulp	39·6	
						Soyabeans	34·0	
Djouvinov *et al.*[50]	9 × 14	16	3	24 (C) 72 (S)	Two equal portions/d 4 kg lucerne hay, 4 kg meadow hay and 1 kg barley	Barley	49·4	Machine wash for 10 min
						Grass	71·6	
						Wheat bran	71·3	
						Brewer's grain	48·2	
						Soyabean meal	76·5	
						Sunflower meal	70·2	
						Fishmeal	28·4	
Kristensen *et al.*[51]	7·5 × 10	36	1	48	*Ad libitum* hay	Soyabean meal	73	Washed manually under tap water 40°C
						Cotton-seed meal	73	
						Fishmeal	50	
Noziere & Michalet-Doreau[52]	5 × 9 cm	53	3	23	7 kg DM/d 57% grass hay, 12% wheat straw, 31% barley pellets	Two grass hays:		Washed manually in salt solution
						Regrowth	55–60	
						Late harvested	35–45	

DCP, degradable crude protein; C, concentrate; S, silage.

similar variations in practice will also cause variations in DM and organic matter degradability values.

Beside microbial contamination within the bag, there are numerous other sources of errors that affect the *in sacco* DM and N disappearance from feeds. The importance of sample weight in a given bag size has been emphasised by Bullis *et al.* [53] who observed reduced DM digestibility with increased weight in the bag. This finding agreed with the finding of Van Keuren & Heineman[42] who showed that sample weight influenced DM digestibility, at least when short incubation times were used; the difference tended to disappear with longer periods of incubation. Also, oven drying of silage samples at high temperatures was found to reduce N degradability and solubility[54] of these samples. Additionally, Noziere & Michalet-Doreau[55] reported that grinding and pre-wetting underestimates degradation rates due to the increased microbial colonisation. Machine washing of residues overestimates solubles and particulate losses but it is less subjective than hand washing[56]. Huntington & Givens[57] reported that bag pore size less than 15 μm can reduce degradation by restricting microbial colonisation and diversity and trapping fermentation gases. However, bag pore size of more than 40 μm can cause losses of solubles and undegradable particles. Furthermore, the animal effects and bag incubation sequence also contribute to the variation in results among laboratories[58].

The disappearance of DM is also affected by the diet fed to the host animal[59]. While these effects make it difficult to compare feeds for degradation across studies, DM or protein degradation of a feed is not entirely a function of the feed, but also affected by the ruminal conditions, so variation across studies is expected. More troublesome aspects of the *in sacco* method do exist. The pH inside the nylon bag has been shown to be lower than that outside the bags, especially when small pore-sized bags were used[60]. The microbial population inside the bags also differed, both in composition and concentration, from that of the outside of the bag. For example, both protozoa and bacterial populations were found to be lower inside the bags[60–61]. This could be due to the limited micro-environment that existed within the bag involving a single ingredient of smaller size with limited exposure to rumen microbes perhaps due to the bag size and its pores.

Analysis of digesta from nylon bags incubated *in vitro* showed that some nutrients escaped the nylon bags before being digested[62]. The microbial attachment to feeds incubated *in sacco* is frequently not measured, though several studies have shown high levels of contamination of incubated feed with rumen microbes[57]. All these sources of errors increase variability of predictions of degradability among laboratories. In spite of being widely used and standardised, the application of this methodology needs to address two points: first, the fraction assumed to be completely degraded, and the DM and N disappearance during this step could simply be due to DM or N washed out of the bag; second, the microbial contamination of feeds within the bags. The first point would overestimate degradation and the second point would underestimate it[63]. The significance of these two factors would be important depending on the type of feed being analysed. However, the *in sacco* method is still the reference method in most countries; the reason is probably that the degradability is measured in the rumen and, therefore, from a biological point of view it is more reliable than those of the *in vitro* methods[64]. But as *in vivo* and *in sacco* methods require fistulated animals they cannot be accepted as methods for routine screening of feedstuffs. Therefore, there is a need for a viable and accurate *in vitro* method to estimate the degradability of feeds in the rumen.

Tables 1 and 2 indicate some of the variations of the most commonly tested methods using fistulated sheep or cattle, i.e. the same feeds give different values in different laboratories. For example, Cottrill & Evans[44] reported effective crude protein (CP) degradability (ECPD $= a + (b \times c/c + k)$, where c is degradation rate of b and k is rumen outflow rate) in fistulated sheep of 51 and 82 % for fishmeal and soyabean respectively. In an unrelated study, Djouvinov *et al.* [50] used fistulated cattle and reported ECPD of 28 and 77 % for fishmeal and soyabean respectively. In contrast, Kristensen *et al.* [51] used fistulated cattle and reported ECPD of 50 and 73 % respectively for different samples of the same feeds. Tables 1 and 2 show that different amount of feeds were incubated using various dimensions of bags in each of the above three studies. Additionally, Cottrill & Evans[44] reported ECPD of 33 % for maize using fistulated sheep compared with 52 % reported by De Smet *et al.* [49] for another sample of maize using fistulated cattle. On the other hand, variation was also observed in ECPD for cattle where variable ECPD of 28 *v.* 50 % for fishmeal were reported by different authors[50–51]. Similar variations for the ECPD of 34 *v.* 77 % for soyabean meal were also reported[49–50]. This clearly shows the inconsistency between laboratories which may be because different diets offered to the same host animal have different effects on degradability of the same feed. Additionally, different time (h) of incubation, different sample size and different dimension of *in sacco* bags gave different degradability values for the same feeds. Nevertheless, Table 3[65] presents variation in the *in sacco* ECPD results for the same feed in the same laboratory. Madsen & Hvelplund[65] reported different values for feeds using fistulated cattle. In their study they tested a minimum of three samples for the same feed obtained from different sources and the ECPD was calculated as the average of all values obtained. Table 3 shows the variations that could occur in the *in sacco* method for the same feed, although feeds were

Table 3. Variation in the *in sacco* data for the same feed within the same laboratory[65]

Feeds	CP in DM (%)	ECPD (%)
Barley	12·9	74
Barley	12·1	60
Fishmeal	75	53
Fishmeal	77	22
Peas	22·9	80
Peas	23·2	73
Cotton seed	44·2	62
Cotton seed	44·3	39
Soyabeans	40·5	81
Soyabeans	35·3	66
Maize	9·8	33
Maize	9·8	26

CP, crude protein; ECPD, effective crude protein degradability.

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selected on the basis of their almost similar CP contents in DM. These variations could partly be attributed to the differences in variety, agronomic conditions and processing methods to obtain these feeds. Such variations in the *in sacco* estimates raise the question: when formulating a ration for ruminants, which of the reported values should be followed? This question necessitates the need to either standardise the *in sacco* method by following a standard protocol with minimum sources of variations or more preferably develop alternative methods which are more consistent and which do not require the use of surgically modified animals to estimate degradability of ruminant feeds.

In vitro *methods to estimate nutrient degradation*

Numerous *in vitro* methods have been used in the past as alternatives to the *in sacco* method. These methods involve buffers, chemical solvents, rumen fluid and enzymes that are either commercially available or extracted from rumen contents. Another approach is to use gas production as an indirect measure of *in vitro* digestion. *In vitro* techniques are considered less expensive than the *in vivo* and *in sacco* methods, and they offer the possibility of analysing both the residue and the metabolites of microbial degradation. In contrast, more physiological techniques are complicated by the fluxes out of the rumen or out of the incubation bags. *In vitro* methods may ultimately allow for the control of various factors that alter the feed degradation (microbial, animal, environment) and, therefore, allow for the uniform characterisation of feeds for DM and protein degradation.

Tilley & Terry[17] developed an *in vitro* method to estimate the apparent DM digestibility of feeds for ruminants in the laboratory. The method has two stages. In the first stage, a feed sample is incubated at 38°C in rumen fluid, which is diluted with a buffer solution resembling saliva and saturated with carbon dioxide. After 48 h, the incubation is stopped and the incubation mixture filtered. The feed residues are subsequently incubated for another 48 h with pepsin-HCl. The main disadvantage of the method is that rumen fluid is required, which is obtained from fistulated animals, and may not be available in all laboratories. Since the use of fistulated animals is not desirable, the need for alternative *in vitro* methods has arisen. There are many difficulties that are associated with the *in vitro* fermentation studies. These include the requirements to standardise the fermentation process, measurement of fermentation profiles and the access to fistulated ruminants to obtain rumen inocula. Therefore, several methods have been developed to measure nutrient degradation by using various enzyme preparations involving cellulases, proteases, lipases and amylases individually or as mixtures. Ruminal protease was enriched from *Bacteroides amylophilus* and used for studies of degradation of several protein sources[43]. Alternatively, other methods have used commercially available proteases. Some of these *in vitro* methods are discussed in the following sections.

In vitro *methods involving solubility in solvents and buffers*

Several researchers have attempted to characterise feed nutrients according to their solubility in aqueous solutions such as saline and buffers, autoclaved rumen fluid (ARF) and water (cold, hot or distilled)[8–10,48,66–71]. However, most of the available reported literature on feed solubility focuses on protein solubility. N solubility varies greatly for different feedstuffs. For example, while N of brewer's grains was only 3 % soluble in borate–phosphate buffer, oat N was 55 % soluble[69]. Buffer-soluble N is comprised mostly of NPN, such as ammonia, urea, nitrates, amino acids and small peptides[66–69]. Nucleic acid N is the major NPN fraction that is not soluble in neutral buffer, but generally it is low in quantity and it is underestimated by N analysis using the Kjeldahl procedure[72]. In addition to NPN, some true protein is soluble to varying degrees among feeds.

Protein solubility is influenced by various factors associated with the solvent or extraction procedure. Changes in chemical composition of solvent can have pronounced effects on N solubility. For example, substitution of ammonium chloride with sodium chloride in Burroughs' mineral mixture (BMM) resulted in increased N solubility for several concentrates[70]. It is difficult to draw meaningful conclusions as to the effect of solvent composition on N solubilisation, because of the interaction that may exist among feedstuffs, methodologies and solvents. For example, in one study, N solubility was found to be higher in BMM or 0·15 M-sodium chloride than in ARF[67], whereas some researchers have reported the opposite response[73] and others found insignificant difference among three solvents[74]. It is noteworthy that similar concentrate feeds were analysed in each of the above studies.

Comparisons of ARF, NaCl, BMM and hot water revealed that the differences among feeds were most pronounced for ARF, suggesting that this method may best separate different feeds according to protein solubility[73]. However, while homogeneous variance within samples was detected in the above experiment, the use of ARF may not be repeatable from laboratory to laboratory, or from time to time, as ARF composition can very depending upon the diet and other biological differences between donor animals.

The pH of a solvent has been shown to influence N solubility of concentrate feed protein[9,73]. About 85 % of soyabean meal protein was soluble in aqueous HCl (pH 2·0) and water (pH 7·2), while less than 10 % was soluble at pH 4[73]. Significant differences in solubility of isolated soyabean protein and casein were also observed when pH of ARF and BMM varied from 5·5 to pH 6·5[9]. However, the effect of pH on protein solubility often is measured in neutral pH solutions, even though rumen fluid is slightly acidic when donor animals consume high-grain-based diets. As sodium chloride has almost no buffering capacity and bicarbonate–phosphate buffer has an unstable pH, a borate–phosphate buffer has been suggested as an appropriate substitute to measure N solubility without variation due to fluctuation in pH[75].

Buffer-soluble CP appears to be more readily degraded in the rumen than insoluble N. There was a close association between buffer-soluble CP and N disappearance from synthetic fibre bags suspended in the rumen, especially when feeds were incubated *in sacco* for short periods of 1 h[57,71]. In addition, the amount of buffer-soluble N in a feed was correlated strongly to increases in rumen ammonia

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concentration after feeding[76]. However, other researchers found no consistent relationship between N solubility in various aqueous solvents and ammonia concentration[68]. N that is available to microbes is converted to ammonia which is used for microbial protein synthesis which is considered as true protein due to its high amino acid profile. Soluble NPN is utilised by rumen bacteria and solubility in mineral buffer of pure proteins was correlated strongly to degradation rate in rumen fluid[77].

Some researchers have reported a poor correlation between N solubility in buffer and *in vivo* protein degradation across several feeds[71]. This result was not surprising given that the amount of soluble feed protein appears to have no relationship with degradation rate of the remaining insoluble protein fraction of a given feed[48,70]. Therefore, one can conclude that estimation of N solubility in neutral buffers may predict the amount of N immediately available to bacteria, but it may not be accurate for all feeds as discussed above and N solubility in aqueous solutions is not sufficient to determine protein degradation rate or protein degradability. However, as suggested by Chaudhry & Webster[10], positive correlations between some soluble protein fractions and degradation of feeds do require further investigations to test this approach for its much wider application.

In vitro *methods using enzymes*

Rumen digestion is mainly linked to the cellulolytic activity of the microbial flora, which represents its specificity and advantage to utilise cellulose-rich feeds. Researchers have used commercially available cellulolytic enzymes, often extracted from fungi, to reproduce this activity; hence many enzymic methods have been proposed for their use to predict feed digestibility. These methods differ on the basis of the nature and level of the enzymes and the target feeds and whether a pre-treatment (chemical or enzymic) is necessary or not[78]. These methods are widely used for forages, and have been applied to by-products, concentrates and mixed feeds produced by the agro-food industry. For various types of forages, prediction involving enzymes is higher, perhaps due to the enzyme specificity and greater activity than the chemical methods and comparable with that obtained *in vitro*[79]. In addition, cellulase methods can be used for mixed rations and permanent pastures. With forages containing tannins, organic matter digestibility prediction is generally poor when cellulolytic enzymes are used. This can be due to the fact that enzymes are used at pH values that are different from those prevailing in the rumen, enabling possible release of tannins and their subsequent linkage to protein. Another possible explanation is that some tannins might have inhibited the enzyme activity, especially on cellulose. Malestein *et al.*[80] showed clear differences in starch degradation upon incubation with α-amylase or rumen fluid. However, Cone & Vlot[81] concluded that it was not possible to accurately predict the rate of starch degradation by rumen fluid as with enzymic degradation. Addition of non-amylolytic enzymes, such as cellulase, protease, lipase, xylanase and pectinase, did not enhance starch degradation. Starch granules contain components other than amylose and amylopectin and possibly need non-amylolytic enzymes for full degradation. These enzymes may be found in the rumen micro-organisms.

Indeed, the procedures involving the use of commercial proteases offer potential advantages over other techniques, particularly in terms of the labour and speed of operation. Proteases from different origins have been tested by several researchers to estimate ruminal protein degradation; however, the most commonly used is the one obtained from *S. griseus* as reported by Chaudhry[14,15] and Krishnamoorthy *et al.*[69]. Table 4[9,13–15,69,82–87] gives the list of enzymes studied in some recent publications on the topic.

CP degradation using five different commercially available proteases was compared with that of the *in sacco* method for several concentrate feeds[13]. The solubility of CP was determined by filtering after separate incubation with *S. griseus* protease, papain, bromelain, ficin and *Aspergillus oryzae* protease. Though absolute degradation with protease was different from that observed *in sacco*, all enzymic degradations were significantly correlated to the *in sacco* data, supporting the possible use of enzymes to detect relative differences among feeds for degradation. *S. griseus* protease has become popular for prediction of protein degradation of feeds but the optimal pH for the

Table 4. Summary of the selected commercial enzymes used by different authors to estimate *in vitro* degradation of feeds

Enzyme used	pH	Time (h)	References
Protease (*Streptomyces griseus*)	8·0	1	Krishnamoorthy *et al.*[69]
	6·7	18 (C) and 48 (F)	Roe *et al.*[82]
	8·0	18 (C) and 48 (F)	Licitra *et al.*[83]
	7·5	48 (F)	Wohlt *et al.*[9]
	6·7	18 (C); 30 and 48 (F)	Licitra *et al.*[84,85]
	6·8	0 to 30	Chaudhry[14,15]
	7·4	26	Kopecny *et al.*[86]
	5–7	1, 4, 8 and 24	Poos-Floyd *et al.*[13]
	8·0	1, 6, 24 and 70	Cone *et al.*[87]
Ficin (*Ficus glabrata*)	5–7	1, 4, 8 and 24	Poos-Floyd *et al.*[13]
Papain (*Corica papaya*)	6·8	0 to 30	Chaudhry[14]
	5–7	1, 4, 8 and 24	Poos-Floyd *et al.*[13]
Bromelain (*Ananas comosus*)	5–7	1, 4, 8 and 24	Poos-Floyd *et al.*[13]
Protease (*Aspergillus oryzae*)	5–7	1, 4, 8 and 24	Poos-Floyd *et al.*[13]

C, concentrate; F, forage.

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enzyme is 8, which is greater than the rumen pH. However, it has been used at both the rumen pH with either reduced activity[88,89] or reasonable response[14,15] or higher or optimum activity at the higher pH[69].

Protease extracted from *S. griseus* was used because its activity was comparable with the protease of *B. amylophilus*. Blackburn[90] selected a pure culture of *B. amylphilus* strain H18 to study the nature of ruminal microbial proteases. This micro-organism was selected due to its ability to hydrolyse casein rapidly, its simple nutrient requirements and its utilisation of ammonia in preference to preformed amino acids and peptides. All these characteristics simplify the measurement of the endproducts of protein degradation[69].

In the *in vitro* method as proposed by Krishnamoorthy *et al.* [69], 6·6 enzyme units (IU) of *S. griseus* protease were used per g of sample DM to break down the peptide bonds of the feedstuffs. The sample (0·5 g) was incubated in 40 ml of a borate–phosphate buffer (pH 8·0) for 1 h, then 10 ml protease solution (0·33 IU/ml) were added. Roe *et al.* [82] observed a fixed ratio of enzyme:CP and pH of phosphate buffer solution of 6·7 more effective for studying the enzyme degradation of feeds. These researchers assumed that all the protein that remained insoluble after an incubation of 18 h for concentrate feed and 48 h for forages was potentially rumen undegradable. The procedure then consisted of incubation of the test feed for the appropriate time and then filtration of the whole contents through a filter paper. After washing the residues with distilled water, the N in the residue was estimated by the Kjeldahl method. Degradability of the feedstuffs was then calculated as percentage of the total CP as follows:

$$\text{Degraded (g/kg)} = \frac{\text{CP in feed} - \text{CP in feed}}{\text{CP in feed}} \times 1000.$$

A later modification of this method was proposed by Licitra *et al.* [85] who used a fixed ratio of enzyme:true protein as determined by tungstic acid precipitation. The authors found a significant effect on the estimate of degradable N when compared with the original procedure. They also reported a significantly higher degradation when the buffer solution had a pH of 8 instead of 6·7. This finding differed from that of Krishnamoorthy *et al.* [69] where optimum pH for enzyme activity was 8. In fact, Licitra *et al.* [84] suggested that the pH of the buffer solution should be similar to rumen conditions despite the pH of 8 required for the optimum enzyme activity.

A drawback in relying on the proteolytic activity of just one specific bacterium, as opposed to a group of micro-organisms, is that the whole range of activities towards the different nitrogenous substrates found in the rumen may not be present in a single enzyme. Russell[91] showed that not all species of bacteria can degrade and utilise nitrogenous substrate to the same extent. This suggests that the use of a single enzyme, even if extracted from a rumen micro-organism, may not be appropriate for an accurate estimation of the total proteolytic activity of the whole rumen fluid. Luchini *et al.* [92] compared the activity of a mixture of trypsin, carboxypeptidase B, chymotrypsin and carboxypeptidase A with that of the strained rumen fluid in incubations with fifteen feeds. Degradation rates using strained rumen fluid ranged from 0.008 to 0.250/h. However, results using the enzyme mixture as the inoculum source, detected no differences in degradation rates among feeds. This indicates that the commercial enzymes employed did not mimic the activity of the strained ruminal fluid.

The use of commercial enzymes has not provided consistent results. Theoretically, there may be a problem that only one of the many microbial sources of proteases that exist in the rumen is used to prepare a purified enzyme. More serious, however, is the possibility that the protease activity present in the purified enzyme does not exist in the rumen. A comparison of protein degradation by crude extract from the rumen and by *S. griseus* protease showed variations in degradation for several feeds[37]. Furthermore, some researchers have questioned the use of any commercial protease for the estimation of ruminal protein degradation due to differences in specificity and mode of action of proteases[86]. However, Chaudhry[14,15] has supported the use of *S. griceus* due to its ability to predict protein degradation of both purified substrates or commonly used feeds. In fact, the enzyme-based estimates for these feeds were reasonably compared with their *in sacco* counterparts. Therefore, this enzyme deserves further attention in standardising and validating its use to estimate rumen degradation of feeds.

In vitro *methods involving gas production*

A number of reports have described adaptations of the first stage of the *in vitro* digestibility method of Tilley & Terry[17] to permit the measurement of the volume of gas produced by fermenting feedstuffs[11,12,93–100]. The procedure used for gas collection and measurement ranges from the use of calibrated syringes[11] and pressure transducers[94] to computerised monitoring[96]. Rumen fluid obtained from fistulated cattle or sheep was used for fermentation of substrates. The rumen fluid had been diluted in either bicarbonate or phosphate or bicarbonate–phosphate buffers or in a modified medium[93] which was a complex buffer containing micro-minerals, cysteine, resazurin as well as both phosphate and bicarbonate mineral mixtures. According to Pell & Schofield[96], gas is produced from metabolic energy sources, and they measured the potential of different sources (monosaccharides, polysaccharides, pectin, starch, cellulose and hemicellulose) for conversion to CO_2 and CH_4. Gas produced has been reported to be primarily from the fermentation of digestible carbohydrates by the activity of rumen microbes. However, differences were found between researchers who suggested that gas production was also affected by other factors such as the nature of the buffer, and source and/or handling of the fermenting micro-organisms[94,100–107]. This aspect requires further attention when such *in vitro* studies are involved to estimate degradation of ruminant feeds.

The advantage of the gas production systems is that they can be automated, thus reducing the labour input. However, automated gas production methods are expensive and may not handle large numbers of samples. While manual methods are considered cheap, they are labour intensive and restricted in capacity. The results obtained from automated

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or manual systems are dependent on several procedural details. Table 5[94,96–102] shows the effect of several factors on gas production. In addition to these factors, the results varied with the type of system and the source, activity and consistency of the rumen fluid used[69].

In vitro *method involving faeces*

It is well established that certain amounts of cellulose and hemicellulose are fermented in the large intestine, because many bacterial species in the rumen are also represented in the hind gut from where bacterial residues are subsequently passed in the faeces[103]. Therefore, the suspension of sheep faeces in buffer might be capable of acting as an inoculum for the initial fermentation of feed samples, in place of rumen liquor. *In vitro* digestibility determined by using sheep faeces as an inoculum correlated well with the *in vivo* digestibility[104]. These researchers demonstrated the potential of using micro-organisms from faeces instead of rumen fluid in the two-stage procedure of Tilley & Terry[17]. Akhter *et al.*[105] showed potential for cattle faeces to be used as an alternative to rumen liquor that is collected from rumen-fistulated sheep for use in the *in vitro* digestibility assay of forages. When a 48 h acid pepsin digestion, the second stage of the Tilley & Terry technique[17], was included, the organic matter digestibility values and the ease of filtration of undigested residues were increased. They also investigated the accuracy of estimating the organic matter digestibility of eight forages determined by using rumen liquor from three sheep (y) and faeces from two cows (x). All regressions between sheep rumen fluid and cow faeces-based organic matter digestibility were significant ($P < 0{\cdot}001$) with residual standard deviations of between $\pm 0{\cdot}019$ and $\pm 0{\cdot}022$. In another study Akhter & Hossain[106] concluded that fresh or frozen freeze-dried cow faeces were satisfactory and repeatable substitutes for rumen liquor as a source of micro-organisms for *in vitro* digestibility assay for forages. If proven to work under most conditions and satisfactorily standardised among laboratories, this method provides an opportunity to overcome the main disadvantage of the methods that require surgically prepared animals to obtain fresh rumen liquor.

Harris *et al.*[107] successfully demonstrated an experiment using dairy cow faeces rather than using the traditional rumen fluid in the gas pressure transducer technique. It was observed that cumulative gas production from the test feeds for faecal inoculum showed a correlation of R^2 0·95 with data obtained from the use of rumen fluid. Nsahlai & Umunna[108] compared rumen fluid inocula with reconstituted sheep faeces to predict *in vivo* digestibility and intake and concluded that gas production using faecal inoculum was positively related to gas production using rumen fluid inoculum particularly at 48 h (R^2 0·85) of incubation. It was further confirmed by these authors that *in vitro* DM digestibility estimated using reconstituted sheep faecal inoculum had a positive correlation (R^2 0·88) with *in vitro* DM digestibility measured using rumen fluid. Since sheep faeces are much more easily obtained than rumen fluid, the faecal inoculum method would seem to have a distinct advantage in use. This advantage may be of special value as the use of fistulated animals for the purpose of nutritional studies has been criticised due to the cost and animal welfare implications of keeping fistulated animals.

Summary and conclusions

The information on rumen degradation of different feeds before their use to formulate nutritious diets for ruminant animals is essential. The *in sacco* method has been useful for decades to obtain this information. However, its continuous use is limited as it is laborious, inconsistent and costly. As the *in sacco* method requires surgically modified animals, its routine use to study degradation of ruminant feeds has undesirable implications for animal welfare and management. The *in vitro* methods offer advantages over the *in sacco* method as these are simple and speedy. However, the results from the *in vitro* studies were variable when compared with those from the *in sacco* method. While the *in vitro* methods involving rumen fluid-based proteases offered some interesting observations, the protein degradation rates from these methods were either greater or lower than the published estimates for the *in sacco* methods. These findings suggest that the faster or slow protein degradation with commercial and extracted enzymes compared with the *in sacco* degradation may have been due

Table 5. Factors affecting the accuracy of *in vitro* gas production technique involving rumen fluid (RF)

Factor	Effect	Reference
Sample form	Wilting increases fermentation rate and freeze-drying and milling increases gas production relative to chopped or unchopped fresh forage	Sanderson *et al.*[98]
Oven-drying samples	Eliminates volatile constitutes from fermented substrates thus reducing the indirect gas produced from the reaction of feeds with the buffer	Deaville & Givens[99]
Buffer composition	High-phosphate buffers reduce gas production by utilising protons that would have been used for CO_2 production	Schofield[97]
RF inoculum:buffer ratio	When greater than 1:2, blanks no longer truly represent the contribution of the inoculum to gas production	Cone *et al.*[100]
Size of liquid–gas interface	Determines the potential for gas saturation and solubilisation which reduces gas production	Theodorou *et al.*[94]
Prevailing pH and temperature	Decreases gas production if below optima for cellulolytic bacteria growth	Russell & Dombrowski[101]
Atmospheric pressure	Determines actual gas volumes, yet it is often omitted such that it is difficult to compare results from different laboratories	Williams[102]
Stirring	Reduces CO_2 supersaturating which causes erroneous volume and pressure readings	Pell & Schofield[96]

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to the variations between the enzymic activities depending upon the origin and amount of these enzymes. The gas production technique has been used as another alternative but it has been criticised as there was no firm agreement either about the gas data obtained or their relevance to the degradation of ruminant feeds. These *in vitro* methods require rumen fluid for the incubation of target feeds for either the estimation of degradation or to use these estimates for comparisons with the enzyme-based estimates. The use of rumen fluid is advantageous as it simulates the dynamic and specificity of the *in sacco* microbial degradation. However, keeping fistulated animals for obtaining rumen fluid is unacceptable on animal welfare grounds. Therefore, either rumen fluid from freshly slaughtered animals or fresh or thawed faeces could be used as alternative sources of microbes instead of rumen fluid from fistulated animals for the *in vitro* incubation of feeds to estimate rumen degradation (Chaudhry[109,110]). However, it would be essential to standardise the protocols for each method (*in sacco* and alternatives). This standardisation should aim to minimise the potential sources of variations among different laboratories that estimate degradation of feeds being used to formulate nutritious diets for ruminants.

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