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Nutrient digestibility and balance studies

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Introduction

The nutritional value of a feed for cattle depends on its nutrient and energy contents, the extent of rumen fermentation and degradation, and the post-ruminal digestibility [1]. Efficiency of digestion depends on different factors, for example, the apparent digestibility (estimated by subtracting the nutrients contained in faeces from the nutrients contained in dietary intake – unlike true digestibility where the endogenous and microbial amount is taken into account and corrected in final outcome) usually decreases when the level of intake increases. This decrease is due to a more rapid passage of feed through the rumen-intestinal tract, which reduces the retention time and thus the time for fermentation, digestion and absorption [2]. Additionally, various processing techniques, which alter the physical form of feed e.g., drying, grinding, freezing, heating, or pelleting, can change the fermentation and digestibility characteristics. Chemical processing techniques, such as treatment with NaOH and/or NH_3, and biological additives, such as bacterial additives or enzymatic compounds, can also alter nutrient digestibility in cattle [3], [4], [5]. Different methods at the *in vivo*, *in vitro* or *in situ* level are available to measure the digestibility of nutrients. The total collection method *in vivo*, also known as a conventional digestion trial, is the most accurate method, but is time-consuming and costly to carry out. However, there are *in vitro* and *in situ* methods, which are inexpensive and can produce outcomes correlated to *in vivo* results [6]. Digestion with microorganisms and/or gas production techniques are among the common *in vitro* methods introduced in the 1960s and 1970s [7], [8]. Some researchers have concluded that the *in situ* nylon bag method ensures a more standardised and repeatable alternative to the *in vitro* techniques [9], but it requires the use of fistulated animals. Both microbial digestion and nylon bag techniques are based on the disappearance of the substrate i.e., can be measured gravimetrically, whereas the gas production method focuses on the appearance of fermentation end products.

It is important to consider the passage rate of different feedstuffs. The fractional passage rate is defined as the proportion of a representative digesta pool that passes a point along the digestive tract in a given time or leaves a particular pool e.g. the reticulo-rumen (RR) [10]. Digesta is usually considered in the solid (particulate) and the fluid (solute) phase, where the solute phase has a higher rate of passage through the digestive tract than the particulate phase. The fractional passage rate is estimated separately for the liquid and solid phase, with the latter divided into small (concentrates) and large (forages) particles. Fractional passage rate can be expressed as h^{-1} (passage rate= 1/retention time) or %/h (passage rate = 100/retention time). The passage rate is known to be feed specific and influenced by feed quality, forage-to-concentrate ratio, forage type, intake level and other animal-related factors [11], [12], [13], [14]. Understanding the retention time of nutrients in the digestive tract is a basic requirement for different feeding evaluation programs [15], [16]. Measurement of fractional passage rate can be

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executed by using non-toxic markers, which are not fermentable, digestible or absorbable. Markers can be grouped into externally applied (rare earth and chromium markers) or inert internal (acid-insoluble ash and indigestible fibre fractions) markers. It is important to note that ruminal content, i.e. particulate and solute portions of the digesta, are constantly mixed, and that ruminal microbes 'move' between these fractions; however, a marker may not. External solid markers have been shown to favour binding small particles, inhibiting the digestibility of distinct feed particles, and may migrate to rumen fluid [17]. This could raise questions regarding the outcome of fractional digesta passage studies using these markers. Recent studies have shown the potential advantages of stable isotopes as internal digesta markers in comparison to external markers [18], [19].

Protein sources are among the most expensive feed components in cattle rations, particularly for dairy cows. In general, nutritionists aim to create a ration, which maximises the conversion of dietary plant proteins into proteins of animal origin, like milk and meat. Dietary proteins can undergo microbial degradation in the rumen, be absorbed by the small intestine and deposited in tissues, secreted in milk or excreted in urine and faeces. Minor nitrogen (N) losses may also occur through eructating or exhaling volatile N components or by scurf and hair losses. The efficiency of N utilisation in dairy cattle is generally low [20]. It is known that for higher potential N utilisation, more N has to be partitioned into milk and meat, yet roughly 70–80% of dietary N is excreted via urine and faeces in dairy cattle [21], [22]. Improving the efficiency of protein N utilisation by ruminants, and subsequently lowering N excretion, has become a focus in many countries [23]. In addition to the dietary N intake, the type of energy and degradability of ration components, protein degradability in the concentrates, as well as the level of fertilisers applied prior harvesting have been shown to affect N utilisation in cattle [21], [24], [25]. Nitrogen balance experiments in cattle can be carried out to better understand the approaches for improving N utilisation in these animals. Castillo et al. [26] stated that a complete N balance is required to assess the partitioning of total N intake as N output in faeces, milk and urine. They also advised researchers to take any retained or mobilised N into consideration with possible bodyweight changes of the animal. Spanghero and Kowalski [27] sought to identify error sources of N balance measurement and pointed out that incomplete collection of material, volatile losses of ammonia during collection and subsequent drying of the samples could all affect measurements. Scurf and dermal losses also contribute to output sources of N but their contribution to total N excretion is very low and difficult to measure. However, it is possible to calculate scurf and dermal losses using distinct equations [28].

Passage rate and digesta retention time

Prerequisites

This guideline provides a standardised procedure for passage rate measurement in non-fistulated cattle, with points on general feed preparation and sampling for determining passage rate retention time or fractional passage rate in the whole digestive tract. Determination of the passage rate or fractional passage rates in specific compartments of the digestive tract requires fistulated and/or cannulated cattle. However, fistulated animals cannot be kept in every experimental facility, may have different passage rates than intact animals and require ethical permission in most countries, and therefore are not considered in the following. The Animal Trait Ontology (ATOL) and Environment Ontology (EOL) for Livestock numbers associated with present guideline are as follows: **ATOL_0005126, ATOL_0001281, ATOL_0001060, ATOL_0000089, ATOL_0001231, ATOL_0001282, ATOL_0005126, ATOL_0000351, ATOL_0001529, ATOL_0001061**, and **EOL_0001686** (for complete list of ATOL, please visit https://www.atol-ontology.com/en/erter-2/).

A – Planning and preparing for the experiment

1. A prerequisite for digestion trials is digestive tract stability. Depending on the deviation between the currently fed diet and the test diet, 14–20 d should be dedicated to the adaptation of the animals to the test diet with extreme diet changes taking longer.
2. After the adaptation phase, the subsequent sample collection period should last for 4–7 d, depending on the size of the animal, diet composition and diet digestibility. Thereby, each distinct experimental period should run for 18–27 d. Daily ration during the trial should be offered once, twice or more often per day to the animals. The time of feeding and the individual dry matter (DM) intake should be recorded daily during the collection period.
3. Feed offered should be sampled and analysed for chemical composition and, if applicable, particle size distribution, dependent on whether the mean or fractional particulate passage rate, is aimed to be determined.

B – Preparation and pulse dose application of markers

1. Generally, markers used for the study should be non-toxic, should not interfere with digestive processes, be neither left nor absorbed within the digestive tract, and may be specific of the phase they are expected to trace, and orally applied as a bolus. Feedstuff, including internal markers, should not be used for pulse dose application.
2. Bolus ingestion can be achieved by offering a small known amount of the marker-containing feed (100–500 g) before the morning feeding (when feeding is done twice per day). Intake of the marker-containing feed should be completed within 30–60 min. As an alternative, the marker-containing feed or water can be applied via an oral gavage.
3. For the assessment of the mean passage rate of whole digesta, the marker needs to be thoroughly mixed with feed or a feed component. In case of feeding a partial mixed ration (PMR), the marker should be added into the concentrate/supplemental feed. Putting the mixture in pellets for easier handling is optional. The marker dosage may be variable, according to the marker and the precision of its measurement. For instance when TiO_2 is utilized, 20 mg/kg bodyweight of the animal (10 g/d) can be applied, but Yb marker can be administered at 2–4 mg/kg bodyweight (1–2 g/d) of the animal. Therefore, the bodyweight of the animal must be measured.
4. For the measurement of fibre mean retention time (MRT), the fibre portion of the feed needs to be mordanted. This should be performed as follows: **a)** soluble particles should be removed with neutral detergent solution; **b)** fibre should be soaked in water overnight and washed to remove any soluble; **c)** fibre-containing feedstuff should be dried at 40°C and milled (2–10 mm); soaking in sodium or potassium dichromate should be applied to Chromium (Cr)-mordant fibre; **d)** the mordant should be washed several times in water so that loosely bound marker molecules are removed (suspending in ascorbic acid to give acidic pH is also an alternative to particularly eliminate incompletely attached Cr), dried at 65°C for 24 h, and **e)** dried mordant should be ground to pass 1–5 mm screen.
5. The mordant feedstuffs with appropriate markers should be analysed for the marker content (g/kg DM of fibre) and stored at −20°C before administering. A marker dosage of 0.1–0.2 g/kg bodyweight should be applied.
6. For the measurement of the solute MRT, the solute marker should be dissolved in drinking water prior administrating to the animal. The marker dose should be in the range of 0.002-0.010 g/kg of animal's bodyweight, dependant on the sensitivity of the instrument for marker analysis.
7. Detailed procedure of labelling feed nutrients with stable isotopes (e.g. ^{13}C or ^{15}N) and dose enrichment assessment of the internal marker should be carried out as described by Pellikaan [29], Warner [30] or Huhtanen and Hristov [18]. Total mixed ration (TMR) should be provided to the animal in order to avoid isotopic fractioning in the digestive tract over time [31].
8. Different markers assessing whole digesta, fibre and solute MRT may be administered at the same time. The time of any oral marker administration/ingestion needs to be recorded. In repeated experiments/periods on the same animal, the operator must ensure the wash-out of any previously dosed marker. Ideally a background sample should be taken before repeated marker administration.

C – Faecal sampling, analysis and calculation of MRT in the digestive tract upon pulse marker administration

1. Before marker administration, a faecal grab sample has to be taken (roughly 200–600 g fresh) to analyse the baseline content of the marker.
2. After administrating or feeding the marker, faecal grab samples (200–600 g fresh) have to be collected in 4- to 12-hourly intervals, e.g., 8, 12, 16, 24, 30, 42, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156 and 168 h. Faecal samples should be stored at −20°C prior to marker analysis.
3. Faecal samples should be dried at 60°C for 36–72 h, dependent on the spread of the sample and the ventilation of the oven. Following this, they should be ground through a 1 mm screen, to allow determination of DM content for further analysis of the applied markers [32].
4. A standard curve of different marker amounts e.g., 0.2–15 mg TiO_2, as described by Brandt and Allam [33], should be established. The recovery of the marker requires validation by adding various amounts (mg/g DM) of the marker to equally-sized portions of dried faeces (e.g. 2 g), obtained from animals that did not receive any marker. Subsequently, the amount of TiO_2 has to be analysed and determined against the standard curve and the values obtained have to be compared with the added amount of TiO_2 [32].

5. The MRT (h) of the respective marker in the total gastrointestinal tract can be calculated as the area under the excretion curve. The method by Thielemans [34] separates the total area under the excretion curve into two equal parts: $MRT = \frac{\Sigma(t_i \times dt \times c_i)}{\Sigma(dt \times c_i)}$
where t_i represents the time after marker application [h], dt the time interval represented [h] by marker content (calculated as $\frac{(t_{i+1}-t_i)+(t_i-t_{i-1})}{2}$ and c_i the faecal marker content at t_i [mg/kg DM]. This method assumes that the flow of digesta is continuous.
The solute MRT from the RR is also possible to compute, using the calculation proposed by Grovum and Williams [35]. This calculation is based on the decrease in faecal solute marker content c_i [mg/kg DM] with time post marker administration t_i [h] according to the equation:
$lnc_i = -k \times t_i + b$
where k represents rate constant [h^{-1}] and b the intercept.
Another method has also been described in literature to calculate the MRT in the whole gastrointestinal tract according to the equation proposed by Blaxter [36]: $MRT = \frac{\Sigma_{i=l}^{n}(t_i \times m_i)}{\Sigma_{i=l}^{n} m_i}$
where m_i is the amount of excreted marker [g] at the i_{th} sample and t_i is the time, elapsed between dosing [h] and the mid-point of the i_{th} collection interval [h].

In vivo nutrient digestibility and N balance

Prerequisites and principles

This guideline seeks to highlight key steps when performing a digestibility and N balance experiment in cattle (also see the review by Hristov [37]). The main focus of the document will be on feeding trials (ration comparisons). This guideline can be used for the N balance experiment, which will be performed on mature or growing cattle including male and female animals.

The faeces consist of the undigested fraction of the ration, having successively escaped the microbial degradation in the RR, the digestion in the abomasum and the small intestine and finally the microbial fermentation in the large intestine, as well as undigested products of endogenous or microbial origin. Urine consists primarily of nutrient degradation products, post-absorptively formed by tissues of the host. Urinary N comes mainly from urea excretion, the latter resulting from both rumen ammonia detoxification in the liver and hepatic amino acid catabolism. Other minor urinary N products originate from rumen microbial metabolism (purine derivatives, hippuric acid, etc.), and from the metabolism of the host (amino acids and related compounds, creatine, creatinine, and endogenous purine derivatives, etc.).

When correctly conducted, the measurement of the quantities of feed ingested (i.e. offered minus refused), and of the quantities of faeces excreted, both on a DM basis, allows estimating the apparent digestibility of the DM of the ration, and of any constituent that can be measured in feed and faeces (organic matter, N, fibre, starch, etc.). Estimating the true digestibility would need to separate the endogenous and microbial products from the feed residues in the faeces.

Nitrogen balance is the difference between nitrogen input and output. Often, the quantities of N consumed with water or lost via hair, scruff or eructation are minor and are often neglected. Thus, N balance is often referred to the difference between N that is ingested with feed and the N excreted with faeces and urine and the N secreted in milk. A correctly conducted N balance will allow the operator to exploit the N balance data as a tool to gain knowledge on N partitioning in different contexts e.g., urinary N to milk or protein retention ratio, N digestibility, or faecal N excretion. The guideline focusses on total collection procedures and ignores marker-assistant techniques.

Nutrient digestibility and N balance trials are mostly performed to compare feed or animal characteristics or the interaction between both. For feeding trials, differences between individuals oblige to carry out the measurement with a minimum of animals per treatment (3 to 8, the minimum value most often quoted being 4, [38]. Day-to-day variation, in intake, faecal and urine excretion, and experimental errors, constrain the measurement period to be conducted for a minimum of days (5 to 14 according to the authors, [38]). These numbers may vary, especially depending on the accuracy sought. Also, if gestation is not a key objective to be studied, animals in their third trimester of pregnancy should be avoided, due to important N retentions of the foetus and the uterus.

The Animal Trait Ontology (ATOL) and Environment Ontology (EOL) numbers associated with present guideline are as follows: **ATOL_0001509, ATOL_0001829, ATOL_0001419, ATOL_0000351, ATOL_0000491, ATOL_0001682, ATOL_0000138, ATOL_0000088, ATOL_0001032, ATOl_0000835, ATOL_0000837, ATOl_0000779, ATOL_0001282, ATOL_0000772, ATOL_0001529, ATOL_0001518, ATOl_0005125, ATOl_0001491, ATOL_0001393, EOl_0001905** and **EOL_0001754** (for full list of ATOL, please visit: https://www.atol-ontology.com/en/erter-2/).

A – Total collections and sampling

1. For total individual urine and faecal collections, animals should be transferred to individual tie stalls and be given sufficient time to adapt to the change in housing environment so that behaviours (standing, lying, eating and ruminating) are normalised to pre-measurement levels. The cattle should be allowed sufficient time (this period can differ between dairy and growing cattle) to adapt to the environment. Adaptation may need to be longer prior to the first measurement experience in tie stalls (e.g. period 1 of a change over study).
2. The animals should be fed the same diet throughout each measurement period and sampling phase, avoiding any change in batch, silo or clamp. Total mixed ration is recommended to be offered to the animal, although care must be taken to ensure good mixing ingredients and homogeneity of particle size distribution on daily basis **[see the guideline on "Feed intake measurement in indoor-feeding system (TMR and PMR)"]**. If the TMR has a wide range of particle size or if a partial mixed ration (PMR) is offered, then it is likely that food sorting or mixing between concentrate and forage sources will occur. In this situation, it will be necessary to quantify and analyse the sorted uneaten feed (refusals) to account for changes in feed N and other nutrient concentration.
3. Animals should be weighted at the start and end of each measurement period or sampling phase in order to determine changes (loss or gain) in their bodyweight. Animals should be weighed at the same time each day in relation to feeding.
4. The duration of the experimental period normally should take 19–28 d, of which 14–18 d should be spent on adapting the animal to the experimental diet (usually two weeks) and to the tie stall (usually a few days). The final 5–10 d should be dedicated to quantitative measurements (g, kg, or L per day) of daily feed and water intake (if possible, but not mandatory), milk yield, faecal and urine excretions as well as sampling of feed, refusals, faeces, urine, milk and scruff and hair (the latter optional, nevertheless mandatory in particular cattle breeds, e.g. Highland) to determine N concentrations. If the sampling period is intended to be performed in a metabolic cage, the duration of the trial must not be longer than 5 d according to the DIRECTIVE 2010/63/EU (Suppl. VIII, sec. III, 2, h) on the protection of animals used for scientific purposes. Possible derogation can be obtained when more than 5 d are required for accuracy of measurements, in particular in growing animals, or when the minimum enclosure dimensions and space allowances of the ‘cage’ are fulfilled.
5. During the adaptation period to the experimental diet, animals should also be trained to accept harnesses, chutes, urinals or separators used for urine and faeces collection when applicable.
6. The timing of feed offered during the experiment period should be consistent, with animals fed at the same time or times in relation to sampling and milking events. Depending on the research aim, animals can be fed either *ad libitum* or restrictively. For *ad libitum* intake, animals should be fed with 5–10% refusal (on dry matter basis). An alternative would be to calculate the offered feed level based on the average daily dry matter intake during the week prior to the sampling phase and to provide a constant daily feed allowance based on actual intake.
7. Sample of feed offered and refusals should be collected daily during the sampling phase and kept frozen at −20°C for further processing prior to nutritional analysis (see the Feed and Water intake chapter). The thawed samples can be pooled, mixed, subsampled and subjected to DM and nutritional composition analysis (Organic matter, N, fibre, starch content, etc.). For determining the N content, Kjeldahl N analysis or other relevant methods and techniques such as Dumas method or Elemental Analysis can be performed. In case of rations containing fermented forages, such as silages, it is necessary to correct the DM content for losses of volatile compounds during drying. It is recommended to perform N analysis on fresh or frozen but non-dried samples.
8. If the N content of drinking water is not known, a sample from the drinking water should be taken and analysed for total N content. In case the N content of drinking water is high and not negligible relative to feed N intake, water intake should be measured.
9. During the trial, animals should be milked *in situ* in their tie stalls usually 2–3 times per day at the same time(s) each day, and ideally with equal time distances between each milking, as the time between milking may affect milk composition. Milk yield by weight should be determined at each

milking with care taken to make sure that no milk remains in the transfer tubing. Milk samples should be collected at each milking for the whole sample phase. Thoroughly mixed samples should be stored at 4°C with the addition of an appropriate preservative (e.g. Bronopol) until compositional and N analysis. Note: Bronopol and other conservatives contain N, which needs to be corrected for. Also, the maximum duration of storage before analysis is 1 week. If the analysis is done later than 1 week from sample collection, then samples should be cooled down as soon as possible after milking and stored at −20°C before analysis (Kjeldahl N or any relevant method as mentioned in point 7).

10. Total faecal excretion must be recorded daily, and care must be taken to account for any faeces that were not cleanly collected; the resulting weight should be added to the total. Faecal collection from each day of the collection phase should be weighed, thoroughly mixed and subsampled (3–5%) into a pooled container of appropriate size with a tight fitting lid and stored and frozen at −20°C. In practice, every morning before feed distribution, the faeces are weighed completely and homogenized. It is advisable to use an electric mixer to obtain a homogeneous sample and to take the sample quickly after the mixing to avoid the decantation. Each day, a subsample should be taken. At the end of the sampling phase, the pooled faecal excretion should be thawed and thoroughly mixed. Representative subsamples of appropriate size (e.g. 500 g) for archiving and analysis should be taken and frozen at −20°C for later nutritional and Kjeldahl N (other relevant methods mentioned in point 7) analysis.
11. Urine collection should be conducted at the same time each day, relative to feeding and milking. The absence of contamination (faeces, hair, etc.) must be as strict as possible. For urine collection and sampling, the urine collection container need to be filled with N-free acid (e.g. sulfuric or hydrochloric acid) added prior to start of collection. The weight of acid added needs to be recorded. The used quantity of acid furnished should reduce the urinary pH <3. The pH of the urine collected into the acid should be checked twice a day and more acid added, if required to maintain urinary pH <3. The total weight of urine (including the acid) excreted, needs to be recorded every day. The tare weights of all containers should be accounted for.
12. At the end of collection phase, urine samples from each day's output should be thawed, combined by weight, thoroughly mixed and subsampled into containers of appropriate volume (e.g. 500 ml) for archiving. Alternatively, a constant percentage of urine excreted can be sampled daily and pooled by period and animal throughout the measurement. Once processed, the urine samples should be stored at −20°C for analysing the Kjeldahl N or other methods for N content determination as mentioned in point 7.
13. In respiration chambers equipped with ammonia analysers or traps for quantifying ammonia in exhaust air, and if air conditioner condensate is collected, the amount of volatilized N can be determined to measure volatile N losses. Throughout the balance period, samples of condensed water (e.g. collected from the heat exchanger and samples of acid solutions through which air is led to trap aerial ammonia) can be collected and analysed for ammonia-N concentration [39]. In addition, losses of N as N_2O can be accounted for if representative samples of incoming and exhaust air can be obtained and analysed [40].

B – Nutrient digestibility and N balance calculation and corrections

1. The obtained data from digestibility trial should be outlined as the feed intake (feed offered minus feed refused) and faecal excretion in g/d of DM or of any other measured constituent. The apparent digestibility of constituent X is computed as: $d(X) = \frac{I(X) - F(X)}{I(X)}$ with *d* = apparent digestibility, *I* = intake and *F* = faecal excretion.
2. The obtained data from the N balance trial should be outlined as the feed N intake (g/d) (eventually plus water N intake (g/d)), total faecal N excretion (g/d), total urinary N excretion (g/d), and – for lactating animals – total milk N secretion (g/d), to compute the N balance as an estimation of retained or released N (g/d).
3. If volatile N losses were measured in parallel to the collection period, the amount of N excreted with urine and faeces should be corrected for volatile N losses [37].
4. If the dermal and scruff N (DSN) losses were not measured but aimed to be considered in the calculation of N balance, DSN can be estimated using the following equation: $DSN(\text{g of N/d}) = \frac{0.2 \times \text{live-weigth (kg)}^{0.6}}{6.25}$ [41].
5. The retained N during final calculation should not be above 10% of the N intake within the treatments (particularly in dairy or maybe late-fattening animals) as this may indicate a loss of N compounds from the collection system and/or errors in sample mixing, subsampling and analysis

[28]. In growing animals, the calculated protein retention (N retention × 6.25) can be regarded in relation to the average daily gain observed before or alternatively during the N balance trial, the latter calculated from the two bodyweight measures covering the adaptation and measurement period. Protein gain varies with bodyweight and breed and represents on average around 15 to 25% (depending on the breed can be lower) of bodyweight gain.

6. If the experiment contains dietary treatments with high N variations between diets, the interpretation of N balance data should be done carefully due to risk of enhanced overestimation of the retained N, particularly in diets with high N availability compared to control [27].
7. It is highly recommended to conduct the N analysis from fresh faecal samples directly. Nevertheless, if the faecal samples were dried in the experimental unit prior to N analysis, appropriate adjustments have to be conducted in order to avoid erroneous results. The following relationship in terms of crude protein (CP) determination in wet and dry faeces samples (drying at a temperature range of 100–105°C seems to result in minimum DM losses) was depicted [42]: $Y = 1.1252X + 0.12$, where Y = CP determined on wet faeces and X = CP determined on dried faeces. Besides, the operator is highly urged to consider the method of faecal drying as faecal N losses are much lower during lyophilisation than oven drying.

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