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IN SITU AND *IN VITRO* TECHNIQUES FOR STUDYING RUMEN FERMENTATIONS: METHODOLOGY AND APPLICATIONS

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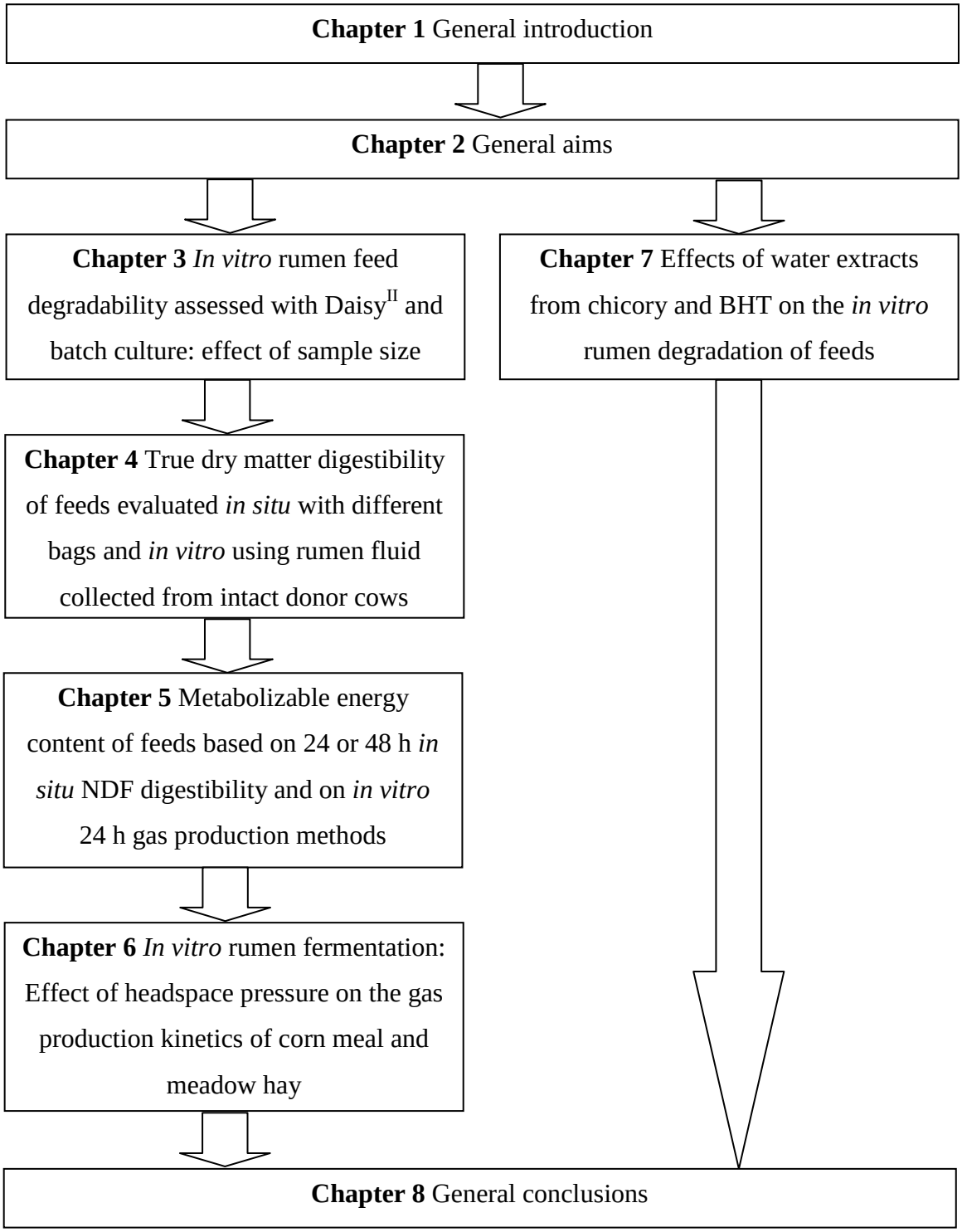
The outline of the thesis

The present PhD thesis includes a general description of the techniques (*in vivo*, *in situ* and *in vitro*) which are commonly employed for studying rumen fermentation process. The techniques are described focusing on methodologies, equipment, main sources of variation, strength and weakness points of each technique (Chapter 1). In Chapter 2 the general aims of five scientific contributes (4 methodological and 1 applicative) are given. In Chapters 3, 4, 5 and 6, the core of the thesis, four methodological contributes are given. Chapter 7 provides the results of an applicative contribute. Three of the five contributes have been already published in scientific papers (Chapters 3, 6 and 7), whereas the other two have been submitted to scientific journals (Chapters 4 and 5). The last chapter (Chapter 8) reports the general conclusions. Figure 1 represents schematically the contents of the present thesis.

Figure 1. The outline of the thesis.



Abstract

The general aim of this thesis was to study rumen fermentation processes using *in situ* and *in vitro* digestibility and *in vitro* gas production techniques. The thesis includes the results of five experiments. The first experiment investigated the validity to reduce the amount of feed sample incubated in filter bags with Daisy^{II} (from 0.50 to 0.25 g feed/bag). This hypothesis was verified by considering the results of two separate trials (conducted on 4 and 7 feeds, respectively) in which the digestibility values provided by Daisy^{II} were compared with those achieved using a conventional batch culture. Results evidenced that, by using the lower sample size (0.25 g feed/bag), digestibility values provided by Daisy^{II} resulted more similar, less variable and better correlated with those achieved with the batch culture.

The second experiment was conducted on 11 feeds and compared the digestibility values achieved: i) *in situ* and *in vitro* using two different kinds of bag (nylon and filter) with a different pore size (40 and 25 µm); ii) *in situ*, placing the bags in the rumen environment, and *in vitro* using rumen fluid collected from intact cows by a suction technique. Results of the experiment showed that the use of filter bags induced a systematic, but predictable, underestimation of digestibility values compared to the nylon bags. Rumen fluid collected from intact cows provided digestibility values directly proportional to those obtained *in situ*. This latter result is relevant, as the validity of using rumen fluid collected from intact cows can be of interest for many research centers, in order to overcome ethical and public concerns related to the use of surgically treated animals.

The third experiment was conducted on the same feeds analysed in the second experiment and it was aimed to compare the energy value of feeds estimated using three different equations: i) one equation based on feed chemical composition and *in situ* digestible NDF at 48 h of incubation (ME_{NRC}); ii and iii) two equations based on feed chemical composition and *in vitro* gas production at 24 h of incubation (ME_{Menke} and ME_{UCD}). A further aim was to evaluate the validity of reducing the *in situ* incubation time from 48 to 24 h. Results of the trial evidenced that, when ME_{NRC} estimates were taken as reference, ME_{UCD} equation provided feed energy estimates more accurate and precise compared to ME_{Menke}. However, the accuracy and precision of ME_{Menke} estimates increased when four feeds rich in protein (CP > 16% DM) were excluded from the analysis. As the *in situ* digestibility values achieved at 24 and 48 h were highly correlated and showed a comparable reproducibility, the reduction of *in situ* incubation time from 48 to 24 h seems can be proposed for reducing the cost of the tests.

The fourth experiment was aimed to compare the effects of two different systems of gas release (venting at fixed times or at fixed pressure) on gas production (GP) kinetics provided by two feeds with a different degradability (meadow hay and corn meal), using an innovative GP system. Results of the experiment showed that the venting system exerted significant effects on GP kinetics. The effects were particularly accentuated for corn meal and for venting at fixed times. Therefore, when venting at fixed times is applied, the headspace volume of GP system, the venting frequency and the amount of fermentable matter incubated must be carefully balanced to avoid the generation of high pressures, which could alter GP kinetics. However, venting at fixed pressure is preferable to that at fixed times, as it can strongly reduce these shortcomings.

The fifth experiment aimed to evaluate the effects of increasing dosages of two antioxidants (BHT and a red chicory extract) on some *in vitro* rumen parameters, when they are incubated with meadow hay and corn meal. Results of the experiment showed that the two antioxidants exerted only small effects on rumen degradability, GP kinetics and volatile fatty acids profile. At the highest dosage, the red chicory extract showed to improve the efficiency of nitrogen utilization in the rumen, by increasing the amount of nitrogen captured by rumen microbes and utilized for microbial protein synthesis.

Riassunto

Lo scopo generale del presente lavoro di tesi è stato quello di studiare le fermentazioni ruminali mediante la tecnica *in situ*, e le più moderne tecniche *in vitro* che valutano la digeribilità e la produzione di gas degli alimenti zootecnici. La tesi comprende i risultati di 5 diverse prove sperimentali. L'obiettivo della prima prova è stato quello di valutare la possibilità di ridurre la quantità di campione alimentare incubato all'interno dei filter bags previsti dal sistema Daisy^{II} (da 0,50 a 0,25 g di alimento/filter bag). La suddetta ipotesi è stata verificata analizzando i risultati ottenuti in due differenti prove sperimentali, condotte rispettivamente su 4 e 7 alimenti, il cui comune obiettivo era il confronto dei valori di digeribilità forniti dal Daisy^{II} e da un sistema *in vitro* tradizionale. I risultati ottenuti hanno dimostrato che, con l'incubazione di 0,25 g di alimento/filter bag, i valori di digeribilità forniti dal Daisy^{II} risultavano complessivamente meno variabili, meglio correlati e comparabili con quelli forniti dal sistema *in vitro* tradizionale.

La seconda prova sperimentale è stata condotta su 11 alimenti e si poneva l'obiettivo di confrontare i valori di digeribilità ottenuti: i) *in situ*, attraverso l'impiego di due diversi tipi di sacchetto (nylon e filter bags) caratterizzati da una differente porosità (40 e 25 µm); *in situ*, incubando i campioni alimentari direttamente nel rumine dell'animale, ed *in vitro*, incubando i campioni alimentari con liquido ruminale prelevato mediante sonda esofagea da bovine non fistolate. I risultati della prova hanno evidenziato che l'utilizzo dei filter bags, in sostituzione dei nylon bags, induceva una sottostima sistematica, ma comunque stimabile, dei valori di digeribilità degli alimenti. I campioni alimentari incubati *in vitro* con liquido ruminale prelevato da bovine non fistolate hanno fornito valori di digeribilità direttamente proporzionali a quelli incubati *in situ*. Un tale risultato è sicuramente soddisfacente: la possibilità di utilizzare liquido ruminale prelevato da bovine non fistolate può rappresentare infatti un'importante opportunità per molti centri di ricerca, consentendo di superare le problematiche di natura etica legate all'impiego di bovine sottoposte ad operazione chirurgica per l'applicazione di fistole ruminali permanenti.

La terza prova sperimentale è stata condotta sugli stessi alimenti impiegati nel secondo contributo e si poneva l'obiettivo di determinare il valore energetico degli alimenti applicando tre differenti equazioni: i) un'equazione basata sulla composizione chimica dell'alimento e sul valore di digeribilità della fibra misurato *in situ* dopo 48 h di incubazione (ME_{NRC}); ii e iii) due equazioni basate sulla composizione chimica dell'alimento e sulla produzione di gas misurata *in vitro* dopo 24 h di incubazione (ME_{Menke} e ME_{UCD}). Un secondo scopo era quello di valutare la possibilità di ridurre il tempo di incubazione *in situ* da 48 a 24 h. I risultati della prova hanno dimostrato che, prendendo come riferimento le stime di valore energetico ottenute applicando l'equazione ME_{NRC},

l'equazione ME_{UCD} forniva stime più accurate e precise rispetto all'equazione ME_{Menke}. Tuttavia, il livello di accuratezza e di precisione dell'equazione ME_{Menke} è migliorato notevolmente escludendo i quattro alimenti proteici (PG > 16% SS) dall'analisi. Poiché i valori di digeribilità ottenuti *in situ* a 24 e a 48 h sono risultati fortemente correlati e hanno evidenziato una riproducibilità comparabile, la riduzione del tempo di incubazione *in situ* da 48 a 24 h può rappresentare una valida soluzione al fine di contenere i costi di analisi.

La quarta prova sperimentale ha valutato gli effetti di due diverse modalità di rilascio del gas (a tempi fissi oppure a pressione fissa) sulle cinetiche di produzione di gas fornite da due alimenti caratterizzati da un differente livello di degradabilità ruminale (un fieno di graminacee e una farina di mais). Ai fini della prova è stato utilizzato un sistema innovativo per la misurazione del gas prodotto dalla fermentazione di matrici alimentari. I risultati della prova hanno evidenziato che la modalità di rilascio del gas ha condizionato significativamente le produzioni di gas fornite dai due alimenti. Tali effetti sono risultati particolarmente rilevanti nel caso della farina di mais e nel caso del rilascio a tempi fissi. Alla luce di questi risultati, quando si adotta la modalità di rilascio a tempi fissi, lo spazio di testa del sistema, la frequenza di rilascio del gas e la quantità di campione alimentare incubato dovrebbero essere opportunamente bilanciati per evitare lo sviluppo di pressioni molto elevate all'interno del sistema, le quali potrebbero alterare le normali cinetiche di produzione del gas. Comunque, al fine di ridurre tali rischi, l'impiego di sistemi che rilasciano il gas a pressione fissa è da ritenersi preferibile.

La quinta prova sperimentale ha valutato gli effetti di due sostanze antiossidanti (il BHT ed un estratto di radicchio) dosate a due livelli differenti ed incubate con farina di mais e fieno di graminacee, sui principali parametri ruminali. I risultati della prova hanno evidenziato che i due antiossidanti hanno esercitato effetti poco rilevanti sulla degradabilità ruminale, sulle produzioni di gas e sui profili acidici forniti dai due alimenti. Al dosaggio più elevato, l'estratto di radicchio ha tuttavia migliorato il bilancio azotato ruminale, favorendo un aumento significativo della quota di azoto impiegato dai microorganismi ruminali per la sintesi di proteina batterica.

CHAPTER 1

General introduction

1. General aspects

Feeding can account for up to 60% of the costs for animal farms, so the nutritional evaluation of ruminant feeds and the formulation of well-balanced diets which can meet requirements of animals represent important challenges in livestock production (Adesogan, 2002). The evaluation of a feed is defined as "*the description of a feed for its ability to sustain different types and levels of animal performance*" (France et al., 2000). The ability of a feed to sustain animal performance depends mainly on its digestibility. Feed digestibility is influenced by its chemical (carbohydrate, protein and fat content) and physical (feed particles size) characteristics, as these properties affect capability of digestive enzymes to colonize and digest the feed particles (Kitessa et al., 1999). The digestibility of ruminant feeds can be evaluated using different techniques. These techniques can be classified as: i) biological methods, which involve the direct use of the animals, by digesting feeds in the rumen of the animals (*in vivo* and *in situ* methods); ii) laboratory methods, which do not require the use of animals and simulate rumen environment and digestion process using rumen fluid collected from donor animals (*in vitro* methods).

1.1. In vivo technique: principles of method and shortcomings

The reference method to evaluate the nutritional value of feeds is the *in vivo* digestibility, which estimates the digestibility from total collection of faeces (McDonald et al., 1981). This method is known as "total collection technique" and involves to fed a known amount of the tested feed to a group of animals, which are housed in individual cages to allow a measurement of daily feed intake and the total collection of faeces. Faeces are chemically analyzed to estimate digestibility of feed dry matter or of specific nutrients. Although this method is the most reliable for evaluation of feed digestibility, it appears laborious, time-consuming and expensive, and so not suitable for a routine and large-scale feed evaluation (Stern et al., 1997). An alternative technique is the "marker technique", which does not require the total collection of faeces, as it estimates feed digestibility by using markers (Stern and Satter, 1982). Feed digestibility is computed as ratio between marker concentration in the faeces and in the feed, so the results can be influenced by kind of marker used (McDonald et al., 1981). A great limit of the total collection and marker techniques is that they provide information about the extent of digestibility, but not about kinetics of feed digestion

(Kitessa et al., 1999). Moreover, some sources of variation can influence the results, as: i) animal characteristics (sex, age, breed, and so on...); ii) feed characteristics (particle size, chemical treatments, associative effects between feeds, and so on…) (McDonald et al., 1981; Kitessa et al., 1999).

1.2. In situ technique: principles of method and shortcomings

The *in situ* technique (known also as *in sacco* or nylon bag technique) was originally introduced by Quin et al. (1938), and studied degradability of ruminant feeds by incubating feed samples into silk bags placed in the rumen of fistulated sheep. Subsequently, the silk bags were replaced with nylon bags (Erwin and Elliston, 1959; Johnson, 1966; Rodriguez, 1968). According to the method of Quin et al. (1938), nylon bag technique involves the incubation of feed samples into nylon bags which are placed in the rumen of fistulated animals. The protocol involves to extract and weigh the bags at fixed times (after 0, 2, 4, 8, 12, 24, 36, 48, 72 and 144 h of incubation) for measuring the progressive disappearance of feed from the bags, providing information both about rate and extent of feed digestion (Mehrez and Ørskov, 1977; Kitessa et al., 1999). Over the years, the nylon bag technique has been largely employed to evaluate rumen degradability of feeds (Emanuele and Staplens, 1988; Trabalza Marinucci et al., 1992; Spanghero et al., 2003; Gosselink et al., 2004; Damiran et al., 2008) and was found to predict well the *in vivo* digestibility (Demarquilly and Chenost, 1969; Fonseca et al., 1998). However, the technique still appears poorly standardized and plagued by low repeatability and reproducibility (Michalet-Doreau and Ould-Bah, 1992; Madsen and Hvelplund, 1994). As reviewed by several Authors (Michalet-Doreau and Ould-Bah, 1992; Vanzant et al., 1998; Kitessa et al., 1999), a first important source of variation is represented by bag porosity. The appropriate bag porosity should allow influx of rumen fluid and, efflux of digested feed particles, and retention of feed particles not yet fermented (Mehrez and Ørskov, 1977). When the bag porosity is too small (< 35 µm), microbial population could be sorted by size (Meyer and Mackie, 1986) and the digested particles could be retained in the bags, with a consequent occlusion of bag pores and accumulation of gases inside the bags (Uden et al., 1974; Nocek et al., 1979; Nocek and Hall, 1984; Uden and Van Soest, 1984). On the opposite, a too large porosity (50-60 µm) could potentially facilitate the escape of feed particles not yet digested from the bags (Vanzant et al., 1998). Several studies (Playne et al., 1978; Lindberg and Knutsson, 1981; Michalet-Doreau and Cerneau, 1991) reported that the escape of feed particles from the bags increased with bag porosity. Currently there is still a debate about what is the optimal bag pore size for *in situ* studies. In relation to counts of protozoa and bacteria, Meyer and Mackie (1986) indicated that a bag pore

size of 30-53 µm could be an appropriate range, whereas Vanzant et al. (1998) recommended a pore size included from 20 to 60 µm. On the basis of the bag porosity, an appropriate grinding size of feed samples should be chosen (Michalet-Doreau and Ould-Bah, 1992). Feed samples are commonly ground to pass a sieve prior to *in situ* incubation. However, despite of several attempts to standardize the procedure, a wide range of different grinding sizes has been used (Stern et al., 1997). Nocek (1985) indicated that the grinding procedure should reproduce the effects of rumination and that feed sample size should be more similar as possible to the size of ruminated feed. Generally, a coarse grinding size (4-5 mm) is associated with lower and slower digestion rates, whereas a fine grinding size (< 2-3 mm) is supposed to facilitate the escape and the loss of feed particles from the bag (Michalet-Doreau and Ould-Bah, 1992). Lindberg and Knutsson (1981) observed that escape of feed particles from the bags increased by reducing the grinding size from 4.5 to 1 mm, whereas Others (Michalet-Doreau and Cerneau, 1991; Damiran et al., 2008) indicated that the material losses could be influenced by the distribution of feed particles inside the bags. Moreover, Dewhurst et al. (1995) argued that losses from the bags could be higher for concentrates and by-products, as these feeds contain a larger proportion of soluble materials. The guideline is to grind feeds on the basis of their chemical composition: Nocek (1988) suggested to use a 2 mm-screen for high-protein feeds, and a 5 mm-screen for more fibrous feeds (grains, by-products and roughages). Vanzant et al. (1998) reported that *in situ* studies used commonly a grinding size ranging from <1 to 6 mm and, from the review of 53 experiments, they found that the large part of the published experiments used a 2-mm screen (34) or a 1-mm screen (11). Generally, the choose of 1-mm screen should be preferred to simplify and standardize the laboratory procedures, as chemical analyses of feeds are commonly performed on feed samples passed to a 1-mm screen. The choose of an appropriate grinding size appears important because this factor can influence the ratio between feed sample size and bag surface area (SS:SA, expressed as mg/cm^2). Literature has well documented that a change of SS:SA can alter influx of rumen fluid, efflux of digested feed particles and retention of those not yet fermented (Nocek, 1985; Michalet-Doreau and Ould-Bah, 1992; Vanzant et al., 1998). Despite of several attempts to standardize SS:SA, literature reports a large number of different ratios. From the comparison between 73 *in situ* studies, Vanzant et al. (1998) observed that the largest part of trials (41) used a SS:SA ranging from 10 to 20 mg/cm^2, but also SS:SA > 20 mg/cm^2 and < 10 mg/cm^2 were tested (in 12 and 20 trials, respectively). This is a great shortcoming of nylon bag technique, as SS:SA was found to affect significantly degradability measures. Several Authors (Van Keuren and Heinemann, 1962; Figroid et al., 1972; Van Hellen and Ellis, 1977; Varga and Hoover, 1983) noted that *in situ* degradation of feeds decreased significantly by increasing SS:SA, whereas Mehrez and Ørskov (1977) found that degradability values resulted

higher and less repeatable by decreasing SS:SA from 54 to 16 mg/cm^2. Mehrez and Ørskov (1977) suggested that use of large SS:SA could reduce feed degradation, as influx of rumen fluid and efflux of digested particles from the bag could be limited. On the basis of these results, it is difficult to establish what SS:SA allows to achieve the most accurate degradability measures. From results reported by literature, Vanzant et al. (1998) suggested to use a low SS:SA (10 mg/cm^2), as greater differences and variability were found by using high SS:SA. However, a possible recommendation could be that the choose of SS:SA should vary on the basis of bag and sample characteristics. As confirmation of that, Playne et al. (1978) observed that *in situ* degradation of different roughages did not change when sample size was triplicated and SS:SA was kept constant by increasing bag size.

1.3. In vitro techniques

In the last years the use of *in situ* technique has been strongly criticized by public opinion for the need of fistulated animals and has raised ethical and moral issues about animal welfare (Stern et al., 1997). Moreover, high associated costs and limited analytical capacity of *in situ* technique have led to the development of alternative *in vitro* techniques, which carried out some important advantages: i) they do not involve the direct use of animals; ii) they are less laborious and more suitable for a large-scale evaluation of ruminant feeds. The *in vitro* techniques can be classified as: i) methods which measure the digestibility of feeds (Tilley and Terry, 1963; Goering and Van Soest, 1970; Czerkawski and Breckenridge, 1977); ii) methods which measure gas production from feed fermentation (Menke et al., 1979).

1.3.1. Digestibility techniques: equipment and shortcomings

The first digestibility techniques consisted in batch cultures (Tilley and Terry, 1963; Goering and Van Soest, 1970). These methods involved a first 48-h incubation of feed samples into individual vessels with buffered rumen fluid, followed by a second 48-h incubation with pepsin in an acid solution (Tilley and Terry, 1963), or with a neutral detergent solution (Goering and Van Soest, 1970). Although these methods have been largely validated with *in vivo* values (Van Soest, 1994), they are plagued by several disadvantages: i) they give an end-point measurement; ii) they do not provide information about kinetics of feed digestion; iii) they are laborious and time-consuming; iv) they involve the use of rumen fluid collected from fistulated animals and, hence,

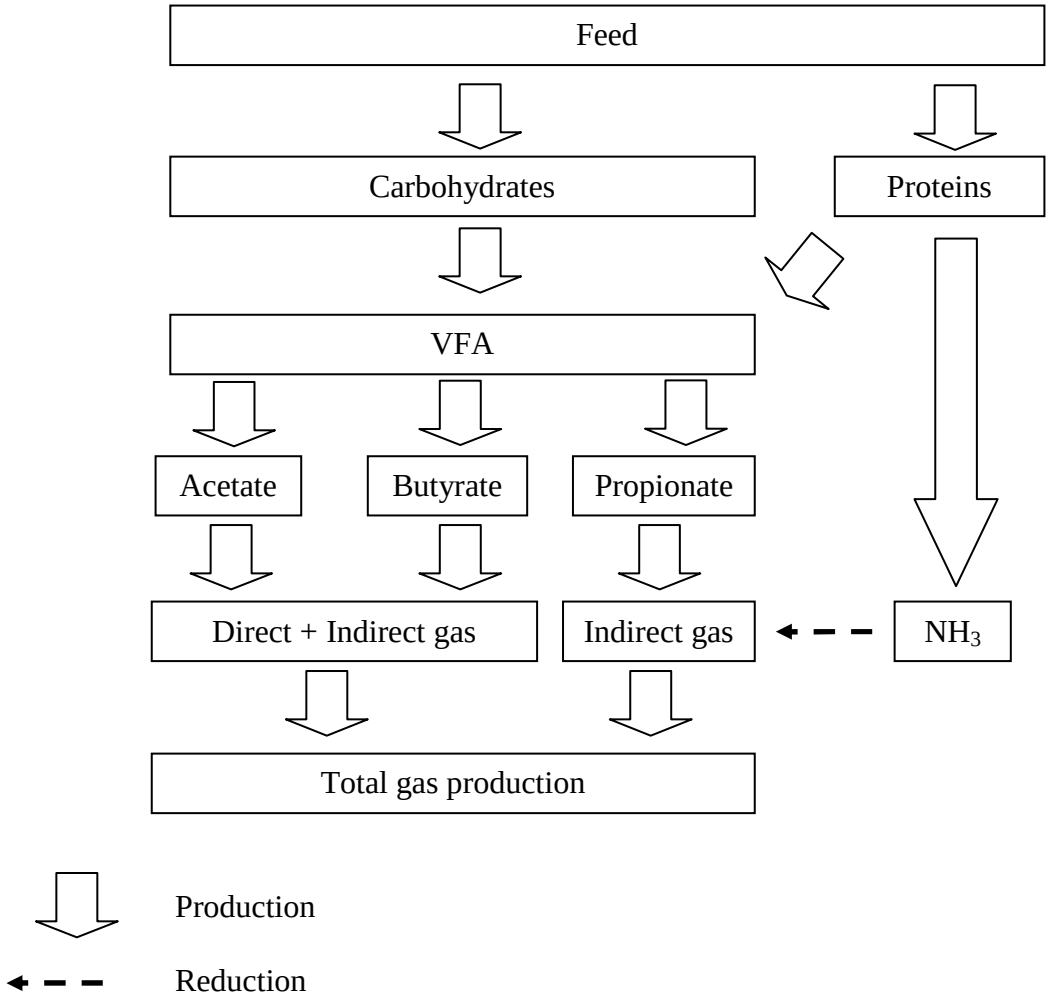
they do not allow to solve public ethical concerns about animal welfare. Afterwards, Czerkawski and Breckenridge (1977) have developed a long-term rumen simulation technique (RUSITEC), consisting in eight vessels in which feed samples (20 g) were placed into nylon bags and digested with rumen fluid collected from fistulated animals. The particularity of RUSITEC is that a solution of artificial saliva (McDougall, 1948) is continuously infused in the system to maintain pH and to reproduce the continuous influx of saliva in the rumen. This technique has been widely employed for different applications, as for evaluating forage digestibility (Akhter et al., 1996; Tejido et al., 2002), changes in rumen microbial population (Newbold et al., 1998) , microbial growth in the rumen (Carro and Miller, 1999), effects of essential oil compounds on rumen fermentations (Newbold et al., 2004). However, RUSITEC appears to be a labor and time-consuming technique, so it has a low suitability for a routine analysis of ruminant feeds (Stern et al., 1999). To overcome some of these shortcomings, the research for more efficient and less animal dependent techniques has led to the development of alternative methods, as Daisy^{II} incubator (Ankom Technology®, Macedon, NY, USA). Compared to Tilley and Terry (1963) and Goering and Van Soest (1970) methods, the Daisy^{II} one leads to an improvement of labor efficiency, as it allows to analyze simultaneously up to 100 feed samples. Several Authors (Holden, 1999; Wilman and Adesogan, 2000; Spanghero et al., 2003; Spanghero et al., 2007; Damiran et al., 2008) found that this instrument provided digestibility measures well correlated with the nylon bag and other *in vitro* techniques. An important similarity between Daisy^{II} and the nylon bag technique is the incubation of feed samples into bags. As consequence, also the results achieved with Daisy^{II} can be significantly influenced by the bag and the sample characteristics, as described for the nylon bag technique. Tagliapietra et al. (2008), from a comparison between Daisy^{II} and a conventional batch culture, noted that the digestibility values achieved with Daisy^{II} were constantly lower and less repeatable, and attributed this result to a barrier effect exerted by the bags, which altered the normal income and outcome of rumen fluid and feed particles. Despite of many attempts to standardize the procedure, there is still a confusion about what sample size allows to achieve the most accurate measures with Daisy^{II}, as some Authors (Holden, 1999; Mabjeesh et al., 2000; Tagliapietra et al., 2008) incubated 0.50 g feed sample/bag, whereas Others (Robinson et al., 1999; Spanghero et al., 2003) preferred 0.25 g. The bags incubated with Daisy^{II} are smaller than those commonly used for *in situ* trials and have a different texture and porosity (F57 filter bags; 4.5x4.0 mm; proved to retain particles > 25 µm; Ankom Technology®, Macedon, NY, USA). Recently, Damiran et al. (2008) compared F57 filter bags and standard nylon bags, and the two feed sample sizes more commonly used (0.25 and 0.50 g feed sample/filter bag): they observed that the two kinds of bag provided digestibility values highly correlated, and that the incubation of 0.50 g feed sample/filter bag

provided digestibility values better correlated with those achieved with the nylon bag technique. Adesogan (2002, 2005) confirmed that the results achieved with Daisy^{II} can be affected by the amount of sample incubated into the filter bags, and observed that the replacement of F57 filter bags with alternative bags allowed to improve the correlation with digestibility values obtained *in situ*.

1.3.2. Gas production techniques: principles of the method

Differently from the digestibility techniques which aim to evaluate the substrate disappearance, gas production (GP) method measures the appearance of fermentation products (gases, volatile fatty acids, NH_3) (Blummel and Ørskov, 1993; Makkar et al., 1995). When a feed is incubated with buffered rumen fluid, it is degraded, and the degraded matter is partitioned to yield gases (mainly CO_2 and CH_4), end-products of fermentation (volatile fatty acids and NH_3), or microbial biomass. The assumption that GP is linearly related to the rate and the extent of feed digestion is questionable, as GP is strongly affected by feed chemical composition (Makkar, 2005). It is generally accepted that gas is mainly produced from carbohydrate fermentation, whereas GP is small for proteins and negligible for fats (Menke and Steingass, 1988; Getachew et al., 1997). Gas produced from feed fermentation is also affected by fermentation patterns and is influenced by the molar proportion of volatile fatty acids (VFA) in the rumen, as highlighted in Figure 2. The butyrate and acetate formation release large amounts of CO_2 and CH_4, whereas propionate production does not release gas of fermentation (Stern et al., 1997). However, GP originated from feed fermentation, defined as “direct GP”, does not represent total GP, as a notable part of gas, defined as “indirect GP”, is produced from buffering of VFA. From each mol of VFA produced, the bicarbonate buffer releases about 1 mol of CO_2. Makkar (2005) indicates that a starch-rich feed produces only 40% of gas in a direct way, whereas the remaining 60% is produced indirectly from buffering of VFA. Wolin et al. (1960), and afterwards Blummel et al. (1999), indicated that GP and VFA are stoichiometrically related, so GP could be estimated from amount and proportion of VFA. However, Cone et al. (1998) specified that the stoichiometrical relationship did not fit for protein-rich feeds. According to this, Cone and Van Gelder (1999) observed that each percentage of protein caused a reduction in GP of 2.48 ml and that the stoichiometry was altered for protein-rich feeds, and attributed these results to the fact that protein fermentation produces NH_3, which has an inhibitory effect on the release of indirect gas. These findings were confirmed by Schofield (2000).

Figure 2. Release of gas from rumen fermentation.



1.3.3. Gas production techniques: description and shortcomings

The first GP equipment consisted in batch cultures and were introduced by McBee (1953) and Hungate (1966). Afterwards, other equipments have been developed, consisting in open vessels equipped with a water manometer (Trei et al., 1970; Jouany and Thivend, 1986; Beuvink and Spoelstra, 1992; Beuvink et al., 1992; Waghorn and Stafford, 1993) or in glass syringes equipped with a plunger (Czerkawski and Breckenridge, 1975; Menke et al., 1979; Blümmel and Ørskov, 1993), in which GP was determined by measuring the displacement of the water or of the plunger at regular time intervals. The main shortcoming of these systems is that their accuracy depends on the accuracy of reading of gas volumes inside the syringes and, moreover, they require a heavy work for manual manipulation of syringes (Cone et al., 1996). Wilkins (1974) described a different approach, in which feed samples were incubated in closed vessels, and GP was determined using a pressure transducer which measured the accumulation of pressure in the vessel headspace. This equipment was subsequently developed by Others (Pell and Schofield, 1993; Cone et al., 1996;

Mauricio et al., 1999; Davies et al., 2000). Pell and Schofield (1993) proposed a system consisting in closed bottles in which gas is accumulated in the bottle headspace and pressure is measured semi-automatically every hour. With this system, GP is calculated from pressure changes inside the bottles. The system proposed by Pell and Schofield (1993) is not vented, so the gas is left to accumulate inside the bottles and headspace pressure is recorded at fixed times. Afterwards, Theodorou et al. (1994) introduced a system similar to that proposed by Wilkins (1974), in which headspace pressure is measured manually and GP is calculated as the amount of gas needed to be released to restore atmospheric pressure inside the bottles. The main innovation introduced by this system, compared to that of Pell and Schofield (1993), is that the gas accumulated in the bottle headspace is vented at fixed times (usually at 2, 4, 8, 12, 24, 48 and 72 h of incubation). This device was introduced as the lack of venting in the system of Pell and Schofield (1993) could lead to an underestimation of GP measures, because: i) with highly and rapidly fermentable feeds (i.e. concentrates), high pressures can be generated inside the system, which could disturb and reduce the microbial activity of rumen fluid; ii) a given proportion of gas could remain dissolved in the inoculum without being released (Theodorou et al., 1998). Despite of these arguments, several Authors (Mertens and Weimer, 1998; Moss et al., 1998; Pell et al., 1998) did not observe a significant reduction of GP measures using Pell and Schofield (1993) system. Moreover, Pell et al. (1998) argued that the reach of high pressures inside the GP system could be prevented by increasing headspace of vessels or by reducing the amount of substrate incubated. A shortcoming of the system proposed by Theodorou et al. (1994) is represented by potential errors due to the manual measure of GP. Therefore, more recently other Authors (Cone et al., 1996; Mauricio et al., 1999; Davies et al., 2000) introduced fully-automated systems, which released gas accumulating in the headspace with a higher frequency compared to the manual systems. More recently, an innovative GP system has been developed (Ankom Technology®, Macedon, NY, USA), which consists in a kit of bottles equipped with a pressure detector and wireless connected to a PC. Pressure values are recorded at a set interval time and transmitted to the PC, and gas accumulating in the headspace of bottles is automatically released by an open-closed valve when a set threshold pressure has been reached. This instrument, which allows to measure also feed degradability, is very innovative and the development of a draft protocol is still not complete.

2. Feed evaluation techniques: effect of quality of rumen fluid

As outlined in the previous subchapters, many different factors can potentially influence or alter digestibility and GP measures. Each technique has its own limitations and weakness points, and all the different sources of variation must to be taken in account. However, it is largely recognized that a common source of variation of *in vivo, in situ* and *in vitro* techniques is represented by the quality of rumen fluid (Mould et al., 2005). Literature indicates that the quality of rumen fluid, in terms of number and kind of microbial population, is mostly influenced by diet composition (Bryant and Burkey, 1953; Makir and Foster, 1957) and by feeding frequency (Thorley et al., 1968). *In vivo* and *in situ* trials should be preferably conducted in animals consuming the feeds or the diets of interest, to limit the diet effects and to achieve a rumen fluid "ideal" in terms of microbial population (Vanzant et al., 1998; Kitessa et al., 1999). As confirmation of that, some Authors (Lindberg, 1981; Cronje, 1992) noted that *in situ* digestion of forages was higher in rumens of animals usually fed forage-based diets compared to that of animals receiving high-concentrate diets. Among forage diets, the kind of forage offered to the animals was found to influence rate and extent of *in situ* digestion (Van Keuren and Heinemann, 1962; Hopson et al., 1963; Vanzant et al., 1996). Despite of these shortcomings, the great advantage of *in situ* technique, compared to *in vitro* techniques, is that this method digests feeds in the actual rumen environment. However, as previously mentioned, the adoption of *in situ* techniques is declining for ethical and moral issues related to animal welfare (Adesogan, 2002). Currently, it is becoming increasingly difficult for research centres to obtain the licenses required to surgically prepare the animals and, as consequence, many laboratories are moving toward *in vitro* techniques which use rumen fluid collected from intact animals. When rumen fluid is taken from intact cows, the greatest challenge is to achieve a representative sample in terms of microbial population and concentration (Mould et al., 2005). It is largely accepted that a correct sampling procedure should avoid oxygen and saliva contamination of rumen fluid (Mould et al., 2005). Saliva contamination could modify pH and alter fermentation patterns, as indicated by Raun and Burroughs (1962), whereas oxygen contamination could reduce or completely inhibit activity and growth of anaerobic rumen microorganisms (Hungate, 1966). Similarly to what observed *in situ*, literature reports that *in vitro* digestibility of forages decreased when rumen fluid was collected by donor animals consuming high-concentrate diets (Russell and Wilson, 1996; Tejido et al., 2002; Vargas et al., 2009). To this regard, some Authors (Bryant and Burkey, 1953; Makir and Foster, 1957) observed that high-concentrate diets significantly affected microbial count of rumen fluid. Differently from what reported for digestibility values, the diet fed to donor animals was found to exert small effects on GP kinetics (Schöner et al., 1981; Menke and Steingass, 1988; Cone et al., 1996; Nagadi et al., 2000). Nagadi et al. (2000) argued that the real challenge should be

feeding diets which allow to attain the minimal microbial activity to ensure GP. Cone et al. (1996) found that microbial activity of rumen fluid was significantly affected by the timing of collection, as they noted a greater microbial activity in rumen fluid collected after feeding. Menke and Steingass (1988) hypothesized that the minimum microbial activity should be ensured by using rumen fluid collected from animals fed within the previous 16 h. However, the same Authors suggested to use rumen fluid collected before feeding, as it could have a less variable composition compared to that collected after feeding.

3. Possible applications of feed evaluation techniques

Literature reports that feed evaluation techniques (*in vivo*, *in situ* and *in vitro*) can be employed in several applications, as: i) the prediction of voluntary dry matter intake (Ørskov et al., 1988; Van Soest, 1994; Blummel et al., 1997); ii) the study of the effects of lipids on microbial activity and rumen fermentation (Chalupa et al., 1986; Ferguson et al., 1990; Michalet-Doreau et al., 1993; Getachew et al., 2001); iii) the study of the effects of different additives on rumen fermentation (Hino et al., 1993; Naziroğlu et al., 2002; Cardozo et al., 2004; Busquet et al., 2006; Alexander et al., 2008); iv) the evaluation of associative effects between ruminant feeds (Rosales et al., 1998; Liu et al., 2002; Sandoval-Castro et al., 2002). However, the main efforts are currently focused on the accurate estimation of energy value of feeds and on the determination of rumen nitrogen balance, in order to evaluate the efficiency of rumen fermentation process.

3.1. Estimation of feed energy value

The accurate evaluation of energy value of ruminant feeds represents a great challenge in order to formulate well-balanced diets, which can meet animal requirements and support their performance. Over the years, different energy systems have been developed for estimating energy content of ruminant feeds, and different equations have been proposed. Weiss et al. (1992) suggested to predict energy content of ruminant feeds from their chemical composition. The main shortcoming of this approach is that it is a theoretical method and it does not take in account neither the animal nor the feed characteristics (Kitessa et al., 1999). Alternative approaches proposed to estimate energy value of feeds from analytical data about chemical composition (crude protein, ether extract, ash content) and from:; i) the *in vivo* organic matter (OM) digestibility (INRA, 1988); ii) the *in situ* or *in vitro* NDF digestibility (NDFD) at 48 h of incubation (NRC, 2001); iii) the *in*

vitro NDFD at 30 h of incubation (Robinson et al., 2004); iv) the *in vitro* GP at 24 h of incubation (Menke and Steingass, 1988; Robinson et al., 2004). These approaches present significant differences, as they involve the use of values obtained from different procedures of analysis (*in vivo, in situ* or *in vitro*). The energy system proposed by INRA (1988) is complex, as the energy value of feeds is predicted using *in vivo* OM digestibility values which were established from a database composed by more than 300 feeds. The main US energy system (NRC, 2001) suggested to use NDFD measured after 48 h of incubation, but it is not totally clear what technique should be used for the determination (*in situ* or *in vitro*). To this regard, NRC (2001) simply indicated that *in vitro* techniques should be preferred as more standardized. Also the predictive equations using GP measures (Menke and Steingass, 1988; Robinson et al., 2004) are based on different approaches. The European system (Menke and Steingass, 1988; Givens et al., 1989) proposed equations in which the energy value of single nutrients is determined from *in vivo* digestibility studies with sheep. Differently, the North-American system (Robinson et al., 2004) developed more general and summative equations in which the energy contribution of each nutrient is computed in relation to the total energy content of feed. Moreover, the two systems attributed to GP a different energy value. The equations based on *in situ* or *in vitro* NDFD (NRC, 2001) usually consider 48 h as reference incubation time, whereas those based on *in vitro* GP use 24 h (Menke and Steingass, 1988; Robinson et al., 2004). Currently, there is still a debate about what incubation time (24 or 48 h) allows to achieve the most accurate predictions of feed energy value. In fact, 24 h appears a time closer to the retention time of feeds in the rumen, whereas the adoption of a longer incubation time (48 h) can allow to achieve more accurate and more repeatable predictions, especially for those feeds, as roughages, which are characterized by slower and more variable fermentation rates. Recently, some Authors (Robinson et al., 2004; Spanghero et al., 2010) evaluated the possibility to adopt an intermediate incubation time (30 h) for the estimation of in *vitro* NDFD. Spanghero et al. (2010) found that predictions of feed energy content using NDFD at 30 h were less repeatable and reproducible compared to those using NDFD at 48 h, whereas Robinson et al. (2004) found that the use of *in vitro* NDFD measures at 30 h provided predictions better correlated with *in vivo* digestibility, compared to those at 48 h.

3.2. Determination of rumen nitrogen balance and evaluation of efficiency of fermentation process

As described above, when a feed is degraded in the rumen, the degraded matter is partitioned to yield microbial protein, volatile fatty acids (VFA) and gases. The sum of microbial protein and VFA represents the amount of feed energy which can be potentially used by the animals to meet their requirements, whereas gases are the proportion of energy which is lost by the animals. The accurate estimation of microbial protein synthesized in the rumen represents a great challenge in animal feeding, as it is the most important protein source for ruminants (Beever, 1993; Leng, 1993). Blümmel et al. (1997) have demonstrated that the combination of *in vitro* degradability and GP measures can provide important information about the partition of feed energy in the rumen, and can allow to estimate the *in vitro* microbial protein. As suggested by Blummel et al. (1997), the amount of microbial protein synthesized *in vitro* can be calculated as the ratio between the total amount (mg) of substrate degraded (TSD) and the GP (ml) adjusted for a stoichiometric factor, to take in account for VFA production. This allows to estimate the efficiency of microbial protein synthesis (EMPS), as ratio between the microbial protein (mg) and the TSD (mg). The accurate estimation of EMPS is crucial in animal nutrition, and several ruminant feeding systems (NRC, 1996, 2001; Offer et al., 2002) include EMPS to predict animal performance. Blummel et al. (1999) obtained a satisfactory correlation between EMPS estimated *in vitro* from GP and *in vivo* from the urinary excretion of allantoin. However, there is still a debate about what incubation time allows to achieve the most accurate estimates of microbial protein and EMPS. Several Authors (Blummel et al., 1997; Blummel et al., 1998; Makkar and Becker, 1999; Getachew et al., 2000) noted that GP and VFA production were inversely related to the amount of microbial protein, and that at increasing incubation times GP and VFA production increased, whereas the microbial protein declined, as reflection of microbial lysis. Therefore, Blummel et al. (1999) suggested to measure the microbial protein at the time at which half of asymptotic GP is produced (defined as "t½"), as it can be supposed that microbial activity is maximum and microbial lysis is minimum at this time. This incubation time was subsequently adopted by Grings et al. (2005), which found a good correlation between EMPS estimated *in vitro* and *in vivo*. However, further research is needed as other findings evidenced that the accurate prediction of EMPS is affected by animal factors (AFRC, 1993), by feed characteristics (Hespell and Bryant, 1979) and by the level of synchronization in the rumen fermentation of N and carbohydrate sources (Sinclair et al., 1993).

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CHAPTER 2

General aims

The general aim of the present thesis is the study of rumen fermentation processes using the *in situ* and the *in vitro* digestibility techniques and the *in vitro* gas production technique. Five experimental contributes will be presented, four of methodological nature (Chapters 3, 4, 5 and 6) and one of applicative nature (Chapter 7). In detail, the aim of Chapter 3 was to investigate the effect of feed sample size on digestibility values provided by two different *in vitro* techniques. The aims of Chapter 4 were: i) to compare the digestibility values achieved *in situ* and *in vitro* using two different kinds of bag; ii) to compare digestibility values obtained *in situ* with those achieved *in vitro* using rumen fluid collected from intact cows. The aims of Chapter 5 were: i) to compare energy value of feeds estimated using equations based on *in situ* digestibility at 48 h or *in vitro* gas production at 24 h; ii) to compare *in situ* digestibility values measured at 24 and 48 h of incubation. The aim of Chapter 6 was to compare the effect of two venting procedures on the GP kinetics provided by two feeds with a different digestibility. Finally, the aim of Chapter 7 was to evaluate the effect of increasing dosages of two additives with antioxidant properties on some *in vitro* rumen parameters provided by two feeds.

CHAPTER 3

In vitro rumen feed degradability assessed with Daisy^{II} and batch culture: effect of sample size

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1. Abstract

In vitro digestibility with Daisy^{II} (D) equipment is commonly performed with 0.5 g of feed sample into each filter bag. Literature reported that a reduction of the ratio of sample size to bag surface could facilitate the release of soluble or fine particulate. A reduction of sample size to 0.25 g could improve the correlation between the measurements provided by D and the conventional batch culture (BC). This hypothesis was screened by analysing the results of 2 trials. In trial 1, 7 feeds were incubated for 48 h with rumen fluid (3 runs x 4 replications) both with D (0.5 g/bag) and BC; the regressions between the mean values provided for the various feeds in each run by the 2 methods either for NDF (NDFD) and *in vitro* true DM (IVTDMD) digestibility, had R^2 of 0.75 and 0.92 and RSD of 10.9 and 4.8%, respectively. In trial 2, 4 feeds were incubated (2 runs x 8 replications) with D (0.25 g/bag) and BC; the corresponding regressions for NDFD and IVTDMD showed R^2 of 0.94 and 0.98 and RSD of 3.0 and 1.3%, respectively. A sample size of 0.25 g improved the precision of the measurements obtained with D.

Abbreviations: NDFD, NDF digestibility; IVTDMD, *in vitro* true dry matter digestibility; BC, batch culture.

Key Words: *In vitro* techniques; Rumen degradability; Feeds; Daisy^{II}

2. Introduction

Daisy^{II} (D) is an equipment for analyzing DM and neutral detergent fiber *in vitro* digestibility (NDFD). The technique entails digesting several feed samples in filter bags within glass jars which are rotated in insulated chamber. The amount of feed sample commonly introduced in the filter bags is 0.50 g (Holden, 1999; Mabjeesh et al., 2000), but Others preferred 0.25 g (Robinson et al., 1999; Spanghero et al., 2003). Damiran et al. (2008) found significant differences of digestibility due to sample size of 0.25 and 0.50 g/bag. It can be possible that a lower sample size could facilitate the release of soluble and fine particulate, while a larger sample size can exert a barrier effect, occluding the bags pores and limiting the rumen fluid passage. This work was aimed to evaluate what sample size (0.25 or 0.50 g/bag) allows to achieve a better correlation between the digestibility values obtained with the D equipment and with a conventional batch culture technique (BC; Goering and van Soest, 1970).

3. Material and methods

The results of 2 previously conducted trials were analyzed. In trial 1, 7 feeds (corn meal, soybean meal, dry sugar beet pulp, corn silage, alfalfa hay, grass hay and wheat straw), milled at 1 mm, were simultaneously incubated (3 incubation runs x 4 replications) for 48 h at 39°C both with D (0.50 g feed/filter bag) and BC (0.50 g feed/bottle), using the same rumen fluid collected from 3 donor cows and a buffer solution. Standard filter bags were used (F57; 4.3x4.8 cm; Ankom). Similarly, in trial 2, 4 feeds samples (concentrate mix; two different corn silages; alfalfa hay) were incubated (2 incubation runs x 8 replications) both with D (0.25 g feed/filter bag) and BC (0.50 g feed/bottle). At the end of each incubation, the residuals in the filter bags were analyzed for NDF content with Ankom^{220} system while the residuals in the BC were filtered in gooch and analyzed with a fibertech analyser (Goering and van Soest, 1970). The *in vitro* true DM digestibility was computed as IVTDMD = 100*[(DMfeed–NDFres)/ DMfeed)], where NDFres was the residual NDF after incubation, and DMfeed was the amount of DM incubated. Data of each trial, either for NDFD and IVTDMD, were subjected to ANOVA using two models in which: i) the effect of technique was evaluated for each single feed; ii) the effect of technique was evaluated considering the various feeds as source of variation. The root of MSE (RMSE) and the coefficient of variations (CV) were used as precision indexes. The mean values of digestibility obtained with D equipment for each feed, in each trial and in each run, were compared by regression with the values obtained with BC (trial 1: 21 pairs of values; trial 2: 8 pairs of values).

Table 1. LS means and variability parameters for NDF (NDFD, % NDF) and *in vitro* true DM digestibility (IVTDMD, % DM) obtained with the Daisy^{II} (D) and the batch culture (BC) techniques in trial 1 and 2.

Technique	NDFD			IVTDMD		
	Daisy^{II}	BC	RMSE^2	Daisy^{II}	BC	RMSE^2
	Mean±SD^1	Mean±SD^1		Mean+SD^1	Mean+SD^1	
Trial 1 (0.50 g/filter bag)^4:						
Corn meal	63.5±12.3	81.4±4.5	11.3**	96.6±1.3	98.2±0.4	3.2**
Soybean meal	99.1±2.1	93.5±2.0	2.1*	100.0±0.6	99.1±0.3	0.6*
Dry sugar beet pulp	76.3±6.0	89.7±1.1	5.5**	87.9±3.3	94.7±0.5	3.1**
Corn silage	38.1±7.4	63.5±1.3	6.8**	73.0±3.5	84.0±0.6	3.2**
Alfalfa hay	48.1±3.3	51.7±2.6	3.2**	76.9±1.8	78.6±1.1	2.3**
Grass hay	46.3±3.6	61.9±2.9	3.4**	69.6±2.2	78.4±1.6	2.2**
Wheat straw	31.4±3.9	53.1±2.0	3.6**	46.4±3.4	63.4±1.6	3.2**
RMSE	6.4	2.6		2.6	1.2	
CV^3	11.1	3.7		3.3	1.0	
Trial 2 (0.25 g/filter bag)^5:						
Concentrate mix	71.3±4.4	75.9±4.4	4.4**	92.9±1.1	94.1±1.1	1.1**
Corn silage 1	62.4±3.0	65.6±1.9	2.5**	80.6±1.5	82.3±1.3	1.4**
Corn silage 2	57.5±1.6	60.4±3.4	3.4**	74.6±1.0	76.4±2.0	1.6**
Alfalfa hay	42.1±2.3	44.0±4.0	3.7	73.5±1.1	74.4±1.7	1.4
RMSE	3.0	3.6		1.2	2.0	
CV^3	5.1	5.9		1.5	1.6	

Data within row for NDFD or IVTDMD significantly differed (**P<0.01; *P<0.05). ^{1}SD=standard deviation; ^{2}RMSE=root of MSE; ^{3}CV= coefficient of variation. ^{4}Each value is a mean of 12 measurements (3 runs x 4 replications); ^{5}Each value is a mean of 16 measurements (2 runs x 8 replications).

4. Results and discussion

In trial 1 (0.5 g feed/bag), the values of NDF digestibility (NDFD) and IVTDMD obtained with D were significantly lower and less repeatable with respect to those obtained with BC (Table 1). The relationships for the NDFD and IVTDMD measurements provided by D (y) and BC (x) were: y = -20.4 + 1.10x (R^2= 0.75; RSD=10.9%) and y = -27.7 + 1.25x (R^2= 0.92; RSD=4.8%), respectively. The CV obtained with the D technique, both for NDFD and IVTDMD, were markedly higher than the corresponding CV achieved with BC. With respect to trial 1, in trial 2 (0.25 g feed/filter bag), the digestibility values obtained with the 2 techniques were much more similar both in term of mean values and CV (NDFD CV=5.14 and 5.85%, IVTDMD CV=1.46 and 1.59%, with D and BC, respectively). In trial 2 the relationships between the NDFD and IVTDMD measurements provided by D (y) and by BC (x) were: y = 3.6 + 0.89x (R^2= 0.99; RSD =3.0%) and y = -1.15 + 0.99x (R^2= 0.98; RSD =1.3%), respectively. Result of this screening analysis indicated that the reduction of the sample size from 0.50 to 0.25 g of feed sample/bag (corresponding to 12 and 6 mg/cm^2 of bag surface) with the D allowed to achieve estimates of NDFD and IVTDMD more correlated to those provided by BC and less variable. This good agreement can be useful to

exploit the advantage of each technique: D allows the simultaneous incubation of a large number of samples, giving benefit in term of labour and cost per determination, while BC gives the possibility of measuring not only the disappearance degree of substances but also the product of fermentations, such as volatile fatty acids and gas production.

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CHAPTER 4

Running title: *In situ* and *in vitro* rumen techniques

True dry matter digestibility of feeds evaluated *in situ* with different bags and *in vitro* using rumen fluid collected from intact donor cows

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1. Abstract

Many methods for determining dry matter digestibility are available, but studies for improving their precision and accuracy, for reducing the cost and for limiting the use of surgically modified animals are required. This study was designed: i) to compare the true dry matter digestibility (TDMD) values achieved with commercially available synthetic filter bags or with traditional nylon bags *in vitro* and *in situ*; ii) to compare the TDMD values measured *in situ* with those achieved *in vitro* using rumen fluid collected by oro-ruminal suction from intact donor cows. Four methods were compared: *in situ* with nylon bags (IS-nylon) or filter bags (IS-filter), *in vitro* with conventional small individual bottles (CB) or with the Daisy^{II} incubator using rumen fluid collected via oro-ruminal suction from intact donor cows. For each method 11 feeds were incubated for 48 h with 8 values for each feed from two separate incubations. Repeatability and reproducibility of methods were computed following official standardized procedures. The reproducibility coefficient (an index of agreement between two single measures made in separate incubations with a specific method on the same feed) of the TDMD measurements was 97.9, 95.1, 98.8 and 96.0 % for IS-nylon, IS-filter, CB, and Daisy^{II}, respectively. The main relationships between TDMD values (g/kg DM) were: IS-nylon = 0.86×IS-filter + 174 (R^2=0.97; root of MSE= 22); IS-nylon = 1.02×CB (R^2=0.90, root of MSE =42); IS-nylon=0.94×Daisy^{II} + 101 (R^2=0.93, root of MSE = 38); IS-filter = 0.98×Daisy^{II} (R^2= 0.95, root of MSE = 34). *In situ* and *in vitro* filter bags induced a predictable underestimation of the TDMD values compared to IS-nylon bags and to CB, respectively. Nylon bags could be replaced by filter bags by adjusting the measurements for the systematic error. The replacement of nylon by filter bags *in situ* could simplify the procedure of analysis, with less manipulation of the residuals of fermentation and less labour, and hence it could allow to increase the number of samples simultaneously analyzed. The direct proportionality between the TDMD values obtained *in situ* and *in vitro* with different techniques (ISnylon vs. CB and IS-filter vs. Daisy^{II}) indicates that rumen fluid collected from intact cows can produce *in vitro* estimates of TDMD at 48 h similar to those obtained *in situ*. The use of fistulated cows as donors of rumen fluid for *in vitro* tests does not seem to be necessary. This is of interest for many research centers for ethical and public concerns related to the use of surgically treated animals.

Abbreviations: IS, *in situ*; CB, conventional bottles; DM, dry matter; CP, crude protein; EE, ether extract; aNDF, neutral detergent fibre; NDFD, aNDF digestibility; TDMD, true dry matter

digestibility; NDICP, neutral detergent insoluble crude protein; ADICP, acid detergent insoluble crude protein; RT, repeatability; RD, reproducibility.

Keywords: *In situ* digestibility, *In vitro* digestibility, Nylon bags, Filter bags, Fistulated cows, Repeatability.

2. Introduction

Feed digestibility and energy content are commonly assessed using chemical assays, *in vivo*, *in situ* and *in vitro* methods. *In vivo* experiments are very expensive, and often inaccurate for evaluating feeds that cannot be the sole ingredient of the diet (Schofield, 2000). The energy content of feeds is more commonly estimated from feed chemical composition (Weiss, 1993) and feed digestibility after 48 h of incubation *in situ* or *in vitro* (NRC, 2001).

The most common *in situ* technique (IS) uses nylon or dacron bags containing the feed samples which are placed and left into the rumen of cows for 48 h (Ørskov and McDonald, 1979). This technique is expensive, not suitable for routine analysis and criticized for the use of surgically treated animals. However it has the advantage that it uses the rumen environment to measure feed degradation and for this reason it is often the standard against which the *in vitro* methods are frequently compared (Kitessa et al., 1999). The replacement of nylon by commercially available, synthetic filter bags *in situ* could simplify the procedure of analysis, with less manipulation of the residuals of fermentation and less labour, and could allow an increase in the number of samples simultaneously analyzed.

In vitro techniques, such as those based on conventional tubes or bottles (CB), are popular (Tilley and Terry, 1963; Goering and Van Soest, 1970) as they permit the simultaneous analysis of a large number of samples (Makkar, 2005) and they can also be equipped with devices for measuring the kinetics of gas production (Tagliapietra et al., 2010a). To better account for post-ruminal digestion, Van Soest et al. (1966) introduced a technique, which measures the *in vitro* true digestibility by replacing the acid-pepsin step of the Tilley and Terry (1963) method with a neutral detergent digestion step. This method is used as reference for evaluating the energy value of the feeds by NRC (2001). With the *in vitro* Daisy^{II} system (Ankom Technology®, Macedon, NY, USA) up to 100 feed samples can be simultaneously incubated into synthetic filter bags placed in large jars with rumen fluid and buffer (Ammar et al., 1999; Adesogan, 2005).

The major criticisms about the use of the various *in vitro* techniques (CB, Daisy^{II}), compared to IS, are in regard to possible alterations of the fermentation properties of rumen fluid due to collection, manipulation, dilution with buffer and to the accumulation of end products during fermentations (Mould, 2003). The public opinion and the lawmakers arise concerns about the use of fistulated cows. These concerns could be reduced if the rumen fluid for *in vitro* test is collected from intact cows with suction techniques, avoiding the need of surgical operation. Surprisingly, no information about collection of rumen fluid with suction techniques is available in the literature. Goering and van Soest (1970) only indicated that rumen fluid should be "preferably" collected from fistulated animals.

The present study was designed: i) to compare the true dry matter digestibility (TDMD) values achieved with commercially available synthetic filter bags or with traditional nylon bags *in vitro* and *in situ*; ii) to compare the TDMD values measured *in situ* with those achieved *in vitro* using rumen fluid collected by oro-ruminal suction from intact donor cows.

3. Material and methods

3.1. Feeds

Eleven feeds were tested: wheat straw, meadow hay, 3 corn silages collected from different farms, 2 alfalfa hays, dry sugar beet pulp, a commercial mixed feed (composed by corn grain 25.9%, barley 19.3%, soybean meal 17.2%, wheat grain 15.4%, sugar beet pulp 14.2%, mineral mix 8.0%), corn grain and soybean meal. Corn silage samples were previously dried at 60 °C until constant weight. All the tests, including those performed *in situ*, were conducted with feed samples that had been ground by a hammer mill (Pullerisette 19, Fritsch GmbH, Laborgeratebau, D) with a screen size of 1 mm.

3.2. In situ *digestibility with nylon and filter bags*

The tests were completed following the procedures proposed by Ørskov and McDonald, (1979) and the indications proposed by Nocek, (1988) and Vanzant et al. (1998).

Two dry Holstein-Friesian fistulated cows (housed at the experimental farm of Department of Animal Science of the University of Milan) that had been fed meadow hay *ad libitum* and 2 kg/d of concentrate for 2 weeks were used. The feed samples were incubated in two separate periods of

incubation into the rumen of the two cows for 48 h, using simultaneously the two types of bags. For each kind of bag the experimental design was: 2 incubations × 2 cows × 11 feeds × 2 replications, giving a total of 8 values for each feed, plus sixteen empty bags as blanks (4 blanks/cow/bag type). The two types of bags used were: nylon bag (40 µm of pore size; 10.5 × 8 cm of net surface area) and synthetic filter bag (F57, Ankom Technology®, Macedon, NY, USA; which has been tested by ANKOM to retain particles with size >25 µm; 4.5 × 4.0 cm of net surface area). The ratio between the amount of feed sample introduced in each bag and the corresponding net surface area (excluding the tissue areas not in contact with the feed) was 14 mg/cm^2 (Nocek, 1988). Thus, the amount of sample introduced into the nylon and filter bags was 1.150±0.005 g and 0.250 ±0.001 g, respectively. Nylon and filter bags were bound to plastic strings and introduced into the rumen of the cows 2 hours after the morning feeding. After 48 h the bags were extracted from the rumen, washed in cold water and weakly centrifuged in a washing machine (2 minutes rinse + 2 minutes spin, repeated 5 times), stored at 4°C for about 2 h which was the time required for transferring the samples to the laboratory.

3.3. In vitro digestibility with conventional bottles

The tests were completed following the procedures proposed by Tilley and Terry (1963) modified by Goering and Van Soest (1970).

The buffer mineral solution, prepared according to Menke and Steingass (1988), was heated in a waterbath at 39°C and purged continuously with CO_2 for 30 minutes. Sodium sulphite was used as reducing agent and it was added (0.33 g/l solution) to the buffer solution (Menke and Steingass, 1988).

Rumen fluid was collected two hours after morning feeding from 3 dry Holstein-Friesian cows (housed at the experimental farm of the University of Padova, Italy) that were fed meadow hay *ad libitum* and 2 kg/d of concentrate for 2 weeks. Rumen fluid was collected using a vacuum pump connected to a glass vacuum container in turn connected to a semi-flexible oro-ruminal probe equipped with a steel strainer fixed on the top. The steel strainer (15 cm of length, 3.5 cm of diameter and 600 g of weight) had 32 holes of 0.5 cm of width. The vacuum pump was activated when the top of the probe was positioned in the rumen. The first 100 to 200 ml of rumen liquor collected from each cow was discarded to limit contamination with saliva (Raun and Burroughs, 1962). The pH of the rumen liquor was measured immediately after collection and only fluids with pH lower than 6.8 were kept for the tests. The rumen fluid was poured into two thermal flasks

preheated to 39.0 ± 0.5 °C and immediately transferred to the laboratory. At the laboratory the rumen fluid was filtered through 3 layers of cheesecloth to eliminate feed particles and mixed with the buffer mineral solution in a ratio 1 to 2. All the operations were conducted under anaerobic conditions by flushing carbon dioxide. The time required for all the operations was less than 30 minutes.

Two incubations were performed in two successive weeks. The experimental design was: 2 incubations × 11 feeds × 4 replications, giving a total of 8 values per feed, plus 8 bottles without feed sample as blanks. Each bottle (280 ml) was filled with 0.5000±0.0010 g of feed and equipped with a commercial system (Ankom RF gas production system, Ankom Technology®, Macedon, NY, USA) that allows the release of gas produced during the fermentation and maintains a low positive pressure in the bottle headspace avoiding the entrance of air (Tagliapietra et al., 2010). The bottles were preheated to 39 °C and filled with 75 ml of buffered rumen fluid under anaerobic conditions by keeping the bottle headspace continuously flushed with CO_2. The bottles were incubated in an air ventilated chamber at 39 °C. After 48 h of incubation the fermentation liquor was filtered into crucibles where the residue was collected and immediately analyzed.

3.4. In vitro digestibility with Daisy^{II} incubator

A Daisy^{II} incubator (Ankom Technology®, Macedon, NY, USA), consisting in a thermostatic chamber (39 °C) with 4 rotating jars, was used to determine *in vitro* NDF an true DM digestibility following the Tilley and Terry (1963) approach modified by Goering and Van Soest (1970) as described by Ammar et al. (1999).

Two incubations were performed in two successive weeks. The experimental design was: 2 incubations x 2 rotating jars × 11 feeds × 2 replications, giving a total of 8 values per feed, plus 8 empty bags as blanks. Buffer mineral solution, collection and manipulation of rumen liquor were performed as previously described for the CB test. The feed samples (0.2500 ± 0.0010 g/bag) were weighed into the filter bags (F57, Ankom Technology®, Macedon, NY, USA). The filter bags were placed into the jars filled with 2.4 l of buffered rumen fluid. After 48 h of incubation the bags were extracted from the jars, washed in cold water and immediately analyzed as described later.

3.5. Chemical analyses

All the chemical analyses were performed in the laboratory of the Department of Animal Science of Padova (Italy) by the same operator.

The feeds were analyzed in triplicate for dry matter (DM, AOAC method 934.01, 2003), crude protein (CP, AOAC method 976.05, 2003), ether extract (EE, AOAC method 920.29, 2003) and ash (method 942.05, AOAC, 2003). Neutral detergent fibre (aNDF) was determined (Mertens, 2002) with a treatment with α-amylase and sodium sulphite using the Ankom^{220} Fiber Analyzer (Ankom Technology®, Macedon, NY, USA). ADF and sulphuric acid lignin (Lignin sa) contents were determined sequentially after aNDF determination (Robertson and van Soest, 1981). The measurements of aNDF, ADF and $Lignin_{(sa)}$ were corrected by subtracting values obtained from blanks. The feeds were also analyzed for neutral detergent insoluble crude protein (NDICP) and acid detergent insoluble crude protein (ADICP) (Licitra et al., 1996). The content of metabolizable energy of feeds (ME, Mj/kg DM) was computed from the actual chemical composition following the lignin-based approach proposed by NRC (2001).

All the residuals of fermentation obtained from the *in situ* and *in vitro* incubations were analyzed for aNDF (Mertens, 2002) and corrected by subtracting values obtained for corresponding blanks.

The nylon bags were dried at 60 °C in an air ventilated incubator and weighed; the residual feed material was transferred from the bag to a weighed crucible (30 ml por. 2, Robu Glasfilter-Geräte GMBH®, Hattert, D) and treated as indicated for the aNDF analysis (Mertens, 2002) with a Fibertech Analyzer (VELP® Scientifica, Usmate, Milano, I). The transfer of the fermented feed sample from the bag to the crucible can result in incomplete collection of the feed residual. The amount of lost material was estimated by calculating the dry weight of residue remaining in the bags (as difference between the weights of the bag with the dry residue and the empty bags) and that collected in the crucible (as difference between the weights of the crucible with the dry residue minus that of the empty crucible). Therefore, the residual aNDF found in each crucible was adjusted for the DM losses computed for each bag. These losses accounted for 7.9 ±2.6 % of the dry matter contained in the nylon bags. A further adjustment for blank crucibles was applied.

Filter bags from *in situ* and *in vitro* Daisy^{II} incubations were analyzed using the Ankom^{220} Fiber Analyzer following the user's instruction (Ankom Technology, 2005), dried at 60 °C until constant weight and weighed. The removal of the feed residual from the bag was not applied with

this procedure, as only the dry weight of the bags before the incubation (empty bags) and after the aNDF treatment is required. Data were corrected for values obtained from blanks. .

The residual fluids of fermentation obtained from CB were directly transferred from the bottles to the crucibles and later analyzed for aNDF (Mertens, 2002) using the Fibertech Analyzer with a correction for blanks.

3.6. Computation of true dry matter digestibility

The aNDF digestibility (NDFD) and the true DM digestibility (TDMD) were computed using the following equations (Goering and Van Soest, 1970):

$$\text{NDFD (g/kg NDF)} = 1000 \times ((\text{aNDF}_{feed} - \text{aNDF}_{res}) / \text{aNDF}_{feed}) \quad (1)$$

$$\text{TDMD (g/kg DM)} = 1000 \times ((\text{DM}_{feed} - \text{aNDF}_{res}) / \text{DM}_{feed}) \quad (2)$$

where: aNDF_{feed} is the amount (g) of aNDF incubated, aNDF_{res} is the amount (g) of aNDF measured on the residue of fermentation, DM_{feed} is the amount (g) of DM incubated.

The TDMD values were also computed from the values of NDFD estimated from the chemical composition of feeds following the lignin-based approach proposed by NRC (2001) on page 14 (eq. 2-4e).

3.7. Statistical analysis

3.7.1. Repeatability and reproducibility of methods

Estimation of variance components was accomplished, separately for measures of NDFD or TDMD provided by different methods, using the mixed procedure of SAS (SAS Inst. Inc.) with two mixed linear models. In the first model the following sources of variation were considered as random effects: incubation (**I**), the feed (**F**), the interaction I×F and the error term ($\mathbf{e_1}$). Additional random effects included in the model were: the cow for the IS-nylon and the IS-filter methods and the jar for the Daisy^{II} incubations. However, as the proportion of variance explained by these factors was very low these effects were included in the error term. The restricted maximum likelihood method (REML) was used as the method of estimation of variance components. The components of variance of each factor, σ^2_I, σ^2_F, $\sigma^2_{I\times F}$ and σ^2_{e1}, were used to compute the repeatability (**RT**), defined as the value below which the absolute difference between two single measures obtained with the

same method and under the same conditions (same incubation, same feed) is expected with a 95% probability and coefficient of repeatability (RT%) (International Organization for Standardization, 1994a,b). These parameters were computed as (International Organization for Standardization, 1994a,b):

$$RT=2\sqrt{2\sigma^2_{e1}} \quad (3)$$

where RT is the repeatability and as:

$$RT\%=\frac{\sigma^2_I+\sigma^2_F+\sigma^2_{I\times F}}{\sigma^2_I+\sigma^2_F+\sigma^2_{I\times F}+\sigma^2_{e1}}\times 100 \quad (4)$$

where RT% is the coefficient of repeatability.

Reproducibility (**RD**) was defined as the value below which the absolute difference between two single measures obtained with the same method of analysis on the same feed in different incubations is expected within a 95% probability, and coefficient of reproducibility (RD%) (International Organization for Standardization, 1994a,b). In this case the components of variance of each method were estimated using the mixed procedure of SAS (SAS Inst. Inc.) running a second mixed linear model which considered only the feed and the residual error ($\mathbf{e_2}$) as random factors. The values of RD and RD% were computed as (International Organization for Standardization, 1994a,b):

$$RD=2\sqrt{2\sigma^2_{e2}} \quad (5)$$

and as:

$$RD\%=\frac{\sigma^2_F}{\sigma^2_F+\sigma^2_{e_2}}.\times 100 \quad (6)$$

3.7.2. Comparison among methods

From a preliminary analysis it was found that, according to the Bartlett's test (Bartlett, 1937) of the SAS (SAS Inst. Inc.), the variances associated to the various methods were not homoscedastic, and so use of ANOVA linear models to compare the effects due to the different methods was not applicable. Thus, the various methods were compared by linear regression of the mean values of TDMD data obtained for each feed. Significant differences of the slope and intercept from unity and zero, respectively, were tested using the regression procedure (proc reg) of

SAS (SAS Inst. Inc.). The root of the mean square error (RMSE) provided as output from the proc reg analysis of SAS (SAS Inst. Inc.) was considered as prediction error. When the intercept did not significantly differ from 0 it was forced through the origin.

4. Results

4.1. Chemical composition of feeds

The chemical composition (Table 1) was close to the NRC (2001) tabled data. Among feeds, the aNDF fraction ranged from 101 to 768 g/kg DM and the ME content computed from chemical composition (NRC, 2001) ranged from 7.3 to 15.5 MJ/kg DM.

Table 1. Chemical composition (g/kg DM) and ME content (MJ/kg DM) of feeds

Feed	DM	CP	EE	Ash	aNDF	ADF	$Lignin_{(sa)}$	ME[1]
Wheat straw	927	52	18	84	768	486	58	7.3
Meadow hay	883	85	16	75	600	341	39	9.2
Corn silage 1	948	71	18	42	514	271	35	9.8
Corn silage 2	937	67	13	44	596	336	47	8.9
Corn silage 3	908	83	20	34	433	233	12	11.2
Alfalfa hay 1	953	165	21	110	457	349	90	8.3
Alfalfa hay 2	916	197	31	109	437	314	77	9.2
Sugar beet pulp	910	92	7	43	502	264	33	10.7
Mixed feed	905	166	20	89	245	106	18	12.0
Soybean meal	898	482	18	64	135	81	-	15.5
Corn grain	900	98	37	14	101	19	-	15.2

[1] Metabolizable energy computed from actual chemical composition of feeds following the lignin-based approach suggested by NRC (2001).

4.2. Digestibility and repeatability

The values of NDFD (Table 2) achieved with IS-nylon bags and CB for the various feeds were on average numerically higher (overall mean 715 and 659 g/kg NDF) compared to those achieved with IS-filter bags and Daisy^{II} (overall mean 540 and 575 g/kg NDF, respectively). The IS methods based on nylon or filter bags showed a repeatability of 119 and 174 g/kg aNDF, respectively, the repeatability of Daisy^{II} was intermediate (135 g/kg aNDF) and that of CB was good as indicated by the low values of RT (66 g/kg aNDF). The reproducibility values were in general similar or slightly higher than the corresponding values of repeatability.

The TDMD values provided by IS-nylon bags were always numerically higher compared to those provided by the IS-filter bag technique (847 and 778 g/kg DM respectively). With respect to IS-nylon bags, IS-filter bag showed slightly less repeatability. The TDMD values provided by CB were in general close to those obtained with IS-nylon bags and the overall means were 834 and 847 g/kg DM, respectively (Table 2). With respect to the other techniques, CB showed the best repeatability, being the RT value only 24 g/kg DM. The TDMD values obtained by Daisy^{II}, which uses the filter bags, were always lower compared to those achieved from IS-nylon bags, but similar to those achieved with the IS-filter bag technique (Table 2). Daisy^{II} and IS-filter bag also provided similar reproducibility, as the RD values were within the range from 79 to 65 g/kg, respectively.

Table 2. *In situ* and *in vitro* aNDF (NDFD) and true DM (TDMD) digestibility at 48 h (mean ± SD).

	NDFD, g/kg aNDF				TDMD, g/kg DM			
	In situ		*In vitro*		*In situ*		*In vitro*	
Feed[1]	Nylon bag	Filter bag	CB[2]	Daisy[II]	Nylon bag	Filter bag	CB	Daisy[II]
Wheat straw	473 ±21	351 ±22	511 ±8	371 ±74	595 ±16	502 ±17	625 ±6	517 ±57
Meadow hay	503 ±27	359 ±62	628 ±24	491 ±47	702 ±16	616 ±37	777 ±14	695 ±28
Corn silage 1	723 ±10	512 ±39	637 ±20	618 ±40	857 ±5	749 ±20	813 ±10	804 ±21
Corn silage 2	591 ±82	492 ±27	586 ±34	585 ±12	756 ±49	697 ±16	753 ±20	752 ±7
Corn silage 3	588 ±42	408 ±51	633 ±9	389 ±48	822 ±18	743 ±22	841 ±4	735 ±21
Alfalfa hay 1	566 ±17	386 ±50	414 ±31	422 ±28	802 ±8	719 ±23	732 ±14	736 ±13
Alfalfa hay 2	624 ±31	445 ±100	506 ±15	480 ±29	835 ±14	757 ±44	784 ±7	773 ±12
Sugar beet pulp	1000 ±1	830 ±17	894 ±14	761 ±56	1000 ±1	915 ±9	947 ±7	880 ±28
Mixed feed	800 ±110	631 ±48	706 ±70	703 ±57	951 ±27	910 ±12	928 ±17	927 ±14
Soybean meal	1000 ±1	959 ±86	935 ±5	932 ±25	1000 ±1	994 ±12	994 ±1	991 ±3
Corn grain	1000 ±1	572 ±162	802 ±22	572 ±86	1000 ±1	957 ±16	981 ±2	957 ±9
Mean	715	540	659	575	847	778	834	797
Repeatability, RT[3]:	119	174	66	135	50	44	24	57
RT%	95.9	91.3	98.0	92.8	98.2	99.0	99.5	97.8
Reproducibility, RD[3]:	128	206	92	142	55	65	35	79
RD%	95.2	87.7	96.1	92.1	97.9	95.1	98.8	96.0

[1] Each value is the mean of 8 values;[2] Conventional bottles;

[3] Repeatability: $RT = 2\sqrt{2\sigma^2_{e1}}$ and $RT\% = \frac{\sigma^2_I + \sigma^2_F + \sigma^2_{I\times F}}{\sigma^2_I + \sigma^2_F + \sigma^2_{I\times F} + \sigma^2_{e1}} \times 100$; Reproducibility: $RD = 2\sqrt{2\sigma^2_{e2}}$ and $RD\% = \frac{\sigma^2_F}{\sigma^2_F + \sigma^2_{e2}} .\times 100$

where: σ^2_I, σ^2_F, $\sigma^2_{I\times F}$ and σ^2_{e1} σ^2_{e2} are variance components for incubation (I, n=2), feed (F, n=11), incubation × feed, e_1 is the error term for RT and e_2 is the error term for RD.

4.3. Relationships between TDMD values achieved with different methods

The TDMD data provided by the two IS techniques were highly correlated (Table 3). The regression between the IS-nylon bag and the IS-filter bags data showed a slope (P=0.02) and an intercept (P<0.01) significantly different from unity and zero, respectively; the RMSE was only 22 g/kg and the CV was 2.6%. The regression between IS-nylon bags and CB had a slope not different from the unity (1.09; P=0.47) and an intercept not different from zero (-61 g/kg DM; P=0.56). Forcing the intercept to zero the slope became 1.02, the RMSE was 42 g/kg DM and the CV was 5.0%. The relationships between the TDMD values achieved with IS-nylon bags and Daisy^{II} showed, as observed for IS-nylon vs. IS-filter bag, a slope lower than one (0.94), an intercept of 101 g/kg DM (P=0.19) and a RMSE of 38 g/kg DM. When the intercept was forced to zero the slope became 1.06 and differed from the unity (P<0.01). The relationships obtained using the CB-TDMD values as dependent variable and the IS-filter bag or the Daisy^{II} TDMD values as independent variables were similar to those where the IS-nylon bags data were used as predicted variable. Likewise, the TDMD values calculated from IS filter bags were linearly related with the TDMD values from Daisy^{II} filter bags with a slope of 1.09 (P=0.26) and an intercept of -89 g/kg DM (P=0.17). Forcing the intercept to the origin the slope became close to the unity (0.98) and the RMSE was increased only from 32 to 34 g/kg DM. The results for the IS-nylon bag TDMD data regression against the TDMD values achieved from the sole chemical analysis using the lignin based approach suggested by NRC (2001) are also shown in table 3 for comparison. This relationship showed slope higher than 1, a low R^2 and a high RMSE.

Table 3. Relationships between TDMD values (g/kg DM) estimated using different methods[1].

Methods[2,3]		Equation	SE (*P-value*)		R^2	RMSE[5]	CV[6] %
Dependent (y)	Independent (x)		Slope	Intercept			
IS-nylon bag	IS-filter bag	y=0.86x + 174	0.05 (0.02)	37 (<0.01)	0.97	22	2.6
"	CB [4]	y=1.02x	0.02 (0.27)	-	0.90	42	5.0
"	$Daisy^{II}$	y=0.94x + 101	0.09 (0.49)	72 (0.19)	0.93	38	4.5
"	$Daisy^{II}$ [4]	y=1.06x	0.01 (<0.01)	-	0.90	40	4.7
CB	IS-filter bag	y=0.73x + 262	0.07 (<0.01)	56 (<0.01)	0.92	34	4.1
"	$Daisy^{II}$	y=0.81x + 190	0.09 (0.06)	71 (0.03)	0.90	38	4.6
IS-filter bag	$Daisy^{II}$ [4]	y=0.98x	0.01 (0.13)	-	0.95	34	4.4
IS-nylon bag	Chem. analysis	y=1.14x – 88	0.23 (0.88)	181 (0.58)	0.69	82	9.7

[1] Eleven pair of observations (n.=11), each observation is the mean of 8 measurements.
[2] IS-nylon bag: TDMD values of feeds incubated *in situ* for 48 h into nylon bags; IS-filter bag: TDMD values of feeds incubated *in situ* for 48 h into filter bags; CB: TDMD values of feeds incubated *in vitro* 48 h in conventional bottles (without use of bags); $Daisy^{II}$: TDMD values of feed incubated *in vitro* for 48 h using filter bags; Chem. analysis: TDMD values computed from actual chemical analysis of feeds following the lignin-based approach proposed by NRC (2001).
[3] All the *in vitro* techniques were performed using rumen fluid collected by oro-ruminal suction from intact cows; [4] Equation obtained forcing the intercept to zero; [5] Root of the mean square error.
[6] Coefficient of variation.

5. Discussion

5.1. General considerations

Ideally, *in situ* and *in vitro* methods for evaluating feed digestibility should be tested against *in vivo* measurements but these also have methodological deficiencies (White and Ashes, 1999; Mould, 2003; Damiran et al., 2008). The experiments which compare feed digestibility values achieved using different methods presume that estimates determined *in vivo* are accurate and that errors in the prediction are due to errors in the alternative techniques. Clearly these assumptions cannot be correct and the extent of the resulting error cannot be determined (Robinson et al., 2004).

The *in situ* method based on nylon bags has been generally found to provide a good comparison with *in vivo* measurements even though it is notoriously difficult to standardize and hence it is often plagued by a low reproducibility and repeatability (Kitessa et al., 1999). A number of parameters can affect *in situ* digestion and the major are: bag porosity, sample particle size, sample size to bag surface ratio (Vanzant et al., 1998), physical nature of the feed (Cozzi et al., 1993; Ramanzin et al., 1994), dietary effects, associative effects (Tagliapietra et al., 2010b), animal effects (Calabrò et al., 2004) and operating procedures (Huntington and Givens, 1997; Michalet-Doreau and Ould-Bah, 1992). In spite of these shortcomings, the IS-nylon bag method has the advantage over *in vitro* methods in that it uses

the rumen environment to measure feed degradation, thus it is one of the preferred methods for some feeding systems and it is commonly considered the standard against which the *in vitro* methods are compared (Kitessa et al., 1999).

In this trial the repeatability achieved for the IS-nylon bag TDMD measurement (50 g/kg), if expressed in term of residual standard deviation ($\sqrt{\sigma^2_{e1}}$=18 g/kg) was good and comparable with those found by others (see the review of Kitessa et al., 1999). At least, in part, this value might also have been influenced by the manual transfer of feed samples from the bags to crucibles, required for the aNDF analysis, as this transfer was associated to a 7.9 ± 2.6% (30 ±11 mg) of DM losses. It should be noticed that with CB, where the whole content of the bottles was directly filtered in the crucibles, the data were much more repeatable.

The repeatability and reproducibility values obtained with CB (24 and 35 g/kg, respectively) were comparable, when expressed in terms of residual standard deviation ($\sqrt{\sigma^2_{e1}}$=8.6 and $\sqrt{\sigma^2_{e2}}$=12.3 g/kg) to those observed for the rumen liquor-pepsin method, where the residual standard deviations between replicates within an incubation and between incubations were ±6.6 and ±11.8 g/kg of apparent DM digestibility, respectively (Tilley and Terry, 1963). The good repeatability and reproducibility of the CB methods (Tilley and Terry, 1963; Goering and van Soest, 1970; Mould, 2003) is one of the reasons why they are recommended as reference for evaluating the energy value of feeds by the NRC (2001) energy system.

Results of this work are also in agreement with those of Wilman and Adesogan (2000), who found that the repeatability of the NDFD and TDMD measurements was slightly better with CB compared to Daisy^{II}. Spanghero et al. (2007), in a trial conducted on 162 hay samples, obtained a limited repeatability of the Daisy-NDFD measurements (SEM=4.8% of the mean) and attributed this result to unidentified filter bags characteristics and preparation (porosity, sample size, amounts of substrate,…). However, the lower repeatability of the filter bags can be overcome by increasing the number of replicates, as 3 filter bags can give, as shown in this research, approximately the same standard error of the mean of 2.5 nylon bags and of 2 CB measurements.

5.2. The effect of filter bags

To our knowledge no research has compared nylon to filter bags *in situ*. Damiran et al. (2008) found that filter bags *in situ* tended to overestimate forage digestibility compared to Tilley and Terry (1963) and to Daisy^{II}, but the incubation was preceded by a 48 h acid pepsin treatment. Cattani et al. (2009) found that NDFD and TDMD values measured *in vitro* with CB were higher than those achieved with Daisy^{II} filter bag and this was influenced by the sample size of the feed introduced in the bags. Two previous findings (Robinson et al., 1999; Spanghero et al., 2003) obtained higher digestibility values for Daisy^{II} compared to IS-nylon bags, but in these trials feed samples incubated with IS-nylon bags were more coarsely ground (2 and 4 mm, respectively for Daisy^{II} and IS-nylon bags). Lindberg and Knutsson (1981) noticed that the escape of fine particulate matter from nylon bags more than doubled when using a grinding size of 1 mm compared to 4.5 mm, but this occurred during the first 24 h of digestion. Vanzant et al. (1998), when reviewing the literature found that feed samples for IS-nylon bag incubations are commonly ground using screen size ranging from <1 to 6 mm, and that, among the 53 reviewed papers, 34 used a 2-mm screen, whereas 11 used a 1 mm screen. However, it is generally assumed that after 48 h of incubation the degradation of these escaped particles is complete (Setälä, 1983; Vanzant et al., 1998).

Results of the current experiment showed that the TDMD values achieved *in situ* from nylon or filter bags were very well correlated. However, the significant differences of the regression slope from one and of the intercept from zero suggest the presence of a systematic underestimation due to the filter bags, as graphically evidenced in Figure 1. This effect was also evidenced by the relationship between the IS-nylon bag and Daisy^{II} filter bag data. This relationship was very similar with respect to slope and intercept, to that relating the two kind of bags *in situ*. In addition, the two methods based on the use of filter bags but operating in different environments, *in situ* and *in vitro,* were linearly related ($R^{2=}0.95$) with a slope not different from one (0.98; P=0.13). Therefore, considering that in the present trial the ratio between sample weight to bag surface area (14 mg/cm^2) and the grinding size (1 mm) were the same in the two kind of bags, the most probable reason for these effects is due to the different texture and pore size of the nylon (40 µm) and the filter (25 µm) bags. Meyer and Mackie (1986) suggested the use of nylon bags with 30 to 53 µm pore size to allow for maximal activity of rumen microorganisms and generally the recommendations range from 20 to 60 µm (Vanzant et al., 1998). It is widely recognized that texture and pore size of bags used for *in situ* incubations can influence the efflux of digested material, the exchange of fluid with

the rumen content and the loss of digested material (Kitessa et al., 1999). According to this, the results of the present trial indicate that filter bags depressed feed degradation and induced a lower repeatability of the measurement compared to those achieved from IS-nylon bags and from CB.

However, the very close correlation (R^2=0.97) found between IS-nylon and IS-filter bags suggests that filter bags can be used to predict IS-nylon bag digestibility at 48 h by adjusting the data for the systematic error. The equation found in this trial, being based on a limited number of feeds, should be used with caution and only as a first indication. Reliable equations based on a wider range of feeds should be developed as it could be useful to replace nylon with filter bags *in situ* for simplifying the procedure of analysis, reducing the manipulation of the residuals for the chemical analysis, and processing a larger number of samples in a short time.

5.3. Use of in vitro methods with rumen fluid collected from intact cows for TDMD determination

In contrast to common practice, the *in vitro* tests in this research were completed using rumen fluid collected via oro-ruminal suction from intact donor cows. Very few attempts to compare the effect of the method of rumen fluid collection have been published so far (Spanghero et al., 2010). Collection of rumen fluid should avoid, as much as possible, salivary and oxygen contamination and exposure to ambient temperature, because of their possible effects on the microbial population and activity (Mould et al., 2005). Raun and Burroughs (1962) reported that total volatile fatty acids tended to be lower and pH was significantly higher in samples taken using a suction strainer technique than in those taken via rumen fistula and attributed this result to salivary contamination. Lodge-Ivey et al. (2009) recently described an oral lavage technique for aspirating rumen fluid from intact sheep. They showed that rumen samples collected via oral lavage or rumen cannula had similar contents of ammonia and volatile fatty acids and no difference in the bacterial community as determined by gradient gel electrophoresis. Whatever the method of rumen fluid collection, oxygen contamination and exposure to ambient temperatures are unavoidable, but they can be minimized by reducing the collection to incubation time. In the current trial incubations

started within 30 minutes from rumen fluid collection and the first 100-200 ml of rumen fluid collected from each cow was discarded to avoid salivary contamination.

In the current experiment there was a good correspondence between the TDMD values provided by the IS-nylon bag and CB, as the value of the slope (1.02) did not differ significantly from one and a R^2 value of 0.90 was acceptable. In addition, it was found that the TDMD values calculated from the IS-filter bags are predictable with a good accuracy (R^2=0.95) from the TDMD Daisy^{II} values. The slope of this regression did not differ significantly from one, strengthening the evidence of this relationship. The feed samples incubated with IS-nylon or CB differed by the presence of a bag and by the different types of rumen fluid (oro-collected or in the rumen). Feed samples treated with filter bags *in situ* or *in vitro* differed only by the types of rumen fluid. For all the other conditions they were subjected to the same treatments (grinding size, sample weight:bag surface area, procedure of analyses). The direct proportionality between the TDMD values obtained *in situ* and *in vitro* with different techniques suggests that the fermentation properties of the rumen liquor collected by suction from intact cows can be considered similar to that of the *in situ* rumen environment at least in terms of ability to degrade the feeds in 48 hours of incubation.

This result is important as it implies that the use of fistulated cows for collecting rumen fluid for *in vitro* tests appears not to be necessary, providing that all the operations from fluid collection to the start of the incubation are conducted by avoiding salivary and oxygen contamination and minimizing the exposure to ambient temperatures. *In vitro* tests comparing the fermentation properties of rumen fluid collected from the same cows through a surgically placed cannula and with the oro-ruminal probe should be conducted to support these results.

6. Conclusions

The use of filter bags induced a systematic, but predictable, underestimation of the TDMD values compared to nylon bags. The replacement of nylon by filter bags *in situ* could simplify the procedure of analyses, with less manipulation of the residuals of fermentation for chemical analysis and associated errors. This technique requires less labour and equipment than other conventional techniques, and like Daisy^{II} offers the possibility to process a large number of samples in a short time. The lower repeatability provided by the filter bags can be overcome increasing the number of replicates: 3 filter bags give approximately the same standard error of the mean of 2.5 nylon bags and of the mean of 2 CB measurements. Rumen fluid collected from intact cows and used for *in vitro* test produces estimates of TDMD at 48 h directly proportional to those obtainable *in situ*. Therefore, the use of fistulated cows for collecting rumen fluid for *in vitro* tests may not be necessary. The use of rumen fluid collected from intact cows is of interest for many research centers for ethical and public concerns related to the use of surgically treated animals.

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CHAPTER 5

Running title: Use of NDF digestibility and gas production for feed evaluation

Metabolizable energy content of feeds based on 24 or 48 h *in situ* NDF digestibility and on *in vitro* 24 h gas production methods

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1. Abstract

The true digestible DM (TDMd) and ME contents of feeds are frequently assessed from the amount of digestible aNDF (dNDF) measured *in situ* or *in vitro* after 48 h of incubation, using the summative approach proposed by NRC. Alternative ways for determining the ME content of feeds are based on the gas produced after 24 h of *in vitro* incubation (GP24) and the chemical composition of feeds. The present study was aimed to: i) evaluate the validity to reduce the *in situ* time of incubation from 48 to 24 h for determining TDMd; ii) to study the relationship between the ME values estimated from dNDF and from GP24. Eleven feeds, with an aNDF content ranging from 101 to 768 g/kg DM, were used. The feeds were simultaneously incubated in the rumen of two cows for 24 and for 48 h with two replications. The entire incubation was repeated a week later. Following the NRC approach, the amounts of digestible aNDF measured after 24 and 48 h of incubation were used to compute the true DM digestibility values (TDMd24 and TDMd48, respectively) and the ME (ME_{NRC}) estimates. The same feeds were incubated *in vitro* and GP24 was measured by an automated gas production system with 4 replications. The entire incubation was repeated at a later time. Estimates of ME from GP24 were computed using the Hohenheim (ME_{Menke}) and the UC Davis (ME_{UCD}) equations. The $ME_{NRC,}$ ME_{Menke} and ME_{UCD} estimates were compared by regression. The repeatability of TDMd24 and TDMd48 was comparable (SED=12 g/kg DM) and they were related as: TDMd48= 0.74×TDMd24 + 260 (R^2=0.98; RMSE=21.5 g/kg DM). The results suggest that, for *in situ* tests, the incubation time can be shortened from 48 to 24 h, so that the labor and the costs for feed evaluation can be reduced. The measures of GP24 and TDMd48 were also well correlated (R^2=0.97), but only for feeds with less than 16% of CP/DM. The repeatability of ME_{NRC}, ME_{Menke}, and ME_{UCD}, expressed in terms of SED, were 0.35, 0.46 and 0.46 MJ/kg DM, respectively. The ME_{NRC} was predicted as 1.04×ME_{UCD} (RMSE=0.63 MJ/kg DM) and as 1.11×ME_{Menke} (RMSE=1.16 MJ/kg DM). When the ME_{NRC} values are taken as comparison terms, the UC Davis equation is more accurate and precise than the Hohenheim ones. However, the precision and the accuracy of ME predictions based on GP24 depend on: i) the availability of standardized procedures for measuring GP; ii) the availability of reliable equations relating GP and feed chemical composition (with special regards for CP) to predicted or *in vivo* measured energy contents of feeds; iii) the set of equations that a given energy system proposes to predict the ME of feeds from their digestible nutrient contents.

Keywords: Feed energy; Nylon bags; Automated gas production system; *In situ* digestibility;

2. Introduction

Energy content of ruminant feeds is often estimated from feed chemical composition (Weiss, 1993) or alternately using *in situ* or *in vitro* methods that evaluate the feed digestibility (NRC, 2001), or even the gas production (GP) (Menke and Steingass, 1988, Robinson et al., 2004). The traditional *in situ* method (Ørskov and McDonald, 1979) has the advantage that it uses the actual rumen environment to measure feed degradation and that it is the standard against which the *in vitro* methods are often compared (Kitessa et al., 1999). However, this technique has some shortcomings because: ii) it is expensive in terms of labour and analytical costs; ii) it measures the feed disappearance and not the actual amount of fermented substrate; iii) it has raised public concerns about animal welfare (Lindberg, 1985; Nocek, 1988; Michalet-Doreau and Ould-Bah, 1992; Stern et al., 1997). The *in vitro* batch culture fermentation followed by an enzymatic digestion step is a reliable alternative (Tilley and Terry, 1963). Goering and Van Soest (1970) replaced the enzymatic step with a neutral detergent treatment to determine the amount of digestible aNDF (dNDF) and the true dry matter digestibility (TDMd). NRC (2001) suggests that the ME content of feeds (ME_{NRC}) can be estimated from dNDF determined *in situ* or *in vitro* after 48 h of feed incubation with rumen fluid. Some advantages of this technique, compared to the conventional *in situ* technique, are: i) ease of standardization and low cost (Makkar, 2005); ii) less ethical concerns related to the use of surgically cannulated cows, as rumen fluid can be collected from intact donor cows (Tagliapietra et al., submitted). A reduction of the incubation time from 48 to 30 or 24 h has been previously proposed, because the shorter incubation times are considered to be closer to the retention times of the feed particles in rumen of high producing dairy cows (Hoffman et al., 2003; Goeser and Combs, 2009). However, direct comparisons of TDMd values achieved after 24, 30 or 48 h of incubation are still insufficient and more information is required (Hoffman et al., 2003; Spanghero et al., 2010).

Alternative, reduced cost, ways for determining the energy value of feeds are those based on gas production (GP). The Hohenheim technique (Menke and Steingass, 1988) estimates the feed ME content (ME_{Menke}) using equations based on the amount of gas produced in syringes after 24 h of incubation (GP24) and from the feed chemical composition. The UC Davis approach described by Robinson et al. (2004) is similar, but it uses a different equation for estimating ME (ME_{UCD}). This technique is evolving towards semi-automated (Theodorou et al., 1994; Mauricio et al., 1999) and automated systems (Pell and Schofield, 1993; Cone et al., 1996; Tagliapietra, 2010); both at a reduced cost in terms of time and labor

when compared to the Hohenheim syringe method. The major doubts about the use of these techniques regard: i) the repeatability of the GP measurements; ii) the relationship between GP and feed digestibility; iii) the relationships between the ME values predicted from GP24 and the ME values resulting from the digestibility measurements.

The aims of the present study were: i) to evaluate the repeatability of the TDMd measurements obtained *in situ* after 24 (TDMd24) or 48 h (TDMd48) of incubation and the relationship between these two sets of measurements; ii) to evaluate the repeatability of the ME_{NRC}, ME_{Menke} and ME_{UCD} estimates and the relationships among the various ME estimates.

3. Material and methods

3.1 Feeds

The following 11 feeds were used: wheat straw, meadow hay, 3 corn silages collected from different farms, two alfalfa hays, dry sugar beet pulp, soybean meal, ground corn grain and a commercial mixed feed (composed by corn grain 25.9%, barley 19.3%, soybean meal 17.2%, wheat grain 15.4%, sugar beet pulp 14.2%, mineral mix 8%). Corn silages were previously dried at 60 °C until constant weight. Feed samples were ground to pass a 1-mm sieve using a hammer mill (Pullerisette 19, Fritsch GmbH, Laborgeratebau, D) and analyzed for their chemical composition (Table 1).

Table 1. Chemical composition (g/kg DM) of feeds

Feed	DM	aNDF[1]	ADF	ADL	CP	EE	Ash
Wheat straw	927	768	486	58	52	18	84
Meadow hay	883	600	341	39	85	16	75
Corn silage 1	948	514	271	35	71	18	42
Corn silage 2	937	596	336	47	67	13	44
Corn silage 3	908	433	233	12	83	20	34
Alfalfa hay 1	953	457	349	90	165	21	110
Alfalfa hay 2	916	437	314	77	197	31	109
Sugar beet pulp	910	502	264	33	92	7	43
Mixed feed	905	245	106	18	166	20	89
Soybean meal	898	135	81	-	482	18	64
Corn grain	900	101	19	-	98	37	14

Data are means of three analyses

3.2 In situ technique

The incubations were completed according to the procedure proposed by Ørskov and McDonald (1979), modified by Nocek (1988) and Vanzant et al. (1998).

Two dry Holstein-Friesian fistulated cows (housed at the experimental farm of Department of Animal Science, University of Milan, Italy), that had been fed hay *ad libitum* and 2 kg/d of concentrate for 2 weeks, were used. The feed samples were incubated in two separate periods of incubation in the rumen of the two cows for 24 h and 48 h. The experimental design was: 2 repeated incubations × 2 cows × 11 feeds × 2 incubation times × 2 replications, giving a total of 8 bags per feed and per time, plus 16 empty bags as blank.

Feed samples (1.150+0.005 g) were placed in nylon bags (40 µm of pore size; 10.5 × 8 cm of net surface area) to achieve a ratio between feed sample size and net bag surface area (excluding bag areas not in contact with the feed) of 14 mg/cm^2 (Nocek, 1988). The nylon bags were bound to plastic strings and introduced in the rumen of the cows 2 hours after the morning feeding. After 24 and 48 h the bags were extracted from the rumen, washed in cold water, weakly centrifuged in a washing machine (2 minutes rinse + 2 minutes spin, repeated 5 times) and stored at 4°C for about 2 h, which was the time required for transferring the samples to the laboratory.

3.3 Gas production technique

The *in vitro* gas production analyses were completed according to the procedure proposed by for 24 h (Menke and Steingass, 1988). The feed samples were incubated in two separate periods of incubation. The experimental design was: 2 incubation periods × 11 feeds × 4 replications, giving a total of 8 bottles per feed, plus 8 empty bottles as blank (4 blanks/incubation).

The buffer mineral solution, prepared according to Menke and Steingass (1988), was heated in a waterbath at 39°C and purged continuously with CO_2 for 30 minutes. Sodium sulphite was used as reducing agent in the buffer solution (0.33 g/l solution; Menke and Steingass, 1988). Rumen fluid was collected two hours after morning feeding from 3 dry Holstein-Friesian cows (housed at the experimental farm of the Department of Animal Science, University of Padova, Italy) that were fed hay *ad libitum* and 2 kg/d of concentrate for 2 weeks. Rumen fluid was collected using a vacuum pump connected to a glass vacuum container in turn connected to a semi-flexible oro-ruminal probe equipped with a steel strainer fixed on the top (Tagliapietra et al., submitted). The steel strainer (15 cm of length and 3.5 cm

of diameter, 600 g of weight) had 32 holes of 0.5 cm of width. The vacuum pump was activated when the top of the probe was positioned in the rumen. The first 100 to 200 ml of rumen sample collected from each cow was discarded to limit contamination with saliva (Raun and Burroughs, 1962). The pH of the rumen liquor was measured immediately after collection and only fluids with pH lower than 6.8 were kept for the tests. The rumen fluid was poured into two thermal flasks preheated to 39.0±0.5 °C and immediately transferred to the laboratory. At the laboratory the rumen fluid was filtered through 3 layers of cheesecloth to eliminate feed particles and mixed with the buffer mineral solution in a ratio 1 to 2 (Menke and Steingass, 1988). All the operations were conducted under anaerobic conditions by flushing carbon dioxide. The time required for all the operations was less than 30 minutes.

A commercial wireless GP apparatus (Ankom RF Gas Production System, Ankom Technology®, Macedon, NY, USA) consisting of to 48 bottles equipped with pressure sensors (pressure range: from −69 to +3447 kPa; resolution: 0.27 kPa; accuracy: ±0.1% of measured value) and a receiving base station and computer was used. Each bottle (280 ml) was filled with 0.5000±0.0010 g of feed sample and preheated overnight at 39 °C. On the day of the incubation, each bottle was filled with 75 ml of buffered rumen fluid (headspace volume of 205 ml), keeping headspace of bottle continuously flushed with CO_2. After the inclusion of the feed the bottles were closed and placed in an air ventilated oven at 39±0.4 °C.

During the incubation, the bottle headspace pressure changes, with respect to the atmospheric pressure measured at the start of the incubation (Po), were transmitted via a radio frequency to a PC with a set frequency of 1 minute. Gas in the headspace of the bottles was automatically released by opening a closed valve when a threshold pressure variation of +3.4 kPa was reached. This pressure, previously used by Tagliapietra et al. (2010), is slightly lower than 4.5 kPa used with others automated equipments (Davies et al., 2000; Calabrò et al., 2005). The readings of gas pressure changes after 24 and 48 h of fermentation were cumulated (ΔP) and converted to units of volume (GP, ml) using the ideal gas law:

$$GP = (\Delta P/Po) \times Vo \quad (1)$$

where: ΔP is the cumulated pressure change (kPa) in the bottle headspace; Vo is the bottle headspace volume (205 ml), Po is the atmospheric pressure read by the equipment at the beginning of the trial. The final GP volume of bottles with no feed (blank) was subtracted from the final GP volume for bottles with feed to correct for baseline fermentation in the rumen fluid. Data of GP were expressed as ml/g DM incubated.

At the end of the incubation the fluids of fermentation were analyzed for pH to test the ability of medium to maintain the buffer properties and the values resulted always higher than

the threshold of 6.2, below which the Menke's buffer is exhausted and the release of gas becomes not-linearly related to the production of end products of fermentation (Menke et al., 1979; Beuvink and Spoelstra, 1992).

3.4 Chemical analyses

All the chemical analyses were performed in the same laboratory by the same technician.

The feeds were analysed in triplicate for dry matter (DM, AOAC method 934.01, 2003), crude protein (CP, AOAC method 976.05, 2003), ether extract (EE, AOAC method 920.29, 2003) and ash content (AOAC method 942.05, 2003). Neutral detergent fibre (aNDF) was determined (Mertens, 2002) with a treatment with α-amylase and sodium sulphite using the Ankom^{220} Fiber Analyzer (Ankom Technology®, Macedon, NY, USA). ADF and sulphuric acid lignin ($Lignin_{(sa)}$) contents were determined sequentially after aNDF determination (Robertson and Van Soest, 1981). The feeds were also analysed for neutral detergent insoluble crude protein (NDICP) and acid detergent insoluble crude protein (ADICP) (Licitra et al., 1996).

After incubation and washing, the nylon bags were dried at 60°C in an air ventilated incubator and weighed; the residual feed material was transferred from each bag to weighed crucibles (30 ml por. 2, Robu Glasfilter-Geräte GMBH®, Hattert, D) and treated as indicated for the aNDF analysis (Mertens, 2002) with a Fibertech Analyzer (VELP® Scientifica, Usmate, Milano, I).

3.5 Computation of TDMd and ME_{NRC} values

The digestible NDF (dNDF) and the true DM digestibility (TDMd) were computed using the following equations (Goering and Van Soest, 1970):

$$dNDF\ (g/kg\ DM) = 1000 \times [(aNDF_{feed} - aNDF_{res}) / DM_{feed}]; \quad (2)$$

$$TDMd\ (g/kg\ DM) = 1000 \times [(DM_{feed} - aNDF_{res}) / DM_{feed}]; \quad (3)$$

where: $aNDF_{feed}$ is the amount of aNDF incubated with the feed, $aNDF_{res}$ is the amount of aNDF residual measured after 24 or 48 h of fermentation, DM_{feed} is the amount of DM incubated; these amounts were expressed in grams of DM.

The energy values of feeds were computed as (NRC, 2001):

$$DE\ (MJ/kg\ DM) = (dNDF/1000\times4.2 + tdNFC/1000\times4.2 + tdCP/1000\times5.6 + tdFA/1000\times9.5 - 0.3)\times4.184 \quad (4)$$

ME_{NRC} (MJ/kg DM) = [– 0.45 × 4.184 + 1.01×DE] (5)

where: dNDF is the measurement described before but only at 48 h of incubation, and tdNFC, tdCP and tdFA are the estimated true digestible contents of non-fibre carbohydrates, crude protein and fat (g/kg DM) calculated using the equations proposed by NRC (2001) (Eqs. 2-4a to 2-4e) with an adjustment factor (PAF) for different feeds selected from NRC (2001). For the application of these equations the analytical determination of the following feed constituents is required: aNDF, CP, NDICP, ADICP, EE and Ash.

3.6 Computation of ME_{Menke} and ME_{UCD} values from GP measurements

The Hohenheim gas method was developed using data from 400 *in vivo* digestibility trials mainly conducted on sheep. The feed ME contents were computed considering 15.2, 34.2 12.8, and 15.9 MJ ME/kg of *in vivo* measured digestible CP, EE, crude fiber and nitrogen free extract. These ME values were then regressed against the GP24 and the proximate composition of the same feeds. The resulting equations, proposed for estimating the feed ME_{Menke} values from only GP24 and proximate composition, were the following, respectively for roughages and other feeds (Menke and Steingass, 1988):

ME_{Menke} (MJ/kg DM) = 2.20 + 0.1357×$GP24_{200}$ + 0.0057×CP + 0.0002859×EE^2 (6)

ME_{Menke} (MJ/kg DM) = 1.06 + 0.1570×$GP24_{200}$ + 0.0084×CP + 0.0220×EE – 0.0081×Ash (7)

where: $GP24_{200}$ (ml/200 mg of DM incubated) is the gas production measured at 24 h. All the chemical constituents were expressed as g/kg DM.

In the present research we used the first equation (6) for wheat straw, grass hay, alfalfa hay and corn silage and the second one (7) for the remaining feeds.

Metabolizable energy content was also estimated using the UC Davis unified equation proposed by Robinson et al. (2004) and resulting from a modification of those proposed by Menke and Steingass (1988):

ME_{UCD} (MJ/kg DM) = 1.25 + 0.0292 × $GP24_{DM}$ + [0.0143× (CP-ADICP)] + 0.0246 × EE (8)

where: $GP24_{DM}$ is expressed as ml/g DM.

3.7 Statistical analysis

3.7.1 Repeatability and reproducibility

Estimation of variance components was accomplished separately for estimates of TDMd, GP at 24 and 48 h, $ME_{NRC,}$ $ME_{Menke,}$ and ME_{UCD} provided by different methods, using the mixed procedure of SAS (SAS Inst. Inc.) with two mixed linear models. In the first model the following sources of variation were considered as random effects: the period of incubation

(**I**), the feed (**F**), the interaction I×F and the error term (**e_1**). An additional random effect included in the model was the effect of the different cows for the *in situ* method, but as the proportion of variance explained by this factor was very low, it was included in the error term. The restricted maximum likelihood method (REML) was used as the method of estimation of variance components. The components of variance of each factor, σ^2_I, σ^2_F, $\sigma^2_{I\times F}$ and σ^2_{e1} were used to compute the repeatability (**RT**), defined as the value below which the absolute difference between two single measures obtained with the same method and under the same conditions (same incubation, same feed) is expected with a 95% probability, and the coefficient of repeatability (**RT%**) (International Organization for Standardization, 1994a,b):

$$RT = 2\sqrt{2\sigma^2_{e1}} \quad (9)$$

and

$$RT\% = \frac{\sigma^2_I + \sigma^2_F + \sigma^2_{I\times F}}{\sigma^2_I + \sigma^2_F + \sigma^2_{I\times F} + \sigma^2_{e1}} \times 100 \quad (10)$$

Reproducibility (**RD**) was defined as the value below which the absolute difference between two single measures obtained with the same method of analysis on the same feed in different incubations is expected within a 95% probability, and coefficient of reproducibility (**RD%**) (International Organization for Standardization, 1994a,b). In this case the components of variance of each method were estimated using the mixed procedure of SAS (SAS Inst. Inc.) with a second mixed linear model which considered only the feed as random factor and the residual error (**e_2**). The values of RD and RD% were computed as:

$$RD = 2\sqrt{2\sigma^2_{e2}} \quad (11)$$

$$RD\% = \frac{\sigma^2_F}{\sigma^2_F + \sigma^2_{e2}} \times 100 \quad (12)$$

3.7.2 Comparisons

From a preliminary analysis it was found , using the Bartlett's test (Bartlett, 1937) of the SAS (SAS Inst. Inc.), that the variances associated to the various methods were not homoscedastic, and so the use of ANOVA linear models was not applicable to compare the effects due to the different methods. Thus, the various methods were compared by linear regression of the mean values of TDMd, GP24, ME_{NRC}, ME_{Menke} and ME_{UCD} data obtained for each feed. Significant differences of the slope and intercept from unity and zero,

respectively, were tested using the regression procedure (proc reg) of SAS (SAS Inst. Inc.). The root of the mean square error (RMSE) provided as output from the proc reg analysis of SAS (SAS Inst. Inc.) was considered as prediction error. When the intercept did not significantly differ from 0 it was forced through the origin.

4. Results

4.1 Repeatability of TDMd, GP24 measurements and of ME_{NRC}, ME_{Menke} and ME_{UCD} estimates

The values of TDMd (Table 2) measured from *in situ* digestion for the various feeds were 6.3% lower at 24 h compared to those measured at 48 h of incubation (overall mean 793 and 847 g/kg, respectively). The RT values were 34.1 and 50.5 g/kg DM respectively for 24 and 48 h, corresponding to RT% values of 99.5% and 98.2%, respectively. The values of RD and RD% were slightly higher than the corresponding RT and RT% values.

The GP24 values of the feeds averaged 259 ml/g DM incubated. The RT and the RD values of GP24 were 46.0 and 46.3 ml/g DM, respectively.

Except for wheat straw, the ME_{NRC} values were always numerically higher than the ME_{Menke} values based on GP24. The ME_{UCD} values were intermediate. The reproducibility of the ME_{NRC} estimates (RD=0.98 MJ/kg DM) was better compared to that obtained by the other two procedures based on the GP24 measures, ME_{Menke} and ME_{UCD}, (RD=1.32 and 1.35 MJ/kg DM, respectively).

Table 2. *In situ* TDMd[1] at 24 or 48 h of incubation and estimated feed ME according to NRC (2001) (ME_{NRC}); *in vitro* gas production at 24 h of incubation (GP24) and estimated feed ME (ME_{Menke} and ME_{UCD}) according to Menke and Steingass (1988) and Robinson et al. (2004), respectively; (mean ± SD).

Incubation time	TDMd g/kg DM		GP24 ml/g DM	ME_{NRC} MJ/kg DM	ME_{Menke} MJ/kg DM	ME_{UCD} MJ/kg DM
	24 h	48 h	24 h	48 h	24 h	24 h
Feed[2]:						
Wheat straw	456±12	595±16	149±13	6.3±0.3	6.6±0.3	6.6±0.4
Meadow hay	583±25	702±16	198±17	8.8±0.3	8.1±0.5	8.5±0.5
Corn silage 1	753±25	857±5	273±20	11.3±0.1	10.1±0.5	10.6±0.6
Corn silage 2	687±8	756±49	245±25	9.4±0.9	9.3±0.7	9.5±0.7
Corn silage 3	739±20	822±18	275±20	10.8±0.3	10.2±0.5	10.8±0.6
Alfalfa hay 1	775±9	802±8	199±16	10.0±0.1	8.7±0.4	9.6±0.5
Alfalfa hay 2	816±9	835±14	166±8	11.1±0.2	8.1±0.2	9.3±0.2
Sugar beet pulp	1000±1	1000±1	375±10	15.1±0.0	13.4±0.3	13.6±0.3
Commercial mixed feed	932±2	951±27	305±17	13.2±0.5	11.8±0.5	12.9±0.5
Soybean meal	1000±1	1000±1	250±12	16.3±0.0	12.8±0.4	15.8±0.3
Corn grain	979±6	1000±1	411±17	15.7±0.0	15.5±0.5	15.5±0.5
Mean	793	847	259	11.6	10.4	11.2
Repeatability, RT[3]	34.1	50.5	46.0	0.90	1.31	1.34
RT%	99.5	98.2	96.2	99.0	97.1	97.5
Reproducibility, RD[4]	38.9	54.9	46.3	0.98	1.32	1.35
RD%	99.4	97.9	96.2	98.8	97.1	97.4

[1] True dry matter digestibility; [2] Each observation is the mean of 8 measurements; [3] Repeatability: RT= $2\sqrt{2\sigma^2_{e1}}$ and RT%= $\frac{\sigma^2_I+\sigma^2_F+\sigma^2_{I\times F}}{\sigma^2_I+\sigma^2_F+\sigma^2_{I\times F}+\sigma^2_{e1}} \times 100$; [4] Reproducibility: RD = $2\sqrt{2\sigma^2_{e2}}$ and RD% = $\frac{\sigma^2_F}{\sigma^2_F+\sigma^2_{e2}}$.× 100, where: σ^2_I, σ^2_F, $\sigma^2_{I\times F}$ and σ^2_{e1} σ^2_{e2} are variance components for incubation (I, n=2), feed (F, n=11), incubation × feed, e_1 is the error term for RT and e_2 is the error term for RD.

4.2 Relationships between true digestibility, gas production and ME estimates

The TDMd24 and TDMd48 values were well correlated (R^2=0.98) and the relationship obtained by regressing TDMd48 (y) against TDMd24 (x) evidenced a slope lower than 1 (P<0.01) and a positive intercept (P<0.01) (Table 3).

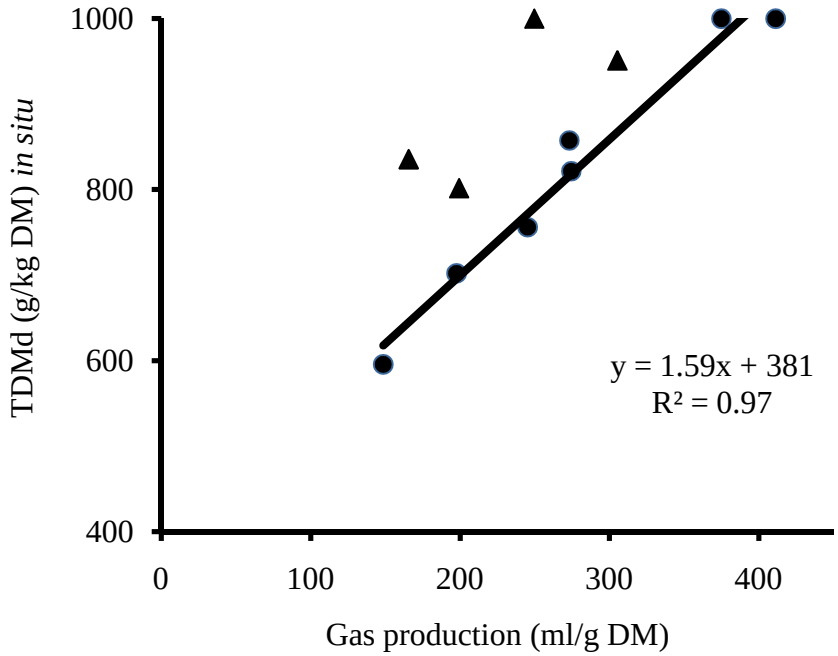
Table 3. Relationships between TDMd[1] (g/kg DM) and GP[2] data (ml/g DM) of eleven feeds measured after 24 and 48 h of incubation.

Methods		Feeds[3]	Equation	SE (*P*-value)		R^2	RMSE[4]	CV[5]
Dependent (y)	Independent (x)	n.		Slope	Intercept			%
TDMd48	TDMd24	11	y=0.74x+260	0.04 (<0.01)	31 (<0.01)	0.98	21.5	2.5
GP48	GP24	11	y=0.92x+53	0.06 (0.20)	16 (<0.01)	0.96	15.2	5.2
TDMd24	GP24	11	y=1.56x+388	0.49 (0.28)	133 (0.02)	0.53	128.0	16.2
TDMd48	GP24	11	y=1.27x+519	0.33 (0.44)	90 (<0.01)	0.62	86.7	10.2
TDMd24 [6]	GP24	7	y=2.10x+164	0.15 (<0.01)	42 (<0.01)	0.98	32.9	4.4
TDMd48 [6]	GP24	7	y=1.59x+381	0.13 (<0.01)	37 (<0.01)	0.97	29.3	3.6

[1] True DM digestibility of feeds incubated *in situ* for 24 or 48 h into nylon bags. [2]Gas production of feeds incubated *in vitro* for 24 h or 48 h. [3] Each observation is the mean of 8 measurements. [4]Root of the mean square error. [5]Coefficient of variation. [6] Equations obtained excluding the four feeds with CP content > 160 g/kg DM (soybean meal, 2 alfalfa hays and the commercial mixed feed).

The TDMd values both at 24 and 48 h of incubation were poorly correlated with the GP24 measurements, being the R^2 always lower than 0.62. However, the R^2 of these equations increased markedly until values of 0.98 when the feeds with CP content > 160 g/kg DM (soybean meal, 2 alfalfa hays and commercial mixed feed) were excluded from the regression analysis (Figure 1).

Figure 1. True DM digestibility values (TDMd) of 11 feeds achieved *in situ* after 48 h of incubation (y) and gas production values measured *in vitro* after 24 h of incubation (x). Four feeds (▲) with > 160 g CP/kg DM were excluded from the regression. The other feeds are indicated as ●.



The ME_{NRC} values were poorly correlated (R^2=0.86) with the ME_{Menke} values and the prediction error of the corresponding regression was 1.16 MJ/kg DM (Table 4). Also in this case, the equation was improved when the four high protein feeds were excluded from regression analysis (R^2=0.97) and in this case the prediction error was 0.63 MJ/kg DM. The ME_{NRC} values were always well correlated (R^2=0.96) with the ME_{UCD} estimates, excluding or not the four rich-protein feeds, and the prediction error of the equation was 0.63 MJ/kg DM.

Table 4. Relationships between ME values (MJ/kg DM) of eleven feeds estimated according to different approaches.

Methods		Feeds[1] n.	Equation	SE *(P-value)*		R^2	RMSE[2]	CV[3] %
Dependent (y)	Independent (x)			Slope	Intercept			
ME_{NRC}[4]	ME_{Menke}[5]	11	y=1.09x+0.27	0.14 (0.55)	1.54 (0.86)	0.87	1.22	10.5
"	" [6]	11	y=1.11x	0.03 (<0.01)	-	0.86	1.16	9.9
	" [6]	7	y=1.06x	0.02 (0.04)	-	0.97	0.63	5.7
ME_{NRC}[4]	ME_{UCD} [7]	11	y=1.04x-0.01	0.08 (0.57)	0.83 (0.99)	0.96	0.68	5.4
"	" [6]	11	y=1.04x	0.02 (0.03)	-	0.96	0.63	5.4
"	" [6]	7	y=1.03x	0.02 (0.13)	-	0.97	0.58	5.2

[1] Each observation is the mean of 8 measurements. [2] Root of the mean square error. [3] Coefficient of variation. [4] ME contents estimated from the digestible aNDF content of feeds incubated *in situ* for 48 h into nylon bags according to NRC (2001). [5] ME contents estimated from gas production of feeds incubated *in vitro* for 24 h according to Menke and Steingass (1988). [6] Equation obtained forcing the intercept to zero. [7] ME contents estimated from gas production of feeds incubated *in vitro* for 24 h according to Robinson et al. (2004).

5. Discussion

5.1 Prediction of the true digestibility of feeds from in situ incubation at 24 or 48 h

For evaluating TDMd and the energy value of ruminant feeds NRC (2001) suggests the use of digestible aNDF data achieved after 48 h of *in situ* or *in vitro* incubation. There is a debate about the validity of reducing the incubation time from 48 to 30 or 24 h. A first consideration is that a shorter incubation time will reduce the cost of feed evaluation tests. Some authors (Hoffman et al., 2003) suggested that incubation times in the order of 24 to 30 h are preferable, as these are closer to the retention times of the feed particles in the rumen of high producing dairy cows. Others authors indicated that a 48-h incubation time is preferable to shorter durations because of a better repeatability of the measures (Hall and Mertens, 2008; Spanghero et al., 2010). Hall and Mertens (2008) compared different types of vessels for measuring the *in vitro* TDMd and observed that the reduction of the incubation time from 48 to 24 h tripled the standard error of the differences (SED=$\sqrt{\sigma^2}_{e2}$; from 13 to 37 g/kg DM,

respectively). In the present work, conducted *in situ* with nylon bags, it was observed that the reduction of the incubation time from 48 to 24 h reduced the RT of the TDMd measurements from 50.5 to 34.1 g/kg DM. These values are in the same range of variation of those reported by Hall and Mertens (2008), when expressed in terms of standard error of the differences (18 and 12 g/kg DM, respectively). However, in contrast to what was observed by Hall and Mertens (2008), the shortest incubation time was associated to the best repeatability. This was mainly due to the fact that the TDMd measures from 2 feeds (a corn silage and the commercial mixed feed) showed low standard deviations at 24 h and high standard deviations at 48 h (Table 2). The repeatability of TDMd24 and of TDMd48 measures was comparable when these two feeds were excluded. The TDMd24 and TDMd48 were strongly correlated (R^2=0.98) in this experiment, which is in agreement with Hoffman et al. (2003). These results, even considering the comparable reproducibility obtained at the 2 incubation times, suggest the validity of developing equations to predict feed digestibility using values measured at 24 instead of 48 h. This could represent a good opportunity for reducing the costs of feed evaluation, but requires further investigations on a larger set of feeds.

5.2 Prediction of ME content of feeds from in vitro gas production

The gas production technique is an alternative and less expensive method than *in situ* studies for evaluating the value of feeds. According to published studies, the GP24 values are commonly found to be less repeatable and reproducible than TDMd data (Gosselink et al., 2004; Rodrigues et al., 2009). Because of the poor repeatability, the GP technique is recommended mainly as a tool for ranking feeds (Valentin et al. 1999; Hall and Mertens, 2008). The poor reproducibility of the GP measurements between laboratories can be partially attributed to the low standardization of the method (Getachew et al., 2002; Spanghero et al., 2010). Instead of measuring the disappearance of insoluble feed components, as occurs in other *in situ* and *in vitro* methods, the GP technique measures the appearance of gaseous products. The gas measured is not only that directly generated by the fermented matter, as additional amounts of gas are released from the buffer in relation to the acid properties of the end-products of fermentation. Thus, although a good correlation between the amounts of OM digested and gas produced is expected (Makkar, 2005), the degree of correlation is influenced by various factors (different equipments, differences in experimental protocol, feed and inoculum characteristics) that alter the release of gas from the medium (Cone et al., 2002;

Mould et al., 2005; Tagliapietra et al., 2010). These factors could also influence the repeatability and the reproducibility of the GP measurements.

In the current trial we used an automated apparatus in which the headspace pressure inside the bottles is maintained always lower than 3.4 kPa by means of automated gas valve venting. This low threshold pressure was adopted in order to prevent underestimation of GP due to supersaturation of the medium, as described by Tagliapietra et al. (2010). In contrast to what is commonly practiced, we used rumen fluid collected by a suction technique from the rumen of intact cows. This procedure was adopted on the basis of a previous trial (Tagliapietra et al., submitted), conducted on the same feeds used in the current experiment, where it was observed that the *in situ* TDMd48 values achieved with nylon bags were predictable as 1.02 (RMSE=42 g/kg DM) of the *in vitro* TDMd48 data achieved from conventional bottles and rumen fluid collected by the suction technique. In this trial the repeatability of the GP24 measurements was similar ($\sqrt{\sigma^2}_{e1}$= 16 ml/g DM) to the values reported by other authors, with standard errors of the differences in the order of 14 ml/g DM (Valentin et al., 1999; Getachew et al., 2002; Gierus et al., 2008). The values of repeatability of TDMd24, TDMd48 and GP24 reported in Table 2 are not directly comparable as they have a different unit of measurement. However, when considering the ratio between RD and the corresponding means, it can be seen that the GP24 measurements were about 3 times less reproducible than the TDMd values computed from the aNDF measurements achieved after 24 and 48 h of fermentation. Nevertheless, once the data were converted in ME terms, the reproducibility of the ME_{Menke} and of the ME_{UCD} estimates was the same and about 30% greater (RD=1.32 and 1.35 MJ/kg DM, respectively) than that computed for ME_{NRC} (RD=0.98 MJ/kg DM). This confirms that GP measurements and feed chemical information must be combined for predicting the ME of feeds, as the release of gas from the medium is influenced by the feed chemical composition (Menke and Steingass, 1988) and likely by the extent of which the feed constituents are fermented. On the other hand, feed chemical information alone are inadequate for a precise ME content prediction (Robinson et al., 2004); in the present work, when the ME_{NRC} values of feeds where regressed against ME values computed following the lignin-based NRC (2001) approach, entirely based on chemical information of feed, the resulting equation was accurate but not precise ($ME_{NRC} = 1.04 \times ME_{lignin} + 0.57$; $R^2 = 0.78$).

The dietary CP content of feeds is a factor which was found to strongly reduce the amount of gas released. Cone and Van Gelder (1999) observed that fermentation of casein produced only 32% of gas volume compared to carbohydrates, and therefore a correction of GP values

for CP content of feeds is required when these values are compared with the corresponding digestibility measurements (Chenost et al., 2001). In agreement with these findings, in the current trial, the correlation between TDMd48 and GP24 measurements was good (R^2= 0.97) when the four feeds containing > 160 g CP/kg DM were excluded from the regression analysis. In the current trial the ME_{UCD} values were well correlated to the ME_{NRC} estimates and their regression showed a prediction error of 0.63 MJ/kg DM. The same did not occur for the ME_{Menke} values as in this case the regression relating ME_{Menke} to ME_{NRC} was neither precise nor accurate, except when protein-rich feeds were excluded. These results are in agreement with those reported by Robinson et al. (2004) and Magalhães et al. (2010). The difference observed between the Menke and Steingass (1988) and the Robinson et al. (2004) approaches are partially due to the different weights attributed to the various independent variables considered in the predicting equations. The intercept of these regression equations do not differ very much nor does the energy content attributed to GP24 [a GP of 100 ml/g DM is equivalent to 3.14 and 2.92 MJ of ME with Menke and Steingass (1988) and with Robinson et al. (2004), respectively] but they substantially differ for the weight attributed to the dietary CP content. Menke and Steingass (1988) allow a contribution of 0.57 and 0.84 MJ of ME for each 100 g of CP/kg DM for forages and concentrates, respectively, whereas Robinson et al. (2004) attribute about 1.43 MJ of ME per 100 g CP/kg DM.

The better precision and accuracy achieved with the Robinson et al. (2004) equation compared to those proposed by Menke and Steingass (1988) also depend on the energy system taken as a reference. In this work we used as a reference the summative equation approach proposed by NRC (2001), based on dNDF measurement and feed chemical analysis. In this approach the DE content is calculated by multiplying the truly digestible nutrient concentrations by their heats of combustion (23.43, 39.74, 17.57 and 17.57, and MJ/kg for CP, EE, NDF and NFC, respectively); summing these products and adjusting for the metabolic fecal energy. The resulting DE is converted into ME with the equation proposed by NRC (2001). At the basis of the GP24-based approach of Menke and Steingass (1988), there is the implicit assumption that ME can be predicted as: 15.2×dCP + 34.2×dEE, + 12.8×dCF + 15.9×dNFE, where dCP, dEE, dCF and dNFE are the digestible contents of crude protein, ether extract, crude fiber and nitrogen free extract, respectively. In the two systems the energetic coefficients used to weigh the variables are very different. Vermorel and Coulon (1998) showed that for feeds identical for chemical composition and digestibility the use of different coefficients in the equations proposed by the different energy systems resulted in marked differences in the predicted ME contents. They observed that NRC (1989)

overestimates the ME content of feeds at maintenance with about 5-7% compared to various European energy systems. They attributed these differences mainly to the equation adopted by NRC (1989) to convert DE in ME (equation n. 5). This equation, established from data obtained with lactating cows at feeding level close to 3 times maintenance (Moe and Tyrrell, 1976), is still adopted by NRC (2001). This overestimation is of the same order of magnitude of those of + 4% and + 6% evidenced by the ME_{NRC} values compared to the ME_{UCD} and the ME_{Menke} , respectively, but in this last case only excluding the protein rich feeds.

6. Conclusions

The results of the present work support the validity of reducing the duration of the *in situ* incubation time from 48 to 24 h, with advantages in terms of saving labour, time and costs for the TDMd evaluation of feeds. The reproducibility of TDMd48 and TDMd24 was comparable (SED=14 g/kg DM) and these measures were related by the following relationship: TDMd48 = 0.74×TDMd24 + 260 (R^2=0.98; RMSE=21.5 g/kg DM).

It was found that the measures of GP24 and TDMd48 are well correlated (R^2=0.97), but only for feeds with less than 16 % of CP/DM, as CP strongly reduces the release of gas produced during fermentation. The *in situ*-based ME_{NRC} content of feeds can be predicted with good precision and accuracy from measurement of GP24 and feed proximate composition, providing that the influence of feed CP content on GP24 is adequately quantified. To this regard the equation proposed by Robinson et al. (2004) was found to be more precise and accurate than those proposed by Menke and Steingass (1988). However, the precision and the accuracy of predictions based on GP24 depend on: i) the availability of standardized procedures for measuring GP; ii) the availability of reliable equations relating GP and feed chemical composition (with special regards for CP) to predicted or *in vivo* measured energy contents of feeds; iii) the set of equations that a given energy system proposes to predict the ME of feeds from their digestible nutrient contents.

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CHAPTER 6

Running head: Effect of venting on *in vitro* gas production

In vitro rumen fermentation: Effect of headspace pressure on the gas production kinetics of corn meal and meadow hay

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1. Abstract

An automated batch system, consisting in 20 bottles equipped with gas pressure sensors and venting valves, was used to test the effects of headspace pressure on the kinetics of gas production (GP). Two venting procedures were compared: with FT (fixed times) the gas accumulated in the headspace of bottles was released after 2, 4, 6, 8, 12, 24, 48, 72 and 144 h of incubation, whereas with FP (fixed pressure) the valves were opened at a threshold of 3.4 kPa. For each procedure, samples of corn meal and meadow hay (0.50 g) were incubated in 4 replications in 310 ml bottles with 25 ml of rumen fluid and 50 ml of medium for 144 h at 39°C. Both with FT and FP, gas pressures at the times of venting, converted in terms of volumes, were adjusted or not for the amount of dissolved gas according to the Henry's law. Data were cumulated and they were best fitted by a first order model the which parameters are the asymptotic GP (A), the time at which half of A is produced (T½) and the sharpness (c) of the curve. The effects of the 2 procedures were evaluated using a Wilcoxon two-sample test. The headspace pressure obtained with FT peaked 18.0±2.84 kPa at 12 h on corn, while peaked 7.5±0.81 kPa at 48 h on hay. For corn, the un-adjusted GP achieved between 12 and 48 h of incubation were 21 and 8% lower with FT compared to FP (P=0.01), and FT also had greater standard deviations. A similar trend, less accentuated, was observed for hay. The T½ values were greater with FT compared to FP (+1.3 and +2.3 h, for corn and hay, respectively; P<0.05), suggesting that FT delayed the release of gas dissolved in the medium. After adjustment, the GP values provided by the 2 procedures continued to be different for corn: compared to FP, FT reduced GP at 12, 24 and 48 h (P=0.01). Adjustments removed all the differences for hay due to the venting procedure. Using the FT procedure, headspace volume, venting frequency and amount of fermentable matter must be carefully balanced to avoid high headspace pressures, lowered gas release and, hence, altered GP kinetics.

Abbreviations: GP, gas production; FT, fixed time; FP, fixed pressure; A, asymptotic GP; T½: time at which half of GP is obtained; c: sharpness of the kinetic curve of GP.

Keywords: *In vitro* rumen fermentation; Gas production techniques; Valve venting; Feed evaluation.

2. Introduction

Gas released from feeds inoculated with rumen fluid reflects the microbial activity. Gas is produced as the fermentation proceeds and the cumulated profile can give information on feed digestibility and fermentation kinetic (Getachew et al., 1998). Kinetics of gas production (GP) are used to rank feeds and GP at 24 h is used to estimate feed energy value (Menke and Steingass, 1988). The main employed techniques are based on the measurement of the volume of gas produced in syringes under atmospheric pressure (Menke and Steingass, 1988) or on the measurement of the headspace gas pressure of incubation bottles (Pell and Schofield, 1993; Theodorou et al., 1994; Cone et al., 1996; Davies et al., 2000). Gas produced during the in vitro fermentation obtained with the technique proposed by Pell and Schofield (1993) is not vented, but the cumulated headspace pressure is recorded at regular times, whereas with the techniques described by Theodorou et al. (1994), Cone et al. (1996) and Davies et al. (2000) headspace gases are released at pre-determined times (FT) or when a pre-set threshold of pressure (FP) is reached. The effect of venting during the incubation on the profile of GP has been the object of many discussions (Rymer et al., 2005), in particular after the observations of Theodorou et al. (1994) who argued that (i) when the gas pressure is left to accumulate in the fermentation bottles, according to the Henry's law, a given proportion of gas remains dissolved in the culture medium, so less gas is released, and (ii) microbial activity could be disturbed if gas pressure exceeds a given threshold (48 kPa). The effect of venting procedure was discussed in papers reporting comparisons between techniques (Rymer et al., 2005; Gierus et al., 2008), but the results were inconclusive, likely because of confounding effects due to the use of different apparatus, diets and donor animals. No efforts have been made to evaluate the effects of venting on GP using the same equipment. The effects of FT or FP venting procedures on the GP kinetics were studied with an automated batch GP system on feeds with different degradability.

3. Material and methods

3.1. Gas production system

A commercial apparatus (AnkomRF Gas Production System, Ankom Technology, NY, USA) consisting up to 50 bottles equipped with pressure sensors (pressure range: −69, +3447 kPa; resolution: 0.27 kPa; accuracy: ±0.1% of measured value) wireless connected to a computer

was used. During incubation the headspace pressure of each bottle is read with a frequency of 1 minute and recorded in a database. Each bottle is equipped with an electromechanical valve that controls the release of gas: for each bottle the operator can establish the venting by fixing a given threshold of pressure (FP) or in alternative a pre-defined sequence of times (FT).

3.2. Experimental design and incubation procedures

Eight samples of corn meal and 8 samples of meadow hay, milled through a 1 mm screen, were incubated in a single run using 16 bottles (plus 4 for blanks) of the system. A buffer solution (2 l), reduced with sodium sulphite according to Menke and Steingass (1988), was placed in a waterbath at 39 °C and purged with CO_2. The rumen fluid was collected by an oesophageal probe from 3 dry Holstein-Friesian cows fed *ad libitum* meadow hay and 2 kg of concentrate (500 g/kg corn meal, 250 g/kg barley meal, 250 g/kg soybean meal). The use of fluid collected with the probe instead of fluid collected from fistulated cows was not considered to be relevant for the purposes of this work. The rumen fluid, strained through 3 layers of cheesecloth, was stored into pre-heated thermos and immediately transferred to the laboratory. Each bottle (310 ml) was filled with 0.5000±0.0010 g of feed, 25 ml of rumen fluid and 50 ml of medium, for a corresponding headspace volume of 235 ml. These procedures were conducted under anaerobic conditions by keeping the bottle headspace continuously flushed with CO_2. The bottles were placed into a ventilated incubator at 39±0.5 °C for 144 h and they were not stirred or shaken. Eight bottles were vented at fixed times (FT), after 2, 4, 6, 8, 12, 24, 48, 72 and 144 h of incubation, as commonly done with manual equipments (Calabrò et al., 2004; Adesogan et al., 2005; Blummel et al., 2005) and avoiding that bottle pressure exceeded 48 kPa (Theodorou et al., 1994). The remaining 8 bottles were vented when the headspace pressure reached the threshold of 3.4 kPa (FP). This pressure, slightly lower than 4.5 kPa used with others automated equipments (Davies et al., 2000; Calabrò et al., 2005), was chosen in order to: i) minimize the effect of headspace pressure on gas release; ii) maintain a good precision of measurements according to the pressure detection ability of the system.

3.3. Computations and statistical analysis

The pressure data measured at the times of each venting were converted in terms of volume and cumulated; correction for blanks was negligible. The data were best fitted by the model proposed by Groot et al. (1996): GP (t) = A/[1+(T½/t)^c], where A= asymptotic GP, T½ = time at which half of A is produced, t = observation time and c = a constant representing the sharpness of the curve. The volumes measured at venting were adjusted by adding the amounts of dissolved gas computed according to the Henry's law, from total gas pressure and CO_2 solubility as extensively described by Pell and Schofield (1993). The adjusted data were fitted with the model of Groot et al. (1996). The Wilcoxon two-samples test (SAS, 2007) was used to test the effects of venting on GP, adjusted or not, at different times and on the GP kinetics parameters of the 8 curves achieved for each feed.

4. Results and discussion

The pressure profile during incubation of corn and hay with the 2 venting procedures is given in Figs 1 and 2.

Figure 1. Effect of the venting procedure ("fixed times": dotted lines; "fixed pressure": solid lines) on the headspace pressure values (kPa) recorded during the incubation of corn meal

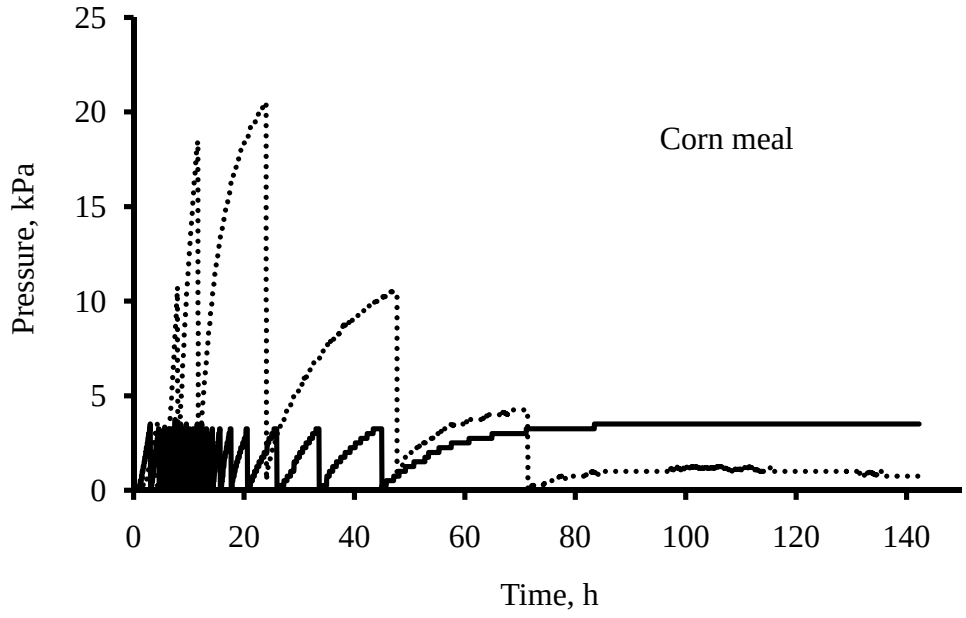
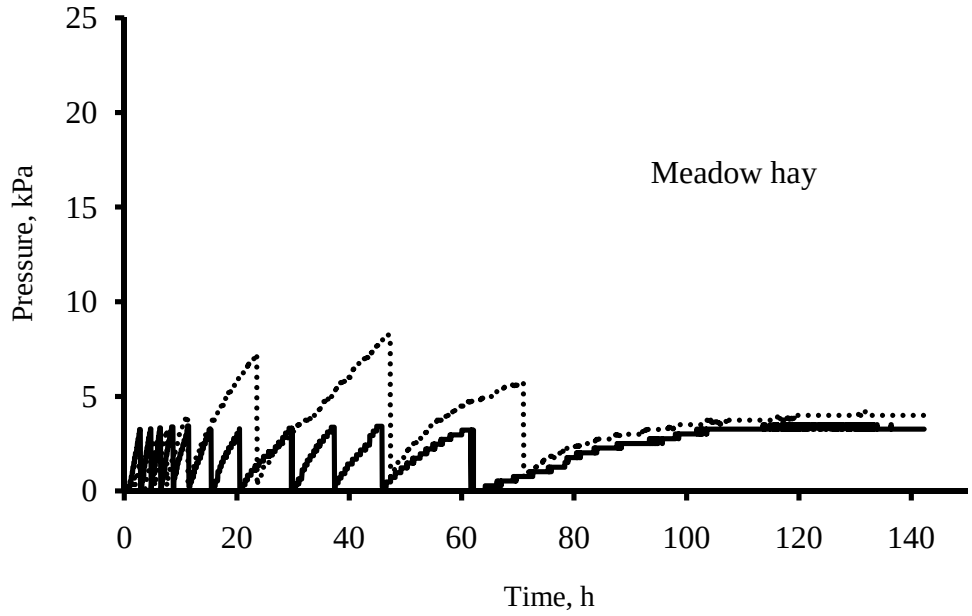
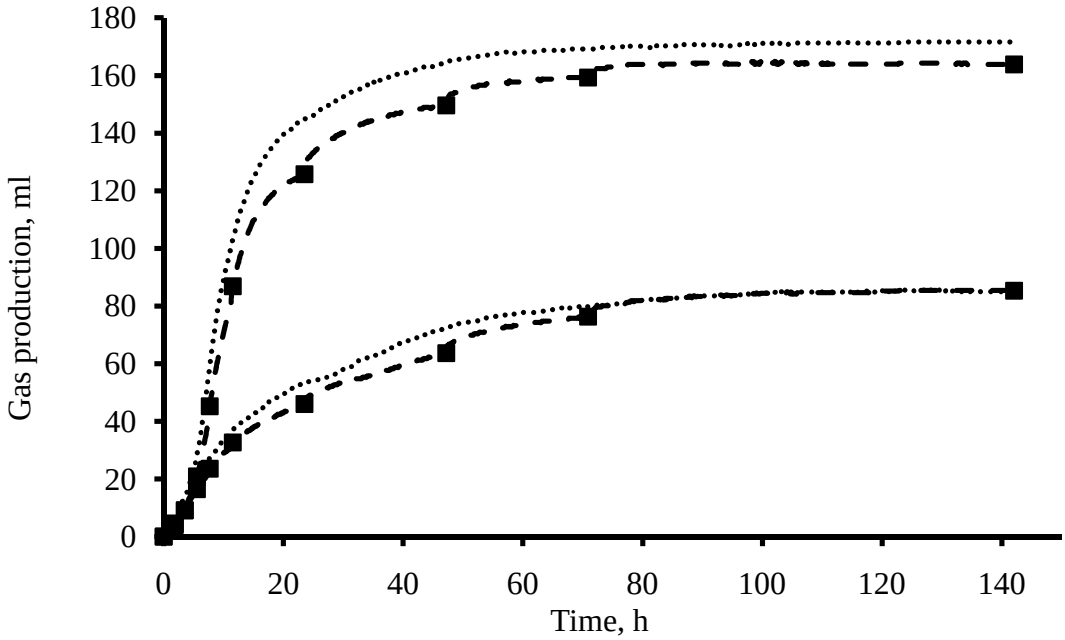


Figure 2. Effect of the venting procedure (“fixed times”: dotted lines; “fixed pressure”: solid lines) on the headspace pressure values (kPa) recorded during the incubation of meadow hay.



With FP the number of venting was 23.0±0.00 and 11.3±0.4 for corn meal and meadow hay, respectively. With FT the headspace pressure obtained with corn peaked 5.6±1.11, 12.0±3.70, 18.0±2.82, 17.6±2.24, 10.5±1.10 kPa respectively at 6, 8, 12, 24 and 48 h, respectively, whereas with hay the pressures were much lower and peaked 2.5±0.51, 3.1±0.44, 4.2±0.52, 7.4±0.40 and 7.5±0.80 kPa at the same incubation times, respectively. The profiles of cumulated pressure, expressed in terms of volume, without adjustment for dissolved gas, are shown in Fig. 3.

Figure 3. Effect of the venting procedure ("fixed times": dotted lines; "fixed pressure": solid lines) on the gas productions recorded during the incubation of corn meal and meadow hay.



All the curves achieved with FT presented the same discontinued pattern. The points of discontinuity reflects the fact that after each venting the internal pressure rapidly increases because of the release of the dissolved gas produced in the previous interval. The profiles achieved with FP using both corn meal and meadow hay did not show signs of discontinuity. For corn, the observed GP at 12, 24 and 48 h (Table 1), were lower with FT compared to FP (-21, -15 and -8%, respectively; P=0.01), and also less repeatable as indicated by the greater standard deviations. A similar trend, less accentuated, was observed for hay. As consequence, differences due to venting were observed for the various kinetics parameters of the 2 feeds (P<0.05). The T½ values, being greater with FT compared to FP (+1.3 and +2.3 h, for corn and hay respectively; P<0.05) indicated that FT there delayed the release of gas, as the gas dissolved at the times of venting was released, and hence read, later.

Table 1. Effect of venting procedure on gas production (GP) and kinetic parameters of GP (mean[1] ± SD) for corn meal and meadow hay using GP data not adjusted for the dissolved gas in the medium.

Feed	Corn meal			Meadow hay		
Venting procedure[2]	Fixed times (FT)	Fixed pressure (FP)	P^3	Fixed times (FT)	Fixed pressure (FP)	P^3
Observed GP:						
at 4 h, ml	17±2.0	13±2.6	0.03	13±2.4	13±2.3	0.44
at 6 h, ml	34±2.3	35±3.9	0.56	18±3.3	20±3.1	0.56
at 12 h, ml	$83±7.8^{B}$	$105±1.6^{A}$	0.01	32±5.0	35±4.6	0.34
at 24 h, ml	$129±10.6^{B}$	$151±2.5^{A}$	0.01	$48±6.0^{b}$	$53±5.1^{a}$	0.05
at 48 h, ml	$149±8.9^{b}$	$162±2.7^{a}$	0.01	64±5.6	70±4.6	0.06
Parameters:[4]						
A, ml	$157±6.6^{a}$	$168±3.0^{b}$	0.17	91±5.6	95±3.5	0.17
T½, h	$11.4±0.88^{b}$	$10.1±0.25^{a}$	0.01	$22.5±4.04^{b}$	$20.2±3.11^{a}$	0.05
c	2.04±0.242	2.37±0.196	0.01	$1.09±0.077^{a}$	$1.19±0.02^{b}$	0.01
RSD [5]	4.1	2.4		1.5	1.4	

[1] Each data is the least square mean of 4 observations. [2] Fixed times, the bottles were vented after 0, 2, 4, 6, 8, 12, 24, 48, 72, 144 h from the beginning of incubation; fixed pressure: the bottles were vented when the headspace pressure reached the value of 3.4 kPa. [3] Wilcoxon Test, couples of values are statistically different when $P<0.05$. [4] *A*, asymptotic GP; T½, time at which half of *A* is produced; c, sharpness of the kinetic curve of GP. [5] Residual standard deviation of fitting procedure.

After adjustment (Table 2), the gas released by the 2 venting procedures continued to be different for corn at 12, 24 and 48 h ($P=0.01$), but not for meadow hay ($P>0.05$), where the adjustment accounted for around 50% of the differences. These results indicate that with FT the adjustment of the pressure for dissolved gas at times of venting was useful for a better representation and interpretation of the gas production profiles, both when forages and concentrates were used. In the case of hay, where the headspace pressure was always lower than 7.5 kPa, only numerical differences of GP between venting procedure were observed. In the case of corn, where high pressures were reached, the adjustment alone was not sufficient for removing the differences of GP due to the 2 venting procedures, and GP remained lower ($P<0.01$) and less repeatable with FT compared to FP.

Table 2. Effect of venting procedure on gas production (GP) and kinetic parameters of GP (mean[1] ± SD) for corn meal and meadow hay using GP data adjusted for the dissolved gas in the medium.

Feed	Corn meal		P^3	Meadow hay		P^3
Venting procedure[2]	Fixed times (FT)	Fixed pressure (FP)		Fixed times (FT)	Fixed pressure (FP)	
Adjusted GP:						
at 4 h, ml	17±2.2	14±2.7	0.10	13±2.6	14±2.4	0.44
at 6 h, ml	36±2.6	36±3.9	0.44	19±3.6	21±3.2	0.44
at 12 h, ml	91±9.0[b]	106±1.6	0.01	35±5.3	36±4.5	0.24
at 24 h, ml	136±11.0[b]	152±2.5	0.01	51±6.1	54±5.1	0.35
at 48 h, ml	152±9.8	163 ±2.8	0.01	66±5.6	71±4.6	0.17
Parameters:[4]						
A, ml	158±6.6	165±3.0	0.06	92±5.7	97±3.6	0.10
T½, h	10.5±0.78[a]	9.6±0.25	0.10	20.6±5.50	19.5±3.40	0.44
c	2.20±0.277[b]	2.72±0.190[a]	0.01	1.11±0.083	1.12±0.008	0.56
RSD [5]	3.9	2.4		1.5	1.4	

[1, 2, 3, 4, 5] see Table 1 for explanations.

A first hypothesis to explain these results is that in the bottles with high pressure (FT) a supersaturation of CO_2 in the medium could have occurred. To this regard it can be observed that with both the venting procedures the bottles were not stirred. Pell and Schofield (1993) have suggested that, in their closed system, agitation prevents supersaturation of solution with CO_2. Morris (1983) and Lowman (1998) indicated that if the bottles are continually vented (at threshold pressure of 4.5 kPa) supersaturation of solution is unlikely to occur. Moreover, Lowman (1998) found that gas production was higher in bottles that were not shaken compared to those that were shaken intermittently after every gas reading or continuously on an orbital shaker at 115 rpm. Davies et al. (2000), tried to explain these results suggesting that microbes subjected to shaking could not be well attached to the feed particles as in not shaken bottles, but this explanation is not fully convincing and further research is required. An additional consideration is that high amounts of dissolved CO_2 can affect the pH of the medium and, consequently, can alter the microbial activity (Mould et al., 2005) and perhaps GP. To this regard Theodorou et al. (1994) indicated that the microbial activity can be disturbed when the pressure was around 48 kPa, and it cannot be excluded that lower pressures can also inhibit the microbial activity. Proper trials, including gradients of pressures and corresponding yields of volatile fatty acids, should be performed to test if this is true. However, it is unlikely that headspace pressure had relevant influences on volatile fatty acids yield in this trial. More likely, in this experiment the Henry's Law was not fully appropriate to quantify the CO_2 dissolved in the rumen fluid, particularly in FT where high headspace

pressures were reached. As no reliable methods for accounting variations of dissolved CO_2 are available, systems operating at low pressure and frequent valve opening should be preferred.

5. Conclusions

Venting exerts a critical role for the correct evaluation of GP during *in vitro* fermentation, particularly when high pressures are generated from feed fermentation. It was proved that different venting procedures influence the GP kinetics, and reasonably this could also have consequences on feed ranking. In situation where only the FT procedure can be applied, the headspace volume, the venting frequency and the amount of fermentable matter must be carefully balanced to avoid high headspace pressures, less gas releasing and consequent alterations of the GP kinetics. When high pressures are generated inside the bottles adjustments for the amount of dissolved gas at the time of venting could not be sufficient for a proper evaluation of the GP profile. With FT the frequency of GP reading should be as high possible; for fibrous feeds the range should be more frequent in the range from 12 to 24 h when the rate of gas production is highest. However, this is not commonly done for reasons of labour and convenience. Techniques with automated devices for gas release at low threshold pressure can strongly reduce such shortcomings, moreover they provide repeatable measurements of the GP profile and they also are less labour consuming.

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CHAPTER 7

Effects of water extracts from chicory and BHT on the *in vitro* rumen degradation of feeds

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1. Abstract

Effects of Butyl-Hydroxyl-Toluene (BHT) and of Red Chicory Extract (RCE) on kinetics of gas production (GP) and rumen digestibility values (OMD, NDFD and *in vitro* true OM digestibility - IVTOMD) of two feeds (meadow hay and corn meal) were evaluated using an *in vitro* automatic batch system. For each feed 2 increasing dosages (0.15 and 1.5 mg/g of feed) of BHT and RCE and a Control (C) were tested in 4 replications and 2 incubations. First incubation lasted 72 h, the 2nd one was stopped at the times on which half of GP was produced ($T_{½}$), which were 9 and 16 h for corn and hay, respectively. From the supernatants of the 2nd incubation, VFA, NH_3, N content of the residual NDF were analyzed and the microbial N balance was computed. The 2 feeds significantly affected rumen fermentation parameters; BHT significantly increased asymptotic GP, $t_{½}$ and IVTOMD (P<0.01), decreased the proportion of butyrate (P<0.01) but did not affect microbial N balance; RCE did not influence any of the parameters measured with respect to C, except for a significant increase of the estimated N available for microbes at the higher dosage.

Abbreviations: OMD, organic matter digestibility; NDFD, NDF digestibility; IVTOMD, *in vitro* true organic matter degradability; BHT, Butyl-Hydroxyl-Toluene; RCE, red chicory extract; VFA, volatile fatty acids; GP, gas production; A, asymptotic GP; T½, time at which half of A is produced; c, sharpness of the curve profile.

Key words: Gas production, *In vitro* rumen digestibility, Natural extracts, Antioxidants

2. Introduction

In the North East area of Po valley red chicory is enjoying a great success and in the year 2005 the local market demand reached about 250,000 ton/year. Increasing amounts of by-products are made available. Red chicory has been shown to contain considerable amounts of phenolic compounds with antioxidant properties (Rossetto et al., 2005).

Extraction of bioactive substances from by-products is receiving growing interest for human and animal nutrition, also for the opportunity to replace synthetic compounds. Some studies suggested that natural extracts from vegetables can be used to manipulate the rumen fermentations, selecting or promoting the growth and the activity of microbes, changing the amount and the ratio of the end products of fermentation (Naziroğlu et al., 2002; Busquet et al., 2006; Alexander et al., 2008). However, limited data are available about the effect of antioxidants and natural extracts on rumen fermentations. This study was aimed to screening the effect of Red Chicory Extracts (RCE) and Butyl-Hydroxyl-Toluene (BHT) on some parameters of rumen fermentations when incubated *in vitro* with different feeds.

3. Material and methods

In vitro rumen fermentations were conducted using an automatic batch gas production (GP) system (RF, Ankom Technology®) for 72 h at 39°C. In each jar, 25 ml of rumen fluid, collected from 3 dry cows, 50 ml of buffer (Menke *et al.*, 1979) and 0.55 g/batch of meadow hay or corn meal, milled at 1 mm, were used. For each feed 5 treatment groups were tested in 4 replications: 2 increasing dosages (0.15 and 1.5 mg/g of feed) of BHT (BHTL; BHTH, respectively) and RCE (RCEL; RCEH, respectively) and a Control (C) group. Four blanks without feeds were also included. Note that the maximum dosage of BHT permitted by law in compounds feeds is 0.15 mg/g. Chicory extracts were achieved as described by Rossetto *et al.* (2005). GP at various times (*t*) was measured by mean of a pressure detector every minute. GP kinetics were fitted with the model: $GP = A/[1+(T_{½}/t)^{c}]$, where A is the asymptotic GP, $t_{½}$ is the time at which half of the asymptotic GP is produced, c is a constant representing the sharpness of the switching characteristics of the curve profile. At the end of incubation OM, NDF and the *in vitro* true OM digestibility (IVTOMD) were computed from chemical analysis of feeds and residues as proposed by Grings *et al.* (2005). The T½ values resulting

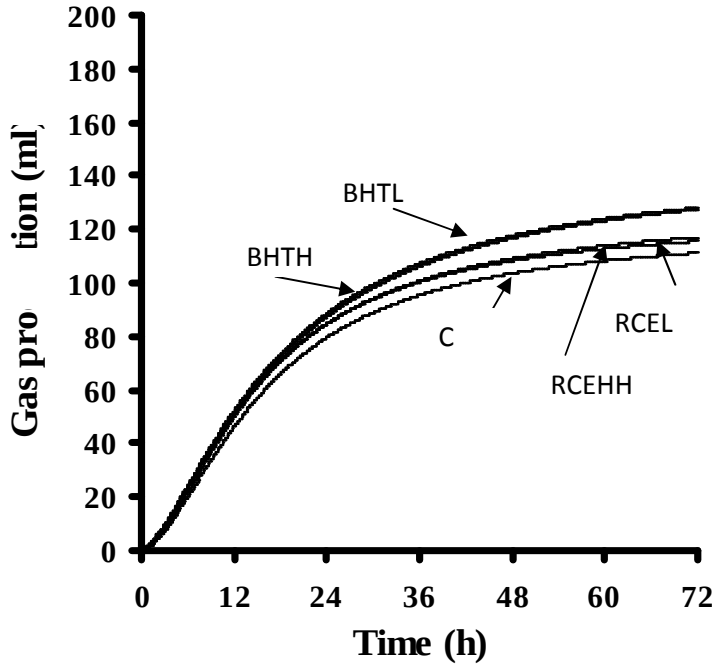
from the first incubation for the 2 feeds (9 and 16 h for corn and hay, respectively) were used to establish the times for stopping a 2nd incubation performed with the same criteria described above. The supernatant fractions, obtained from the 2nd incubation, were analyzed for volatile fatty acids (VFA), NH_3 and N content of the undegraded NDF and the microbial N balance was computed (Grings *et al.*, 2005). Data were analyzed for the effects of feeds, additives at different dosages and their interactions by ANOVA.

4. Results and conclusions

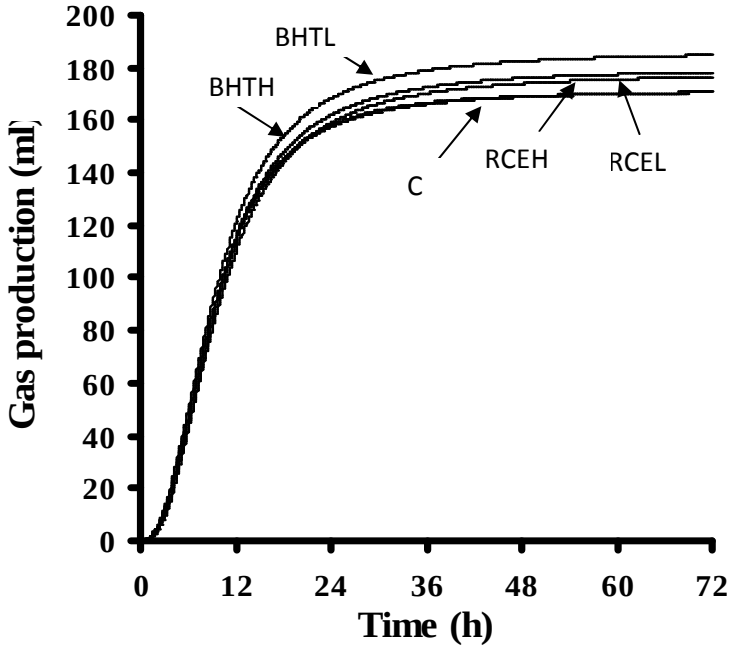
The GP kinetics are graphically described in Figure 1.

Figure 1. Kinetics of gas production (72 h) of meadow hay (a) and corn meal (b) samples incubated with 0 (C), 0.15 (L) and 1.5 (H) mg/g feed of BHT or Red Chicory Extract (RCE).

a) Meadow hay



b) Corn meal



The kinetic parameters and the digestibility values are given in table 1. In general, the residual variability within treatment was low (coefficients of variation always lower than 7%, except for c). Hay and corn significantly differed (P<0.01) for almost all the various kinetic and digestibility parameters. With respect to Control, BHT significantly increased asymptotic GP, $t_{½}$ and IVTOMD, without any difference between the two dosages. No significant influence of RCE was observed on the various parameters of GP and digestibility parameters with respect to Control. In agreement with literature, the ratio between the truly degraded OM and GP at 72 h (TOMD/GP) ranged from 2.47 to 2.70 mg/ml. The former parameter was not significantly influenced both by feeds and additives.

Table 1. Kinetics of gas production (GP), OM, NDF and *in vitro* true OM digestibility of feeds incubated with 0 (C), 0.15 (L) and 1.5 (H) mg/g feed of BHT or Red Chicory Extract (RCE)

		Feed		Additive					Root MSE
		Hay	Corn	Control C	BHT L	BHT H	RCE L	RCE H	
Kinetic of GP:									
c		1.59^{B}	2.43^{A}	2.04	1.91	1.82	2.12	2.16	0.27
A	ml	132^{B}	176^{A}	147^{B}	161^{A}	167^{A}	148^{B}	148^{B}	11
$T_{½}$	h	16.0^{A}	8.7^{B}	12.0^{B}	13.0^{A}	13.4^{A}	11.8^{B}	11.3^{B}	0.8
Digestibility (72 h):									
OMD	%	66.3^{B}	95.8^{A}	81.4	80.6	80.1	82.1	80.8	1.3
NDFD	“	59.6^{B}	83.8^{A}	71.2	73.4	72.7	70.6	70.6	1.9
IVTOMD	“	70.0^{B}	98.0^{A}	83.5^{b}	84.4^{a}	84.7^{a}	83.8^{ab}	83.6^{b}	0.4
TOMD/GP	mg/ml	2.51	2.70	2.70	2.47	2.52	2.68	2.68	0.20

Numbers on the same row for feeds or additives with different letters significantly differed: A,B P<0.01; a,b P<0.05. c= sharpness of the curve profile; A= asymptotic GP; $T_{½}$= time at which half of the asymptotic GP has been formed; TOMD/GP= truly digested OM/GP at 72 h.

As expected, results of the 2nd incubation showed significant differences of VFA profile between hay and corn (P<0.01) (Table 2). Hay produced higher proportions of acetate and lower proportions of propionate and *n*-butyrate with respect to corn. At $t_{½}$ hay and corn significantly differed also for the microbial N balance. With hay, amount of N in form of ammonia found after 16 h ($T_{½}$ for hay) of incubation was similar to the value measured at the beginning of incubation, while for corn after 9 h of incubation the amount of N from ammonia was reduced by half, with respect to the initial value. The estimated amount of N available for microbial growth for hay was about 3 times lower than that observed for corn at $T_{½}$. BHT significantly decreased the proportion of butyrate and significantly increased the remaining

VFA (P<0.01), but no significant effects were observed for the microbial N balance, with respect to C. RCE did not influence any of the parameters measured with respect to C, except for a significant increase (P<0.05) of the estimated N available for microbes at the higher dosage. In conclusion, the results of this work did not evidence a significant effect of RCE on rumen fermentation when incubated with different feeds at different dosages, whereas BHT significantly influenced GP kinetics, degradability parameters and VFA profile.

Table 2. VFA profile and microbial N balance of hay and corn incubated for 9 and 16 h ($T_{½}$), respectively, with 0 (C), 0.15 (L) and 1.5 (H) mg/g feed of BHT or Red Chicory Extract (RCE).

		Feed		Additive					Root MSE
		Hay	Corn	Control C	BHT L	BHT H	RCE L	RCE H	
Acetate (Ac)	%	73.5^{A}	66.6^{B}	66.7^{B}	74.5^{A}	74.6^{A}	68.2^{B}	66.4^{B}	0.6
Propionate (Pr)	"	15.9^{A}	20.8^{B}	17.6^{b}	19.0^{a}	19.5^{a}	17.9^{b}	17.6^{b}	0.8
n-Butyrate (Bu)	"	7.2^{A}	9.6^{B}	13.1^{A}	2.9^{B}	2.4^{B}	10.6^{A}	13.0^{A}	1.1
Others VFA	"	3.4^{A}	3.0^{B}	2.8^{B}	3.6^{A}	3.5^{A}	3.3^{AB}	3.0^{AB}	0.3
(Ac+Bu)/Pr ratio		6.4^{A}	4.8^{B}	5.7^{a}	5.4^{b}	5.3^{b}	5.7^{a}	5.7^{a}	0.2
Microbial N balance (mg/jar)									
N from feed (F)		4.3	6.8	5.6	5.6	5.6	5.6	5.6	-
N from NH_3 at t=0 (N0)		10.4	10.4	10.4	10.4	10.4	10.4	10.4	-
N from NH_3 at $T_{½}$ (Nt)		10.4^{A}	5.4^{B}	9.3^{a}	8.9^{a}	7.4^{ab}	8.1^{ab}	5.8^{b}	1.6
N content of NDF at $T_{½}$ (N_NDF)		1.2^{B}	1.9^{A}	1.5	1.3	1.7	2.0	1.3	0.5
Microbial N at $T_{½}$		3.1^{B}	9.9^{A}	5.2^{b}	5.8^{b}	6.9^{ab}	5.9^{b}	8.6^{a}	1.9

^{A,B} P<0.01; ^{a,b} P<0.05. Microbial N at $t_{½}$ was computed as: (F + N0) – (Nt + N_NDF).

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CHAPTER 8

General conclusions

The main conclusions to be drawn from of this dissertation are:

- With Daisy^{II} equipment, the use of 0.25 g feed sample/bag should be preferred to 0.50 g/bag, as this sample size seemed to provide digestibility estimates more correlated to those achieved with a conventional batch culture and less variable
- For *in situ* studies commercial synthetic bags could replace nylon bags, as the two kinds of bag provided digestibility values highly correlated; the replacement of nylon bags with commercial bags could allow a simplification of procedure of analysis
- For *in vitro* studies the use of rumen fluid collected from fistulated animals seems to be not strictly necessary, as the digestibility estimates obtained *in vitro* using rumen fluid taken from intact cows were directly proportional to those achieved *in situ*
- As the digestibility measures at 24 and 48 h were highly correlated and showed a comparable reproducibility, it seems to be possible to reduce the duration of the *in situ* incubation time from 48 to 24 h, with advantages in terms of saving labour and costs for feed evaluation.
- Once the GP values were converted in terms of ME, taking into account the effects of feed chemical composition, the repeatability of the ME estimates from GP24 was only 20% higher than the ME resulting from digestibility measurements
- The precision and accuracy of feed energy estimates from GP strongly depends by the equations used to convert GP values in energy values
- The venting procedure can affect significantly GP kinetics, especially when high pressures are generated from feed fermentation
- With venting at fixed times the headspace volume and the amount of fermentable matter incubated should be carefully balanced; the venting frequency should be higher in correspondence to the highest GP rate
- When highly and rapidly fermentable feeds are incubated, high pressures could be generated into the GP system, and the adjustment of GP measures for the amount of dissolved gas could not be sufficient for allowing a proper evaluation of GP kinetics
- GP techniques equipped with automated devices for releasing gas at a fixed pressure should be preferred

- The red chicory extract (RCE) did not exert a significant effect on rumen fermentations, although it was found to improve the efficiency of microbial protein synthesis; with respect to RCE, BHT showed more accentuated effects, as it significantly influenced GP kinetics, degradability parameters and VFA profile.

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