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Comparison of in sacco, Daisy^{II} incubator and Gas production to evaluate digestibility of silage in ruminants

Sammenligning av in sacco, Daisy^{II} Incubator og gassproduksjon for vurdering av fordøyelighet av surfôr hos drøvtyggere

Elise Hatch Fure
Master of Science in animal nutrition

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Elise Hatch Fure

Summary

The aim of this thesis is to compare three different methods to evaluate digestibility of silages. The methods are in sacco, the Daisy^{II} Incubator and the gas production system. This is achieved by a literature review and by own experiments.

Several methods exist to estimate the degradation of a feed in the digestive tract. In vivo methods involve measuring the disappearance from feed to faeces and is the reference method to describe the biological value of a feed. In vivo is expensive, time consuming and requires high quantities of feed. In sacco is a method where a feed sample are inserted into small bags and incubated in the rumen, losses are related to degradation. The in sacco method is cheaper, less time consuming and require less feed. In sacco is also dependent on cannulated animals, and therefore more labours than many in vitro methods. In vitro methods are characterized by simulating the digestion process in a laboratory environment. The Daisy^{II} Incubator and the gas production system are equipment's that can be utilized the execution of the in vitro methods. Daisy is based on losses after incubation. Gas production system measures the gas production during incubation and relates it to degradation of a feed.

In total, 22 silages with a variation in chemical composition were studied. The silages are a part of a larger experiment by TINE, aiming for testing around 100 silages of different quality. In sacco, Daisy and Gas production produced results with good correlations between methods. In general, higher correlation between methods was observed for dry matter degradation than for NDF degradation. In addition, better correlation between Daisy NDF degradation was observed with in sacco dry matter degradation than in sacco NDF degradation. This indicate that the measurements of NDF degradation is challenging and depending on methods. The highest correlation between gas production and the other methods was observed when using the 72-hour incubation. Digestibility of early harvested silages appeared underestimated by the gas production method.

Despite the good correlation between the methods used in this thesis, a validation and comparison to in vivo measurements are needed. All feeds used had been exposed to an in vivo digestibility using sheep. Unfortunately, these results were not available within the time limit of this thesis.

The silages studied in the thesis displayed a variation in digestibility measurements in all methods tested. Assuming that the variation is also reflected in vivo, this imply that equations to determine digestibility between in vivo and the methods studied can be constructed.

Sammendrag

Formålet med denne masteroppgaven er å sammenligne tre ulike metoder til å vurdere fordøyeligheten av surfôr. Metodene er in sacco, Daisy^{II} Incubatoren og gassproduksjonssystemet. Dette er gjort ved å skrive en litteraturdel og ved å utføre egne forsøk.

Flere ulike metoder er utviklet for å estimere nedbrytelsen av et fôr i fordøyelsessystemet. In vivo metoder består i å måle hva som blir borte fra fôr til avføring. Det er dette som er referansemetoden for å beskrive den biologiske verdien av et fôr. In vivo er dyrt, tidskrevende og krever store mengder fôr. In sacco er en metode der fôrprøver er plassert i små poser og inkuberes i vomma. In sacco metoden er billigere, mindre tidskrevende og har et lavere behov for fôr enn in vivo metoder, men den er avhengig av fistulerte dyr og er mer arbeidskrevende en mange in vitro metoder. In vitro metoder kjennetegnes av at fordøyelsesprosessen er simulert i et laboratoriemiljø. Daisy^{II} Incubatoren og gassproduksjonssystemet er apparater som kan benyttes til å utføre in vitro forsøk. Daisy er basert på å måle tap etter inkubering, mens gassproduksjonssystemet måler gassproduksjonen under inkubering og relaterer det til nedbrytingen av fôr.

Totalt ble 22 surfôr med varierende kjemisk sammensetning studert. Surfôrene er en del av et større forsøk av TINE, hvor de ønsker å teste omtrent 100 surfôr av ulik kvalitet. In sacco, Daisy og gassproduksjon ga gode korrelasjoner mellom metodene. Generelt ble det observert høyere korrelasjoner for nedbrytelsen av tørrstoff, enn nedbrytelsen av NDF. Det ble også observert bedre korrelasjoner mellom nedbrytelsen av NDF i Daisy og nedbrytelsen av tørrstoff fra in sacco, enn mellom nedbrytelsen av NDF i Daisy og nedbrytelsen av NDF fra in sacco. Dette indikerer at nedbrytelsen av NDF er utfordrende å måle, og er avhengig av metodene. Den høyeste korrelasjonen mellom gassproduksjon og de andre metodene, fremkom ved bruk av 72-timers inkubasjon. Fôrverdien av tidlighøstet fôr ble undervurdert av gassproduksjonssystemet i forhold til de andre metodene

Til tross for en god korrelasjon mellom metodene som ble brukt I denne oppgaven, trengs det en validering opp mot resultater fra in vivo. Alt fôr som er brukt har vært gjennom ett in vivo fordøyelsesforsøk. Uheldigvis er ikke resultatene fra dette forsøket tilgjengelig innen tidsfristen for denne oppgaven.

Fôret som ble studert i denne oppgaven viste en variasjon i fordøyelighet for metodene som ble brukt. Forutsatt at variasjonen også forekommer I resultatene fra in vivo, vil ligninger for fordøyeligheten mellom in vivo og de andre metodene kunne skapes.

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Introduction

In modern animal production, feed holds a position of major importance. In addition to be a considerable cost for the farmer, the feed influence production and health of an animal. Consequently, it is desired to construct a well composed diet that sustenance health and production of an animal at lowest cost possible. To achieve this, it is not only the chemical composition of the feed that is important, but also the actual degradation of nutrients in the digestive tract.

It exists several methods to study feed degradation in the digestive tract. The most apparent is to measure the disappearance from feed to faeces, known as in vivo methods. This is expensive and time consuming but recognised as the reference method to describe the biological value of a feed. Alternatives are the in sacco method, based on rumen incubation of feed material, or in vitro methods. In vitro methods are often based on incubation in various devices using enzymes or rumen liquor. In vitro methods estimate feed degradation either by disappearance of feed material or by gas production after incubation. In sacco and in vitro methods are generally cheaper and less time consuming than the in vivo method (Stern, Bach, & Calsamiglia, 1997), but their accuracy is dependent upon their ability to resemble the biological processes that take place in the living animal (Van Soest, 1994).

The objective of this thesis is to compare three different methods to evaluate digestibility; the in sacco method, the Daisy^{II} Incubator and the gas production system. This is done by a literature review and by own experiments. The literature review gives an overview and background information needed to evaluate the different methods, while the experiments gives data needed for the comparison. The feed used in the experiments are silages with a wide range of dry matter content and chemical composition. Silages are a major constitution of a ruminant diet, especially in countries with a short grazing season (Mo, 2005). Therefore, the determination of digestibility in silages are important. The three evaluated methods are expected to reflect digestibility of nutrients in a comparable way.

1 Literature review

The literature review opens with a description of grasses, and the importance maturity have one the grasses chemical composition. This is followed by methods to preserve grasses, with a focus on silages. Afterwards, a short review of the benefits of the ruminant digestive system and its structure is followed by an overview of the degradation of carbohydrates, proteins and lipids in the ruminant's digestive system. At last, there is an introduction of different methods to estimate the digestibility of a feedstuff.

1.1 Grasses

Grasses is the common name of the large and diverse family named *Poaceae* (also known as *Gramineae*). Timothy, ryegrass and meadow fescue are examples of grass species, but also cereal crops like maize, wheat and rice are included in the family of *Poaceae*. Many grasses are perennial, but there are great diversity of durability between species (Skår, 1999). They often have cylindrical, hollow stems that is plugged at the nodes. The leaf consists of leaf sheath and leaf blade, where the leaf sheath starts at the node. The ligule is placed in the junction between sheath and blade (Figure 1). The shape and visibility of the ligule can variate widely, and even be missing. Because of the variation of the ligule shape, it is often used to spot the difference between species. The roots usually only penetrate the top 15-20 cm of the soil, making most grasses exposed to drought (Skår, 1999). Flowers of grasses are commonly hermaphroditic and simple built (Mossberg & Stenberg, 2014).

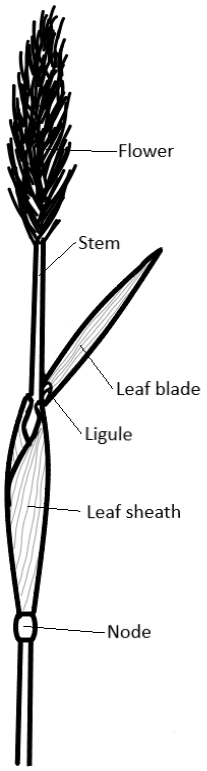

Figure 1. Schematic drawing of a generalised grass

The ratio between stem and blades variate between species, and is connected with how the grasses are commonly used (Skår, 1999). Grasses with a lot of blades are more suited for grazing and multiple harvestings, because they generally have faster regrowth after harvest and keep their high nutritional content longer. While grasses with a lot of stem are more suited for meadows, since they keep the plants up from the soil, thus making it easier to harvest and dry.

Chemical composition of grasses can variate widely. The largest factor affecting chemical composition is the maturity of grasses (McDonald et al., 2011). When grasses grow, there is a rapid production of leaf before the growth transitions to the stem. Towards the end, flowers emerge, and seeds are formed. At increasing stages of growth, the amount of carbohydrates increases (hemicellulose, cellulose, lignin and sugar) at the expense of protein, lipids and minerals (Mo, 2005). Lignin develop strong chemical bonds with cellulose, hemicellulose and proteins, making them unavailable for digestion (McDonald et al., 2011). A delayed time of harvest will therefore give more feed (dry matter/acre), but a decrease in degradability (Mo, 2005). An example of changes in chemical composition based on different times of harvest are presented in Table 1.

Table 1. Chemical composition of silages at different harvesting times. Including dry matter (DM), neutral detergent fibre (NDF), indigestible NDF (iNDF), ash, crude protein (CP), soluble crude protein (sCP), sugar and crude fat (CFat). DM is given in g/kg, iNDF in g/kg NDF, sCP in g/kg CP and the others in g/kg DM. Values are obtained from the NorFor Feed Table (NorFor, n.d.)

Time of harvest	DM	iNDF	NDF	Ash	Sugar	CFat	CP	sCP
Early	224	115	507	69	32	31	168	679
Medium	237	144	566	64	35	28	140	661
Late	260	169	610	67	114	23	121	735
Blooming	224	244	570	55	63	17	97	698

The content of cellulose is usually within the range of 200-300 g/kg dry matter, while the content of hemicellulose can variate between 100-300 g/kg dry matter (McDonald et al., 2011). Water-soluble carbohydrates can vary between 25-300 g/kg dry matter, and consist merely of fructans, starches and sugars (McDonald et al., 2011). Crude protein can for instance range from 30 g/kg to 300 g/kg (McDonald et al., 2011). The amino acid composition of grasses holds a high biological value for growth, compared to grains (McDonald et al., 2011). And the composition of amino acids is quite stable, regardless of maturity or grass species. Young grasses usually have a very high degradability of proteins, but it decreases as the forage mature because of the lignification. Grasses have generally low contents of lipids, which rarely exceeds 60 g/kg dry matter (McDonald et al., 2011). The chemical composition of grasses is also influenced by soil, fertilisers, weather and the species chosen.

1.1.1 Silage

Preservation of grasses are often done by drying (hay) or fermentation (silage), where silage is the most used method to preserve grass today (McDonald et al., 2011). Preserved grass can pose an important part of the diet to ruminants, especially in countries with a limited growth season. If correctly conducted, the preservation will maintain quality, nutritional value and palatability of the grass. The principles behind fermentation are quite simple. An anaerobic environment and a low pH will inhibit microbial growth, and thereby preserve the feed. A low pH is obtained by acid production from microorganisms. The growth of lactic acid bacteria is desired, because it gives a relatively large reduction of pH compared to the associated energy loss. In addition, the undissociated lactic acid contributes to the inhibition of microbial growth (Mo, 2005). The inhibition of microbial growth is also affected by the dry matter content and the temperature of the feed.

Silage mainly consist of grasses but can also have a fluctuating content of legumes. For example, a mix of grass and clover generally gives an increased quantity of forage, higher content of protein and a better composition of minerals. Clovers also fixate nitrogen and have a good soil structuring effect. However, clover are harder to dry and/or ferment, and therefore more difficult to preserve (Skår, 1999).

1.2 The ruminant

Grasses and other plants contain β-linked polysaccharides, like cellulose and hemicellulose. These polysaccharides can't be broken down by the enzymes produced by the animal itself (McDonald et al., 2011). However, herbivores have evolved large chambers in the digestive tract containing microorganisms. The microorganisms are able to break down the β-linked polysaccharides, as well as other nutrients, and the end-products are available to be digested by the host-animal (Sjaastad, Sand, & Hove, 2016). Herbivores can be divided into ruminants and simple-stomached herbivores, depending on whether the microbe-containing chamber is located respectively before or after the stomach.

In addition to the ability to utilise β-linked polysaccharides, the rumen microorganisms alter a feed with a relatively poor nutritional composition to be of a better value to the ruminant. Rumen microorganisms produce vitamin B and K in quantities sufficient to the ruminants needs (McDonald et al., 2011). Rumen microorganisms also synthesise essential amino acids, and thereby ensure an amino acid composition that's often sufficient to the nutritional needs of the host animal (McDonald et al., 2011). However, high producing ruminants sometimes needs a different composition of nutrients than what is given by the microorganisms. In these cases, there is developed feed that's

more resistant to microbial degradation, letting a greater part of the feed reach the stomach untouched by the microorganisms in the rumen.

1.2.1 The structure of the ruminant digestive system

An overview of the digestive system of a ruminant are shown in Figure 2. It starts at the oral cavity, where the feed is chewed and mixed with saliva. Unlike omnivores and simple-stomached herbivores, the saliva of ruminants does not contain amylase, and thereby the degradation of starch does not start until the forestomachs (Sjaastad et al., 2016). The oesophagus leads the chewed feed to the forestomachs, in addition to transport ruminal content back up to the oral cavity for additional chewing – rumination. Fibrous feed is repetitively ruminated until the particles are small enough to move on.

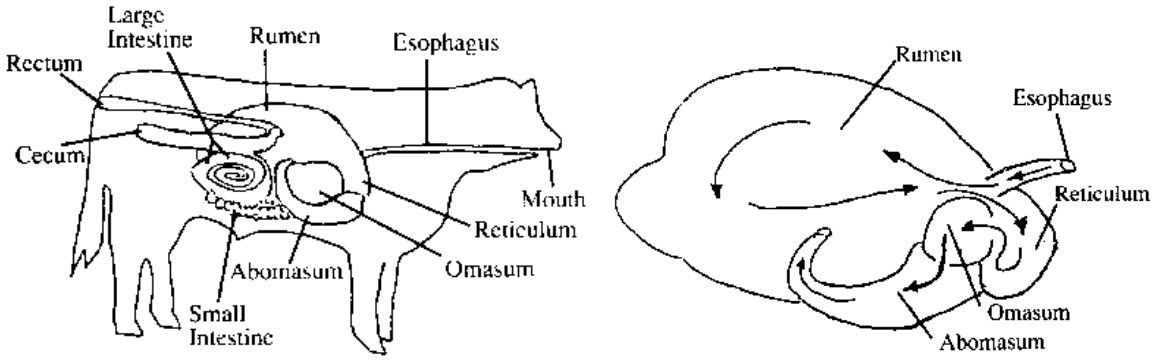

Figure 2. Overview of the digestive system of bovine. Copyright by Crown copyright (Hamilton, 2015).

The forestomachs consists of the rumen, the reticulum and the omasum. The rumen and the reticulum are linked with a big opening, making the ruminal content able to easily pass back and forth between the compartments (Sjaastad et al., 2016). In the forestomachs there is no production of digestive enzymes by the ruminant itself, but there is microbial enzymes and mechanical processing (Sjaastad et al., 2016). The microorganisms start the enzymatic degradation of carbohydrates, proteins and lipids in the rumen. Ingesta are transferred into the omasum regulated by a sphincter (a circular muscle). The omasum contains a considerable absorptive surface by rows of tissue folds. Some of the digesta flows between the folds, while some goes directly to the ruminant stomach, called abomasum. The abomasum contains glands that produce pepsinogen and hydrochloric acid. The pH of the abomasum are low enough to kill most of the microorganisms that's followed the ingesta from the rumen (Sjaastad et al., 2016).

From the abomasum the digestive system continues to the small intestine, where nutrients are additionally degraded and absorbed. Pancreatic juice and bile are excreted at the duodenum, the beginning of the small intestine. Pancreatic juice are produced in the pancreas, and neutralises the acid content entering the small intestine in addition to contain enzymes to degrade carbohydrates,

protein and fat (Sjaastad et al., 2016). Bile are produced by the liver and holds an important role in the degradation and absorption of fat. In many species, like cat, dog and human, the storage of bile in the gall bladder is important. Ruminants have a more continuous flow of digesta through the intestines in addition to a lower content of fat in the feed. Resulting in a lessen need for a periodic contribution of bile, and therefore a gall bladder of impaired function (Sjaastad et al., 2016).

The large intestine is important to absorb water and ions, in addition to house microorganisms that digest and absorb carbohydrates and proteins that's escaped former digestion. Microorganisms from the rumen are an important nutritional contribution to the host animal, since they are digested in the small intestine. Microorganisms from the large intestine are excreted in the faeces, and therefore not utilized. However, the end products from the microbial fermentation in the large intestine are available for absorption.

1.3 Degradation of nutrients in the ruminant digestive system

Normally, nutrients are divided into carbohydrates, proteins, lipids, mineral, vitamins and water. In the following chapters the ruminant's degradation of carbohydrates, proteins and lipids are described.

1.3.1 Carbohydrates

The ruminal microorganisms attack all types of carbohydrates, but not lignin (McDonald et al., 2011). Thus, the carbohydrates masked or bound to lignin, are indigestible. Complex carbohydrates are digested to simple sugars by extracellular microbial enzymes. Simple sugars are then rapidly absorbed by the microorganisms and digested further by intracellular enzymes. Thus simple sugars are seldom detectable in the rumen fluid, because it is absorbed consecutively by the microorganisms (McDonald et al., 2011). The major end products from rumen degradation of carbohydrates are VFA (mainly acetic acid, propionic acid and butyric acid), carbon dioxide and methane.

The proportions of acetic acid, propionic acid and butyric acid will variate according to the feed that's degraded (McDonald et al., 2011). Forages at a late stage of development gives more acetic acid, while younger forages tend to give more propionic acid. The degradation of concentrates also gives more propionic acid at the expense of acetic acid. In addition, young grass and concentrates gives a larger production of VFA than fibrous feeds. In total, the amount of VFA produced can be up to 8 kg for a high-yielding cow (Sjaastad et al., 2016). Most of the VFA are absorbed through the ruminal wall, however 10-20 % might pass the forestomachs to be absorbed in the small intestine (McDonald et al., 2011). In addition, the microorganisms utilise some of the products from

carbohydrate digestion to make their own cellular polysaccharides. But the quantities of cellular polysaccharides passing to the small intestine are probably trivial (McDonald et al., 2011).

As mentioned, carbohydrate degradation also leads to the production of gas. The gas production are highest immediately after feeding, and the rate can surpass 30 litres/hour (McDonald et al., 2011). Normally the gas consists of 40 % carbon dioxide and 30-40 % methane, in addition to 5 % hydrogen (McDonald et al., 2011). The gas produced in the rumen also consists of small amounts of oxygen and nitrogen, however these compounds originates from ingested air (McDonald et al., 2011). The production of carbon dioxide is partly a by-product from fermentation, and partly from a reaction between organic acids and bicarbonate from saliva. Methane is produced from the reduction of carbon dioxide. Gas production are mostly lost by eructation, and about seven percent of the energy from the feed are lost as methane (McDonald et al., 2011). However, about 20% of the produced gas are absorbed through the ruminal wall (Sjaastad et al., 2016).

The degradation of carbohydrates continues in the small intestine. Amylase from the pancreas juice degrade polysaccharides into oligosaccharides. Ruminants have little amylase in their pancreas juice compared to simple-stomached animals except horses (Sjaastad et al., 2016). A further degradation to monosaccharides occurs by enzymes produced by the epithelial cell. Nondigested carbohydrates continue to the large intestine, where the degradation is similar to the degradation in the rumen and reticulum.

1.3.2 Protein

The degradation of protein starts in the rumen. Microorganisms hydrolyses protein into peptides and amino acids. A small portion of the amino acids are deaminated into fatty acids, while some amino acids are degraded further, making organic acids, ammonia and carbon dioxide (McDonald et al., 2011). Degradation of protein are done extracellular until small peptides are transported into the microorganisms to be further degraded. The rumen microorganisms utilise ammonia, small peptides and free amino acids to synthesise microbial protein. A part of the microbial protein are broken down and reused in the rumen by microorganism (McDonald et al., 2011). However, the major part of the protein reaching the small intestine is microbial protein, whereas a minor part will be undigested food protein (Sjaastad et al., 2016).

Ruminal ammonia concentration is important in the microbial degradation and synthesis of protein. Therefore, a diet with a low amount of rumen degradable protein will lead to a slow growth of microorganisms in the rumen. A slow growth of microorganisms would in turn lead to a slower degradation of carbohydrates (McDonald et al., 2011). Nevertheless, a surplus of ammonia will be absorbed, and carried with the blood to the liver where it is converted to urea. Some of the urea are

reused in the rumen through recycling in saliva, but the majority are wasted by excretion to urine and milk (McDonald et al., 2011). In times of poor protein intake, the reuse of urea can lead to a higher amount of protein in the intestine compared to what's eaten (McDonald et al., 2011). It is important to note that dietary protein is not the only contributor to ammonia in the rumen. Up to 30% of the nitrogen in ruminant feed can be non-protein nitrogenous compounds, like amino acids, amides, amines and nitrates (McDonald et al., 2011).

Pepsinogen are produced by the gastric glands in the abomasum, and thereby starts the ruminant's own degradation of protein. However, the amount degraded in the abomasum are usually modest (Sjaastad et al., 2016). The degradation of protein continues in the small intestine by proteases from the pancreas juice. This degrade proteins into short-chain peptides and minor quantities of amino acids. During absorption the short-chain peptides are further broken down to amino acids, dipeptides and tripeptides (Sjaastad et al., 2016). Any undegraded protein left goes to the large intestine, where the degradation is similar to what's done in the rumen and reticulum. The absorption of nitrogen in the large intestine, is considered to give insignificant contribution to the protein status of the animal (McDonald et al., 2011).

1.3.3 Lipids

Rumen microorganisms have a limited capacity to digest lipids. If the lipid content of a diet exceeds 100 g/kg, the activity of ruminal microorganisms are reduced (McDonald et al., 2011). An reduction in activity can cause a retardation of the fermentation that take place in the rumen, and can lead to a reduction in feed intake (McDonald et al., 2011). However, if the content of lipids is below this threshold, they are at a large extent hydrolysed in the rumen by extracellular lipases from microorganisms. Unsaturated fatty acids are hydrogenated, where both cis- and trans-fatty acids can arise (Sjaastad et al., 2016). The rumen microorganisms also synthetises lipids, which eventually can be found in the milk and body fat of ruminants (McDonald et al., 2011).

Short-chain fatty acids are absorbed directly from the rumen. Whereas long-chained fatty acids travels to the small intestine, where they are hydrolysed by the ruminant's own enzymes and absorbed. Lipases from the pancreas juice and bile from the liver, is important to digest lipids. Bile reduces the droplets of lipids, making a larger surface between water and lipids. This is important since the degradation only occurs at the interface between water and lipids. During degradation, degradation products will occur. To maintain the maximal surface of undegraded lipids and water, bile salts transports degradation products between the lipid droplets and the surface of the epithelial cells (Sjaastad et al., 2016). Any undegraded lipids are available to microbial degradability in the large intestine.

1.4 Methods to estimate degradation

The feeding value of a feedstuff is determined by both chemical composition and digestibility. The chemical composition is relatively easy to find through chemical analysis. However, the digestibility of the same feedstuff is more labours to obtain. It is often estimated by the degradability of a feedstuff. This can be done in several ways, but the methods can be categorised into in vivo, in sacco and in vitro methods. The next chapters describe these methods more in detail.

1.4.1 In vivo methods

In vivo digestion terms the amount of nutrients who is absorbed in the living animal. Usually it describes what disappeared in the total tract from the feed to the faeces (Weisbjerg & Hvelplund, 2003), but it can also be used to estimate the digestibility at certain places in the digestive system (MacRae & Armstrong, 1969; MacRae & Ulyatt, 1974). However, the determination of digestion in vivo does have some issues that needs to be addressed. Firstly, in ruminants there is a loss of methane by eructation, which consequently gives an overestimation of digestibility. Secondly, the faeces do not merely consist of undigested feed residues. There is an excretion of certain minerals, like calcium, in the gut (McDonald et al., 2011). This excretion gives an overestimation of undigested minerals in the faeces, and thereby an underestimation of mineral digestion. Lastly, faeces also contain endogenous and undigested microbial content, which does not directly originate from the feed. If the endogenous and microbial content of the faeces are corrected for, true digestibility can be calculated (Equation 1) (Weisbjerg & Hvelplund, 2003). But the content of endogenous and microbial matter is difficult to quantify for most nutrients. Therefore, apparent digestibility (Equation 2), is more easily obtained. In theory, these calculations can be used to find the digestibility of dry matter, organic matter or even every constituent of the dry matter (McDonald et al., 2011). However, they are quite meaningless on some mineral elements because of what's previously stated (McDonald et al., 2011)

$$True\ digestibility = \frac{feed - (faeces - (endogenous\ matter + microbial\ matter))}{feed} \quad \text{(Equation 1)}$$

$$Apparent\ digestibility = \frac{feed - faeces}{feed} \quad \text{(Equation 2)}$$

As evident in the equations, the quantity and composition of both the feed and faeces are needed to measure digestibility. The amount of faeces can be decided either with complete manure collection or with faeces samples using markers to calculate the total excretion (Weisbjerg & Hvelplund, 2003). The digestibility at a certain place of the digestive system can be found by using markers to estimate the amount of dry matter or a specific nutrient before and after the place in question. To estimate the digestibility at a certain place is easiest measured using cannulated animals (McDonald et al.,

2011). An important aspect to the estimation of digestibility is that both the amount of feed given and the composition of the ration affects the digestibility of the feed (Weisbjerg & Hvelplund, 2003). Because of this, there is developed a standardised method for in vivo, where digestibility is measured on sheep fed at maintenance level (Weisbjerg & Hvelplund, 2003).

1.4.2 In sacco methods

The in sacco method is also termed the in situ method, or the nylon bag technique (Cattani, 2011). The method is most commonly used to measure rumen degradation of protein (McDonald et al., 2011; Weisbjerg & Hvelplund, 2003) But in sacco is also frequently used to measure rumen degradation of NDF and starch (Åkerlind et al., 2011). The principle is to enclose a feed sample into a nylon bag and let it incubate in the rumen. The bags can be incubated at various times, usually 0, 2, 4, 8, 16, 24, 48 and either 72 or 96 hours depending on what's determined (Åkerlind et al., 2011). Feed samples are kept separated from the surrounding content of the rumen by the bags, while microbes and degradation products can go through (Weisbjerg & Hvelplund, 2003). The bags pore size is a compromise between letting microbes in, whilst not losing undegraded feed particles (Van Soest, 1994). In the standardised in sacco protocol used in the Nordic countries, a pore size of 38 µm is used (Åkerlind et al., 2011). After incubation, the bags are washed, dried and the residues are noted (Weisbjerg & Hvelplund, 2003).

Disappearance from the bags gives a degradation profile, following the time points used (Weisbjerg & Hvelplund, 2003). This profile usually follows first order degradation kinetics and is used to determine a soluble fraction, a degradable fraction and a degradation rate of the degradable fraction, as described by Ørskov and McDonald (1979). Loss of feed particles from the in sacco bags is a substantial problem with the method. If not corrected for, these particles are assumed soluble and rapidly degraded in the rumen. Assuming these particles are degraded in a similar patters as particles left in the bags, the loss can be corrected for as described by Weisbjerg and Hvelplund (2003). The in sacco method is also used to decide the level of indigestible NDF. If so, a pore size of 11-15 µm and an incubation time of 288 hours is used (Åkerlind et al., 2011).

Another similar method to in sacco, is the mobile nylon bag technique. Here bags of feeds are inserted freely into the digestive system, and later removed through a cannula (McDonald et al., 2011) or from excreted faeces. The nylon bag technique can therefore be used to measure digestion at different locations in the digestive tract, and not only the ruminal digestion like the in sacco method.

1.4.3 In vitro methods

In vitro terms methods where the digestion process is simulated in a laboratory environment, i.e. not in the living animal. This is often faster and have a better replication than in vivo or in sacco methods (Van Soest, 1994). However, the success of in vitro methods are dependent of their degree to reflect the biological processes which occurs in the digestive tract (Van Soest, 1994). There is developed an assortment of different in vitro methods. But they are often based on incubating a feed sample in a solution with buffer and either rumen inoculum or commercial enzymes (Weisbjerg & Hvelplund, 2003). It is also possible to use secretion from other places in the digestive tract than the rumen, or to use faeces as a source of digestive microorganisms (Abdouli & Attia, 2007; Coles, Moughan, & Darragh, 2005; Edmunds et al., 2016). The buffer should both ensure a sustainable pH and access to ammonia, where the latter is more important in poor-quality forage (Van Soest, 1994). To estimate digestibility, the duration of incubation have been seen to variate between 3 to several hundred hours (Van Soest, 1994). An incubation time of 48 hours are often used, as it is assumed that the degradation has reached its potential by then, although this is not always the case, e.g. with poor quality forage (Wood & Badve, 2001).

The two-stage method of Tilley and Terry (1963) has been noted to be the most commonly used in vitro method (Stern et al., 1997). The method consists of a 48-hour incubation of the feed sample in a buffer solution added rumen inoculum. After the incubation the residues are left in pepsin hydrochloric for additional 48-hours to degrade microbial protein and situationally any left-over feed protein (Weisbjerg & Hvelplund, 2003). The explanation for the necessity of a two-stage method is that the fibre digestion are usually ended by 48 hours, while the degradability of protein is not (Tilley & Terry, 1963).

Goering and Van Soest (1970) developed a modified method to the two-stage method of Tilley and Terry. In the modified method, the residues were treated with neutral-detergent solution instead of the second incubation in pepsin. Thereby shortening the procedure. Both of these methods have shown to give results comparable to in vivo values (Van Soest, 1994). However, it is important to note they have some disadvantages, like being laborious (Cattani, 2011). One reason for this, is that they are executed in an individual container for each feed.

To simplify, it has since been developed methods with the possibility to incubate multiple samples in the same container. An example of this is the ANKOM Daisy^{II} Incubator (ANKOM Technology, Macedon, NY), who gives the opportunity to incubate 25 samples in one container by sealing each sample in individual filter bags (ANKOM Technology, 2017). The ANKOM Daisy^{II} Incubator has shown to measure digestibility that correlates well with in sacco and various in vitro methods (Damiran, DelCurto, Bohnert, & Findholt, 2008; Holden, 1999; Mabjeesh, Cohen, & Arieli, 2000; Robinson, Mathews, & Fadel, 1999; Spanghero, Boccalon, Gracco, & Gruber, 2003; Spanghero, Gruber, & Zanfi, 2007; Wilman & Adesogan, 2000). Although, some of these articles also express less accurate values from the Daisy incubator compared to other in vitro and in sacco methods (Damiran et al., 2008; Spanghero et al., 2007; Wilman & Adesogan, 2000).

Figure 3. A picture of the ANKOM Daisy^{II} Incubator during incubation.

An alternative is the in vitro gas production method. McBee (1953) proposed a method based on measuring gas production during incubation and relating it to the fermentation of a feed. In other words, the digestibility is assumed to be proportional to the gas production (Weisbjerg & Hvelplund, 2003). This assumption is questionable, since the gas production is dependent of the chemical composition of the feedstuff. A considerable amount of the gas production derives from the interaction between produced VFA and the buffer solution, where the production of one mol VFA releases 0.8-1.0 mol carbon dioxide from the buffer solution (Makkar, 2004). Gas production is therefore mainly due to the fermentation of carbohydrates, which is also described in 1.3.1 Carbohydrates. Fermentation of proteins on the other hand, produces ammonia, who have an inhibitory effect on the release of gas from the buffer solution (Cone & van Gelder, 1999). Protein-rich feeds will therefore lead to an underestimation of the fermentation of the feed if it is measured by the production of gas.

Since McBee (1953) , other methods utilizing the same principle been developed. Examples are Czerkawski and Breckenridge (1975), Menke et al. (1979), Jouany and Thivend (1986), where water manometers and glass syringes were used to measure the gas produced. In the method of Menke et al. (1979), they found a high correlation between gas production and in vivo measurements. The feed was incubated with buffer and rumen inoculum in a syringe closed with a piston which are pushed out by the gas produced.

Beuvink, Spoelstra, and Hogendorp (1992) introduced an automated method to measure gas production every 25 minute. Today the development of pressure gauges and electronical data collection have led to an increased use of the gas production method (Weisbjerg & Hvelplund, 2003). When using pressure gauges to measure gas production instead of expandable syringes, high pressures could be generated inside the system. During high pressure, some of the gas remain dissolved in the solution without being released. Thus, giving an underestimating of gas production measurements. Eventually automated valves to release gas pressure during incubation have been developed (ANKOM Technology, 2018; Cone, van Gelder, Visscher, & Oudshoorn, 1996; Davies et al., 2000).

Figure 4. Bottles and heads containing pressure sensor modules for the ANKOM Gas production system. The knob on the right side of the head is an automated valve to realise gas during incubation.

Normally, in vitro and in sacco methods are based on end-point values for each incubation time. Whereas the gas production system have the advantage of monitoring the full fermentation process, without disturbance (Wood & Badve, 2001). The gas production method is also the only in vitro technique that's adequate to determine the degradation of water-soluble material (Wood & Badve, 2001).

2 Materials and Methods

The objective of this thesis is to compare three different methods to evaluate rumen digestibility; the in sacco method and two in vitro methods. The in vitro methods are based on rumen inoculum, but are using different devices. The first method is using the ANKOM Daisy^{II} Incubator, where the residuals after incubation is measured. The other in vitro method is using the ANKOM RF Gas Production System, where the gas production is continually measured throughout the incubation in combination with reviewing the residues afterwards.

All the experiments were conducted at Stoffskifteavdelingen (SSA) at the Norwegian University of Life Sciences (NMBU). The in sacco data was obtained by the staff at SSA in 2018, according to the standardised in sacco protocol of NorFor (Åkerlind et al., 2011). The two in vitro methods were conducted at the same site, from January to March 2019.

The feed used were 22 samples of silages from different round bales, which were collected all over Norway the summer of 2017. They therefore express a comprehensive variation of nutrient composition and degradability. The silages were dried in two ways, in a heating cabinet at 59°C and by freeze-drying. The samples who was dried at 59°C were grinded through a 1.0 mm sieve and used for the Daisy^{II} Incubator and the gas production system. While the freeze-dried samples were grinded through a 1.5 mm sieve and used for the in sacco experiment.

2.1 ANKOM Daisy^{II} Incubator

The ANKOM Daisy^{II} Incubator is a digestion chamber containing four digestion jars with the capacity of 25 bags of samples each. The digestion chamber maintains a temperature at 39.5°C during incubation, while the digestion jars slowly rotate to agitate the content. To get a degradation profile, each digestion jar was assigned to an incubation time, 12, 24, 48 and 96 hours. It was performed two runs with each feed, and eleven feeds in each run. Consequently, four runs was needed to examine the 22 feeds. Each feed had two replicas in each digestion jar. In addition, there were added 3 samples of standard feeds and an empty bag in each jar for the correction factor.

The procedure used derived from the method described by the manufacturer of the Daisy Incubator, ANKOM Technology (2017), with some adjustments. The adjustments apply to the amount of sample in each bag and the collection and treatment of the rumen inoculum. ANKOM Technology filter bags #F75 were pre-rinsed in acetone for five minutes, and then air-dried before being filled with one gram of sample each. After this the filter bags were heat sealed and partitioned on each side of the divider in the associated digestion jar. Each jar was then filled with 1600 ml buffer solution and 400 ml rumen inoculum.

The buffer solution was made according to the measures in Figure 5, using distilled water to dilute. These measures were obtained from the analytical method proposed by ANKOM Technology (2017), but turned out to be incorrect. The unit of solution B are supposed to be in g/100 ml (Marten & Barnes, 1979). Possible effects of this is described in the discussion. Solution A and B were heated to 39°C in a water bath before being mixed at a 5:1 ratio. The exact ratio was adjusted to achieve the final pH of 6,8 at 39°C. The jars, including samples and buffer solution, were then placed in the pre-heated Daisy Incubator with agitation. The digestion jars got 30-60 minutes to equilibrate in the Daisy, while the rumen inoculum was gathered.

Solution A:

10.0 g/litre	KH_2PO_4
0.50 g/litre	$MgSO_4*7H_2O$
0.50 g/litre	NaCl
0.10 g/litre	$CaCl_2*2H_2O$
0.50 g/litre	Urea

Solution B:

15.0 g/litre	Na_2CO_3
0.55 g/litre	Na_2S*3H_2O

Figure 5. Composition of solution A and B to make the buffer solution.

Ruminal inoculum was obtained from two ruminally cannulated Norwegian Red cows throughout the four runs. These cows were fed a standard diet, meaning they got a hay and straw to concentrate ratio of 67:33 and a crude protein contend higher than 120 g/kg dry matter (Åkerlind et al., 2011). In practice they got 1.8 kg hay, 1.1 kg straw and 1.5 kg of the concentrate "drøv energirik høy". Inoculum was retrieved 4-4.5 hours after morning feeding, and directly put into a pre-heated thermos bottle. This was immediately taken to the laboratory and strained through an 200µm nylon filtration mesh. Before being purged with CO_2, closed with a bottle-top dispenser, and put in a water bath at 39°C. The inoculum was then added in the digestion jars, who were purged approximately 30 seconds with CO_2 gas before the lid were secured. It is important that the CO_2 gas does not bubble the inoculum, but rather lay on top of the content. The time from starting the collection of ruminal digesta to dividing it into the digestion jars and starting the run was less than half an hour.

After incubation for each time frame, the associated digestion jar was taken out of the Daisy, while trying not to disturb the other jars. The contents of the finished jar were then poured into a bucket with holes and rinsed with running water for 10 minutes (see Figure 6). The water flowed with 24,3 litres per minute and held a temperature of 5-7°C. Afterwards the bags were dried at 45°C for 48 hours. Both the immediate and equilibrated weight (after 24 hours) were noted.

Figure 6. Washing the bags after incubation

2.2 ANKOM RF Gas Production System

The ANKOM RF Gas Production System consist of multiple bottles with heads containing pressure sensor modules. Feed sample, buffer and rumen inoculum is placed into each bottle, and the pressure sensor modules measures the gas production as pressure (psi) during incubation. The readings are sent to a computer with radio frequency (RF) transmissions. The heads energy supply is rechargeable batteries who were charged 2.5-3 hours the day(s) before the start up and flushed 30 minutes the day of use. It is possible to choose the recording interval, which were set to 10 minutes in this experiment. The build-up pressure in the bottles during incubation were set to automatically realise at 0.75psi. It is also possible to measure methane emissions with this system, although it was not used in this thesis.

One gram of forage sample was put in each bottle, and each feed had three replicas in each run. Like the Daisy method, eleven of the feeds were included in each run. Four bottles were used for the standard feeds in addition to three blank bottles, making a total of 40 bottles for each run. Also, 1-2 extra standard feeds were added in some of the runs, due to early trials for methane production. The gas production and residues of these were included in the results to get more observations. The blank bottles contained the buffer and inoculum, but no feed sample. This is to correct for the gas produced by the inoculum and a slight gas loss caused by the permeability of CO_2 (ANKOM Technology, 2018). The bottles with samples were covered with parafilm and kept in a cabinet incubator at 39°C overnight.

The buffer of Goering and Van Soest (1970) was used according to the measurements in Figure 7, using distilled water to dilute the solutions. Because of their small contribution, the resaruzin solution and the micromineral solution was made to last throughout the experiment, while the others were made for each run. In order to make the final buffer solution, 0.413ml micromineral solution, 825ml buffer solution, 825ml micromineral solution and 4.125ml resaruzin solution, in this order, were mixed. 1650ml distilled water were added throughout the mixing to give the total of 3.3 litres of final buffer solution. This gave enough buffer solution to run 40 bottles. The final buffer solution was heated in a water bath of 39°C and was bubbled with CO_2 for two hours. After this 172ml reducing solution were added and stirred vigorously. Before adding reducing solution, the buffer solution holds a blue/purple colour.

Resaruzin 0.1% solution:

0.01 g/litre	resaruzin

Buffer solution:

4.0 g/litre	NH_4HCO_3
35.0 g/litre	$NaHCO_3$

Reducing solution:

6.25 g/litre	Cystein HCl
40 ml/litre	1N NaOH
6.25 g/litre	Na_2S*9H_2O

Macromineral solution:

5.7 g/litre	Na_2HPO_4
6.2 g/litre	KH_2PO_4
0.6 g/litre	$MgSO_4*7H_2O$

Mikromineral solution:

132 g/litre	$CaCl_2*2H_2O$
100 g/litre	$MnCl_2*4H_2O$
10 g/litre	$CoCl_26H_2O$
80 g/litre	$FeCl_3*6H_2O$

Figure 7.Composition of the Goering and Van Soest buffer

When adding the reducing solution, the buffer solution immediately changes colour to pink. After some time, it gradually becomes transparent. When the colour was transparent, the container was purged with CO_2 and closed with a bottle-top dispenser. The buffer solution was kept in the water bath throughout the distribution into the bottles, to maintain a temperature of 39°C. The bottles were brought out from the incubator in two turns, with 20 bottles each time. 66ml of the final buffer solution was added to each bottle before putting them back in the incubator.

Figure 8. The colour change in the buffer solution from the original blue/purple to transparent. This takes approximately 30 minutes.

While the bottles with the buffer solution equilibrated in the cabinet incubator, the rumen inoculum were gathered. This was done similar to what's described in 2.1 ANKOM Daisy^{II} Incubator, with some exceptions; it was taken from three cows and they were on a more fibrous diet. They got 2.5kg hay, 1.5kg straw and 0.5kg of the concentrate "drøv energirik". 33ml of the strained inoculum were added to each bottle in two turns such as when adding the buffer solution. Each bottle was purged with CO_2 before the heads with the pressure sensor modules were attached. To minimize the lag time from the first to the last bottle, and to avoid a large drop in temperature, this prosses of filling the bottles were done as quickly as possible. After putting everything back in the incubator the registrations started. To ensure that the feeds had met their degradation potential at 48 hours, the feeds were first incubated for 72 hours. Consequently, it was needed with 4 runs to complete the 22 feeds.

After incubation the data were saved, and the bottles were taken out of the cabinet incubator. The content of each bottle was poured into a 12 µm nylon bag, using distilled water to rinse. The nylon bags were then closed with a rubber band and washed in a washing machine at 25°C using the wool program without centrifugation. This is the same washing method that is used for in sacco (Åkerlind et al., 2011). After washing, the bags were dried at 45°C for 48 hours. Both the immediate and the equilibrated weight (after 24 hours) were obtained. The bags were reused after another cleansing in the washing machine, using the same settings as above. The bottles were washed in warm soapy water and rinsed thoroughly between runs.

2.3 Chemical analysis and preparation

Both the freeze-dried silages and the residues after incubation were analysed for dry matter, ash, NDF and CHNS. The dry matter content was found by multiplying the dry matter content found by freeze-drying, with the dry matter value found by analysing the dried material. The dry matter content was not corrected for fermentation acids.

The analysis for NDF had to be done on samples grinded to 1mm, but no less, to prevent loss of particles (Berg, 2018b). While the analysis for dry matter, ash and CHNS could be grinded a bit more. The residues from the ANKOM Daisy^{II} Incubator had two replicas for each feed from the same run and time point. One of these was send (still in the bag) for the NDF analysis, while the other replica was taken out from the bag and grinded in a ball mill with a vibrational frequency of 30 for 55 seconds. The residues were then sent to be analysed for dry matter, ash and CHNS. The residues from the ANKOM Gas Production System had three replicas from each run. These were mixed together using a coffee grinder, making sure not to make the particle size to small. 330-470 mg were bagged in ANKOM Technology filter bags #F75 and send to NDF analysis. The rest was grinded in a ball mill with a vibrational frequency of 30 for 55 seconds and sent to analyse for dry matter, ash and CHNS.

The analysis was done by LabTek, a laboratory at NMBU. The NDF analysis was done as aNDFom, meaning it was treated with amylase and corrected for residual ash. This is the recommended method when publicising NDF values (Udén, Robinson, & Wiseman, 2005). This was achieved by heating the samples in a neutral detergent solution, and deciding NDF gravimetric (Berg, 2018b). To correct for ash, the samples were burned at 550°C (Berg, 2018b). Dry matter was decided after drying at 103°C ± 2°C until the samples reached a constant weight (Berg, 2018c). Ash was analysed from the same sample who had gone through dry matter analysis. It was burned at 550°C for 4-20 hours (Berg, 2018a). Analysis for CHNS gives the total amount of carbon, hydrogen, nitrogen and sulphur. This was done by burning the sample at a high temperature in an oxygen rich environment, followed by removing the other elements and excess oxygen. The total amount of CHNS were then decided with a hot-wire detector (Johnsen, 2018).

2.4 Calculations and statistical analysis

2.4.1 In sacco

Loss of dry matter was calculated as $Loss\ of\ DM = \frac{(DM\ before\ incubation - DM\ after\ incubation)*100}{DM\ before\ incubation}$.

The loss of NDF was calculated using the same method but using NDF instead of DM. Soluble (A), rate of degradation (C) and total degradable (D) were estimated using the Proc NLIN statement in SAS software (SAS 9.4, SAS Institute Inc., Cary, NC). Potentially degradable (B) were calculated as $B = D - A$.

A, B and C were used to calculate the degrees of degradation using the formula: $Degree\ of\ degradation = A + \frac{B*C}{C+kp}$. Where kp is the rate of passage from the rumen. A kp of 0.08 were used for dry matter and protein, while a kp of 0.03 were used for NDF. Soluble (A) NDF were forced to 0. Before this intervention, most of the samples had a soluble NDF fraction between -1 to -3, although the lowest value was -6.28, and two feeds had a positive soluble NDF fraction. Because of this B and D for the NDF fraction are identical.

2.4.2 Daisy

Dry matter loss was calculated as In Vitro True Digestibility ($IVTD_{DM}$) according to the formula ANKOM Technology (2017) suggest: $\%\ IVTD_{DM} = 100 - \big(W_3 - (W_1 *\ C_1)\big) * 100\ (W_2 * DM)$. Where W_1 is the tare weight of the bag, W_2 is the weight of the sample, W_3 is the bag weight including the sample after incubation and C_1 are the blank bag correction calculated as the blank bag weight after incubation divided on tare weight of the blank bag. This was calculated for the individual bag and have since been averaged for each feed.

The loss of NDF and nitrogen were calculated in a similar way. The W_1 was multiplied with C_1, and both the sample weight before and after incubation was multiplied with the corresponding NDF or nitrogen content to get the loss of a specific nutrient.

2.4.3 Gas Production

Outputs from the ANKOM Gas Production System were pressure measured in psi, and had to be converted to gas produced in ml. This was done according to the recommendation of ANKOM Technology (2017), using ideal gas law and Avogadro's law. Ideal gas law was used to convert the gas production to moles of gas produced: $n = p\ \frac{V}{RT}$, where n is the number of moles of gas, p is the pressure in kPa, V is the head-space volume in litres, T is the temperature in Kelvin and R is the ideal gas constant (8,314472). Psi was converted to kPa using the correction factor of 6,894757293. V was calculated as the volume of the bottle subtracted the volume of the sample/inoculum/buffer, in this case $0{,}31 - 0{,}1 = 0{,}21$. The average of the blank bottles was subtracted from the others for each

time unit to do the blank correction, as mentioned earlier. Avogadro's law is used to convert gas production in moles to millilitres (mL), since 1 mole occupies 22,4 litres at standard conditions (2,73.15°K and 101,325 kPa). $Gas\ produced\ in\ ml = n * 22{,}4 * 1000$.

The gas production in mL was divided on the dry matter of sample weight (g) in each bottle. This was done to the individual bottles at every time point. SAS software was used to calculate A, B and C according to the method of Groot, Cone, Williams, Debersaques, and Lantinga (1996). A is the asymptotic gas production, B is the time of incubation where half of A have been produced and C is the shape of the curve. A, B and C were averaged for each feed, and from this the gas production models were created. The fractional rate of gas production (R) was calculated as: $R = \frac{Ct^{C-1}}{B^C + t^C}$ (Groot et al., 1996).

2.4.4 Comparing in sacco, Daisy and gas production

Obtained measurements from in sacco, the Daisy[II] Incubator and the gas production system were compared using correlations by the proc corr statement in SAS. The measurements compared are listed and explained in Table 2. Some correlations were decided unimportant, and thus omitted henceforth. Interesting correlations, on the other hand, were investigated further using the proc reg statement in SAS.

Table 2. Explanations of the abbreviations used to compare the in sacco, daisy and gas production methods.

Abbreviation	**Meaning**
DigDM8, DigNDF3	In sacco digestibility of dry matter (DM) and NDF, with 8 or 3 percent of passage rate.
ADM, BDM, CDM, DDM ANDF, BNDF, CNDF, DNDF	Soluble (A) nutrient (DM or NDF), potentially degradable (B) nutrient (DM or NDF), rate of degradation (C) of nutrients (DM or NDF) and total degradable (D) of nutrients (DM or NDF). Obtained by in sacco.
DM0, DM2, DM4, DM8, DM16, DM24, DM48, DM96	Loss of dry matter (DM) at 0, 2, 4, 8, 16, 24, 48 and 96 hours of in sacco incubation.
NDF0, NDF2, NDF4, NDF8, NDF16, NDF24, NDF48, NDF96	Loss of NDF at 0, 2, 4, 8, 16, 24, 48 and 96 hours of in sacco incubation.
DaisyDM12, DaisyDM24, DaisyDM48, DaisyDM96	Loss of dry matter (DM) at 12, 24, 48 and 96 hours of incubation in Daisy.
DaisyNDF12, DaisyNDF24, DaisyNDF48, DaisyNDF96	Loss of NDF at 12, 24, 48 and 96 hours of incubation in Daisy.
GASA72, GASB72	Calculated A and B value of Groot et al. (1996) after 72 hours of incubation. Obtained by the Gas production system.
GASA48, GASB48	Calculated A and B value of Groot et al. (1996) after 48 hours of incubation. Obtained by the Gas production system.

3 Results

3.1 Chemical composition of the feed

The chemical composition of the different feedstuffs is presented in Table 3. The table shows a wide range in dry matter and nutrient concentration between feeds. Dry matter varies from 185 to 659 g/kg with an average of 343 g/kg, whereas NDF varies from 396 to 628 g/kg DM with an average of 534 g/kg TS.

Table 3. Chemical composition of the individual feedstuff, including the total average with minimum and maximum values. Dry matter (DM) are given in g/kg feed, while the others are given in g/kg DM.

Feed No.	Dry matter	NDF	Crude protein[1]	Ash	Residue[2]
94	203	606	107	49	238
95	222	527	196	80	198
96	231	545	148	87	220
103	433	536	109	72	283
106	659	574	113	80	233
111	352	400	156	104	339
113	269	527	185	85	203
114	371	579	132	82	206
115	420	396	179	139	285
120	222	476	164	106	254
121	332	397	172	74	356
133	391	585	122	43	249
142	354	615	119	66	199
143	618	628	83	54	235
144	203	558	111	88	242
145	185	531	182	76	212
146	252	491	200	100	209
147	273	576	152	96	175
152	450	530	128	69	272
153	239	562	97	67	274
154	356	618	115	63	205
160	519	494	100	67	338
Average	343	534	140	80	247
Minimum	185	396	83	43	175
Maximum	659	628	200	139	356

[1] Crude protein are calculated by multiplying the nitrogen content of the feeds with 6.25.

[2] Residue equals dry matter subtracted NDF, crude protein and ash. It consists mainly of lipids, fermentation products and carbohydrates except NDF.

3.2 Results from in sacco

Figure 9 and Figure 10 shows the degradation profile of dry matter and NDF, respectively, of all feeds studied. The profiles are in general well adapted to an exponential curve with few inter-crossings between timepoints.

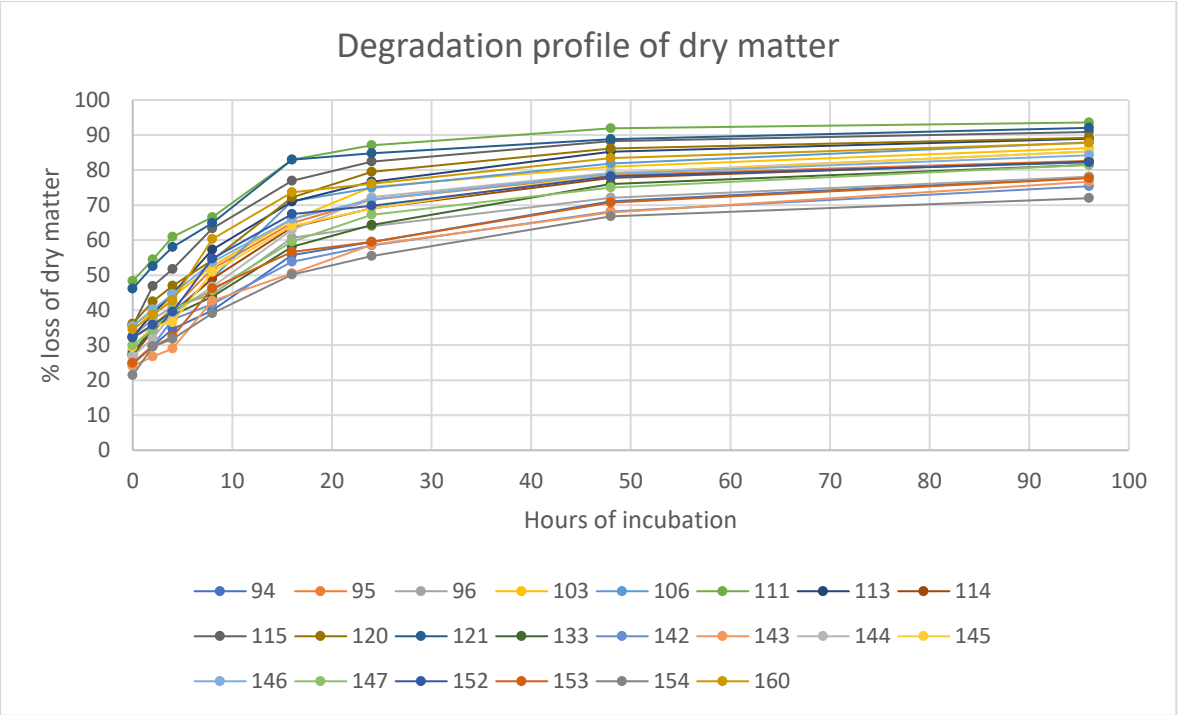

Figure 9. Degradation profile of dry matter from increased incubation time in sacco.

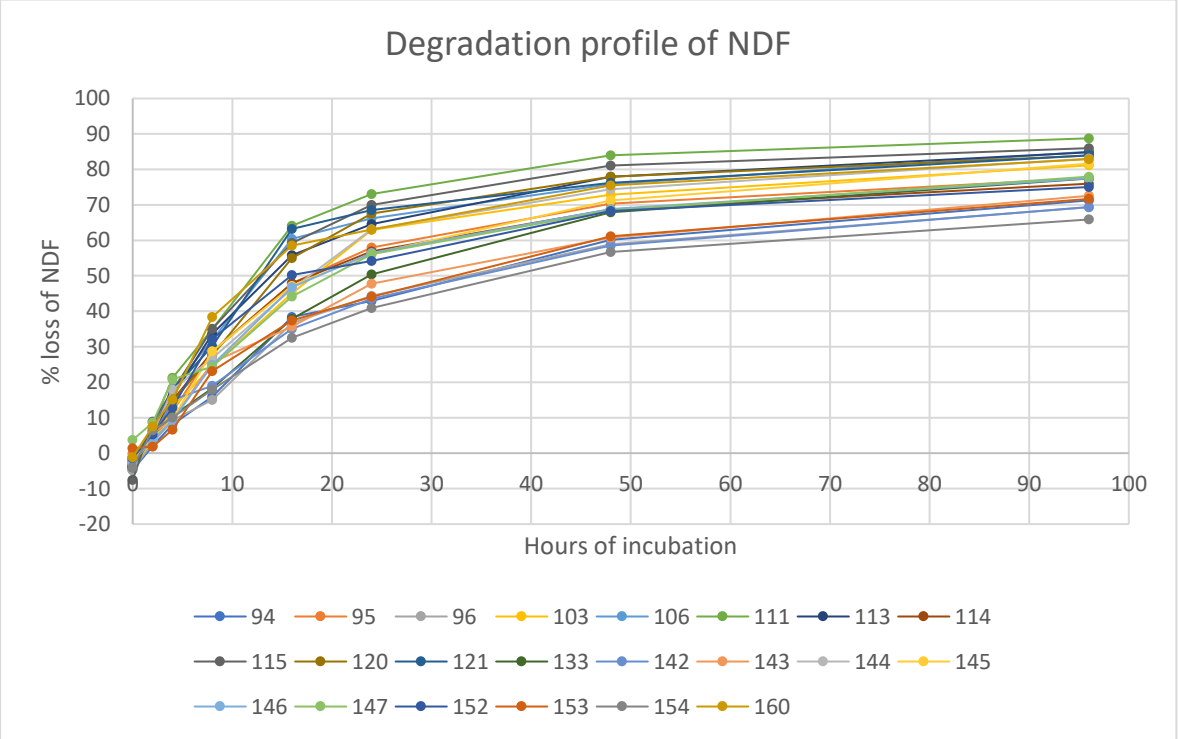

Figure 10. Degradation profile of NDF from increased incubation time in sacco.

Using the degradation profiles, degradation values can be calculated. Table 3 shows the estimated effective rumen degradation of dry matter (DigDM8) and NDF (DigNDF3). It is assumed a passage rate of 8 %/hour for dry matter, and 3 %/hour passage rate for NDF. Table 4 also lists the loss of dry matter and NDF after 96 hours of incubation in the rumen (DM96 and NDF96). The estimated effective rumen degradation of dry matter (DigDM8) have an averaged value of 53 %, with a variation from 42 to 71 %, whereas the estimated effective rumen degradation of NDF (DigNDF3) have an overall average of 50 percent, with variations from 66 to 89 percent. A higher degradation from dry matter compared to NDF are also evident from the dry matter and NDF losses after 96 hours (DM96 and NDF96).

Table 4. Calculated effective rumen degradation of dry matter (DigDM8) and NDF (DigNDF3), in addition to the loss of dry matter (DM96) and NDF (NDF96) after 96-hour incubation in the rumen. Overall average with standard deviation and minimum and maximum values are also presented. All values are given in percentage.

Feed No.	DigDM8[1]	DM96[2]	DigNDF3[3]	NDF96[4]
94	45^{K}	78^{H}	41^{GHI}	71^{GH}
95	54^{FG}	83^{G}	50^{E}	78^{E}
96	50^{IJ}	78^{H}	40^{HI}	69^{H}
103	56^{E}	86^{DE}	51^{DE}	81^{D}
106	54^{EF}	88^{CD}	57^{BC}	85^{BC}
111	71^{A}	94^{A}	62^{A}	89^{A}
113	58^{D}	89^{C}	57^{C}	85^{CB}
114	52^{HI}	83^{G}	50^{E}	76^{EF}
115	64^{C}	91^{B}	60^{B}	86^{B}
120	60^{D}	89^{C}	56^{C}	84^{BC}
121	69^{B}	92^{AB}	58^{BC}	84^{BC}
133	49^{J}	82^{G}	45^{F}	77^{E}
142	45^{K}	75^{I}	41^{GHI}	69^{H}
143	42^{L}	77^{HI}	43^{FG}	73^{G}
144	51^{HI}	85^{EF}	53^{D}	83^{CD}
145	52^{GH}	85^{EF}	50^{E}	82^{D}
146	56^{E}	84^{F}	49^{E}	78^{E}
147	50^{IJ}	82^{G}	49^{E}	78^{E}
152	54^{EF}	82^{G}	50^{E}	75^{F}
153	46^{K}	78^{H}	42^{GH}	72^{G}
154	42^{L}	72^{J}	38^{I}	66^{I}
160	59^{D}	88^{CD}	57^{C}	83^{CD}
Average	53	84	50	78
Minimum	42	72	38	66
Maximum	71	94	62	89
Std Dev	7.9	5.8	7.0	6.3

$^{A-L}$ different letter indicate significant difference between feeds within parameter.

[1] DigDM8 is the calculated effective rumen degradation of dry matter when using a passage rate of 8.

[2] DM96 is the loss of dry matter after 96 hours of incubation in the rumen.

[3] DigNDF3 is the calculated effective rumen degradation of NDF when using a passage rate of 3.

[4] NDF96 is the loss of NDF after 96 hours of incubation in the rumen.

3.3 Results from Daisy

Figure 11 and Figure 12 shows the degradation profile of dry matter and NDF, respectively, of all the feeds studied. The time point used are 12, 24, 48 and 96 hours. There are few inter-crossings between timepoints, especially for the degradation profile for dry matter.

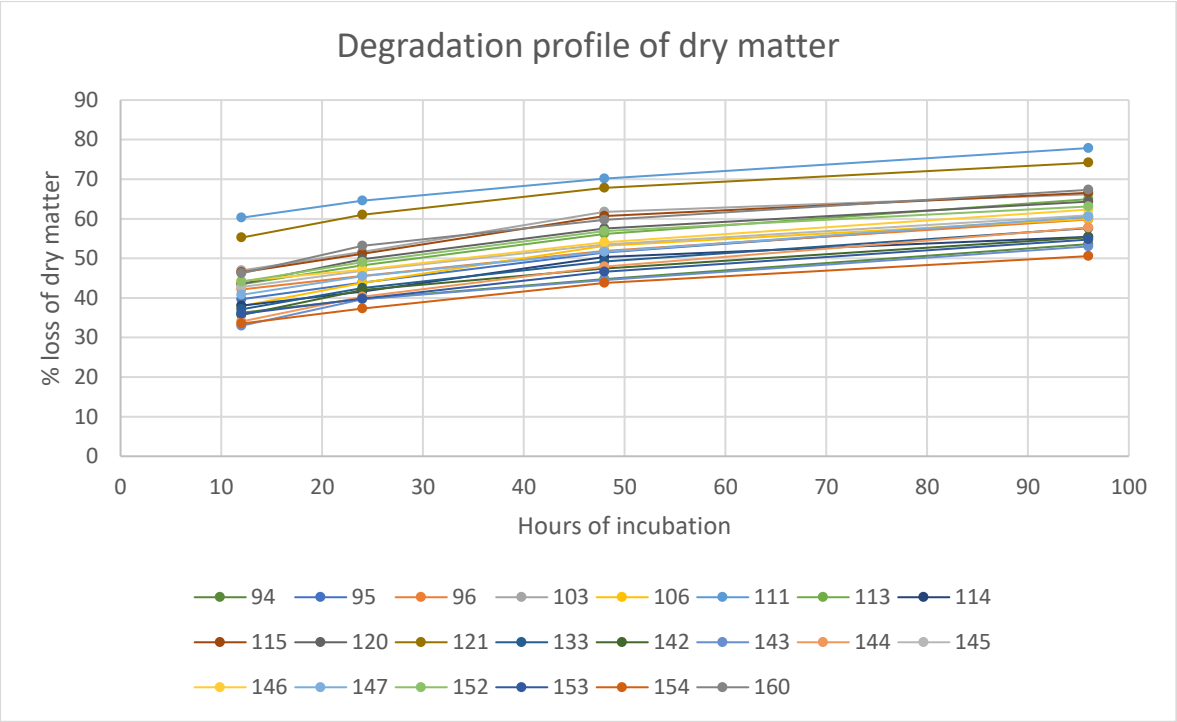

Figure 11. Degradation profile of dry matter from 12, 24, 48 and 96 hours of incubation in the Daisy^{II} Incubator for all feeds studied.

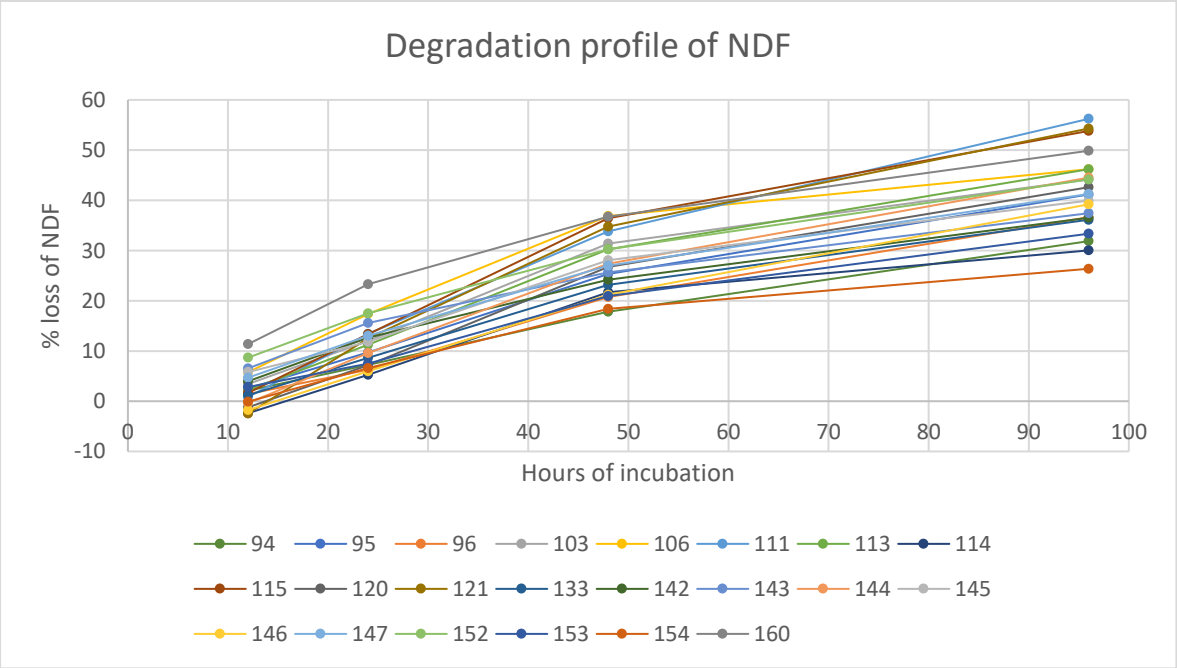

Figure 12. Degradation profile of NDF using 12, 24, 48 and 96 hours of incubation in the Daisy^{II} Incubator for all feeds studied.

All feeds were incubated in the Daisy^{II} Incubator in two separate runs. After 12- and 24-hour incubation, there was some measurements with significant differences between runs. However, most of the measurements had insignificant differences between runs. 48-hour incubations have p values of 0.159-0.292 between runs, while the p values between runs at 96 hours were 0.300-0.978.

Table 5 shows the loss of dry matter and NDF of all feeds studied after 96 hours of incubation in the Daisy^{II} Incubator. The values are given in percentage and A-J indicate insignificant differences between feeds within parameter. Compared to Table 4, there is a larger difference between dry matter and NDF values.

Table 5. Loss of dry matter and NDF after 96-hours of incubation for each feed studied, together with overall averages, standard deviation and the maximum and minimum loss of dry matter and NDF. The values are given in percentage.

Feed No.	DaisyDM96[1]	DaisyNDF96[2]
94	54^{JK}	32^{GHI}
95	60^{GH}	41^{CDEF}
96	60^{GH}	37^{EFGH}
103	66^{CD}	44^{CDE}
106	60^{GH}	46^{BCD}
111	78^{A}	56^{A}
113	65^{CDE}	46^{BCD}
114	56^{IJ}	30^{HI}
115	67^{CD}	54^{AB}
120	64^{CDEF}	43^{CDE}
121	74^{B}	54^{AB}
133	58^{HI}	36^{EFGH}
142	55^{IJ}	37^{EFGH}
143	53^{JK}	37^{DEFGH}
144	58^{HI}	45^{CDE}
145	61^{FGH}	40^{DEFG}
146	62^{EFG}	39^{DEFG}
147	61^{FGH}	41^{CDEF}
152	63^{DEFG}	44^{CDE}
153	55^{IJ}	34^{FGHI}
154	51^{K}	26^{I}
160	67^{C}	50^{ABC}
Average	61	41
Minimum	51	26
Maximum	78	56
Std Dev	6.7	7.8

$^{A-K}$ different letter indicate significant difference between feeds within parameter.
[1] DaisyDM96 is the loss of dry matter after 96 hours of incubation in the Daisy^{II} Incubator.
[2] DaisyNDF96 is the loss of NDF after 96 hours of incubation in the Daisy^{II} Incubator.

3.4 Results from gas production

The calculated profile based on accumulated gas production and the calculated fractional rate of gas production are presented in Figure 13 and Figure 14, respectively. Figure 13 and Figure 14 are based on the 72 hour runs, because these appeared to have higher correlations with the results from in sacco and Daisy.

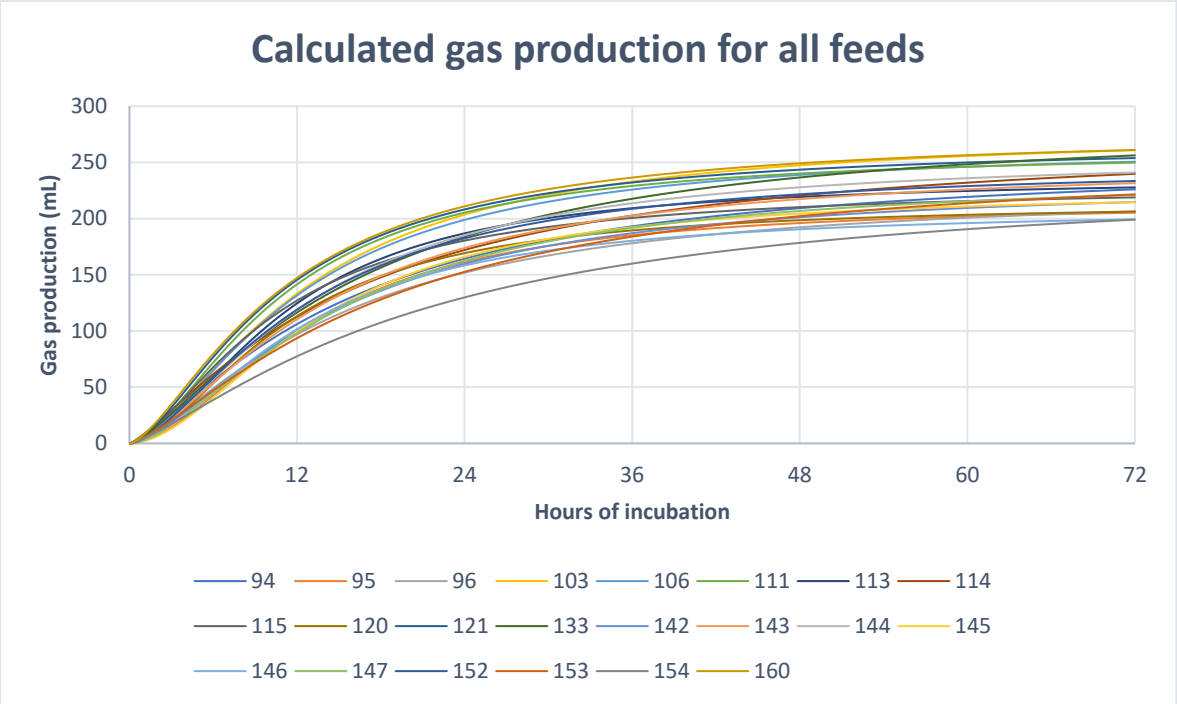

Figure 13 Calculated gas production for all feeds studied using the method of Groot et al.

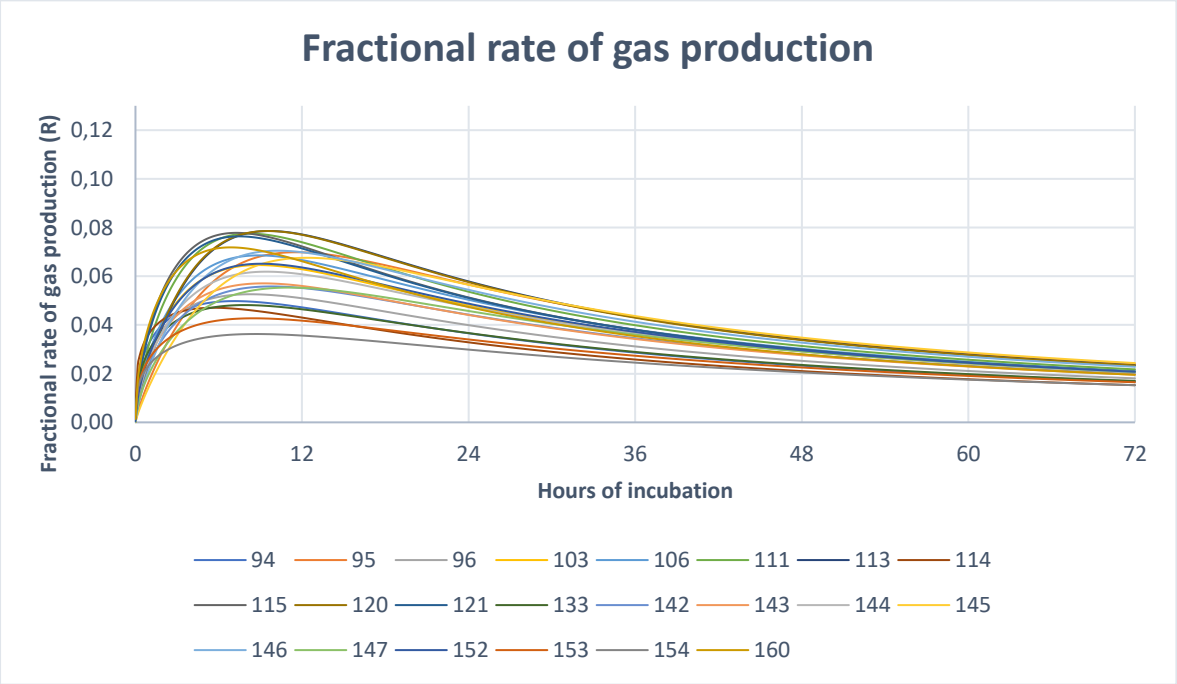

Figure 14 Fractional rate of gas production for all feeds studied using the method of Groot et al.

There is no significant difference between the runs in the gas production system. The asymptotic gas production (A) had a p value of 0.215 between runs, while the halftime to reach the asymptotic gas production (B) had a p value of 0.654 between runs.

Table 6 shows the calculated A and B for 72 and 48 hours of incubation, together with the overall average, standard deviation, minimum and maximum values. Half of the observations for feed 133-160 at 48 hours were excluded in the calculations of A and B because of an error in the experiment. The values of these feeds are therefore based on fewer replicas, and 143 are missing entirely. The calculated asymptotic gas production (A) is higher at 48 hours compared to 72 hours, and the 48-hour incubation had larger difference between the minimum and maximum values than the 72-hour.

Table 6. Calculated asymptotic gas production (A) and the halftime to asymptotic gas production (B) for 72- and 48-hour incubation. Overall average, standard deviation, minimum and maximum values are included.

Feed No.	GASA72[1]	GASB72[2]	GASA48[3]	GASB48[4]
94	244^{DEF}	15^{BC}	264^{EFG}	15^{F}
95	213^{JK}	13^{DEF}	217^{M}	13^{GH}
96	216^{J}	15^{BCD}	239^{JK}	15^{F}
103	267^{AB}	13^{EFG}	289^{BC}	13^{GH}
106	255^{BCD}	12^{EFG}	275^{DE}	12^{HI}
111	246^{DEF}	11^{GH}	276^{D}	11^{IJ}
113	233^{FGHI}	12^{FGH}	241^{JK}	11^{IJ}
114	272^{A}	17^{AB}	280^{CD}	16^{EF}
115	226^{GHIJ}	11^{GH}	233^{KL}	10^{J}
120	212^{JK}	12^{FGH}	216^{M}	11^{IJ}
121	262^{ABC}	10^{H}	271^{DEF}	11^{IJ}
133	270^{AB}	14^{CDE}	316^{A}	19^{C}
142	221^{IHJ}	13^{DEF}	250^{HIJ}	17^{DE}
143	251^{CDE}	14^{CDE}	-	-
144	259^{ABCD}	14^{EFCD}	255^{GHI}	13^{GH}
145	222^{IJH}	13^{CDEF}	233^{KL}	15^{F}
146	199^{K}	12^{FGH}	224^{LM}	13^{G}
147	235^{EFGH}	14^{CDE}	248^{IJ}	17^{D}
152	244^{DEF}	13^{EFG}	262^{FGH}	13^{GH}
153	238^{EFG}	15^{BC}	280^{CD}	22^{B}
154	218^{IJ}	18^{A}	299^{B}	30^{A}
160	272^{A}	11^{G}	292^{BC}	12^{GHI}
Average	240	13	260	15
Minimum	199	10	216	10
Maximum	272	18	316	30
Std Dev	21.9	2.0	27.9	4.6

$^{A-M}$ different letter indicate significant difference between feeds within parameter.
[1] GASA72 is the asymptotic gas production using 72 hours of incubation in the gas production system, given in mL gas.
[2] GASB72 is the halftime to reach the asymptotic gas production using 72 hours of incubation, given in hours.
[3] GASA48 is the asymptotic gas production using 48 hours of incubation in the gas production system, given in mL gas.
[4] GASB48 is the halftime to reach the asymptotic gas production using 48 hours of incubation, given in hours.

3.5 Comparison between methods

In the next chapters the three methods investigated are compared to each other. A selection of correlations between digestibility parameters are given, and especially interesting correlations are presented in fit plots.

3.5.1 Daisy compared to in sacco

Table 7 gives correlations between dry matter parameters from in sacco and all digestive parameters from Daisy, whereas Table 8 shows the correlations between NDF parameters from in sacco and all digestive parameters from Daisy. Table 8 does not include soluble NDF (ANDF) or totally degradable NDF (DNDF), because ANDF are forced to zero and DNDF equals potential degradable NDF (BNDF).

In general, longer incubation times in Daisy give better correlations with the in sacco parameters, except for soluble dry matter (ADM) and potentially degradable dry matter (BDM). BDM had low correlations with Daisy parameters, compared to the other parameters from in sacco. There were also low correlations between dry matter and NDF measurements from in sacco, and NDF loss after 12- and 24- hour incubation in Daisy (DaisyNDF12 and Daisy NDF24).

Table 7. Correlations between digestibility parameters from in sacco, soluble dry matter (ADM), potentially degradable dry matter (BDM), rate of degradation (CDM), total degradable dry matter (DDM) and calculated rumen degradability of dry matter with a passage rate of 8 %/hour, and the loss of dry matter and NDF after 12, 24, 48 and 96 hour incubation in Daisy[II] Incubator. All correlations are given together with the corresponding p value.

	ADM[1]	BDM	CDM	DDM	DigDM8
DaisyDM12	0.96601 <.0001	-0.42788 0.047	0.7339 0.0001	0.78808 <.0001	0.91527 <.0001
DaisyDM24	0.95566 <.0001	-0.36451 0.0953	0.76014 <.0001	0.81949 <.0001	0.92819 <.0001
DaisyDM48	0.93738 <.0001	-0.26237 0.2382	0.81507 <.0001	0.86834 <.0001	0.95658 <.0001
DaisyDM96	0.94948 <.0001	-0.26165 0.2395	0.79913 <.0001	0.88232 <.0001	0.96044 <.0001
DaisyNDF12	-0.28156 0.2043	0.24932 0.2632	-0.01458 0.9487	-0.14523 0.519	-0.17079 0.4473
DaisyNDF24	0.14367 0.5236	0.20549 0.3589	0.43736 0.0418	0.29964 0.1755	0.29959 0.1756
DaisyNDF48	0.54975 0.008	0.23446 0.2936	0.79364 <.0001	0.77249 <.0001	0.74711 <.0001
DaisyNDF96	0.77566 <.0001	0.00507 0.9821	0.83218 <.0001	0.86912 <.0001	0.88694 <.0001

There was a high correlation (r=0.960) between the calculated rumen degradability of dry matter obtained from in sacco (DigDM8) and the loss of dry matter after 96 hours of incubation in Daisy (DaisyDM96). At shorter incubation times in Daisy, the correlation with DigDM8 decreases, but it did not fall below r=0.915. Soluble dry matter (ADM) measured in sacco had high correlation with the dry matter loss from Daisy (r=0.937 to r=0.966), whereas potentially degradable dry matter (BDM) had low correlations with all parameters from Daisy (r<0.430).

Table 8. Correlations between digestibility parameters from in sacco, potentially degradable NDF (BNDF), rate of degradation (CNDF) and calculated rumen degradability of NDF with a passage rate of 8 %/hour, and the loss of NDF and NDF after 12, 24, 48 and 96 hour incubation in Daisy[II] Incubator. All correlations are given together with the corresponding p value.

	BNDF	CNDF	DigNDF3
DaisyDM12	0.64765 0.0011	0.6704 0.0006	0.71014 0.0002
DaisyDM24	0.68716 0.0004	0.71849 0.0002	0.7569 <.0001
DaisyDM48	0.74413 <.0001	0.77266 <.0001	0.81851 <.0001
DaisyDM96	0.77267 <.0001	0.76089 <.0001	0.82724 <.0001
DaisyNDF12	-0.12172 0.5895	0.11263 0.6178	-0.01112 0.9608
DaisyNDF24	0.27335 0.2184	0.56779 0.0058	0.44814 0.0365
DaisyNDF48	0.73699 <.0001	0.84922 <.0001	0.85621 <.0001
DaisyNDF96	0.8153 <.0001	0.81907 <.0001	0.88199 <.0001

The loss of NDF after 96-hour incubation in Daisy (DaisyNDF96) had similar correlations to the calculated dry matter rumen degradability (DigDM8) and the calculated NDF rumen degradability (DigNDF3), respectively r=0.887 and r=0.882. Dry matter loss after 96-hour incubation in Daisy (DaisyDM96) also had a high correlation with the calculated rumen degradability of NDF (DigNDF3) (r=0.827).

Correlations between different incubation times in sacco (DM0 to DM96) and different incubation times in Daisy (DaisyDM12 to DaisyDM96), was high (r=0.783 to 0.970), but highest between the Daisy parameters and the dry matter loss after 0-hour incubation in sacco. For the loss of NDF at different incubation times, Daisy measurements had highest correlation with the loss of NDF after 16-hour incubation in sacco (NDF16).

Figure 15 shows a fit plot between calculated rumen degradability of dry matter from in sacco (DigDM8) and the loss of dry matter after 96 hours of incubation in Daisy (DaisyDM96). This was the highest correlation from Table 7 and Table 8, except the correlation between soluble dry matter obtained from in sacco (ADM) and the loss of dry matter after 12 hours of incubation in Daisy (DaisyDM12) (r=0.966).

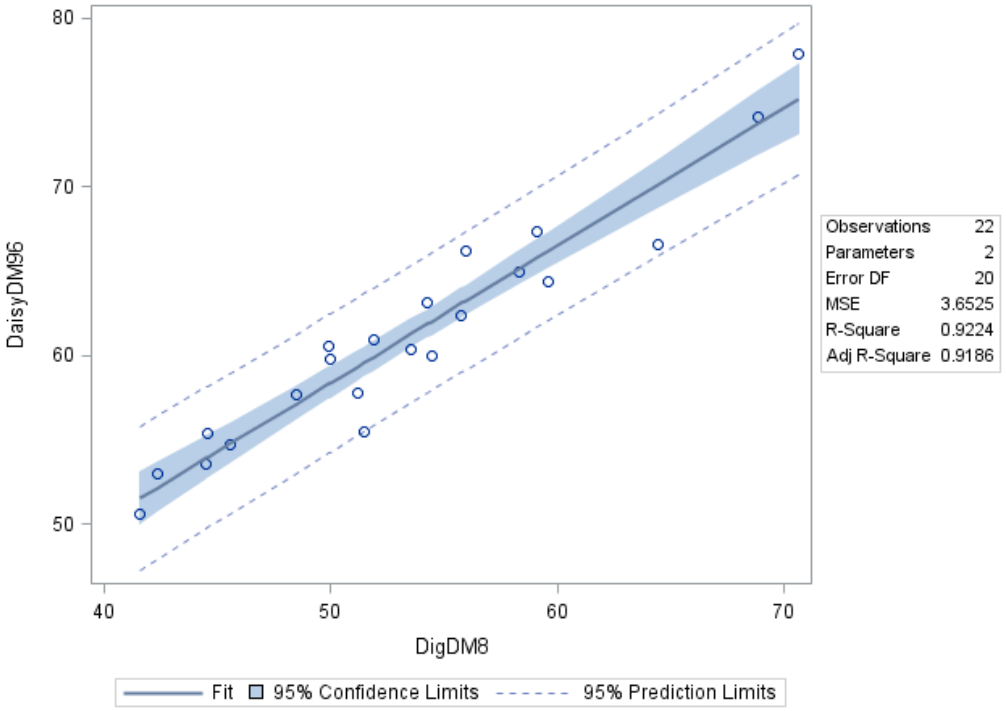

Figure 15. Fit plot for dry matter disappearance after 96-hour Daisy incubation (DaisyDM96) and effective dry matter degradation calculated from in sacco using 8 %/hour passage rate (DigDM8).

A fit plot between the loss of NDF after 96 hours of incubation in Daisy (DaisyNDF96) and the calculated dry matter degradability from in sacco (DigDM8) are presented in Figure 16. Figure 17 shows a fit plot between DaisyNSD96 and the calculated NDF degradability obtained from in sacco (DigNDF3). The correlation between DaisyNDF96 and in sacco DigDM8 was slightly higher than the correlation between DaisyNDF96 and NDF DigNDF3, r=0.887 and r=0.882 respectively.

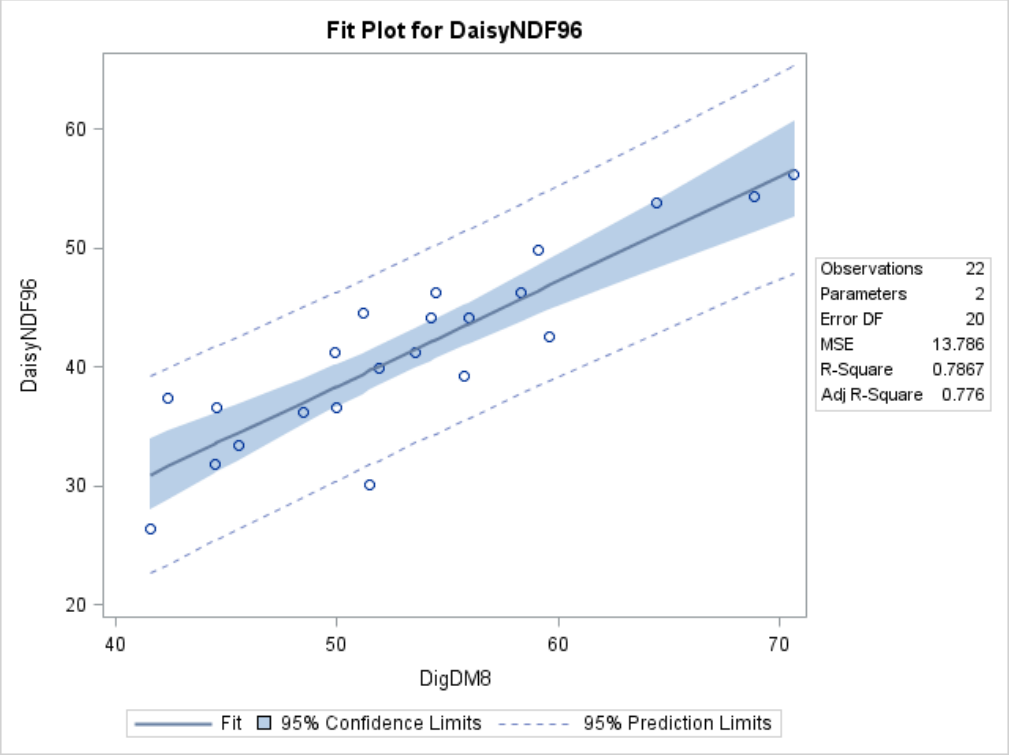

Figure 16. Fit plot between loss of NDF after 96 hours of incubation in Daisy (DaisyNDF96) and calculated rumen dry matter degradation calculated from in sacco using a passage rate of 8 %/hour.

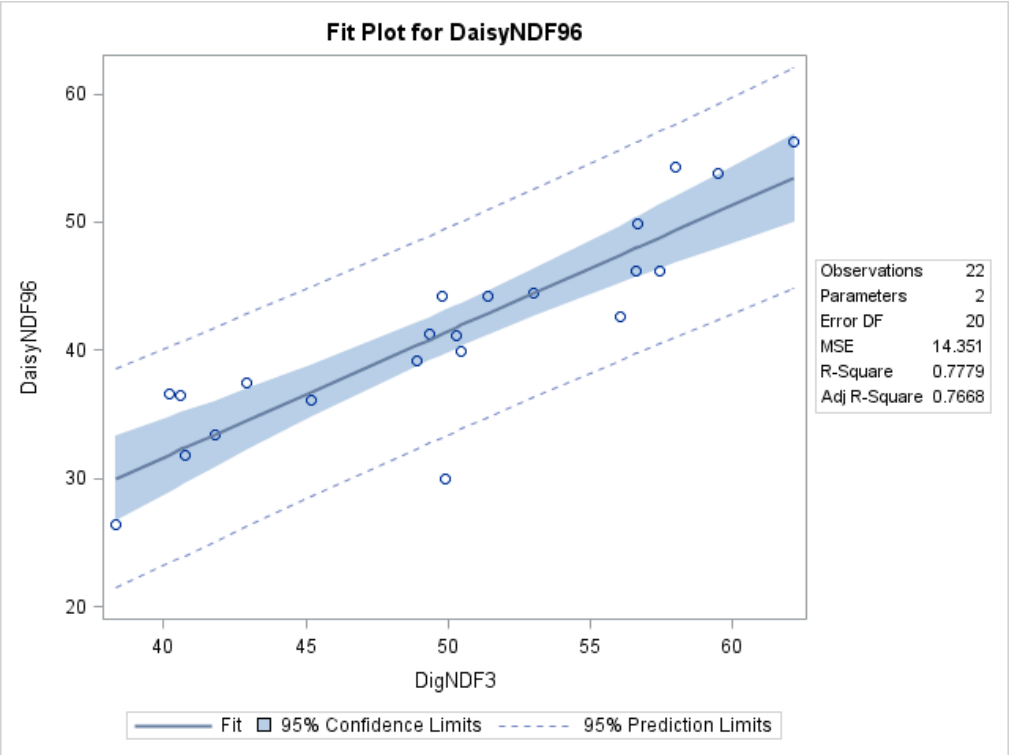

Figure 17. Fit plot between loss of NDF after 96 hours of incubation in Daisy (DaisyNDF96) and calculated rumen NDF degradation calculated from in sacco using a passage rate of 3 %/hour.

3.5.2 Gas production compared to in sacco

There was a higher correlation between in sacco measurements and the half time to the asymptotic gas production (GASB), than the asymptotic gas production itself (GASA). In addition, all correlations between in sacco measurements and asymptotic gas production (GASA) were not significant ($p>0.05$). Because of this, the correlations for asymptotic gas production (GASA) are not presented.

The halftime to asymptotic gas production calculated from 72 hours incubation (GASB72) had higher correlation with every parameter, except the potentially degradable dry matter (BDM), compared to 48 hour incubation (GASB48) (Table 9). The correlations between potentially degradable dry matter (BDM), and GASB72 and GASB48, were low. While the correlations between potentially degradable NDF (BNDF), and GASB72 and GASB48 was relatively high (r=-0.752 and r=-741, respectively).

Table 9. Correlation matrix between potentially degradable dry matter and NDF (BDM and DNDF), total degradable dry matter (DDM), calculated rumen degradability of dry matter from in sacco (DigDM8), calculated rumen degradability of NDF (DigNDF3), and the halftime to asymptotic gas production when having 72 and 48 hours incubation time (GASB72 and GASB48).

	BDM	DDM	DigDM8	BNDF	DigNDF3	GASB72	GASB48
BDM	1	0.15777 0.4832	-0.13793 0.5405	0.3203 0.1462	0.2658 0.2319	0.0073 0.9743	-0.21081 0.359
DDM	0.15777 0.4832	1	0.93893 <.0001	0.96245 <.0001	0.95956 <.0001	-0.811 <.0001	-0.80321 <.0001
DigDM8	-0.13793 0.5405	0.93893 <.0001	1	0.83027 <.0001	0.90213 <.0001	-0.82401 <.0001	-0.74334 0.0001
BNDF	0.3203 0.1462	0.96245 <.0001	0.83027 <.0001	1	0.92324 <.0001	-0.75216 <.0001	-0.74076 0.0001
DigNDF3	0.2658 0.2319	0.95956 <.0001	0.90213 <.0001	0.92324 <.0001	1	-0.79033 <.0001	-0.76626 <.0001
GASB72	0.0073 0.9743	-0.811 <.0001	-0.82401 <.0001	-0.75216 <.0001	-0.79033 <.0001	1	0.81056 <.0001
GASB48	-0.21081 0.359	-0.80321 <.0001	-0.74334 0.0001	-0.74076 0.0001	-0.76626 <.0001	0.81056 <.0001	1

GASB72 had better correlation with the rumen degradability of dry matter (DIGDM8) (r=0.824), compared to the rumen degradability of NDF (DigNDF3) (r=0.790). This is visualised in Figure 18 and Figure 19, respectively.

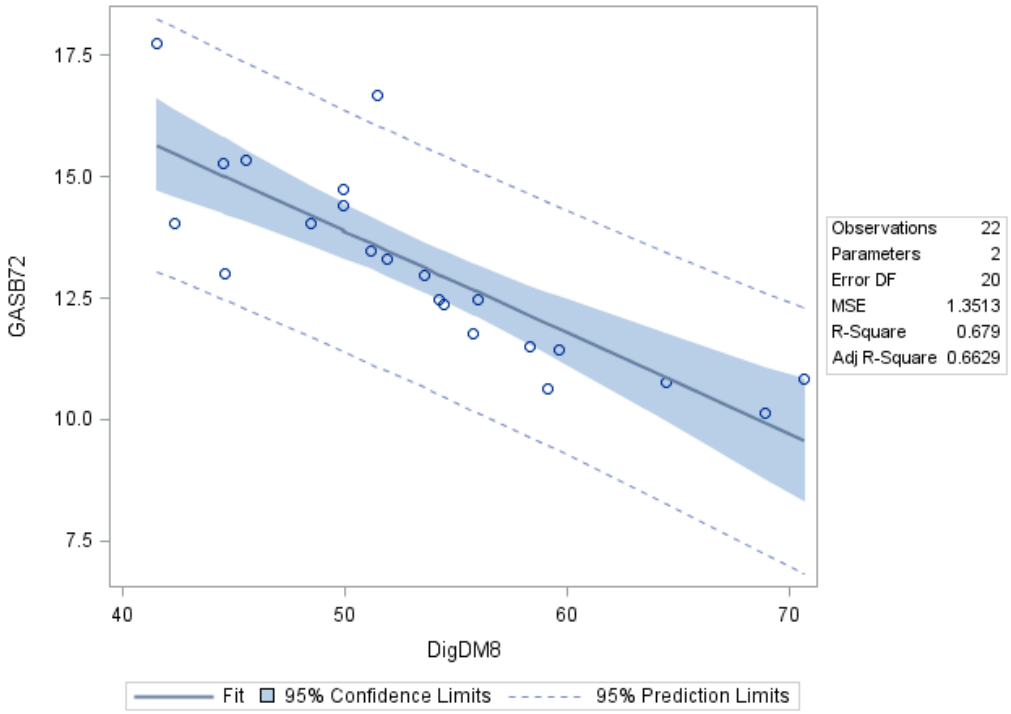

Figure 18. Fit plot between the halftime to asymptotic gas production (GASB72) and the calculated rumen degradability of dry matter obtained from in sacco.

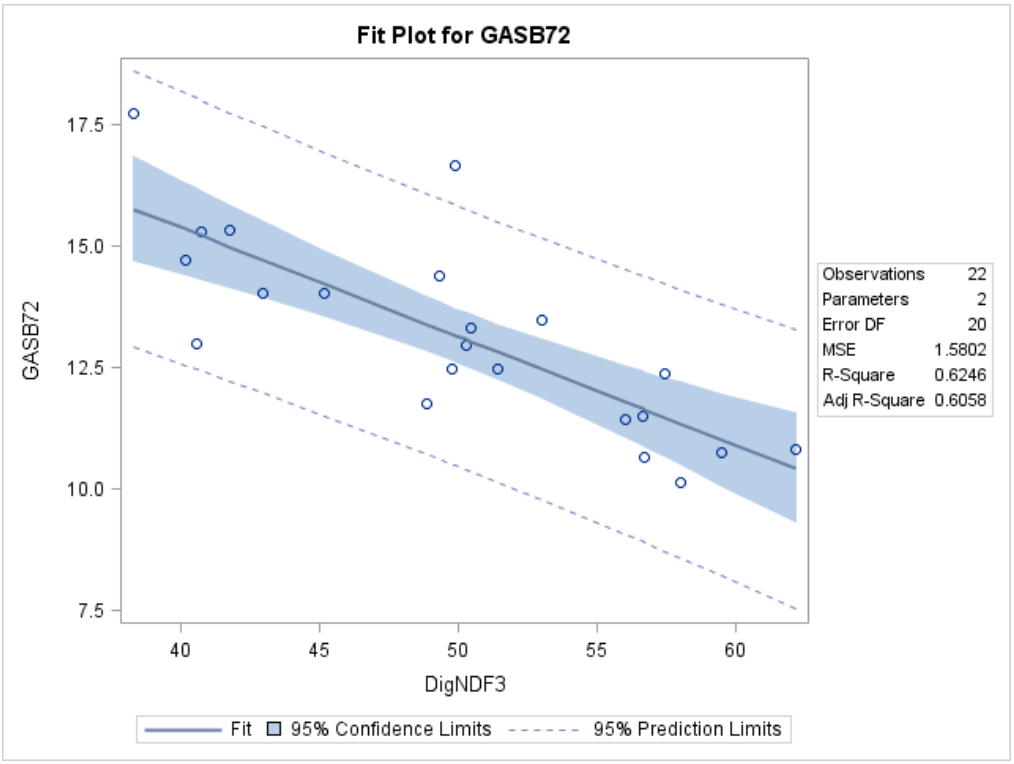

Figure 19. Fit plot between the halftime to asymptotic gas production (GASB72) and the calculated rumen degradability of NDF obtained from in sacco.

3.5.3 Daisy compared to gas production

There was an increasing correlation between gas production and in sacco loss of dry matter and NDF at longer incubation times in Daisy. Table 10 shows the correlations between dry matter loss and NDF loss from the Daisy incubator after 48 and 96 hours, and the gas production after 72 and 48 hours. Gas production after 72 hours had a higher correlation with Daisy, compared with the gas production after 48 hours. Such as in the comparison between gas production and in sacco, the asymptotic gas production (GASA72 and GASA48) had low correlations with high p values.

Table 10. Correlation matrix between in sacco dry matter loss and NDF loss at 48 and 96 hours, and gas production after 48 and 72 hours.

	DaisyDM48	DaisyDM96	DaisyNDF48	DaisyNDF96	GASB72	GASB48
DaisyDM48	1	0.98342 <.0001	0.75472 <.0001	0.86274 <.0001	-0.7992 <.0001	-0.66837 0.0009
DaisyDM96	0.98342 <.0001	1	0.74456 <.0001	0.89864 <.0001	-0.83759 <.0001	-0.69865 0.0004
DaisyNDF48	0.75472 <.0001	0.74456 <.0001	1	0.90525 <.0001	-0.79635 <.0001	-0.66189 0.0011
DaisyNDF96	0.86274 <.0001	0.89864 <.0001	0.90525 <.0001	1	-0.90942 <.0001	-0.77099 <.0001
GASB72	-0.7992 <.0001	-0.83759 <.0001	-0.79635 <.0001	-0.90942 <.0001	1	0.81056 <.0001
GASB48	-0.66837 0.0009	-0.69865 0.0004	-0.66189 0.0011	-0.77099 <.0001	0.81056 <.0001	1

Gas production had a higher correlation with the loss of NDF in Daisy, compared to the loss of dry matter in daisy. While the comparison between gas production and in sacco had a higher correlation between gas production and dry matter, instead of gas production and NDF. Figure 20 and Figure 21 shows fit plots of gas production (GASB72) compared to loss of NDF or DM after 96 hours incubation in Daisy, respectively. The figures support the correlations, where Figure 20 have observations closer to the line than Figure 21.

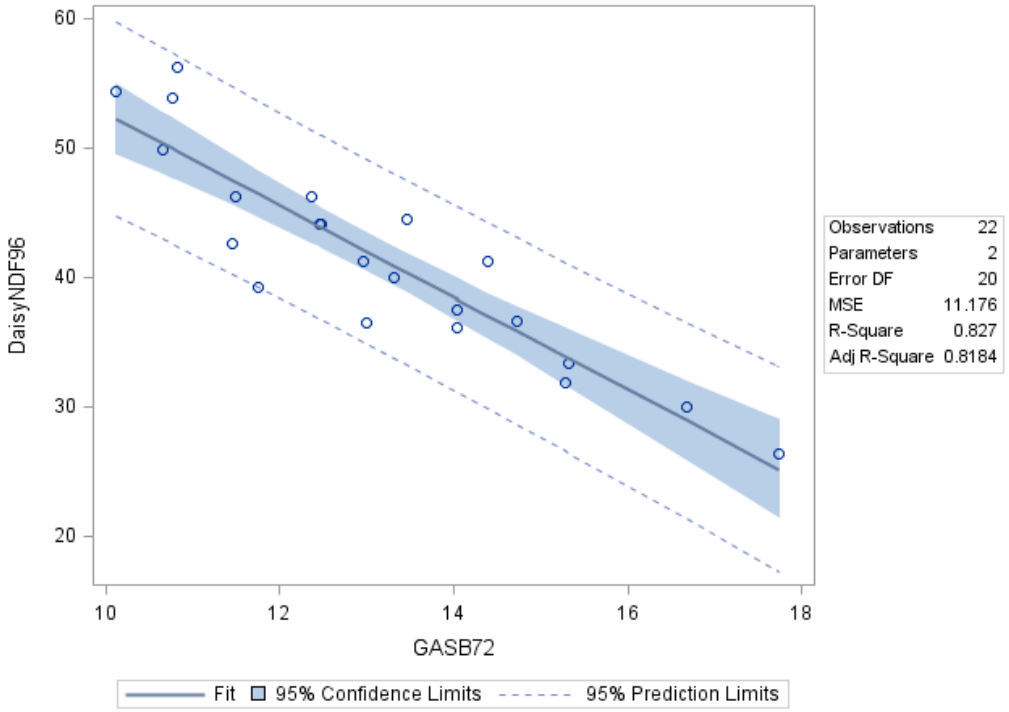

Figure 20. Fit plot between NDF loss after 96-hour incubation in Daisy (DaisyNDF96) and the halftime to asymptotic gas production using 72 hours incubation (GASB72).

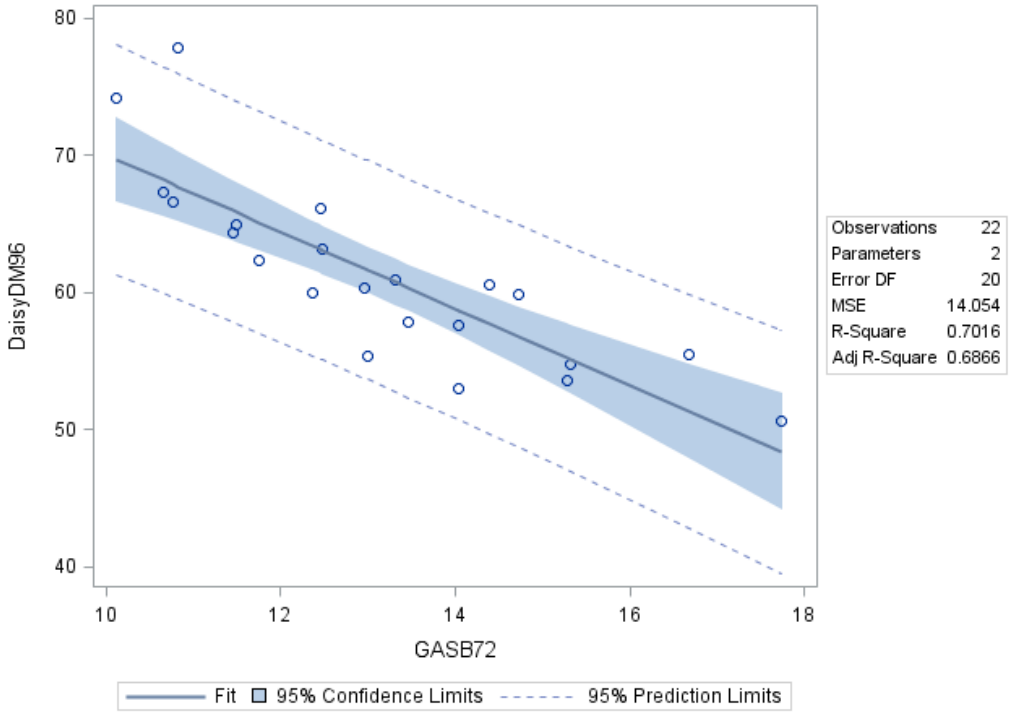

Figure 21. Fit plot between dry matter loss after 96-hour incubation in Daisy (DaisyDM96) and the halftime to asymptotic gas production using 72 hours incubation (GASB72).

4 Discussion

Before discussing the results, some methodical differences need to be addressed. For the in sacco method the grinding were done with a sieve size of 1.5 mm, while a sieve size of 1.0 mm was used for the Daisy incubator and the gas production system. The effect grinding size has on the estimated digestibility is not clear. Michalet-Doreau and Cerneau (1991) changed sieve size between 0.8, 3.0 and 6.0 mm, and found a decrease in degradability at increasing sieve size. However, to change from 0.8 to 6.0 mm are excessive. Wilman and Adesogan (2000) looked at the effect a sieve size of 0.5, 1.0 and 1.5 mm had on digestibility, and found no significant effect. Richards, Pedersen, Britton, Stock, and Krehbiel (1995), on the other hand, found a trend (p value<0.10) to lower digestibility when changing the sieve size from 1 mm to 2 mm. And Damiran et al. (2008) found a significant reduction in digestibility when increasing the sieve size from 1.0 mm to 2.0 mm. From this it probably can be concluded that a difference in sieve size of 1.0 and 1.5 mm probably had no or a minor effect on digestibility.

Wilman and Adesogan (2000) argued that the reason they did not find an effect of sieve size might have been due to the use of incubation bags with small pore sizes. A bag with small and evenly distributed pores would probably reduce particle loss when using small sieve sizes, and thereby reflect digestibility better than when using bags with larger pore size. However, a to small pore size would inhibit microorganisms to penetrate the bag, and thereby reduce the degradability. A difference in pore size could also enforce a different microbial population inside the bags, and thereby give a unlike potential for digestion. The ANKOM F57 filter bags used in Daisy have a pore size of 25 µm (ANKOM Technology, n.d.-b), while the bags used in sacco and for collecting the residuals for gas production had a pore size of 38 µm (Åkerlind et al., 2011). In the gas production system, the residues were transported into in sacco bags after incubation, while the in sacco method and Daisy^{II} Incubator were incubated inside the bags.

Damiran et al. (2008) also found that a higher sample weight gave lower digestibility estimates when comparing 0.25 g samples with those of 0.5 g. When talking about sample weight, the ratio between bag surface and sample weight is important. Åkerlind et al. (2011) recommend 10 mg sample/cm^2. ANKOM Technology (n.d.-a) have previously stated that the Daisy^{II} Incubator can handle sample weight from 0.25 – 1.0 grams, and thereby that the ANKOM F57 filter bags can obtain a sample weigh of 1.0 gram. Later, a sample size of 0.25 seems to be advocated (Adesogan, 2005; ANKOM Technology, 2017; Marten & Barnes, 1979). The reason for this this change might be to increase the ratio of sample to surface area. In this thesis, a sample size of 1.0 g was used for both the Daisy^{II} Incubator and the gas production system, whereas a sample weight of 2.0 g was used for in sacco.

The in sacco bags used have a much larger surface area then the F57 filter bags used in Daisy. As seen in the results, the averaged dry matter and NDF loss after 96 hours incubation was lower for Daisy than in sacco. A smaller sample size in the F57 bags might have improved the Daisy method. However, the value itself is not that important for in vitro digestibility measurements, as long as the correlation to in vivo digestibility is high.

Another important aspect is that samples can affect one another when incubated in the same vessel, as done for in sacco and Daisy. During incubation, soluble matter are released into the rumen fluid and could affect microbial activity and thereby the degradation in all samples within the same vessel. Wilman and Adesogan (2000) found higher error values when using the Daisy^{II} Incubator compared to a Tilley and Terry based method, something they partially blamed the shared vessel used in Daisy. One might assume minimal influence between similar feedstuffs when sharing a vessel, but a high-energy silage would probably increase the NDF degradation of a low-energy-silage if they were to be incubated in the same vessel.

Similarly, the diet fed to the donor of ruminal inoculum could influence both the microbial population and the substrates readily available to the microorganisms. Cone, Cliné-Theil, Malestein, and van't Klooster (1989) found a higher degradation of starch if the donor cow was fed concentrates compared to a hay-fed cow. Richards et al. (1995) found the diet of the inoculum donor to affect the rate of starch digestion, but not the relative ranking of the feeds. Many researches states differences in inoculum to be the major source of variation between laboratories (Bueno et al., 2005; Marten & Barnes, 1979). Some recommends to feed donor cows a diet similar to what's going to be incubated in vitro (Yáñez-Ruiz et al., 2016), while others think a grain-free diet are the best to achieve minimum variability for in vitro degradability between laboratories (Marten & Barnes, 1979). Huntington, Rymer, and Givens (1998) found no effect of donor animal diet and dedicated the reason to be sampling of the rumen inoculum before morning feeding. The effect of diet would be strongest shortly after feeding. To collect rumen fluid right before feeding or a long time after feeding (as done in this thesis) would therefore be preferred. In my experiment there was a minor change in donor diet between Daisy and gas production. It is impossible to know the exact influence on the results presented, but it is expected to be minor as the difference in diet was small and rumen inoculum was collected late compared to feeding time. Also, there was used a mixture of rumen inoculum from two animals for Daisy, and a mixture from three animals for the gas production system. Yáñez-Ruiz et al. (2016) recommend using three or more donor cows to reduce extreme values from one animal.

For in vitro systems the buffer solution are supposed to maintain the microbial population by having a stable pH and by offering available nutrients. The thought behind using different buffer solution for Daisy and the gas production system was to stick to the methods proposed. The manual for Daisy^{II} Incubator only suggest the buffer used, while the approach for the gas production system at Stoffskifteavdelingen are using the buffer of Goering and Van Soest (1970). In addition, measurements of the buffer used in Daisy was incorrect. Unfortunately, the pH after incubation were not measured after incubation in the Daisy^{II} Incubator. This would have been the simplest way to validate sufficient buffering capacity. The degradation measurements from Daisy indicate that the incubation went well, but the possibility of suboptimal degradation condition cannot be ignored.

Good adaption to an exponential curve and few inter-crossings between timepoints, probable gave reliable estimates of dry matter and NDF degradation parameters. In calculations for NDF, the loss at 0 hours incubation was forces to be 0, as soluble NDF was assumed to not exist. Deviations from 0 probably originate from errors from particle loss or inaccuracy with the NDF analysis. Although, Figure 10 indicate some variation around 0, but the variation is small and within what could be expected. Thus, it was concluded that the degradation profiles mirror the variation of dry matter and NDF degradation in the feeds well.

The correlations between the loss of dry matter after Daisy incubation and the calculated degradability of dry matter from in sacco was high. In addition, the loss of NDF after 96-hour incubation in Daisy had a quite good correlation with the degradability of dry matter obtained from in sacco. The reason for this is probably that NDF make up a major part of dry matter. This also explains why the correlations between degradability of dry matter from in sacco and the loss of NDF at increasing incubation times in Daisy are increasing. NDF needs time to be degraded, and therefore the loss of dry matter at longer incubation times will increasingly consist of NDF loss. A similar argument can be made for the large correlation between soluble dry matter and the loss of dry matter after 12 hours of incubation in Daisy. Soluble dry matter disappears almost immediately, before any substantial portion of the potentially degradable matter are degraded. Because of the fast degradation of soluble dry matter, it sustains a high correlation with all incubation times from Daisy although being highest for the shorter incubation time.

The correlations are generally higher between dry matter measurements from in sacco and Daisy measurements, than NDF measurements from in sacco and Daisy measurements. The loss of NDF after 96-hour incubation in Daisy (DaisyNDF96) had higher correlations with the estimated digestibility of dry matter (DigDM8) then the digestibility of NDF (DigNDF3) from in sacco. Based on this, NDF measurements seems challenging.

It is interesting that the potentially degradable dry matter from in sacco (BDM) have low correlations with the measurements from Daisy, while the potentially degradable NDF from in sacco (BNDF) have quite high correlations with the Daisy measurements. This might be because potentially degradable NDF (BNDF) is the same as the total degradable NDF, because soluble NDF equals zero. If one look at the total degradable dry matter from in sacco, this value also has high correlations with the Daisy measurements.

Gas production after 72 hours of incubation have higher correlations with in sacco and Daisy compared to the 48 hours gas production. The explanation is probably that the production of gas had not yet reached its plateau and was still increasing at 48 hours. This will make the model of Groot et al. (1996) overestimate the asymptotic gas production. Reflected by higher estimates values for asymptotic gas production when using the data from 48-hour incubation compared to the 72-hour incubation.

The halftime to asymptotic gas production had higher correlations to in sacco and Daisy than the asymptotic gas production itself. This could be explained by claiming the halftime to asymptotic gas production explains a larger part of the degradable characteristics of a feed. Two different feeds could have the same asymptotic accumulated gas production but with very different pattern of fermentation and thus very different halftime to reach their asymptotic gas production. A feedstuff with a high level of soluble matter wold obtain the halftime faster than a feedstuff with the same amount of total degradable nutrients, but with a lower fraction of soluble material as e.g. sugar.

In the Daisy system, the degradation profile of feed 111 and 121 deviate with higher dry matter degradation of than the remaining feeds. The degradation profile of NDF does not show as high difference between 111 and 121, and the other feeds, but they have a higher degradation after 96-hour incubation in Daisy. Degradation profiles from in sacco also show feed 111 and 121 to have the highest dry matter loss, whereas only feed 111 deviate from the others in NDF loss after in sacco incubation, but 121 is also in the upper range of NDF degradation. Accumulated gas production does not show feed 111 and 121 as the highest-ranking feeds. Feed 111 and 121 has the lowest concentration of NDF, and the highest content of calculated residue (Table 3). Low concentration of NDF indicate early harvesting, and thus the observed high degradation of dry matter and NDF is expected. In the gas production method, fermentation of NDF and soluble carbohydrates will yield comparable levels of gas. But early harvested grass usually contains a higher concentration of fat, and because fat does not ferment, it will not contribute to a higher gas production. In combination with a crude protein content above the average of the feeds used, this could be the reason for mediocre results for feed 111 and 121 in the gas production system. In addition, a feed with high

content of fermentation products would probably give lower production of gas. Since the fermentation products from these feeds were not available, one can only speculate if it is the case. The differences in the ranking of feed 111 and 121 indicates methodological differences between in sacco and Daisy at one side, and the gas production system on the other side. For early harvested grass with varying concentration of residual sugars and fermentation products, asymptotic gas production would probably be challenging to use as a prediction of digestibility. In contrast, feed 111 and 121 have low halftimes to asymptotic gas production. As discussed earlier, this indicate that the halftime to asymptotic gas production is a better indicator for evaluate digestibility in early harvested silages, and might be the reason this thesis found better correlations using the halftime instead of the asymptotic gas production. Feed 154 on the other hand, had lowest degradation in sacco and in Daisy, it was lowest at asymptotic gas production, in addition to having the lowest fractional rate of gas production. This indicate better alignment among the methods for evaluating silages with low digestibility. These feeds will normally have a high content of NDF, with a high degree of lignification. In addition, these feeds normally have low concentrations of readily fermented carbohydrates like sugars, explaining this observation.

5 Conclusion and perspectives

The results show good correlations between the methods of in sacco, Daisy and gas production. Dry matter measurements had higher correlations than NDF measurements, indicating NDF measurements to be challenging. Gas production had higher correlations to the other methods when using 72-hour incubation. The gas production appeared to underestimate the digestibility of some early harvested silages.

Even though the methods seem to correlate well to each other, it is important to validate the results by compare the methods to in vivo digestibility. The feeds used in this thesis have been subjected to an in vivo digestibility trial using mature sheep kept at maintenance. Unfortunately, these results were not available within the time limit of this thesis. In sacco, Daisy and gas production shows a variation in the silages tested. If this variation is present in the in vivo measurements, it would give a good foundation to formulate equations. It would be highly desirable if the Daisy or the gas production would prove to be the better alternate method, since they are the cheapest and least time-consuming than in sacco.

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Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway