

Intensively processed silage using Bio-extruder

– Effects on gas production and forage digestibility

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Intensiv bearbetning av ensilage genom extrudering
– Effekter på gasproduktionen och grovfodrets smältbarhet

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Abstract

The dairy cow requires a high feed intake to maintain a high milk production and forage is a crucial component in dairy cows diet. Forage stimulates chewing activity and rumination which contributes to a normal and effective rumen function. However, the forage intake is limited due to a high fibre content and large particle size, compared to concentrate. A reduced particle size of the forage has been shown to increase the forage digestibility and enable an increased forage intake. The objective of this study was to investigate if intensive physical treatment of forage can increase the *in vitro* digestibility. Primary growth of timothy and red clover silage, harvested at two maturity stages (early and late), were processed using a Bio-extruder. A study was conducted using a gas *in vitro* system with liquid displacement to continuously measure the gas production from the silage, as indicative of digestibility. The gas production was measured during four incubations, two with timothy and two with red clover. The incubations included triplicates of each sample, hence in total six replicates of each forage and treatment combination were incubated. Each incubation lasted for 72 hours and the gas production was evaluated for the main effects, extrusion, crop and maturity as well as for their interactions. After the incubation, the fermented residues were treated with neutral detergent solution to estimate the digestion of neutral detergent fibre. The result showed a significantly higher gas production for processed timothy and red clover compared to control silage where the largest difference occurred between 6 – 24 hours incubation. The NDF residue from processed silage was significantly lower compared to control silage for late harvested timothy and early harvested red clover. It was concluded that, there are possibilities to increase the forage digestibility by intensive physical treatment using a Bio-extruder. How the forage intake and milk production are affected by feeding dairy cows intensively processed forage need to be investigated further.

Keywords: Digestion rate, timothy, red clover, gas production, NDF digestibility, extruder

Sammanfattning

Grovfoder är den viktigaste komponenten i mjölkors foderstat och för att upprätthålla en hög mjölkproduktion krävs ett högt foderintag. Grovfoder stimulerar tuggaktivitet och idissling vilket bidrar till en normal och effektiv vomfunktion. Dock är mjölkorns grovfoderintag begränsat på grund av en hög fiberhalt och stor partikelstorlek jämfört med kraftfoder. Studier har visat att en minskad partikelstorlek av grovfodret kan öka fodrets smältbarhet och öka grovfoderintaget. Syftet med denna studie var att undersöka om intensiv bearbetning av grovfoder kan öka *in vitro* smältbarheten. Timotej- och rödklöverensilage med två olika skördetidpunkter (tidig och sen) bearbetades i en Biogas-extruder. En studie genomfördes där ett gasmätningssystem användes för att mäta gasproduktionen från ensilageproverna, där mängden gas påvisar ensilagens smältbarhet. Fyra inkubationer genomfördes, två med timotej och två med rödklöver. Varje inkubation innehöll tre upprepningar av varje prov, därmed genomfördes totalt sex upprepningar från vardera prov. Varje inkubation varade i 72 timmar och från mätningarna av gasproduktionen uppskattades huvudeffekterna bearbetning, gröda och skördetidpunkt, samt interaktioner mellan effekterna. De fermenterade resterna samlades upp efter inkuberingen och behandlades med ND-lösning för att mäta smältbarheten av fiberinnehållet i fodret. Resultatet visade en signifikant högre gasproduktion från bearbetad timotej och rödklöver jämfört med kontrollensilage. Fiberresterna från bearbetat ensilage var signifikant lägre jämfört med kontrollensilage för sent skördat timotej och tidigt skördat rödklöver. Sammanfattningsvis finns det möjligheter att öka grovfodrets smältbarhet genom intensiv bearbetning av fodret. Ytterligare studier behöver genomföras för att undersöka hur grovfoderintaget och mjölkproduktionen påverkas av intensiv bearbetning.

Nyckelord: Nedbrytningshastighet, timotej, rödklöver, gasproduktion, NDF-smältbarhet, extrudering

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Abbreviations

CP	Crude Protein
DM	Dry Matter
ND	Neutral Detergent
NDF	Neutral Detergent Fibre
REC	Red clover Early Control
REP	Red clover Early Processed
RLC	Red clover Late Control
RLP	Red clover Late Processed
TEC	Timothy Early Control
TEP	Timothy Early Processed
TLC	Timothy Late Control
TLP	Timothy Late Processed
VFA	Volatile Fatty Acids

1 Introduction

The dairy cow is considered as an important animal in the world agriculture since it provides dairy products, meat and manure with only grass and cereal by-products as their main source of nutrition (Van Soest *et al.*, 1991). Dairy cows are ruminants with pre-gastric fermentation and have the capability to utilise energy from cellulosic carbohydrates in fibrous forage. This ability is due to the rumen microbes which convert carbohydrates and protein to volatile fatty acids (VFA), allowing the cow to utilise VFA as metabolizable energy. The proportion of various acids produced in the rumen are affected by the feed source.

Forage as a feed source is crucial for the cow to maintain a normal and effective rumen function (Chamberlain, 1996). Forage has a high fibre content and contributes to more chewing and rumination compared with feeding concentrate. This is due to the fibre content and large particle size in forage, which requires more chewing and rumination to reduce the particle size and enable passage through the rumen. Chewing and rumination stimulates saliva production which has a buffering effect that helps to maintain pH values of rumen fluid above 6.2, optimal for fibre degradation (Márquez *et al.*, 2009; Chamberlain, 1996). Feeding high proportion of readily fermentable non-structural carbohydrates instead of fibre will decrease the ruminal pH as a result of lower chewing activity and thus lower saliva flow, and an increased production of VFA (Mertens, 1997). Hence, the same study submits that low rumen pH values (< 6), may result in metabolic disorders, sub-acute or acute acidosis, and in dairy cows reduced milk fat synthesis.

In spite of the benefits of high fibre diets, a high proportion of fibre rich forage limits the feed intake and hence limits the milk yield (Krause & Combs, 2003). Today's dairy cows have the genetic potential for a high milk production and the farmers aims to maximise the milk production for each cow (Volden, 2011; Krause & Combs, 2003). Therefore, the farmers want to provide the dairy cows with feed of high nutritional value to enable a high milk production, but at the same time maintain healthy cows. Several studies have investigated if different physical treatment of forage might increase the digestibility of the forage (Damborg *et al.*, 2018;

Kononoff & Heinrichs, 2003; Krause & Combs, 2003; Broderick *et al.*, 1999; Hong *et al.*, 1988). Physical treatment of forage includes chopping, grinding, milling, extrusion and maceration. The more intensive treatment is extrusion and maceration where the aim is to break open the cell structure and make the nutrients easily available for the rumen microbes (Hong *et al.*, 1988).

An increased forage digestibility can reduce the proportion of concentrate needed to sustain high milk production (Broderick *et al.*, 2002). Replacing a proportion of the concentrate with highly digestible forage could have both environmental and economic advantages. Growing grass and legumes to forage production has less need for mechanical soil cultivation compared to grain production which contributes to reduced carbon dioxide emissions (Fogelfors, 2015). An increased forage intake enables the use of more home-grown crops, and the need for concentrate and imported feedstuff, as soy bean meal and palm kernel expeller can be reduced (Emanuelson *et al.*, 2006).

The aim of this study was to investigate if intensive physical treatment using a Bio-extruder affects the *in vitro* digestibility of dry matter and neutral detergent fibre fractions in timothy and red clover silages. The hypothesis is that processed silage has higher digestibility compared to unprocessed silage, indicated by a higher gas production rate during incubation in Gas Endeavour system and by less remaining neutral detergent fibre (NDF) after incubation.

2 Literature review

2.1 Cows requirements and limitations

The feed ration for dairy cows needs to contain a certain amount of nutrients to fulfill the requirements for a high milk production, but also to keep the cow healthy (Chamberlain, 1996). Forage is a very important component of the dairy cow's feed ration since it is a source of fibre in the diet. The fibre content contributes to a normal rumen function, which is crucial to maintain a high feed intake and milk production.

However, the fibre content needs to be optimized since dairy cows forage intake is negatively correlated to the fibre content in the forage (Van Soest, 1994). A high fibre content requires more rumination and chewing to enable passage through the rumen. In case of high forage level in the feed ration with high fibre content, the passage rate will decrease, which contributes to a reduced feed intake. Physical treatments of the forage, such as chopping or grinding, may enable an increased passage rate and a higher feed intake despite a high fibre content in the diet.

2.2 Structure of the plant

The plant cell can be divided into three main components (cell membrane, cytoplasm and cell wall), each with specific characteristics to maintain the growth of the plant (Fogelfors, 2015). The cell membrane adjusts material transports in and out from the cell. The cytoplasm includes several organelles, such as cell nucleus, chromosomes and mitochondrion, where the energy metabolism and rebuilding of several molecules takes place. It is also in the cytoplasm that nutrients, in form of crude protein, soluble carbohydrates, starch and lipids, are located. The cell wall's function is to protect the cell and keep the plant stable. The cell wall consists of a primary and a secondary cell wall, where the main components are water, pectin, cellulose,

hemicellulose, lignin and cell wall protein. The analytical method “Neutral Detergent Fibre” (NDF) is routinely used to quantify the content of cellulose, hemicellulose, lignin and cell wall protein in the plant. By definition, NDF is the dried residue after boiling feed samples with a neutral detergent solution (ND solution) and heat-stable α -amylase (Mertens, 2002; Van Soest *et al.*, 1991). This procedure removes components such as starch, lipids, easily digested protein, sugars and pectin from the sample, which results in fibre residues in form of NDF. Also, by ashing the samples, the result can be expressed exclusive of ash, which is a more accurate NDF value because ash from soil contamination will not bias the results.

Generally, grass contains a higher proportion of fibre compared to legumes, and while the highest fibre concentration is located in the stems of the plant, grasses has a greater content of fibre in the leaves than legumes (Buxton & Redfearn, 1997). While animals cannot digest NDF because their digestive tract does not have the enzymes capable to degrade cellulose or hemicellulose, microorganisms present in the digestive tract can do so. Therefore, ruminants have the possibility to utilize the energy in the fibre thanks to the rumen microbes. Lignin though, is totally indigestible even for ruminants (Fogelfors, 2015). Although potentially digestible fibre is slowly digested in the rumen and consequently occupies space which can limit the feed intake (Mertens, 1997).

Nutrient content and digestibility differ among constituents of the plant (Chaves *et al.*, 2006). Perennial ryegrass, tall fescue, Yorkshire fog, Bulbous canary-grass and Bahia grass were analysed for chemical composition by Chaves *et al.* (2006). The results showed a higher digestibility in the leaf of the plants compared to the stem and flower head. Also, the leaf contained a higher proportion of protein and less fibre compared to the stem and flower head in all species. It is well established that forage digestibility decreases in relation to maturity (Frame & Laidlaw, 2011). The decreased digestibility is due to an increased lignification of the cell wall in the plant with an increased maturity of the plant (Van Soest, 1994). Hence, these are important aspects to be considered to harvest forages at the right time and achieve a desirable digestibility.

2.3 Forage plants

Forages containing a mixture of grass and clover species are a popular feed source for dairy cows in Scandinavia (Rinne & Nykänen, 2008). A mix of timothy and red clover is commonly used in the forage due to the chemical composition of these species (Hetta *et al.*, 2004). The major differences between timothy and red clover in chemical composition is the content and degradability of NDF at different harvest times. This variation mainly depends on the differences in blooming occasions and

the leaf development between the species. These characteristics complement each other, and a good mixture of these species increase the conditions for a high-quality forage at various harvest occasions.

2.3.1 Timothy

Timothy (*Phleum pratense* L.) is a grass with high tolerance against cold weather, which makes it a popular grass species to grow in northern Europe (Frame & Laidlaw, 2011). The chemical composition of timothy has been studied at different harvest occasions, first harvest (spring growth) and second harvest (summer growth), see Table 1 (Hetta *et al.*, 2004). The NDF content in timothy is rather high and increases with maturity in contrast with crude protein (CP), which decreases with maturity. Because of the increased NDF content and a decreased digestibility of NDF in more mature timothy, the harvest time is an important parameter to consider in order to achieve a desirable forage digestibility.

Table 1. Chemical composition (g/kg DM) in timothy harvest at various occasions in 1996 (Hetta *et al.*, 2004). Early and late harvest at first cut (spring growth) and second cut (summer growth).

Parameter	Spring growth			Summer growth		
	14/6	9/7	Diff ³	24/7	2/9	Diff ³
NDF ¹	504	675	171	465	602	137
CP ²	180	89	91	245	96	149

¹ NDF, Neutral detergent fibre.

² CP, crude protein.

³ Diff, the difference in NDF or CP (g/kg DM) between early and late harvest in spring and summer.

2.3.2 Red clover

Red clover (*Trifolium pratense* L.) is, like other legumes, a good source of nitrogen as the plants have rhizobial nitrogen fixation ability (Frame & Laidlaw, 2011). In the study by Hetta *et al.* (2004), the chemical composition of red clover was also measured at different occasions, see Table 2. Compared to timothy, the NDF content is lower in red clover. The crude protein content is higher in red clover compared to timothy and does not differ with maturity as much as it does in timothy. Red clover is a popular component in silage according to the nitrogen fixation and high digestibility (Frame & Laidlaw, 2011). However, red clover is not so well adapted to wet heavy soils and has a short productive lifespan, which makes the plant considered as a short-lived perennial.

Table 2. Chemical composition (g/kg DM) in red clover harvest at various occasions in 1996 (Hetta *et al.*, 2004). Early and late harvest at first cut (spring growth) and second cut (summer growth).

Parameter	Spring growth			Summer growth		
	14/6	9/7	Diff ³	24/7	2/9	Diff ³
NDF ¹	288	388	100	271	465	194
CP ²	235	175	60	214	149	65

¹ NDF, Neutral detergent fibre.

² CP, crude protein.

³ Diff, the difference in NDF or CP (g/kg DM) between early or late harvest in spring and summer.

2.3.3 Straw

Among all common forages, straw is one that has the highest fibre content (Chamberlain, 1996). The NDF content in wheat straw is approx. 800g/kg DM and the content of crude protein is only approx. 40g/kg DM. Hence, straw is a feed source with low digestibility and is not a suitable feed for high yielding dairy cows. On the other hand, straw can be used to feed dry cows with low nutrient requirements and in some conditions, straw can be used as a fibre supplement in feed rations with too low fibre content.

2.4 Forage digestibility and physical treatments

The energy requirement of dairy cows depends mainly, among other factors, on the animals body weight and physiological status, such as stage of lactation, pregnancy day and daily milk production (Volden, 2011). Today's dairy farmers aim to maximize the milk production for each cow. Therefore, cows with high energy requirement are fed concentrate with high starch content as a supplement to forage to achieve a high milk production (Beauchemin *et al.*, 2003; Krause & Combs, 2003; Krause *et al.*, 2002). However, a diet high in starch and low in fibre content increases the risk of occurrence of metabolic disorders (DeVries *et al.*, 2008; Krause *et al.*, 2002). According to feeding recommendations, the level of starch rich concentrates in the feed ration should not exceed 65 % of the dry matter content (Spöndly, 2003).

2.4.1 Rumen function

Forage is the main source of fibre in dairy cows feed rations and contributes to a normal and effective rumen function (Chamberlain, 1996). Feeding forage to dairy cows involves more chewing and rumination time compared to feeding concentrate. Chewing and rumination contributes to production of saliva, which contains bicar-

bonate and phosphate buffer. Thus, saliva contributes to maintain a rumen environment, pH value above 6.2, which is beneficial to the cellulolytic rumen microbes. However, a high proportion of forage in dairy cow's diet can result in a lower energy intake which limits the milk production (Mertens, 1997). It is the physical characteristics of fibre, such as density and particle size, that effects the rumen environment, metabolism, milk fat production and ruminal fermentation in dairy cows. Besides the content of NDF in the diet, the physical characteristics of the NDF fraction is also important. The term "physically effective NDF" (peNDF), includes physical characteristics of the fibre, such as particle size. Since the particle size affects the chewing activity and the rumen environment, peNDF can be a good measurement to maintain sufficient rumination and avoid metabolic disorders and low milk fat syndrome.

2.4.2 Effects of chopping on forage intake and utilisation

Forage of longer particle size generally causes a slower passage rate and limits the feed intake (Nasrollahi *et al.*, 2015; Van Soest *et al.*, 1991). Hence, several studies have investigated how reduction of the forage particle size affects the feed intake or passage rate. (Beauchemin *et al.*, 2003; Kononoff & Heinrichs, 2003; Yang *et al.*, 2001). Kononoff & Heinrichs (2003) showed no increased passage rate for reduced particle size of haylage. In addition, the reduction of particle size resulted in increased dry matter intake and NDF digestibility. The same study showed a change in VFA production between forage with short and long particle size. Short particle size resulted in a higher VFA production and lower ruminal pH than long particle size diet.

DeVries *et al.* (2008) observed that feeding forage with short particle size (<8mm) lowers the ruminal pH and increases the risk of ruminal acidosis. Their study evaluated the sorting behaviour for cows in early lactation and mid-lactation with different forage to concentrate ratio (45:55 and 60:40). The result showed that the cows were sorting against large particle size, both in early and mid-lactation, which caused a decreased pH level in the rumen. However, when the pH level decreased, the cows changed their sorting behaviour in favour of large particles to stimulate rumination and increase the ruminal pH.

Nasrollahi *et al.* (2015) conducted a study which comprised a meta-analysis including 45 papers where DMI and milk production were measured when feeding forage of various particle size including different forage level, and source. The results showed an increased DMI with decreased forage particle size (FPS), which indicates less fill value in the rumen and an increased passage rate through the rumen. However, a decreased FPS resulted in a decreased digestibility of NDF which can be explained with the increased passage rate. The forage level has a significant

impact on how the forage particle size affected the DMI. Decreasing FPS resulted in increased DMI only with a high forage level in the diet (>50%), which is explained as the gut filling effect has greater impact in a diet with high forage level. Also, the source of forage was shown to have impact on the effect of FPS. A decreased FPS showed less effect on the DMI in non-corn forage-based diet compared to feeding corn-based forage. This could be explained by higher losses of palatable leaves when chopping grass compared to corn-based forage, which may lower the palatability of the feed and hence the voluntary feed intake. Likewise, an increased DMI with decreased FPS occurred when the forage was based on silage, but not on hay, which might also be due to different palatability of the feed. The study showed that the FPS also has effects on the milk production and composition, as decreased FPS resulted in an increased daily milk and protein production. In contrast, the milk fat percentage decreased with decreasing FPS, which resulted in an unaffected daily production of fat-corrected milk (FCM).

Beauchemin *et al.* (2003) investigated how alfalfa-based diets, with different ratio of alfalfa silage or alfalfa hay with different physical form, affected the dairy cows DMI, chewing activity, particle size reduction, salivary secretion, ruminal fermentation and milk production. The diets consisted of 60% forage with a ratio of either 50:50 or 25:75 alfalfa silage and alfalfa hay and the hay was either chopped or ground. The results showed that DMI and eating time were not affected by the particle size, compared to the chewing and ruminating time which primary seemed to be associated to the particle size rather than the proportion of silage and hay. Thus, rumination and chewing time decreased when feeding ground hay compared to chopped hay. The ruminal pH was higher for cows fed with low silage ratio 25:75, compared to high silage ratio 50:50. Likewise, the cows fed chopped hay had higher ruminal pH compared with cows fed ground hay. However, the milk yield and milk composition showed no difference between chopped or ground hay.

A study by Kruse *et al.* (2002) showed no effects of chopping on DMI when feeding dairy cows corn-based diets with a 39% forage level. The study used two total mixed diets containing alfalfa silage, either coarsely chopped (mean particle size (MPS) of 13.6mm) or finely chopped (MPS<6.4mm) and corn, either dry cracked shell corn or high moisture shelled corn. Feeding fine chopped silage with high moisture corn tended to increase the milk yield, while the other three diets had no effect on milk production. Hence, the milk yield was not affected by the FPS, and the authors concluded that this type of feed, with low level of effective fibre and a high proportion of non-structural carbohydrates, did not impair the milk production or digestibility in dairy cows.

2.4.3 Physical treatments of grass in biogas production

Various pre-treatments of forage have been studied to measure the efficiency of using forage in biogas production (Rodriguez *et al.*, 2017). The study focused on different physical pre-treatment such as mechanical, ultrasound and microwave methods. Mechanical treatments in form of chopping, grinding, milling, and extrusion proved to be an effective pre-treatment method due to reduced particle size and improved availability of the substrate. Extrusion by thermo-mechanical pre-treatment of wheat bran and soybean hulls was conducted to evaluate the availability of lignocellulosic substrate (Lamsal *et al.*, 2010). The extrusion processes resulted in increased yield of reducing sugars for wheat bran, but no effect was shown for soybean hulls. This thermo-mechanical method aims to disrupt the crystalline structure of cellulose and enable a greater surface area to the enzymes (Rodriguez *et al.*, 2017). The extruder machine is supplied with screws and kneading blocks, where the crops are heated, mixed and sheared. Subsequently, once the substance exits the extruder, the high pressure is released causing breakdown of the cell structure and hence a more efficient decrystallization of the fibre fraction, which enable an increased biogas production.

2.4.4 Intensive mechanical treatments of forage

Using side-products from bio refineries as fibre-rich feed for ruminants has been investigated (Damborg *et al.*, 2018; Bruins & Sanders, 2012). Damborg *et al.* (2018) used pulp from extracted white clover, red clover, lucerne and perennial ryegrass to analyse how the chemical composition and digestibility changes compared to original plant. Pulp is the fibre-rich residues from processing the plant with extrusion where soluble protein are extracted and can for example be used as a protein supplement to monogastric animals. The results showed no difference in crude protein percentage between the pulp and plant for all species, likewise the digestible organic matter in the dry matter showed no difference between the pulp and plant. Percentage of all fibre components was higher in the pulp compared to the plant. However, the CP concentration in fibre components (aNDF and ADF) was higher in pulp than plant and the pulp had a larger proportion of rumen escape protein, which may result in a more efficient CP utilisation in the intestine.

Another study used mechanical treatment in the form of maceration to increase forage digestibility (Broderick *et al.*, 1999). The study used alfalfa silage where the maceration was performed during harvesting and the control forage was harvested by a conventional mower conditioner. The diets consisted of either 61% macerated alfalfa silage, 61% control alfalfa silage or 50% control alfalfa silage, supplemented with concentrate based on corn and protein from soybeans or fishmeal. Feeding

dairy cows macerated alfalfa silage showed an increased OM digestibility by 3.1%. Milk yield and concentrations of milk protein, fat and solids non-fat (SNF) were greater for macerated silage compared to 61% control silage. However, the 50% control silage had even greater yields of milk, protein, fat, and SNF, which could be explained by the larger concentrate proportion for that diet. Analysis of the silage composition showed a higher NDF content in the macerated silage compared to the control. This was suggested to be due to an improved fermentation in the silage, which was shown by a more rapid pH decrease during ensiling and a larger breakdown of non-structural carbohydrates in the macerated silage. This rapid pH drop may be caused by the potential of improved compression of the crop and hence establishment of an improved anaerobic environment during the ensiling process.

Hong *et al.* (1988) performed a resembling study and compared macerated alfalfa stems with ammoniated and unprocessed alfalfa stems. Immediately after harvest, the alfalfa was sorted for the stems, processed and then dried in a convection oven. The NDF concentration showed to be higher for both macerated and ammoniated alfalfa compared to unprocessed alfalfa. However, the rate of *in vitro* digestion of potentially digestible NDF was significantly higher in macerated alfalfa compared to the ammoniated and unprocessed alfalfa (33.5, 66.4 and 94.2 hours respectively required for 95% degradation of the potentially digestible NDF). The study also included scanning electron microscopy (SEM) which showed most damages of the cell structure for the macerated alfalfa. This results in an increased surface area and the SEM showed more bacterial mass adherent to the surface of macerated alfalfa compared to ammoniated and unprocessed alfalfa. This indicates a higher effective digestion of fibre in macerated alfalfa since more surface area makes the fibre more easily accessible for the rumen bacteria.

Weisbjerg *et al.* (2018) evaluated the effects of physically treated grass-clover silage before ensiling on forage fibre digestibility. The grass-clover was treated with a shredder after harvest to make the fibre shredded without being chopped, and thereafter the material was ensiled in bales. A crossover experiment was performed with four rumen, duodenal and ileal fistulated cows in late lactation. The treatment resulted in forage with higher NDF digestibility. The milk production and DMI were not affected but the daily eating time and ammonia concentration in the rumen were reduced. The rumination time per kg DMI did not differ between treated and untreated silage, neither did the rumen pH level. The results are discussed to be depended on the maturity of the grass, since forage with higher maturity generally shows higher effects on physical treatments and the forage in this study considered to be normal to relatively mature.

2.5 Environmental and economic aspects

Feed production contributes to the greenhouse effect in form of over-fertilization, carbon dioxide emissions and nitrogen leakage in connection with mechanical soil cultivation (Fogelfors, 2015). To reduce the environmental impact, the Swedish government has set up 16 environmental quality objectives, for example “zero eutrophication”, “a varied agricultural landscape” and “a rich diversity of plant and animal life” (Miljomal.se, 2017). Culturing of forages in the form of perennial grass and legumes may be one step in right direction to achieve the environmental objectives. Compared to grains, grass and legumes can grow for several years without any mechanical soil cultivation as the field does not have to be reseeded every year (Fogelfors, 2015). These properties enable reduction of carbon dioxide emissions, soil compaction and nitrogen emissions from soil cultivation. Another advantage of growing grass and legumes for several years, is the increased time of green plants growing on the ground, which contributes to increased photosynthesis and more carbon dioxide being converted to oxygen. Additionally, legumes are nitrogen-fixing plants which contributes to a lower requirement of fertilization, which decrease the risk of nitrogen leakage (Fogelfors, 2015). Using concentrate as a feedstuff will on the other hand contribute to the greenhouse effect since concentrate usually contains imported feedstuff, such as soy bean meal and palm kernel expeller (Emanuelson *et al.*, 2006).

From these aspects, it would be advantageous to produce more forage and replace a proportion of grain in dairy cow diets with forage. However, feed with low digestibility, such as forage with high fibre content, increase the methane production from rumen fermentation (Pinares-Patio *et al.*, 2003). Since methane has a significant impact on the greenhouse effect, it is of interest to keep the methane production from livestock low (Woodward *et al.*, 2004). High proportion of legumes in forage diets shows a higher total methane production compared to grass forage (Woodward *et al.*, 2001). However, legume forage also showed a significant increased DMI and milk yield in dairy cows. This results in a lower methane production per unit of produced milk in diets with high legumes content, compared to grass diets.

The economic benefits in dairy production can be estimated in net income by subtracting total input cost from total income (Total income per animal - Total input cost per animal = Net income per animal) (Guo *et al.*, 2002). Physically treated feedstuff contributes to a higher input cost. Thus, among various physically treatments, chopping is the treatment with highest economic benefits.

Dairy cows are commonly fed concentrate with a high starch content as a supplement to forage to fulfil the requirements to maintain a high milk production (Beauchemin *et al.*, 2003; Krause & Combs, 2003; Krause *et al.*, 2002). However,

maceration of forage has shown a higher digestibility and increased milk yield compared with unprocessed forage (Broderick *et al.*, 1999). This gives opportunities to decrease the proportion of concentrate in the diet and instead increased the forage proportion with physically treated forage and still maintain a high milk production. Emanuelson *et al.* (2006) showed evaluations on possibilities to maintain the milk production with zero imported feedstuff, instead feeding high quality forage and locally cultivated feedstuff, such as brewer's grain, peas, field beans and rape seed. Calculated results showed that an increased proportion of high quality forage with a reduced amount of concentrate in the diet enables a reduced feed cost while still maintaining high milk production. This result agrees with Patel's, (2012) conclusion that high-quality forage can cover 70 % of dairy cows diet and result in a higher profitability due to reduced proportion of expensive concentrate in the diet.

3 Materials and methods

3.1 Plant material and processing

The study was conducted close to Uppsala. The climate in this region shows a mean precipitation at 600-650 mm per year and the yearly mean temperature is 6.5-7.0 °C (measured during the year 1991-2013) (SMHI, 2015). Timothy (*Phleum pratense* L.K) and red clover (*Trifolium pratense* L.), grown at Säby, one of SLU's field research areas in Uppsala (N 59° 50' 20.95", E 17° 43' 9.72"), were harvested at two maturity stages, 5-6 of June (early) and 19-20 of June (late) 2018. All crops were harvested by scythe. The crops were chopped indoors with a stationary chopper and through a compost mill to a theoretical length of 2 – 4 cm. The crops were wilted indoors to a dry matter content of 35-40%, then packed by hand in lab scale silos with a capacity of 4 L. Five silos were used for each crop, packed with approx. 3 kg and ensiled for 15-17 weeks.

Timothy and red clover silage were transported to Alnarp, Lund, where the silos were opened, mixed well and processed. Approximately 800 g of sample from each silo were processed in a bio-extruder (Bio-extruder MSZ-B15e, LEHMANN Maschinenbau GmbH) (see Fig. 1) set at 50% rotation speed and an out-flow passage of 16mm. The bio-extruder is equipped with rotating double-screws (see Fig. 2) which, in combination with high pressure, reduces the silages particle size and breaks open the cell structure (Lehmann, 2018). Approximately 600 g of sample from each crop was kept unprocessed (control), resulting in the sample categories in Table 3. Samples from each crop were packed in plastic bags (approx. 100 g of sample for each bag) and stored frozen at -20°C. The structure of the silage before and after the process are shown in Fig. 3.



Figure 1. Picture of the bio-extruder MSZ-B15e, LEH-MANN Maschinenbau GmbH.



Figure 2. Picture of the screws in the bio-extruder.



Figure 3. Picture of control and processed silage. From left to right, control timothy, processed timothy, control red clover and processed red clover.

Table 3. Abbreviations for the different forage samples.

	Timothy, early harvest	Timothy, late harvest	Red clover, early harvest	Red clover, late harvest
Control	TEC	TLC	REC	RLC
Processed	TEP	TLP	REP	RLP

3.2 *In vitro* incubations

Incubations were performed in a gas *in vitro* system (Gas Endeavour, Bioprocess Control AB, Lund, Sweden) equipped with 15 Duran bottles of 500 mL capacity and customized with an extra neck and a threaded opening. Four *in vitro* incubation batches were run, two batches with timothy samples and two batches with red clover samples. Each batch included triplets of un processed silage from early and late harvest, and triplets of processed silages from early and late harvest (in total, 12 bottles of sample). Beyond these, each batch included a single blank bottle (without sample) and two internal standards, one with high digestibility (sugar) and another with low digestibility (ground straw).

3.2.1 Incubation preparation

The day before incubation, bottles were prepared with samples and stored in the fridge over the night. The samples were randomly allocated to individual bottles to avoid systematic errors. For example, fluctuating temperature in the water bath, which could have an effect on the results. Internal standards were allocated to the same bottles for all batches. The bottles with forage sample and straw were prepared with approximately 4 g of DM each, the high internal standard was prepared with 3 g of sugar and the blank bottle was kept empty. 6 L VOS buffer was prepared with a concentration per litre solution of 5.80 g K_2HPO_4 , 0.50 g $(NH_4)_2HPO_4$, 1.00 g NaCl, 0.50 g $MgSO_4 \cdot 7 H_2O$, 0.01 g $FeSO_4 \cdot 7 H_2O$, 0.10 g $CaCl_2$ and 8.50 g $NaHCO_3$, added with distilled water. On the incubation day, the bottles were placed in water bath at 38°C. The bottles had the same position in the water bath for each incubation since the samples were randomly allocated to the bottles. Approx. 60 min before the start of incubations, the $NaHCO_3$ was added to the buffer solution to achieve a desired pH level and buffer capacity. The buffer was kept in water bath at 38°C with magnetic stirrer and gassed with CO_2 . Rumen fluid was obtained from two ruminally cannulated non-lactating cows of the breed Swedish red and white. This was except for the second batch, where only one cow was available for rumen fluid collection. The cows were at maintenance feeding level and the rumen fluid was obtained approx. two hours after morning feeding. The rumen fluid was poured into prewarmed insulated flasks. Transported to the laboratory and strained through a screen with approx. 1mm openings into a 3 L beaker under constant gassing. The strained rumen fluid was placed in water bath at 38°C with magnetic stirring and gassed with CO_2 . The bottles with samples were gassed with CO_2 to create an anaerobe environment while filling with buffer and rumen fluid. The buffer was kept on magnetic stirring during dispensing 300mL of buffer to each bottle. Thereafter, 100mL of rumen fluid were dispensed to each bottle, still with magnetic stirring and

in water bath. Directly after rumen fluid was added, the bottles were sealed one by one, and the measurement was activating immediately for each bottle. Bottle were equipped with inner stirring devices which were set at 80 revolutions per minute, 60 sec mixers on time and 30 sec mixers off time, with change of direction for every stirring period.

3.2.2 Gas Endeavour incubation

For each batch, gas production was recorded continuously during 72 hours. The Gas Endeavour (Fig. 4) measures the gas flow from each bottle by collecting the gas in a flow cell by water displacement (Fig. 5) (Liu *et al.*, 2018). For every 2 mL of gas produced, the cell opens to release the gas, which is registered in the system. The ambient temperature and pressure are registered for every opening to obtain normalised reading values (0°C, 1 atmosphere and zero moisture content). Thereby, the gas volume are regisered the unit Nml, where N stands for “Norm”. After 72 hours, the registration was stopped and the motor controller for the stirring devices was turned off. Then the recordings were downloaded, with 1 min resolution, to the computer. Immediately after that, 8 mL fluid from each bottle was collected with plastic pipettes into small cap tubes to measure the final pH of each bottle.



Figure 4. The Gas Endeavour with 500 mL bottles and automatic stirring devices in thermostatic water bath, connected with flow cell units.

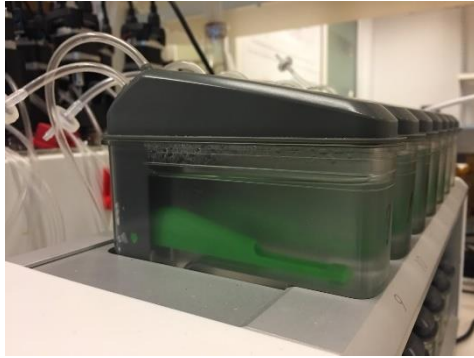


Figure 5. Flow cell unit with 2 mL measurement resolution.

3.2.3 ND solution treatment and NDF residues

Neutral detergent solution (ND solution) was prepared according to Van Soest *et al.* (1991). The residues after 72 h fermentation were collected in filter bags (one bag for each bottle) by rinsing with distilled water. The fabric of the filter bags had pore size of 12 μm and 6 % open surface area (100% polyester, SAATI, Milano, Italy). The bags were sealed and gently knead to squeeze out liquid and save the remaining residue. The residues was treated with ND solution to remove components as starch, lipids, easily digested protein, sugars and pectin from the sample, leaving exclusively NDF (Mertens, 2002). For the first batch, all bags were placed in a 3 L glass vessel and 1650 mL ND solution was added (110 mL ND solution per filter bag). The filter bags with residues were boiled in the ND solution for one hour on a hot-plate. After boiling, the bags were taken out the vessel for washing with hot water. At this stage, it was discovered that the glue that keep the bags sealed were melted and all bags were glued together. After repeated washing with 95 °C hot water, the glue softened, and the bags were able to separate. However, some bags received minor damage with small holes, but no apparent amount of content seemed to leak out. The bags were glued again to preventing any leakage before the second batch. Unfortunately, during collection of fermented residues after the second incubation, small remaining damages of a few bags was detected, and low amounts of forage particle was wasted. This was adjusted by sealing the holes with small cable ties before the ND solution-treatment. To avoid the bags from sticking together, the next batches (2, 3 and 4) were treated with ND solution in separate tubes, added with 150 mL ND solution, and the temperature were lowered according to the protocol of Chai & Udén (1998). Instead of boiling the bags in ND solution, the tubes with filter bags were incubated in a heat cabinet set at 85°C for 22 hours. Furthermore, to ensure that the bags did not leak content, new bags with a more heat-resistant glue were used in the third and fourth batches. After the third incubation, the fermented

residues had to be collected in plastic bags and placed in the freezer, because the new filter bags with the new glue did not get enough time to hardened sufficient when the incubation was finished. Two days later, the frozen residues were taken out of the freezer to thaw and could thereafter be collected in filter bags and treated with ND solution. The transfer of residues from plastic bags into filter bags was not optimal, since pouring from plastic bags was unstable and some residues were wasted during this procedure as well.

After the heat incubation, the bags were repeatedly washed with hot water until the foam from the ND solution were washed out. Thereafter, the bags were dried in 45 °C drying cabinet for 23 hours. The dried bags with remaining sample were taken out of the drying cabinet to cool down before weighing. The weights for each bag were recorded, the bags were emptied by vacuum cleaner and weighed again to estimate the amount of NDF residue for each sample.

3.3 Analysis of chemical composition of timothy and red clover

Timothy and red clover samples used for the *in vitro* incubation were analysed for dry matter content and chemical composition. Forage sample were milled through a 1-mm screen using a Kamasa hammer mill and dried in oven at 100°C for dry matter analysis and were analysed for ash content by incineration at 500°C (Chai & Udén, 1998). A fully automated Kjeldahl method (Kjeltec 1030, Tecator, Höganäs, Sweden) were used for crude protein analysis. Determination of NDF content in the silage was performed according to Van Soest *et al.* (1991) standard method, Procedure A.

Fermentation products (acids, ethanol and 2,3-butanediol) were determined using a gas chromatography (HP 5880A Series, Hewlett Packard, Avondale, PA) (Murphy *et al.*, 2000). Values of pH were measured for the silage and the NH₄-N concentration was determined using flow injection analysis (Fiastar 5010, Tecator, Sweden).

3.4 Statistical analysis and calculations

The results presented are corrected values regarding the gas production from the blank bottle (incubated with only rumen fluid and VOS buffer). Consequently, all results from gas production and NDF residues are subtracted with the results from the blank bottle from respective incubation. Data analysed were gas production after 6, 12, 24, 48 and 72 hours of incubation, residual NDF amount and endpoint pH.

Crop, treatment and harvest were considered as fixed effects and statistical significance was declared for $P < 0.05$. All data was first analysed for the main effects of crop, harvest and treatment with the interactions crop \times harvest, harvest \times treatment and crop \times treatment. Data was then split into separate datasets for timothy and red clover, respectively which were analysed for effects of treatment, harvest and harvest \times treatment. Results are presented as least square means with probability values for the different effects. Statistical analysis was performed using Proc GLM in SAS (SAS, 2018; version 9.4).

Gas volume recordings were subjected to curve fitting to estimate fermentation rate with the MS Excel Solver tool, using a model commonly applied to *in sacco* data (Oerskov & McDonald, 1979):

$$p = a + b[1 - \exp(-ct)]$$

Where p is estimated gas volume at time t , a is estimated volume at time 0, b is asymptotic gas volume at infinite time and c is fermentation rate. Treatment means of data recorded at 0, 2, 4, 8, 16, 24, 48 and 72h were used.

4 Results

The blank samples in each batch showed a relatively low gas production of <35.2 mL, except from the second batch where the blank sample produced 141.1 mL gas in total. The volumes of gas produced from the high and low internal standards (sugar and straw) are illustrated in Fig. 6, corrected by deducting the produced gas of the blank samples from respectively batch. The gas production from the high internal standard showed the highest volumes, 897.9 – 976.3 NmL, compared with all other samples, timothy, red clover and straw. The gas production from the low internal standard was considerably lower, 601.4 – 671.5 NmL. The difference in total gas produced within the samples are 78.4 NmL for sugar and 70.1 NmL for straw, which may be regarded as minor differences and the study can be considered as repeatable.

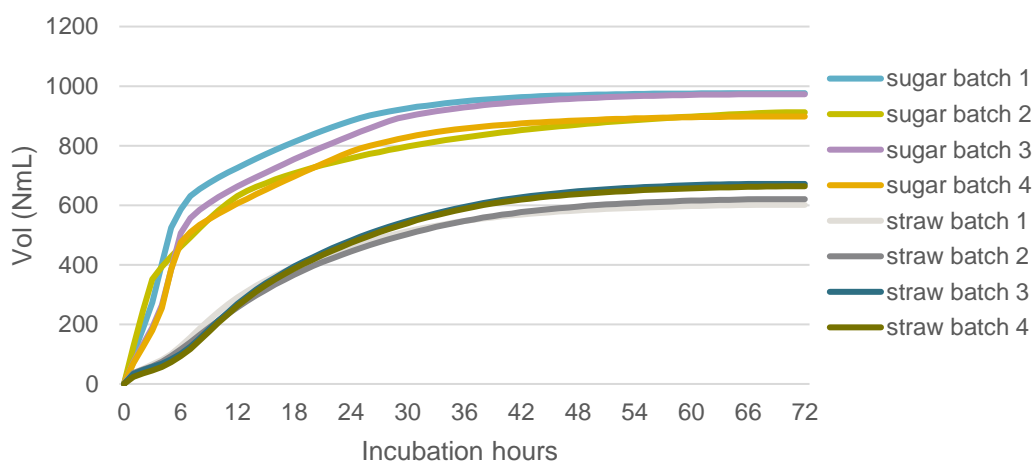


Figure 6. Volumes of gas (NmL) produced from the high standard (sugar) and low standard (straw) during 72 h incubation.

4.1 Chemical composition of timothy and red clover

Dry matter and chemical composition of timothy and red clover samples used for the *in vitro* incubation were analysed and are presented in Table 4. Analysis of VFA, lactic acid, ethanol, 2,3-butanediol, NH₄-N and pH were performed, and values are present in Table 5.

Table 4. Dry matter (DM) and chemical composition for timothy and red clover samples from two different cutting times.

Chemical composition	Timothy				Red clover			
	Early		Late		Early		Late	
	P ¹	C ²	P ¹	C ²	P ¹	C ²	P ¹	C ²
DM ³ , %	37.1	35.0	39.3	39.7	35.7	33.6	34.3	34.6
aNDFom ⁴ , g/kg DM	550	532	539	534	327	324	395	382
CP, g/kg DM	141	156	121	115	183	188	153	162
Ash, g/kg DM	74.7	79.9	71.5	72.0	92.6	92.1	79.4	79.4

¹ P, processed.

² C, control.

³ DM, dry matter content.

⁴ aNDFom, NDF assayed with a heat stable amylase and sodium sulphite, expressed exclusive of residual ash.

Table 5. Content of fermentation products, NH₄-N and pH in timothy and red clover samples from two different harvest times.

Products	Timothy		Red clover	
	Early	Late	Early	Late
Lactic acid, g/kg DM	4.30	2.46	1.84	7.36
Acetic acid, g/kg DM	0.71	0.28	0.69	1.29
Propionic acid, g/kg DM	0.57	1.21	0.99	0.62
Butyric acid, g/kg DM	<0.05	<0.04	<0.05	<0.05
Ethanol, g/kg DM	1.75	2.30	0.79	0.62
Formic acid, g/kg DM	<0.05	<0.04	<0.05	<0.05
2,3-Butanediol, g/kg DM	<0.05	0.48	0.69	0.57
NH ₄ -N ² , % NH ₄ -N/tot N	6.41	9.62	3.84	4.31
pH	4.78	5.51	5.25	4.30

¹ VFA, volatile fatty acid.

² NH₄-N, ammonium-nitrogen.

4.2 Gas production from timothy

Mean values of the gas production after 6, 12, 24, 48 and 72 hours incubation are presented for timothy in Table 6 and illustrated in Fig. 7. The processed timothy had a significantly higher gas production compared to control timothy at both early and late harvest and for all incubation times analysed. The early harvested timothy had significantly higher gas production compared to late harvest timothy for 12, 24 and 48 hours of incubation. TLP had the highest gas production after the first 6 hours incubation and TEC had the lowest gas production at the same incubation time. The mean difference between control and processed timothy after 6 hours incubation was 92.6 mL gas. At incubation endpoint, the highest gas volume was produced from TEP and the lowest from TLC. The mean gas production at 72 hours was 65.4 mL larger for processed timothy than for timothy controls.

Table 6. The volume of gas (NmL) produced from timothy after 6, 12, 24, 48 and 72 hours incubation.

Incuba- tion time (h)	Timothy				SEM ³	p-value		
	Early		Late			Harvest	Treatment	Harvest × Treatment
	P ¹	C ²	P ¹	C ²				
6	295.8	200.7	299.8	209.7	7.45	0.3942	<0.0001*	0.7422
12	489.7	338.3	444.4	330.2	11.38	0.0291*	<0.0001*	0.1170
24	634.3	542.0	591.0	507.7	15.16	0.0187*	<0.0001*	0.7672
48	715.5	652.6	683.4	610.9	16.31	0.0350*	0.0005*	0.7700
72	728.0	669.2	703.7	631.7	16.11	0.0695	0.0006*	0.6864

¹P, processed.

²C, control.

³SEM, Standard Errors of the Means.

*P-values are significant (< 0.05).

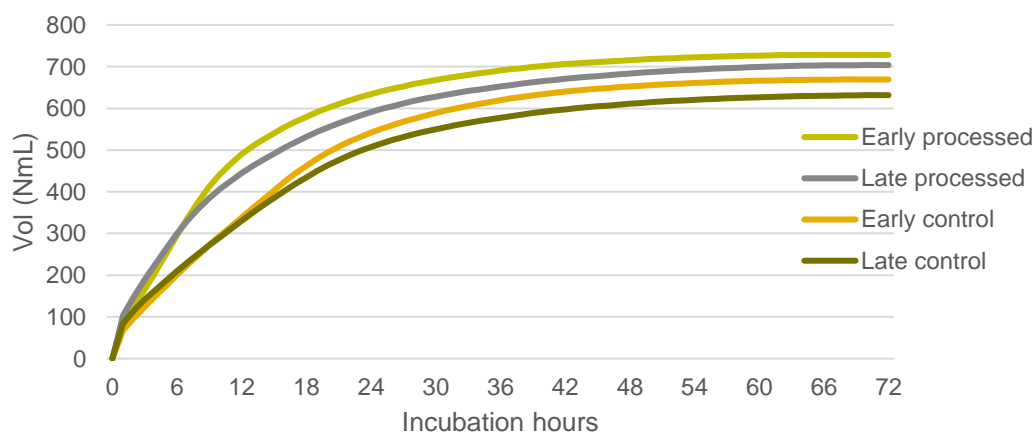


Figure 7. Volume of gas (NmL) produced from timothy during 72 h incubation.

4.3 Gas production from red clover

Mean values of the gas production after 6, 12, 24, 48 and 72 hours incubation time are presented for red clover in Table 7 and illustrated in Fig. 8. The results of the gas production from red clover were similar to the timothy results. The processed red clover had a significant higher gas production compared to control red clover in both early and late harvest and for all incubation times analysed. The early harvest red clover had significantly higher gas production for 24, 48 and 72 hours incubation time, compared to late harvest red clover. REP had produced the highest gas volume after 6 hours incubation and RLC had produced the lowest gas volume at the same time. The mean difference between processed and control red clover after 6 hours incubation was 64.45 mL. At incubation endpoint, REP still had the highest gas production and RLC had the lowest gas production. The mean gas production at 72 hours was 33.85 mL larger for processed timothy than for timothy controls.

Table 7. The volume of gas (NmL) produced from red clover after 6, 12, 24, 48 and 72 hours incubation time.

Incuba- tion time (h)	Red clover				SEM ³	p-value		
	Early		Late			Effects of		Harvest × Treatment
	P ¹	C ²	P ¹	C ²		Harvest	Treatment	
6	261.5	199.7	254.0	186.9	6.80	0.1514	<0.0001*	0.7017
12	522.2	351.1	492.4	344.8	8.78	0.0534	<0.0001*	0.1945
24	659.7	590.6	619.9	563.7	13.97	0.0269*	0.0002*	0.6472
48	715.0	680.6	669.4	633.4	14.46	0.0044*	0.0242*	0.9573
72	721.7	688.6	675.4	640.8	154.89	0.0050*	0.0342*	0.9625

¹ P, processed.

² C, control.

³ SEM, Standard Errors of the Mean.

*P-values are significant (< 0.05).

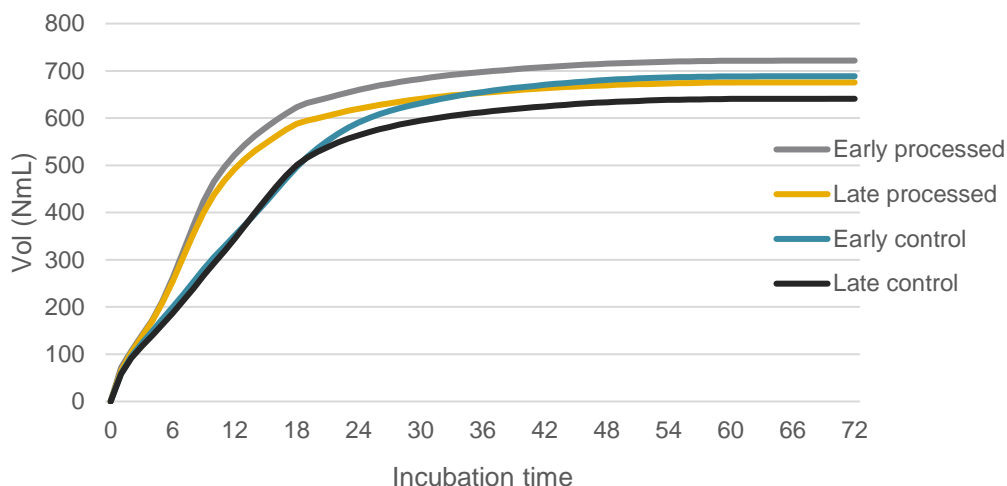


Figure 8. Volume of gas (NmL) produced from red clover during 72 h incubation.

4.4 Effects and interactions for timothy and red clover

Effects and interactions between crop, harvest and treatment after 6, 12, 24, 48 and 72 hours incubation is shown in Table 8. The treatment had significant effect on the gas production for all incubation time analysed (6-72 h). The crop had significant effect on the gas production after 6, 12 and 24 hours incubation and the harvest occasion showed significance after 12, 24, 48 and 72 hours incubation. Significant interactions only occurred between crop and harvest at 24 hours incubation, and between crop and treatment at 6 hours incubation. No significant interactions occurred between harvest and treatment.

The effect of crop, harvest and treatment showed a significant effect of the NDF residues. As well did the interactions between crop and harvest, while no significant interactions were shown between harvest and treatment or treatment and crop.

Table 8. P-values of the interactions between crop, harvest and treatment at 6, 12, 24, 48 and 72 hours of incubation and for the NDF residues after 72 h incubation.

Incubation time (h)	p-value			p-value		
	Effects of			Effects of		
	Crop	Harvest	Treatment	Crop × Harvest	Harvest × Treatment	Crop × Treatment
6	<0.0001*	0.7186	<0.0001*	0.1070	0.9875	0.0079*
12	0.0006*	0.0034*	<0.0001*	0.5475	0.5952	0.1440
24	0.0004*	0.0012*	<0.0001*	0.0405*	0.2294	0.4672
48	0.4121	0.0005*	<0.0001*	0.0722	0.6653	0.7408
72	0.8893	0.0010*	<0.0001*	0.7932	0.7980	0.1577
NDF residues	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.0968	0.0635

*P-values are significant (< 0.05).

4.5 pH-values

Values of pH for the fluid from each sample after the incubation are presented in Table 9. For both timothy and red clover, no significant difference was shown between the pH value from processed and control samples ($P > 0.17$). Likewise, no significant difference was shown between early and late harvest for either timothy or red clover ($P > 0.13$). The mean pH value from the high internal standard (sugar) was 6.42. Hence, the high internal standard had a significantly lower pH value ($P < 0.01$) compared with timothy and red clover, both processed and control samples.

Table 9. pH values in the timothy and red clover samples after the incubation.

Crop	Early		Late	
	P ¹	C ²	P ¹	C ²
Timothy	6.61	6.69	6.65	6.70
Red clover	6.87	6.87	6.84	6.85

¹ P, processed.

² C, control.

4.6 NDF residues

The mean values of NDF residues from each sample after 72 hours incubation and ND-treatment is shown in Table 10. The NDF residues showed a significant difference between harvest occasions for both timothy and red clover. For timothy, there was a significant effect of the treatment on the NDF residues. For both timothy and red clover, there was a significant interaction between harvest and treatment.

As showed in Fig. 9, there is a significant difference between processed and control timothy at late harvest, but not in early harvest. For red clover, a significant difference was showed between the treatment for early harvest but not for late harvest.

Table 10. Mean values of NDF residues (g) from timothy and red clover after 72 hours incubation and ND solution-treatment.

Crop	Early		Late		SEM ³	p-value, effects of		
	P ¹	C ²	P ¹	C ²		Harvest	Treatment	Harvest × Treatment
Timothy	0.783	0.777	0.843	1.008	0.017	<0.0001*	<0.0002*	<0.0001*
Red clover	0.535	0.613	0.833	0.822	0.017	<0.0001*	0.0591	0.0137*

¹ P, processed.

² C, control.

³ SEM, Standard Errors of the Mean.

*P-values are significant (< 0.05).

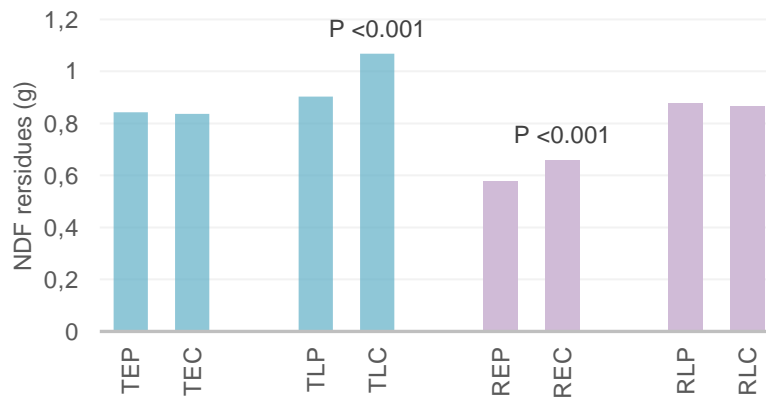


Figure 9. The amount of NDF residues (g) for timothy and red clover. Significant differences between processed silage and controls are displayed with P values.

4.7 Fermentation rate

Results of the estimations with curve fitting describes the fermentation rate per hour incubation for timothy and red clover, shown in Table 11. The processed silage showed a higher fermentation rate per hour for both timothy and red clover in both early and late harvest compared to control silage.

Table 11. Fermentation rate per hour described in percent for timothy and red clover during *in vitro* incubation.

Crop	Processed	Control
Timothy early	8.5 %	6.2 %
Timothy late	9.0 %	6.2 %
Red clover early	8.9 %	6.3 %
Red clover late	9.2 %	6.8 %

5 Discussion

5.1 Results of gas production and NDF residues

Physically processed silage resulted in a higher rate of gas produced compared with control silage for both timothy and red clover. A more rapid gas production during *in vitro* incubation is associated with a higher digestion rate (Liu *et al.*, 2018). An increased digestion rate using intensive physically processed forage agreed with previous studies (Weisbjerg *et al.*, 2018; Broderick *et al.*, 1999; Hong *et al.*, 1988). The highest digestion rate of the forage occurred during the first 24 hours of the incubation, in agreement with the results of Hetta *et al.* (2004). The rapid gas production are mainly due to degradation of readily fermentable carbohydrates in the forage since structural carbohydrates are more slowly digested (Van Soest, 1994; Van Soest *et al.*, 1991). However, physically treatment of the silage showed an increased fermentation rate compared to control silage, which most likely are related with an increased fermentation rate of structural carbohydrate. The higher fermentation rate per hour in processed silage, estimated with curve fitting, are supporting the results of higher digestion rate for processed silage compared to control silage.

Processed silage showed significantly lower NDF residues compared to control silage in both timothy (late harvest) and red clover (early harvest), which indicates a higher NDF digestibility. However, the processed silage also showed higher NDF residues for early harvest timothy (0.007 g) and late harvest red clover (0.012 g) compared to control silage. One possible explanation for this difference in NDF residues between harvest and crop is errors in the method after the incubation. When bottles with residues from the incubation were emptied and the residues were collected in filter bags, there is a risk that minor residues remained in the bottles which may affect the results of NDF residues. During the third batch this error could have an even higher effect since the fermented residues also were collected in plastic bags before pouring over into filter bags. Residues could have remained in both bottles

and plastic bags and some content were wasted during the transfer from plastic bags to filter bags. In addition, there were some errors during the ND-treatment. Damages of the filter bags after the first ND-treatment caused leakages of residues both for batch 1 and 2.

The total errors in the performed method probably affected the results of NDF residues. The amount of wasted residues and from which sample it was wasted is unfortunately unknown. Hence, the results of NDF residues could be considered, at least, as questionable.

5.2 Chemical composition and harvest occasion

The physical treatment showed a small effect on the chemical composition of the silage (Table 9). The processed silage showed a higher NDF content (a difference between 2.5 – 18.0 g/kg DM) compared to the control silage for both timothy and red clover. The CP content was lower for the processed silage (a difference between 5.13 – 15.35 g/kg DM) compared to control silage, except from TLP which had 5.78 g/kg DM higher CP content compared to TLC. The results of a higher NDF content in processed silage agrees with previous studies (Damborg *et al.*, 2018; Broderick *et al.*, 1999; Hong *et al.*, 1988). One discussed explanation of the difference in NDF and CP content are a greater leaf loss during the forage treatment. (Broderick *et al.*, 1999). Resulting in decreased proportion of CP and increased proportion of NDF in the forage.

The harvest occasion had significant effects on the gas production at several incubation times measured for both timothy and red clover (Table 6). Hetta *et al.* (2004) showed that different harvest occasions had substantial impact on the NDF content and digestibility in timothy and red clover. Optimal harvest time occurred at early harvest for red clover compared with timothy where optimal harvest time occurred at late harvest, this consequently complicates the opportunity for an optimal harvest time in mixed leys (Hetta *et al.*, 2004). Present study indicates opportunities to increase or transform the harvest window due to an increased digestion rate of the forage with intensely treatment. This may enable a harvest later in the season which contributes to a longer growth period and an increased amount of green mass harvested. Additionally, physically treatment of the late harvested forage enables an increased digestion rate.

According to Spörndly (2003), the pH value of the silage should be < 4.78, 4.73, 4.57 and 4.55 for TEC, TLC, REC and RLC respectively ($< (0.0257 \times \text{DM } \%) + 3.71$) to obtain a sufficient storage stability. Unfortunately, the silage in the present study had a pH value above this recommendation (4.78 – 5.51), except from late harvested red clover which had lower pH value (4.30) than the recommendations.

However, there was no indications of poor quality of the silage when opening the silos (smelly, mould, etc), but the quality might have decreased during time.

5.3 Passage rate and digestibility

An increased passage rate enables a higher dry matter intake (Van Soest *et al.*, 1991). However, an increased passage rate of the forage also requires a high digestibility. Otherwise, the microbes will not have enough time to digest the feed which will result in an increased passage rate and feed intake, but no increased nutrition absorption (Broderick *et al.*, 1999). Intensive physical treatment of forage had an effect on the digestibility shown by an increased digestion rate but no decreased passage rate (Weisbjerg *et al.*, 2018). According to the present study, the high gas production rate from processed silage indicates a more rapid digestion. Probably, this result occurred due to damages of the forage fibre caused by the treatment which makes the structural carbohydrates more easily accessible for the rumen microbes (Hong *et al.*, 1988).

5.4 Physical treatment of forage before or after the ensiling

The forage in this experiment was physically treated after ensiling. It is possible to perform physical treatment of forage before or after the ensiling and the outcome will probably be affected differently. Broderick *et al.* (1999) indicated that intensive physical treatment of the forage before ensiling might contribute to a more rapid pH drop and a more effective ensiling process. A rapid pH drop is advantageous considering the hygienic quality of the silage. Additionally, this method could be especially advantageous in case of shortage of forage. In such cases, a more rapid ensiling process makes the silage available sooner after harvest which might be crucial for example in seasons with previously low harvest. Intensive physical treatment of the forage before ensiling can also improve the compression of the crop in the silo which may enable a larger amount of silage in each silo. As processing the forage before ensiling contributes to several advantages, processing the forage after the ensiling, as in the present study, could be questioned.

5.5 VFA production and ruminal pH

Studies has showed that a reduced particle size of forage can increase the VFA production in the rumen, which results in increased availability of metabolizable energy (Kononoff & Heinrichs, 2003; Van Soest *et al.*, 1991). However, the proportion of

VFA produced from processed and control silage during *in vitro* incubations would be of interest since various VFA produce different amount of gas (Patel, 2012). In case of differences between the proportion of VFA produced during incubation, it may affect the results of gas produced. Therefore, in case of a repeated study, analysing the proportion of VFA during the incubation would be of interest.

Kononoff & Heinrichs (2003) also showed reduced ruminal pH when feeding forage with reduced particle size. In the present study, all samples incubated had a pH value >6.6, which would indicate a good ruminal environment (Mertens, 1997). However, the pH level in the incubation are affected by the composition and proportion of VOS buffer used and the amount of substrate (silage) incubated. Hence, the pH level in this experiment indicates that the buffer had enough capacity to prevent the pH level to decrease below 6.6, suggesting that the fibre digestion was not limited by the pH level.

5.6 Dry matter content

A mean value of the dry matter was estimated before incubation of early and late harvested timothy and red clover. Dry matter analysis was then performed on the actual forage samples that were used for each incubation. There were differences in g of DM among the samples incubated for the different treatments. On DM basis, TEP had the highest amount (4.24 ± 0.01 g DM) and REC had the lowest amount (3.54 ± 0.06 g DM). The difference between the highest and lowest amount of DM was 0.37 g for timothy and 0.24 g for red clover. This difference could affect the results of gas produced, as well as the NDF residues. The NDF residues from TEP and TEC showed almost the same result, but the sampled DM amount was 0.24 g higher for TEP which could explain the unexpected high amount NDF residues from TEP. However, that theory is not suitable for the results of almost no differences in NDF residues for RLP and RLC since the DM amount for RLP and RLC only differed with 0.03 g.

5.7 Future investigations

For future resemble studies, it would be of interest to treat the forage sample with ND solution before the Gas Endeavour incubation to evaluate the gas production and digestibility of NDF exclusively. This would expose the effect of treatment on NDF digestibility and exclude influences of gas production from easily digested carbohydrates. For future animal studies, effects on forage intake and milk production of dairy cows fed intensively physically treated forage needs to be investigated.

Economical calculations for the cost of the treatment are crucial to estimate if there is profitability in the treatment. The amount of concentrate that can be replaced with processed silage needs to be investigated to calculate the savings from a reduced use of concentrate. In addition, how the intensive physical treatment of the forage can be conducted on farm level have a large impact on the farmers interest of the treatment. Other difficulties for the farmer are to increase the forage production in case of limited cropland available. One suggested solution is a collaboration between dairy farmer and cereal farmer. Forage production on cereal farms contributes to a varied crop rotation which benefits the soil structure and gives potential to an increased yield of harvest in future crop production (Fogelfors, 2015).

5.8 Conclusion

Processed timothy and red clover showed a significant higher gas production rate compared to control unprocessed samples. A high gas production rate indicates a more rapid digestion rate and consequently an expected increased forage fermentation in the rumen. The NDF residues after the incubation showed significant higher amount (g) for the control samples compared to the processed in late harvest timothy and early harvest red clover. This indicates a higher NDF digestibility for processed silage compared to control silage. However, there was minor difference between processed and control samples for early harvest timothy and late harvest red clover in NDF residues. Several problems that occurred in the analytical procedure may explain the results of NDF residues.

According to the results from the gas production, there are possibilities to increase the forage digestibility by intensive physical treatment. However, an increased forage digestibility will not necessary contribute to an increased forage intake. Therefore, further research is needed to investigate if intensive physically treated forage can increase the forage intake, and therefore nutrient supply, to sustain high milk productions. In that case, it would be of interest to investigate whether there is any economic benefit in replacing concentrates by intensive physically treated forage in dairy cows feed ration.

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6 References

- Beauchemin, K.A., Yang, W.Z. & Rode, L.M. (2003). Effects of particle size of alfalfa-based dairy cow diets on chewing activity, ruminal fermentation, and milk production. *Journal of Dairy Science*, 86(2), ss. 630-643.
- Broderick, G.A., Koegel, R.G., Mauries, M.J.C., Schneeberger, E. & Kraus, T.J. (1999). Effect of Feeding Macerated Alfalfa Silage on Nutrient Digestibility and Milk Yield in Lactating Dairy Cows¹. *Journal of Dairy Science*, 82(11), ss. 2472-2485.
- Broderick, G.A., Koegel, R.G., Walgenbach, R.P. & Kraus, T.J. (2002). Ryegrass or Alfalfa Silage as the Dietary Forage for Lactating Dairy Cows¹. *Journal of Dairy Science*, 85(7), ss. 1894-1901.
- Bruins, M.E. & Sanders, J. (2012). Small-scale processing of biomass for biorefinery. *Biofuels Bioproducts & Biorefining-Biofpr*, 6(2), ss. 135-145.
- Buxton, D. & Redfearn, D.D. (1997). Plant limitations to fiber digestion and utilization. *Journal Of Nutrition*, 127, ss. S814-S818.
- Chai, W. & Udén, P. (1998). An alternative oven method combined with different detergent strengths in the analysis of neutral detergent fibre. *Animal Feed Science and Technology*, 74(4), ss. 281-288.
- Chamberlain, A.T. (1996). *Feeding the dairy cow*. Welton: Chalcombe.
- Chaves, A.V., Burke, J.L., Waghorn, G.C. & Brookes, I.M. (2006). Digestion kinetics of leaf, stem and inflorescence from five species of mature grasses. *Journal of the Science of Food and Agriculture*, 86(5), ss. 816-825.
- Damborg, V.K., Stødkilde, L., Jensen, S.K. & Weisbjerg, M.R. (2018). Protein value and degradation characteristics of pulp fibre fractions from screw pressed grass, clover, and lucerne. *Animal Feed Science and Technology*, 244, ss. 93-103.
- DeVries, T.J., Dohme, F. & Beauchemin, K.A. (2008). Repeated ruminal acidosis challenges in lactating dairy cows at high and low risk for developing acidosis: Feed sorting. *Journal of Dairy Science*, 91(10), ss. 3958-3967.
- Emanuelson, M., Cederberg, C., Bertilsson, J. & Rietz, H. (2006). *Närodlat foder till mjölkkor – en kunskapsuppdatering*. Svensk Mjölk, Forskning.
- Fogelfors, H. (2015). *Vår mat: odling av åker- och trädgårdsgrödor: biologi, förutsättningar och historia* Lund: Studentlitteratur.
- Frame, J. & Laidlaw, A.S. (2011). *Improved grassland management New Edition*. Ramsbury: Crowood.
- Guo, T., Sanchez, M.D. & Guo, P. (2002). Animal production based on crop residues. Chinese experiences. *Animal production based on crop residues. Chinese experiences*(149).
- Hetta, M., Gustavsson, A.-M., Cone, J.W. & Martinsson, K. (2004). In vitro degradation characteristics of Timothy and Red Clover at different harvest times. *Acta Agriculturae Scandinavica, Section A – Animal Science*, 54(1), ss. 20-29.
- Hong, B.J., Broderick, G.A., Panciera, M.P., Koegel, R.G. & Shinnors, K.J. (1988). Effect of Shredding Alfalfa Stems on Fiber Digestion Determined by In Vitro Procedures and Scanning Electron Microscopy¹. *Journal of Dairy Science*, 71(6), ss. 1536-1545.

- Kononoff, P.J. & Heinrichs, A.J. (2003). The Effect of Reducing Alfalfa Haylage Particle Size on Cows in Early Lactation. *Journal of Dairy Science*, 86(4), ss. 1445-1457.
- Krause, K. & Combs, D. (2003). Effects of forage particle size, forage source, and grain fermentability on performance and ruminal pH in midlactation cows. *Journal of Dairy Science*, 86(4), ss. 1382-1397.
- Krause, K.M., Combs, D.K. & Beauchemin, K.A. (2002). Effects of Forage Particle Size and Grain Fermentability in Midlactation Cows. II. Ruminal pH and Chewing Activity. *Journal of Dairy Science*, 85(8), ss. 1947-1957.
- Lamsal, B., Yoo, J., Brijwani, K. & Alavi, S. (2010). Extrusion as a thermo-mechanical pre-treatment for lignocellulosic ethanol. *Biomass and Bioenergy*, 34(12).
- Lehmann (2018). Available: <http://www.lehmann-maschinenbau.de/en/biogas-technology/bio-extrusion.html> [2018-10-30].
- Liu, J., Gorp, J.R.v. & Nistor, M. (2018). The new gas endeavour system from bioprocess control AB for in vitro assessment of animal feeds. I: Udén, P., Eriksson, T., Spörndly, R., Rustas, B.O. & Liljeholm, M. (red.) *Proceedings of the 9th Nordic Feed Science Conference*. Uppsala, Sweden.
- Márquez, A., Mendoza, G., Pinos-Rodríguez, J.M., Zavaleta, H., González, S., Buntinx, S., Loera, O. & Meneses, M. (2009). Effect of fibrolytic enzymes and incubation pH on in vitro degradation of NDF extracts of alfalfa and orchardgrass. *Italian Journal of Animal Science*, 8(2), ss. 221-230.
- Mertens, D.R. (1997). Creating a System for Meeting the Fiber Requirements of Dairy Cows. *Journal of Dairy Science*, 80(7), ss. 1463-1481.
- Mertens, D.R. (2002). Gravimetric Determination of Amylase-Treated Neutral Detergent Fiber in Feeds with Refluxing in Beakers or Crucibles: Collaborative Study. *Journal of AOAC International*, 85(6), ss. 1217-1240.
- Miljomal.se (2017). Available: <https://www.miljomal.se/Miljomalen/#> [2018-10-08].
- Murphy, M., Åkerlind, M. & Holtenius, K. (2000). Rumen Fermentation in Lactating Cows Selected for Milk Fat Content Fed Two Forage to Concentrate Ratios with Hay or Silage. *Journal of Dairy Science*, 83(4), ss. 756-764.
- Nasrollahi, S.M., Imani, M. & Zebeli, Q. (2015). A meta-analysis and meta-regression of the effect of forage particle size, level, source, and preservation method on feed intake, nutrient digestibility, and performance in dairy cows. *Journal of Dairy Science*, 98(12), ss. 8926-8939.
- Oerskov, E.R. & McDonald, I. (1979). The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage [sheep]. *Journal of Agricultural Science*(2), ss. 499-503.
- Patel, M. (2012). *Effects of increasing the proportion of high-quality grass silage in the diet of dairy cows*. Diss. Uppsala: Sveriges lantbruksuniversitet.
- Pinares-Patio, C.S., Ulyatt, M.J., Lassey, K.R., Barry, T.N. & Holmes, C.W. (2003). Rumen function and digestion parameters associated with differences between sheep in methane emissions when fed chaffed lucerne hay. *The Journal of Agricultural Science*, 140(2), ss. 205-214.
- Rinne, M. & Nykänen, A. (2008). Timing of primary growth harvest affects the yield and nutritive value of timothy-red clover mixtures. *Agricultural and Food Science*, 9(2).
- Rodriguez, C., Alaswad, A., Benyounis, K.Y. & Olabi, A.G. (2017). Pretreatment techniques used in biogas production from grass. *Renewable and Sustainable Energy Reviews*, 68, ss. 1193-1204.
- SMHI (2015). *Klimatologo Nr 20*. Available: https://data.smhi.se/met/scenariodata/rcp/lansanalyser/rappporter_kartor/03_Uppsala/Rapport/Framtidsklimat_i_Uppsala_L%C3%A4n_Klimatologi_nr_20.pdf [2018-10-30].
- Spörndly, R. (2003). *Fodertabeller för idisslare 2003*. [6. uppl.] Uppsala: Sveriges lantbruksuniv.
- Van Soest, P.J. (1994). *Nutritional ecology of the ruminant Second Edition*. Ithaca: Comstock.
- Van Soest, P.J., Robertson, J.B. & Lewis, B.A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, 74(10), ss. 3583-3597.
- Weisbjerg, M.R., Waldemar, P., Hellwing, A.L.F. & Kristensen, T. (2018). Can we increase digestibility of green forages by physical treatment before ensiling? I: Udén, P., Eriksson,

- T., Spörndly, R., Rustas, B.O. & Liljeholm, M. (red.) *Proceedings of the 9th Nordic Feed Science Conference*. Uppsala, Sweden.
- Volden (2011). *NorFor The Nordic feed evaluation system*: Wageningen Academic Publishers.
- Woodward, S.L., Waghorn, G.C. & Laboyrie, P.G. (2004). Condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) reduce methane emissions from dairy cows. *Proceedings of the New Zealand Society of Animal Production*. Hamilton: New Zealand Society of Animal Production, ss. 160-164.
- Woodward, S.L., Waghorn, G.C., Ulyatt, M.J. & Lassey, K.R. (2001). Early indications that feeding Lotus will reduce methane emissions from ruminants. *Proceedings of the New Zealand Society of Animal Production*. Christchurch: New Zealand Society of Animal Production, ss. 23-26.
- Yang, W.Z., Beauchemin, K.A. & Rode, L.M. (2001). Effects of Grain Processing, Forage to Concentrate Ratio, and Forage Particle Size on Rumen pH and Digestion by Dairy Cows¹. *Journal of Dairy Science*, 84(10), ss. 2203-2216.