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In vitro fermentation and In sacco degradation of the alkaline compound feeds in dairy cow diets

In vitro fermentering og in sacco nedbrytning av alkalisk kraftfôr i rasjoner til melkekyr

X Muqier
Feed Manufacturing Technology

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X Muqier

Summary

The primary aim of this work was to test the *in vitro* fermentation kinetics and *in sacco* rumen degradation of different formulations of alkaline treated local grains in the dairy cattle diet, in contrast to soy-based compound feed. Furthermore, the effect of mixing these compound feeds with three different qualities of grass silages was tested. Hypothesis, and operating procedures are based on the knowledge of ruminant nutrition, physiology, and feed science as introduced in the theoretical backgrounds.

The methods used to study degradation kinetics were: 1) DM disappearance of 4 types of compound feeds (AUMD – alkaline feed with 20% Alka 150 diet containing higher proportion of local ingredients; AUNA- feed with ingredients of AUMD but urea and barely replacing the Alka 150 diet; AUAB- AUMD in a mash form; and DRER- Drøv Energirik which is a standard compound feed for dairy cows with higher level of imported ingredients) using the *in sacco* method, incubated in the rumen for different time intervals; 2) measuring gas production kinetics of 19 dietary treatments (the above four compound feeds, three different quality silages, and their mixtures in the ratio of 45:55% on DM basis, in respective order) fermented in buffered rumen fluid for 48h using the ANKOM RF gas production system.

The alkaline treatment increased the crude protein content of the diets. There were no negative effects on the *in vitro* dry matter degradation (DMD), and *in vitro* gas production (GP) profile of the different formulations of alkaline grains. Besides, there was no difference in DMD and starch digestibility among alkaline feed AUNA and AUAB compared to the DRER using *in sacco* work. The AUMD had higher DMD than DRER ($P<0.0001$). Furthermore, higher digestibility of CP and NDF were observed in the contrast group (AUMD, AUAB, AUNA) compared with the control diet DRER. In addition, the GP and rate of GP of middle cut and late cut silage mixtures increased after they mixed with alkaline grain and soy-based concentrate.

Keywords; ruminant, alkaline grain, feed evaluation, in sacco, in vitro gas production

Sammendrag

Hovedmålet med oppgaven var å undersøke nedbrytingskinetikk i ulike blandinger med alkalisk behandlet norsk korn til melkeku med hjelp av *in vitro* fermentering og *in sacco* nedbrytning, og å sammenligne dette med soyabasert fôr. I tillegg ble effekten av alkalisk behandling målt ved å blande kornblandingene med surfôr. Hypotesene og metodene er vurdert opp kunnskap om drøvtyggerernæring, -fysiologi og fôr framlagt i den teoretiske bakgrunnen.

Metodene som ble brukt for å studere nedbrytningskinetikk var (1) DM tap fra 4 fôrblandinger (AUMD- alkalisk fôr med 20% Alka 150 med høyt innhold av norsk korn; AUNA- fôr tilsvarende AUMD med Alka 150 erstattet med urea og bygg; AUAB- AUMD i mjølform; DRER- Drøv Energirik, en fôrblanding med høyt innhold av importerte råvarer) ved innkubering av nylonposer i vom, og (2) måling av gassproduksjonskinetikk av 19 fôrprøver (fire kornblandinger, tre forskjellige typer surfôr, og disse blandet 45:55% på TS basis) ved fermentering i bufret vomvæske i 48 timer med bruk av ANKOM RF gassproduksjonssystemet.

Alkalisk behandling økte innholdet av råprotein i fôrblandingene. Det var ingen negativ effekt av de forskjellige blandingene med alkalisk korn og deres blandinger på fordøyelighet av tørrstoff (DMD) og gassproduksjonsprofil (GP-profil). Det var ingen forskjell i DMD og stivelsesfordøyelighet for AUNA og AUAB sammenlignet med DRER målt *in sacco*. Fordøyelighet av tørrstoff var høyere for AUMD enn for DRER ($P < 0{,}0001$). Videre var fordøyeligheten av CP og NDF i kontrastgruppen (AUMD, AUAB, AUNA) høyere enn i kontrolldietten DRER. I tillegg økte total gassproduksjon og fraksjonell gassproduksjonshastighet for normalt og seint slått surfôr når de ble blandet med alkalisk behandlede kornblandinger.

Nøkkelord: drøvtyggere, alkalisk korn, fôrvurdering, in sacco, in vitro gassproduksjon

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List of abbreviation

ADF	Acid Detergent Fiber
CHO	Carbohydrates
CP	Crude protein
CF	Crude fiber
DM	Dry matter
DMD	Dry matter digestibility
D-value	Degradation value
EE	Ether Extract
ED	Effective degradability
EDMD	Effective dry matter digestibility
GP	Gas production
MP	Microbial Protein
ME	Metabolizable Energy
NDF	Neutral Detergent Fiber
NDFom	Ash Corrected Neutral Detergent fiber
NPN	Non-protein Nitrogen
OM	Organic Matter
OMD	Organic Matter Digestibility
VFA	Volatile fatty acid

1 Introduction

Feed is one of the major costs (approx. 70%) in animal production. Moreover, nutritional inadequacy for livestock becomes one of the global burning problems, which is caused by the increasing human population, climate change, and deteriorating arable lands, etc. Hence, to meet nutrient requirements of animals, maximize profitability, and reduce negative environmental effects, there is a need to increase the use efficiency of available feed resources. Feed quality evaluation plays an essential role in this effort. However, in ruminant feed evaluation, predicting the nutritive value of feedstuffs is important and the rate and fermentation pattern in the rumen (Hoover, 1986). The rate of digestion is influenced by the intrinsic factor such as the fibers that affecting fill value, moreover the diet composition and concentrate to roughage ratios influencing fermentation pattern, ruminal environment, thereby the digestibility, feed intake and the energy values (Hoover, 1986; Van Soest, 1994).

Currently, various techniques used to evaluate ruminal degradation of feedstuff and mixed rations. *In vivo*: the feed intake and degradability of the feedstuffs are determined by the marker technique and using fistulated animals to measure the rate and differences of nutrient flow, passing from rumen further down to other digestive tracts and in feces (Stern & Satter, 1982). However, this method is unsuitable for routine feed evaluation because it is expensive, time-consuming, and variations associated with markers and animals, etc. (Stern et al., 1997). Alternatively, feed digestion kinetics can be predicted by measurement of nutrients disappearance in porous synthetic bags, suspended in the rumen of cannulated animals (*in sacco*) (Van Vuuren et al., 1989) or determined by the volume of gas production over time of feed samples fermentation in the buffered rumen fluid (*in vitro* gas production) (Pell & Schofield, 1993). These two methods are relatively cheaper and less laborious compared with the *in vivo* and can help the initial screening of the different diets prior to animal experiments.

Ruminants have a large fermentation chamber containing various microbes (bacteria, protozoa, fungi) that enables the ruminants to digest plant cell wall components such as cellulose to obtain chemical energy (Sjaastad et al., 2010). The VFA, ammonia, amino

acids, and some gases (CO_2, CH_4) are the ruminal fermentation end-products of the carbohydrates (e.g., cellulose, starch, fructan) and proteins (amino acid, non-protein N) (McDonald et al., 2011). Thereafter, the VFA, ammonia, and small amounts of amino acids are utilized for microbial protein synthesis, animal maintenance requirements, and production purposes (e.g., weight gain, milk production).

Roughages and concentrate grains are two major feed units of dairy cattle. Forages are supplied to provide energy and to maintain the healthy rumen function. However, the energy value and the digestibility are affected by several factors such as forage quality, feeding level, and the concentrate percentage in the mixed rations (Mertens, 2003). Concentrate grains are usually added into diets to increase the energy density to meet the high energy demands of intensive dairy production. However, nowadays, enough cereal production is becoming far more difficult and unpredictable due to climate change, deforestation, loss of topsoil, etc. In Norway, only 3% of the land is cultivable area, and 30% is used for grain and vegetable production (https://www.tine.no). Barley (～50%) and wheat (～24%) oats (～24%) are the three main groups in grains (https://www.nibio.no/). Based on the Norwegian agricultural report (Status and Trends 2019), 80% of the grain grown in Norway is used in animal feed concentrates. However, annually, around 40% of cereals and 90% of the protein ingredients need to be imported from other places (NFSA. 2015, annual report), which indicates an issue of sustainability. Hence, to overcome these challenges and meet the consumers' requirement (NFSA 2015, annual report), increasing the proportion of locally produced feed and food is envisaged. Therefore, there is the need to increase domestic feed self-sufficiency by including local feed resources and feed technologies to maximize locally available feed usage.

The addition of concentrate or grains such as barley in the diet increases available energy level (McDonald et al., 2011). High-yielding dairy cows require a substantial amount of dietary energy and an adequate supply of metabolizable protein to support milk production. However, with a higher inclusion rate of concentrate or grains in the diet, the concentration of short-chain fatty acids and protons will increase suddenly, which reduces the rumen pH and may impede the cellulolytic activity (Sjaastad et al., 2010). Furthermore, this rumen conditions have negative effects on animal health, such as rumen acidosis, and

may reduce grain degradation (Dixon & Stockdale, 1999). However, these starchy grains usually less in protein content, which is less effective for microbial growth and fiber utilization (Nikkhah, 2012). Therefore, here is the requirement of extra protein addition in the diet to synchronize the available energy and N for microbial protein synthesis. The Alkaline grain technique is developed to overcome these challenges for ruminants, whereby cereal grains are allowed to react with urea and enzyme under the high moisture closed system. It is alleged that alkaline pretreatment may increase the pH in the diet and reduce the ruminal pH fluctuation (McNiven et al., 1995). In addition, urea treatment increases the crude protein content of the grain-based diet, which may create an opportunity of reducing high rate of protein importance. Although there is limited research in this area, recent studies showed positive results of using alkaline grain as cows' diet (Kristensen & Fjeldberg, 2018; Meynadier et al., 2018).

Hence, the objective of this thesis is to compare different formulations of Alka-treated grains in ruminant diets on *in vitro* rumen fermentation and *in sacco* degradation characteristics in the presence of a soya-based standard diet. Furthermore, the interactive effects between these compound feed, and three different qualities of grass/clover silages mixed at a hypothetical dairy cow ration inclusion level were tested for *in vitro* rumen degradation characteristics using the Ankom RF gas production system. It was hypothesized that:

- The alkaline grain diets (AUMD and AUAB) would not differ from DRER in total gas production, rate of gas production, and dry matter degradability using the Ankom RF gas production system.
- The early cut silage will have higher quantity of GP and faster fractional rate of GP, in contrast to middle cut (50% of early cut: 50% of late cut) and late cut silages.
- The mixture different qualities of silages will behave differently to the different compound feeds in GP and fractional rate of GP.
- The *in sacco* degradation of nutrients of the four compound feeds along with intact pelleted feed (AUMD incubated as is) will differ because of their ingredient composition and physico-chemical treatment.

2 Theoretical backgrounds

This section is divided into three parts, introducing the overall picture and basic knowledge of the nutritional value of the ruminant feed and how they affect the digestibility as well as energy supply; the digestion physiology of the ruminant; and the methods to evaluate digestibility of feeds, based on the ruminant digestion physiology.

2.1 Feed characteristic

2.1.1 The classification of nutritional components

According to nutritional compositions, physiochemical properties, and functions, plant feed materials can be divided into different groups (Table 1). Originally, feed materials are grouped into six parts: water, ash, crude protein (CP), ether extract (EE), crude fiber (CF), and nitrogen-free extractives (NFE), which are called proximate analysis of foods (McDonald et al., 2011). For the case of ruminants, during 1960, the fibers (cell walls) were divided into neutral detergent fiber (NDF), acid detergent fiber (ADF) as well as acid detergent lignin (ADL) (Van Soest et al., 1991). However, in the modern Nordic feed evaluation system, the organic matter (OM) is further divided into crude protein, NDF, starch, residual carbohydrates including sugar (Rest CHO), crude fat, and fermentation products (Volden, 2011).

Table 1. Chemical and nutritional fractions of feeds (Mertens, 2003)

Chemical fractions:								
Moisture	Dry Matter							
	Ash	Organic Matter						
		Lipid	Protein	Carbohydrates, Organic acid and Complex polymers				
				-Sugars	Starches	Orgacids	Pectins	Hemicellulse	Lignins	Cellulose
Nutritional Fractions—Incompletely Digested:								
				Cell walls				
				Neutral detergent Fiber				
				Acid detergent fiber				
				Crude fiber				
Nutritional Fractions—Readily Digested:								

| ----------NFE---------- |
| ----------Neutral detergent Soluble---------- |
| Starches |

2.1.2 Major nutrients

Carbohydrates

Carbohydrate (CHO) is the major chemical energy of cattle diet to support microbes and dairy production growth. Also, the rate and extent of CHO digestion affect the energy supply and the ruminal environment and feed utilization. Hence, good balanced diets (such as balancing grains and fibers) are fundamental to maintaining a normal rumen environment and optimal feed utilization.

In general, carbohydrates are divided into structural (cell-wall) carbohydrates (cellulose, hemicellulose, lignin) and non-structural (cell content) carbohydrates such as starch, sugars, fructan, beta-glucans, and some pectin. Cellulose is the polymers of the β-glucose unit, binding with 1-4 bonds. They are usually found in plant cell walls and resistant to digestion by the gut enzymes (McDonald et al., 2011). Hemicellulose is a highly branched polymer, composed of glucose, galactose, mannose, and xylose, while lignin is phenyl-polymers that chemically cross-bind with other fibers hindering chemical digestions (McNeil et al., 1984). These structural carbohydrates in the diets stimulate saliva production, rumen mobility to maintain a normal rumen function, and the fermentation products of those are the main fat synthesis substrate in milk production.

Starch is a complex carbohydrate composed of amylose and amylopectin (account for approx. 80%), which are stored in the granule of the endosperm of cereals. Amylose is a linear molecule of an α-glucose bind by 1-4 linkages, and amylopectin consist of α-glucose chains with 1-4 linkages and a high level of side chains bound by 1-6 linkages (Damodaran et al., 2007). Starch is an important nutritional component in intensive milk production due to its high energy density, high digestibility, and it is the main fermentation source of propionic acid (Huntington, 1997). However, the energy content and the digestibility of the starch depend on their granule size, protein matrix, amylose,

and amylopectin ratio (French, 1973). Pectin is composed of galacturonic acid linked by 1-4 glycosidic bonds, found in the middle lamella and primary structure of the plants (Damodaran et al., 2007). They serve as “glue” that holds the cell wall components together and they are usually highly and rapidly digestible in the rumen. Fructan is consisting of long chains of fructose, which is frequently found in grasses, while simple sugars such as glucose and galactose are low molecular carbohydrates that are rapidly broken by the gut enzyme and microbial fermentation and absorbed by gut cell wall(McDonald et al., 2011). However, excessive non-structural carbohydrates in the diet may slow down fiber digestibility and diet utilizations.

Crude protein and amino acids

Proteins are composed of the 20 different amino acids pool by four levels of structure. The primary structure is the sequence of the amino acid chains binding with peptide bond and the secondary structure are these peptides binding with hydrogen bonds. Tertiary, quaternary structures are bonding with hydrophobic interaction, disulfide bond, ionic bond, etc. (Damodaran et al., 2007). Crude protein (CP) is determined by analyzing nitrogen content, assuming that the protein contains 16 % nitrogen, and the nitrogen content is multiplied by 6,25 to estimate the crude protein content (McDonald, 2002). When nitrogen is not part of the protein is termed as non-protein nitrogen (NPN) such as ammonia, urea. NPN and amino acids are building blocks of microbial protein (MP) synthesis and the MP and dietary amino acid absorbed in the small intestine are affecting milk protein production.

Crude fat and Fatty acids

Fats are esters of fatty acids with glycerol. In cereals, lipids are found as the type of triacylglycerols containing a higher level of linoleic and oleic acid, while in forages, fats present as a form of galactolipids containing mainly α-linolenic acid (Sjaastad et al., 2010). Rumen microorganisms are not able to tolerate a higher level of fat; it is usually recommended to be lower than 50 g/kg (McDonald et al., 2011). Fat is usually added to

the ruminant diet for improving energy content and the fatty acid composition in the milk (Sjaastad et al., 2010).

2.1.3 The ruminant feed: forage and concentrate

Plant (stems, leaves, seeds) and plant by-products (oilseed meals, molasses, milling by-products, etc.) are major sources of ruminant feeds. In general, feeds for dairy cows are classified as forage, concentrates, and other supplements. Roughages and concentrates differ in their fiber content, energy density, moisture content, and particle length, etc.(McDonald et al., 2011). Forages are usually required to be in a coarse form to stimulate rumination and thereby ensure normal rumen function. However, concentrate is added to increase the energy content and achieve better production performance. In the Nordic feed evaluation system, feedstuffs with particle lengths higher or lower than 6 mm are characterized as roughages and concentrate, respectively (Volden, 2011).

Forages

Forages are usually the vegetative parts of the plant, such as grasses (Gramineae), legumes (Fabaceae), and other crop residues. They usually contain low energy and more than 30% of NDF (Collins et al., 2017). The structure of the cell walls, NDF, is a network of cellulose, hemicellulose, and lignin. Moreover, the network between hemicellulose and lignin enhances the inhibitory effects in structural carbohydrates digestion (McDonald, 2002).

Silage: the way of preserving forage

Roughages are grown on-farm, require to be harvested by animals or machines and utilized directly or preserved (hay or silage) (Spedding, 1982). The ensilage process is done by achieving anaerobic fermentation of grasses in the silo or bunker silo to lower the pH (3.8-5.0) to preserve longer time (McDonald et al., 2011). The natural fermentation procedure requires the harvested grass to have correct moisture condition and rapid filling, sealing process to minimize aerobic bacteria and plant enzyme activities. Lactic acid

bacteria produce a mixture of acids (mainly lactic acid) by fermenting the sugars, which increases the hydrogen concentration to discourage the activities of undesirable microorganisms (clostridia and enterobacteria) that produce objectionable fermentation products (Spedding, 1982). However, if the crop condition is suboptimal for natural ensilages, for example high moisture content or lower in water-soluble carbohydrates. The suitable additive is needed, such as lactic acid bacteria, enzymes, formic acid (McDonald et al., 2011).

Concentrates

There is no clear definition of concentrate, which usually means single feedstuff or compound feeds with lo fiber contents and crude protein but higher in energy, except for protein concentrates containing 50% of CP (McDonald et al., 2011). Cereals (Gramineae) are essential in concentrate feeds for ruminants, containing approx. 500-700 g starch/kg DM Table 2. Corn and wheat are the most abundantly produced cereals globally, while in Norway, as mentioned earlier, barley and oats are the most dominant.

Table 2. Chemical composition (g/kg DM) of cereal grain and protein concentrates (McDonald, 2002)

Feed	DM	ME (MJ)	CF	EE	Ash	CP	NDF	ADF	Starch & sugar
Cereals									
Barley	860	12.8	53	17	26	115	201	64	599
Oat	860	12.0	105	49	33	109	290	149	482
Wheat	860	13.6	26	19	21	124	124	30	701
Corn	860	14.2	24	42	13	98	117	28	717
Oilseeds									
Soybean	900	13.3	58	17	62	503	125	91	124
Cottonseed	900	12.3	87	89	74	457	300	0	0

2.1.4 Forage quality, digestibility, and feed intake

Several factors affect the nutritive value of forages, such as species, leaf/stem ratios, maturity stages etc. (Spedding, 1982). For any species, the stage of maturity is the most

important factor regarding the nutritive value and forage quality. Different species have different nutritional values. As shown in Fig. 1, legumes usually contain 10 to 23% crude protein, while grasses typically contain 6 to 18% crude protein (Collins et al., 2017). In addition, Fig. 1 illustrates morphological component differences, and this example clearly shows the leaves contain 2-3 times as much crude protein as stem and the stem has much higher NDF.

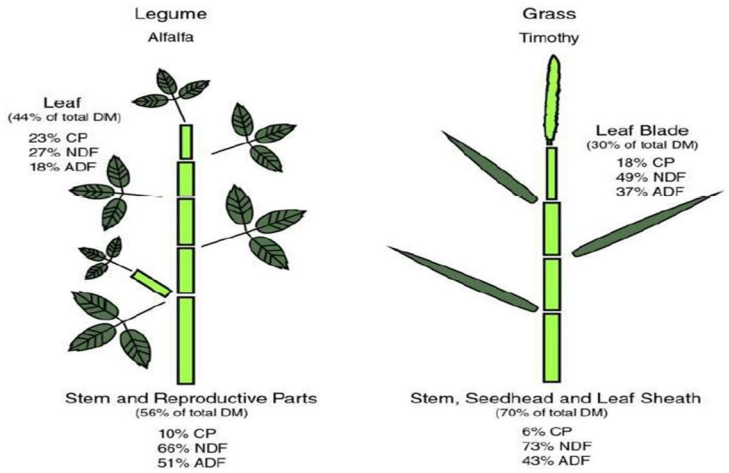

Fig.1 Forage quality analysis of leaf and stem tissue from alfalfa and timothy (Collins et al., 2017) The leaf is usually lower in fiber and higher in crude protein than the stem.

The maturity of the grass and the retention time in the rumen are two main factors affecting the digestibility of the grass and further energy utilization efficiency (Johansen et al., 2017). Forage digestibility and crude protein content declines and cell wall contents increase with advancing maturity, as illustrated in Fig. 2 (Collins et al., 2017).

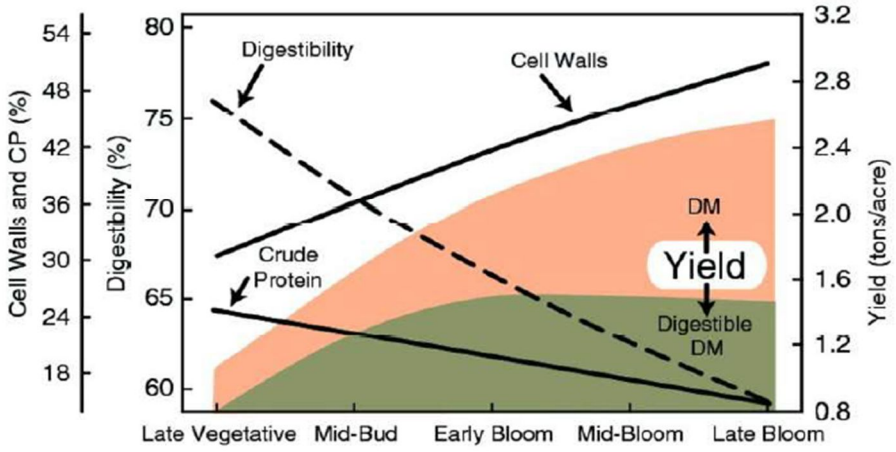

Fig.2 Effects of maturity stage on forage quality the concentration of crude protein and digestibility decline with increase with forage develop (Collins et al., 2017)

The feed intake is affected by the maturity stage of the forages and the animal physiological state. For example, the more mature forages require a much longer time for degradation and moving undigested particles through the digestive system (Sjaastad et al., 2010). Therefore, these rumen-filling effects will reduce feed intake and nutritional values. However, feed intake is also influenced by animal physiological states, for example, non-lactating allows diets containing approx. 65% of cell wall components meet energy needs, while for moderately productive cows with higher energy demands, the NDF contents in rations are recommended below 40% (Collins et al., 2017).

2.1.5 Concentrate to forage ratio and alkaline technique

In intensive dairy production, cows require large proportions of grains to increase energy density and supply nutrients deficient in forages (McDonald et al., 2011). However, when grains, are added up to approx. 65-70%, they may stop forage intake, digestion and frequently cause low efficiency of utilization of grains (Dixon & Stockdale, 1999). In addition, reduced fiber digestion and feed intake due to low rumen pH caused by grains rapid fermentation may also reduce milk fat contents (Sjaastad et al., 2010). Hence, the individual feedstuff does not have a fixed value, and the metabolic energy of mixed

rations forage and grains are determined by the interactions and their metabolism (Dixon & Stockdale, 1999).

Various methods are applied to moderate rumen interaction to ensure satisfactory digestion, microbial protein synthesis, and a healthy rumen environment. The Alkaline technique reduces the sudden pH drops in the rumen, enabling the greater inclusion of grains in the ruminant diet without negative effects on the rumen function and nutrients digestion. Besides, the alkalization process breaks down the linkages between the lignin and other nutrients (Chen et al., 2013; Wyman, 1996). Therefore, this alkaline treatment may bring two benefits: firstly, it works as a buffer to reduce the adverse effect of the high inclusion of grain in the diet (McNiven et al., 1995): secondly, it could increase the availability of the nutrients for microbial digestion and thereby increase the fiber digestibility (Chen et al., 2013; Jackson, 1977).

2.2 The ruminant

2.2.1 Physiological digestion and rumen environment

Ruminants have large fermentation chambers, called rumen (Fig.3), containing microorganisms (bacteria, protozoa, fungi) that can utilize fibers, such as cellulose to provide energy for host animals (Sjaastad et al., 2010). Microbes allow ruminants to eat partly digestible forages, which are resistant to gastric-enzymatic digestion (Dijkstra et al., 2005).

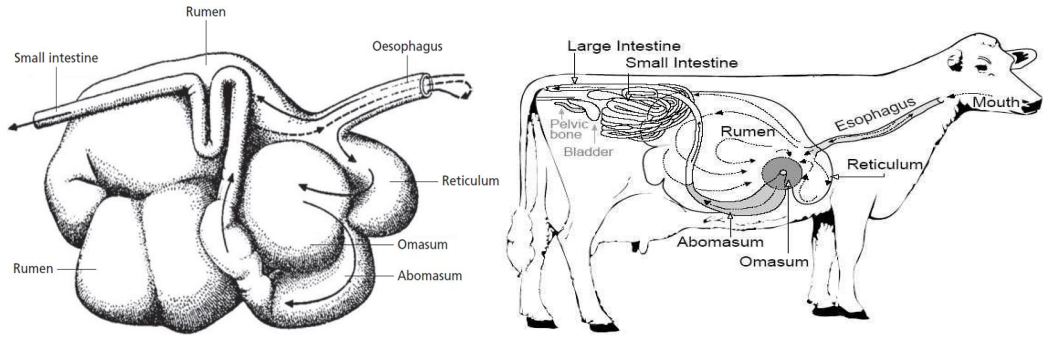

Fig. 3 Diagrammatic description of the ruminant digestive system (rumen, reticulum, omasum, and abomasum) (McDonald et al., 2011)(https://extension.umn.edu)

Feed digestion starts with particle reduction in the mouth and is then swallowed with saliva into the rumen-reticulum chamber. Cows produce approx. 150L saliva per day to dilute digesta when they chewing and ruminating (McDonald et al., 2011). In the rumen, the anaerobic microbes produce enzymes to degrade of the nutrients called fermentation (Sjaastad et al., 2010). Fermentation of carbohydrates and protein produces gases (CO_2, CH_4), volatile fatty acids (VFA), and ammonia as end products, and the VFA are absorbed through the rumen wall and utilized by ruminant (Dijkstra et al., 2005). The gases are lost by eructation, which represents approx. 10% of energy loss from metabolizable energy, by formation of CH_4 (Sjaastad et al., 2010). The produced ammonia is either used for microbial protein synthesis or goes into urea recycle (McDonald et al., 2011). In the fermentation process, the acid production increases the H^+ concentration, resulting in reduced pH in the rumen. The rapid absorption of the VFA through the rumen wall and bicarbonate and phosphate in the saliva help to stabilize the pH, normally 5.5-6.5 (McDonald et al., 2011).
The bacteria, responsible for degrading carbohydrates, are divided into amylolytic bacteria and cellulolytic bacteria (Sjaastad et al., 2010). When cattle ingest large amounts of cereals, the amylolytic bacteria rapidly produce VFA, which causes pH drops quickly. The low pH environment is harmful to cellulolytic bacteria and may reduce the degradation rate of fiber (McDonald et al., 2011). After the fermentation process, the nutrients and the microbial cells enter the abomasum and the small intestine (Fig. 3), where they undergo enzymatic digestions. The large intestine contributes to microbial digestion in the same way as the rumen. While VFA is partly absorbed through the intestinal wall, the microbial cells from the large intestine are excreted in the feces.

2.2.2 Degradation of carbohydrates

Rumen degradation of the carbohydrates is divided into two steps. The initial step is breaking down the complex carbohydrates (NDF, starch) into simple sugar (glucose) by the extracellular bacterial enzyme and then the microbes metabolize the glucose to the intermediate substrate (pyruvate) by glycolysis (McDonald et al., 2011). Secondly, the pyruvate is converted into VFA under anaerobic conditions. The total amount and relative

proportions of the VFA vary with the feed compositions and roughage concentrate ratios (McDonald et al., 2011). When the diet contains a higher level of concentrates, the total VFA production becomes higher and faster and contains a higher propionic acid percentage (Sjaastad et al., 2010). However, the more mature forages produce a high proportion of acetic acid.

The produced acetic acid and butyric acid (as β-hydroxybutyric) are absorbed through the rumen wall and pass into the portal blood, to the liver and to the organs and tissues as a source of energy and for synthesis of fat (McDonald et al., 2011). The propionate, passing from the rumen in the hepatic portal vein to the liver and further used for gluconeogenesis pathway (Dijkstra et al., 2005).

2.2.3 Degradation of proteins

Protein is hydrolyzed to short peptides and amino acids by the protease of proteolytic bacteria. While most amino acids converted to ammonia and VFA through the deamination process, only small amounts are utilized directly by bacteria to synthesize microbial crude protein (MCP) (Sjaastad et al., 2010). The NPN enters the rumen rapidly breaks down to ammonia by ruminal microorganisms. The ammonia, as mentioned, may transfer into the urea cycle, or the rumen organisms catch them with VFA to synthesize microbial protein.

The MCP and rumen-undegradable protein pass into the abomasum, small intestine, hydrolyzed by the gastric pepsin and pancreatic enzymes to amino acids and dipeptides. The amino acids are absorbed into the portal blood and transferred to the liver further used for protein synthesis (McDonald et al., 2011). Some excess amino acids may be used as an energy source, broken down to ammonia and keto acids.

2.2.4 Microbial modification of fat

The fats entering the rumen are hydrolyzed by the bacterial lipase, losing the ester bond to glycerol, galactose, and free fatty acids. While glycerol and galactose are rapidly fermented to VFA, the free unsaturated fatty acids are hydrogenated to saturated fatty acids by ruminal bacteria, for example, to palmitic and stearic acid (D'Mello, 2000).

In the small intestine, the absorption of fatty acids is helped with bile salt and pancreatic enzyme, which is transferred to the lymphatic system used for energy production or used for fat synthesis (McDonald et al., 2011).

2.3 Methods for measuring rumen degradation

The nutritional value of feedstuffs can be determined by the chemical analysis; however, the supply of nutritional value is predicted by digestion studies (*in vivo, in sacco*, and *in vitro*). The common feed evaluation uses a unit of metabolizable or net energy and a metabolizable protein (amino acid absorbed in the small intestine), which are predicted from the digestibility of the feed samples and different calculation equations (see the material and method section).

2.3.1 *In vivo*

In vivo may be the most logical and reliable method to evaluate feed degradation (Mohamed & Chaudhry, 2008). This method is used to detect feed quality and dietary effects directly in the rumen and small intestine helped with cannulated animal and marker techniques for measuring digesta flow from rumen further down to other digestive tracts (Stern & Satter, 1982). However, this method has several disadvantages. For example, the method is expensive, laborious, and carries errors (when sampling, analyzing) associated with markers, inherent animal variations (Stern et al., 1997). Hence, there is always a need for simple, inexpensive, and reliable alternative measurements which have been developed over decades, including in situ, two-stage *in vitro* as well as *in vitro* gas production.

2.3.2 *In Sacco*

In Sacco method is used to measure the disappearance of the feed particles in the digestive tract (e.g. rumen) at different time intervals to assume the digestibility with times (Stern et al., 1997). It is done using nylon bags (containing different feed samples) and suspending these bags in the rumen of living animals. The disappearance of the feed material is calculated as the degraded, which is due to microbial attacking through the small porous to

feed samples and rubbing forces by the movements of the rumen wall (for details, see the material and method). This technique is simple and cheaper compared with *in vivo* intestinally cannulated animals and including a real ruminal digestive environment (Nocek, 1988) compared with other laboratory methods. However, *in sacco* requires the rumen fistulated animals that limit the commercial laboratory routines and suggested this method may not be precise due to particles loss from the bag pore without complete fermentation, which leads to an overestimation of soluble fractions and underestimation of the degradable parts and may also have bacterial contamination (Stern et al., 1997).

2.3.3 *In vitro* and *in vitro* gas production

In vitro is a laboratory method applied to estimate the organic matter digestibility of feedstuff and considered as a model technique of *in vivo* rumen digestion (Beuvink, 1993). Since the degradability is measured in the simulated rumen environment by adding buffered rumen fluid into the sealed container, which is low labor and less harmful to the animals. This method was used for predicting apparent digestibility and selecting good quality silage by measuring the disappearance of dry matter in two stages of incubations (rumen fluid and HCL pepsin) (Tilley & Terry, 1963) with later modifications to measure the volume of gas production from incubation of feed sample rather than DM disappearance to evaluate the digestibility (Menke et al., 1979).

Thereafter, to obtain more information about rumen degradation rates with time, the *in vitro* gas production is registered at set frequent intervals over 48 h (Xiong et al., 1990). A close relationship between rumen fermentation and gas production was found and the volume of gas production is highly related to *in vivo* digestibility (Deinum & Maassen, 1994). To measure gas production from batch cultures of buffered rumen fluid, an automated gas production system is developed, using the computer-linked electronic sensor to record changes in pressure and monitor gas production(Pell & Schofield, 1993; Theodorou et al., 1994).

Gas production is mainly the result of ruminal degradation of carbohydrates, which produces VFA, CO2, CH4, and generally, the gas production level is smaller with

protein-rich feeds (López et al., 1998). The gases include direct gas (CO2 and CH4) production from the fermentation end product, and indirect gases (CO2) are released from the buffer by the produced VFA (Pell & Schofield, 1993). In addition, the gas production from fermentation is influenced by different proportions VFA, which caused by different ratio of fibrous and starchy feeds (Blümmel et al., 1997). *In vitro* shows several advantages compared with the other digestion methods. For example less time consuming, easy to handle, less labor, better animal welfare, and possible routine evaluation (Mohamed & Chaudhry, 2008). However, it has some limitations too: gas production is the total gas production from the diet but not suitable for the individual nutrients; and the method still requires rumen fistulated animals to get the rumen fluid (El Shaer et al., 1987; Omed et al., 2000), among other things.

In summary, for high-yielding cows, predicting the nutritional value of feedstuff is important, moreover, the rate and pattern of digestion in the rumen. Feed (mainly carbohydrates) ingested by ruminant is helped by microbial fermentation transferred into VFA and gases. The ruminal degradability can be estimated by the gravimetrical method based on the disappearance of feedstuff incubated in porous bags in the rumen or alternatively measuring gas production based on microbiological principle, which is directly monitoring rumen fermentation, relatively easy to measure and possible to measure other parameters such as produced VFA and pH. In addition to digestibility estimation, the rate of degradation pattern is crucial to predicting the rumen environment and feed intake. In high-yielding cows, increasing the rate of digestion could enhance the growth of ruminal microorganisms and dry matter intake. Hence, despite the limitations, *in sacco* rumen degradation and *in vitro* gas production techniques are simple and reliable laboratory procedures to examine the rate and extent of the degradation of different feedstuffs. Here, we utilized in vitro gas production method to estimate the rate of substrate degradation, *in vitro* gas production and energy values of different formulations of compound feeds and different qualities of silages and their mixtures. Furthermore, we tested rumen degradation (e.g., kinetics and effective degradation of different nutrients) of the compound feeds using *in sacco* technique.

3 Material and Methods

3.1 Background information

This study included ANKOM RF Gas Production (GP) and *in sacco* rumen degradation to estimate the digestibility of the four types of compound feeds (different formulations of alkaline grain in contrast to soy-based concentrate), three qualities of silages (early cut, late cut, and evenly mixed early and late cut) and combinations of these concentrates and silages. All experiments were performed simultaneously at the Norwegian University of Life Science (NMBU). Chemical compositions of the feed samples and residual analysis were analyzed in LabTek at NMBU.

Six non-lactating dairy cows fitted with rumen cannula were used in this experiment. Three cows were used to collect rumen fluid samples (for gas production) and others used for in sacco method. Table 3 illustrates the information on these animals and their diets. Cows were fed at maintenance level and the diet consisted of hay (3.2 kg/day), straw (2 kg/day), and concentrate (2.5 kg/day). Daily diet was equally divided into two meals with an adaptation period of 14 days and the roughage to concentrate ratio was 67/33, crude protein content of the diet was higher than 120 g/kg DM, according to the experimental description in the Nordic feed evaluation system (Volden, 2011).

Table 3. Information about experimental cows and diet rations

Cow Information						
	In Vitro			In Sacco		
Cow No	1	2	3	4	5	6
Date of birth	11.9.2011	21.11.2010	9.11.2007	17.11.2013	17.9.2012	11.11.2013
Diet Rations						
	In Vitro			In Sacco		
	DM_{kg}	$CP_{g/kg}$	FEm_{Mcal}	DM_{kg}	$CP_{g/kg}$	FEm_{Mcal}
Straw	0.86	54	0.30	0.86	54	0.30
Hay	0.76	83	0.84	0.86	130	0.81
Energirik-høg*	0.87	208	1.09	0.87	208	1.09

**Energirik høg is compound feed for dairy cows produced and supplied by Norgesfôr AS*

3.2 Feed samples preparation

3.2.1 Feed samples for gas production

Nineteen treatments were examined in the Ankom RF gas production procedure, which included 12 mixed rations and 7 pure feeds (Table 4). The rations were four compound feeds (details in Table 4) including DRER , AUMD , AUNA and AUAB mixed with three qualities of grass/clover silages representing early-cut (E-CT) middle-cut (M-CT) and late-cut (L-CT) at a ratio of 45%/55% (compound feed/ silage), on DM basis, which is to simulate the moderate milk production feeding level. Besides, these silages and compound feeds were examined individually as a pure form. The M-CT silage was a mixture of E-CT and L-CT at 50%/50%, creating an average quality grass silage.

Table 4. Description of the compound feeds and silages

Feeds	***Description***
Compound feeds	
DRER	Drøv Energirik → standard compound feed with higher proportion of imported ingredient, formulated for feeding moderate to high producing cows
AUMD	Alka Ultramjølk→ Alkaline compound feed with high proportion of local ingredients (20% Alka 150 diet*)
AUNA	Urea mixed with unreacted parent materials of AUMD
AUAB	Non-heated, unpelleted mixture of parent materials of AUMD
Silages	
E-CT	early cut silage with a lower level of NDF and high CP content
M-CT	mixe of early cut and late cut silage (50:50)
L-CT	late cut silage with a higher level of NDF and low CP content

**Alka 150 diet=15% Home n' dry reacted with 85% Barley+ moisture*

Silages were dried in a 60 °C forced air oven and concentrates were oven-dried at 45 °C. All samples were ground to pass a 1.0 mm sieve in a cutter mill (Retch GmbH SM 200) to obtain a homogenous sample that was used for chemical analysis and further *in vitro* GP.

3.2.2 Feed samples for *in sacco*

In sacco experiment tested five types of compound feeds, including the four types of compound feeds as *in vitro* (DRER, AUMD, AUNA, AUAB), which were dried at 45 °C and ground to pass a 1.5 mm sieve in cutter mill (Retch GmbH SM 200) to obtain a

homogenous sample and mimic the mastication process. Besides, the diet AUMD was incubated as intact pellet form without grinding, used to compare the dry matter difference at different periods in contrast to grounded feed samples.

3.3 ANKOM RF gas production procedure

3.3.1 Ankom gas production system description

The ANKOM RF gas production system consists of incubation bottles (250 mL capacity in our experiment) and the bottle covers or modules containing the pressure sensors (Fig. 4, left). The bottle is used for the incubation of the feed samples with rumen fluid and buffer solution. The pressure sensor in the modules measures the gas production (automatically release at 0.75 psi above atmospheric pressure). The readings are transferred to a computer with radio frequency (RF) transmissions. The pressure reading intervals were set for every 10 minutes of reading intervals used in our experiment.

3.3.2 Feed sample weighing and preparation

The operation procedure followed the ANKOM gas production manual description. Samples were weighed in triplicates (1.0 $\pm$ 0.1g on DM basis) into Ankom glass bottles, sealed with aluminum paper and prewarmed in an incubator (39 °C) overnight (as in Fig. 4, middle).

3.3.3 Preparation of buffer solution and rumen fluid

The buffer solution was prepared according to (Goering & Van Soest, 1970) buffer solution formula (Table 5).

Table 5. Buffer solution and the compositions (Goering & Van Soest, 1970)

Solutions	Chemical compositions
(a) micro-mineral solution	13.2g $CaCl_2 \cdot 2\ H_2O$ + 10.0 g $MnCl_2 \cdot 4H_20$ + 1.0 $CoCl_2 \cdot 6\ H_2O$ + 8.0 g $FeCl_3 \cdot 6\ H_2O$ (Dilute in 100ml distilled water)
(b) buffer solution	3 g NH_4HCO_3 + 35 g $NaHCO_3$ (Bring volume to 1 L using Distilled Water)
(c) macro-mineral solution	5.7 g Na_2HPO_4 anhydrous + 6.2 g KH_2PO_4anhydrous + 0.6 g $MgSO_4 \cdot 7\ H_2O$ (Bring volume to 1 L using Distilled Water)
(d) reduction solution	625mg Cysteine·HCl+4ml 1N NaOH+ 625mg $Na_2S \cdot 9H_2O$ (Bring volume to 1 L using Distilled Water)

- The buffer solutions were made as Table 5 and then continuously flushed with CO_2, under constant stirring with a magnetic stirrer for about 2 h in a 39 °C water-bath.
- Before mixing with feed samples, a reducing solution was mixed with a color change from purple to colorless, indicating a reduced solution.
- Rumen fluid was collected 2 h after the morning feeding. Rumen fluid from each cow was mixed into prewarmed 2 L Thermo bottles and then filtered through 200-micron pore size Nitex cloth into a prewarmed flask (that had been flushed with carbon dioxide), then placed in the 39 °C water bath.

3.3.4 Fermentation procedures, gas measurements and residuals sampling

- The prepared rumen fluid (33.3 ml) and buffer solution (66.7 ml) were injected into the glass bottle containing the feed samples.
- The overhead space of the bottles was flushed with CO_2 to create anaerobic headspace.
- Then, the mixed substrates were incubated in the slow rotating sealed incubator (39 °C) for 48 h, monitoring cumulative gas production (Fig. 4, right).

Fig. 4 Overall Ankom gas production operation procedure (Left: Ankom incubation bottles and modules, Middle: feed sample preparation before incubation, Right: feed incubation)

- Besides, two or three glass bottles containing only the rumen fluid and buffer solution (termed as blanks) and two or three bottles containing an internal standard were incubated together with feed samples, which included the corresponding correction of gas production.
- Each run consisted of 34-36 bottles. The procedure was replicated in two consecutive runs with a total of 4 runs in our experiment.
- After 48h, gas production was recorded, and fermentation was terminated by removing all bottles from an incubator. The endpoint pH of the digesta in the bottles was examined immediately.
- After all procedures, the residues were collected in 11-µm nylon bags and immediately immersed in cold water to minimize microbial activity.
- Then, all bags were washed immediately in a washing machine with cold water without centrifugation and then dried in a drying cabinet (45 °C) following Nor For *in sacco* procedure (Volden, 2011)
- The residues from each sample were ground in a coffee grinder (IKA® A11 Basic Analytical Mill), sent for residual chemical analysis.

3.4 In Sacco methodology

The experiment operation has followed the standard in Sacco procedure in Nor For (Volden, 2011).

- 2.0 g (±10 mg) of pre-dried feed samples were weighed into the nylon bags. The bag size was 6*12cm and the pore opening in the nylon bags was 38μm.
- The bags with feed samples were attached to strings with rubber bands (Fig. 5, left), and the strings were collected and fastened on the rumen fistula lids (Fig.5, middle), which were ready for incubation.

Table 6 Different repetitions in a different incubation period

Bag Number		Incubation hours (h)							
	Cow NO	**2**	**4**	**8**	**16**	**24**	**48**	**72**	**0**
	4	2	2	3	6	6	6	8	4
	5	2	2	3	6	6	6	8	
	6	2	2	3	6	6	6	8	

The bags with the five different compound feeds were incubated (Fig.5, right) for 2, 4, 8,16, 24, 72h, respectively. Repetition was different, depending on the incubation time, 2 to 8 repetitions per animal as in Table 6. Besides, 0 hour incubation was determined by rinsing feed samples in the washing machine with cold tap water for 35 minutes.

Fig. 5 overall description of in Sacco degradation procedure

- After the incubation, the bags were continuously removed from the rumen, rinsed immediately in a washing machine with cold tap water without centrifugation, and then dried for 48h in a drying cabinet (45 °C).
- Then, the residual undegradable part within the bags was weighed for calculating the disappearances of DM and resides from each feed, each incubation period, and each animal was combined into one sample and ground in a coffee grinder, then sent for analysis of N, starch and NDF.

3.5 Chemical analysis of feed samples

The compound feeds were milled through a 1 mm sieve and sent for analysis of DM, ash, CP, EE, NDF and ADF. Besides, the silages were analyzed for all parameters as in compound feed except for starch. The DM content of feed samples was determined by drying the feed sample at 103 ºC overnight and ash content was feed sample burned at 550 ºC for at least 4 h, following (ISO 5984). The CP, EE have used the standard procedures described by (AOAC, 2002). While NDF, ADF was determined with an ANKOM220 fiber analyzer (ANKOM Technology, Fairport, NY, USA) and NDFom is the value of corrected for ash residual. Starch content was analyzed by Total Starch Assay Procedure based on AACC Method 76-13-01. Besides, the chemical composition of the mixed rations was estimated based on the composition of these pure feeds and their inclusion rates in each mixture.

3.6 Calculations and statistical analysis

3.6.1 Calculation of gas production volume

According to ANKOM RF gas production procedure, the gas production over time was recorded as cumulative pressure in pound per square inch (psi). The volume of the gas production is calculated based on the ideal gas law: $\boldsymbol{n = p\ (V*RT^{-1})}$ and Avogadro's law $\boldsymbol{V}$(ml) = $\boldsymbol{n}$∗22.4∗1000. Where $\boldsymbol{n}$ is a gas produced in mol; $\boldsymbol{p}$ is the pressure (kPa), calculated by the conversion factor (6.894757293) *cumulative pressure (psi); $\boldsymbol{V}$ is the head-space volume (L) [calculated as a difference between bottle volume and sample/buffered rumen fluid volume]; $\boldsymbol{T}$ is the incubation temperature in K (273 +39 °C); $\boldsymbol{R}$ is the constant number (8.314472 $L{\cdot}kPa{\cdot}K^{-1}{\cdot}mol^{-1}$). Then the gas produced in (ml) calculated as n∗22.4∗1000: meaning of 1 mol gas will occupy 22.4 L at the standard conditions (273.15°K and 101.325 kPa).

3.6.2 Parameter estimation and fractional rate calculation

The kinetic parameters of gas production were estimated using NLIN procedure in the

SAS software (SAS 9.4), fitting the model GP = A /(1+B^C/t ^{C}) described by (Groot et al., 1996): Where GP (ml g^{-1} DM is the amount of gas produced from gram of dry matter (DM) incubated at t time; A (ml g^{-1}DM) is the asymptotic gas production; B (h) is the time after incubation, when half of the asymptotic gas volume was produced, and C is a factor of defining the shape of the curve. Then mean values of B and C of each feed are used to calculate the fractional rate of the gas production using the equation R= C t $^{C-1}$ / (B^C + t ^{C}) (Groot et al., 1996); where R (h^{-1}) is the relative fractional rate of substrate degradation, and the other parameters as described above. The parameter estimates (i.e., A, B, C, R) were compared among dietary treatments using Proc GLM procedure in the SAS 9.4 (SAS Institute Inc., Cary, NC). Significant Variance was set at $p<0.05$.

3.6.3 Calculations of the OMD and ME

Gas production after 24h was related to the metabolizable energy (ME) content of the feedstuff (Menke et al., 1979). The OMD and ME in *in vitro* gas production was calculated according to (Menke, 1988),: OMD (%) = 14.88 + 0.889×GP + 0.45×CP + 0.0651×ash where GP is *in vitro* gas production (ml/200mg DM), and CP and ash are given as unit of g/kg DM; ME (MJ/kg DM) = 7.81 + 0.07559× GP-0.00384*ash + 0.00565*CP + 0.01898*crude fat -0.00831*ADFom (GfE 2008), where GP is *in vitro* gas production (ml/200mg DM) and the ash, CP, crude fat, ADFom are expressed in g/kg DM.

3.6.4 Parameter estimation and ED calculation (*In Sacco*)

The effective digestibility of the DM, CP, starch and NDF were calculated by the method described by (Ørskov & McDonald, 1979), using the NLIN procedure in the SAS software (SAS, 1994). The *in sacco* data were fitted using the model $\boldsymbol{D = A + B\,(1-e^{-ct})}$ (Ørskov & McDonald, 1979) where: ***D*** is the degradation value of after time ***t***, ***A*** (%) is the immediately degradable fraction, ***B*** (%) is the potential degradable part over incubation times, ***C*** is the fractional degradation rate of ***B***, ***t*** (h^{-1}) is different incubation time intervals. The estimated parameters were used to calculate the effective degradation values of each feed according to the equation $\boldsymbol{ED = A + (B*C/C+K)}$ (Ørskov & McDonald, 1979), where

ED is the effective degradability, ***K*** (%/h) is the assumed passage rate of the nutrients. Our experiment used a passage rate 3% for NDF, 5% for starch and DM and 8% for protein. All Variance analysis were performed by using the GLM procedure in the SAS 9.4 (SAS Institute Inc., Cary, NC). Differences were considered statistically significant when $P < 0.05$ unless otherwise.

4 Results

4.1 Description of feeds

The chemical composition of the parent feeds, i.e., compound feeds and grass silages are provided in Table 7. The CP content varied between 192-215 g kg^{-1}DM for the compound feeds and between 102 and 227 g kg^{-1}DM for the grass silages. The NDF content of all the compound feeds were lower than 189 g kg^{-1}DM, while for silages the NDF varied between 337 and 640 g kg^{-1}DM. The highest NDF and lowest CP content were observed in the late cute silages (L-CT) and conversely, the lowest NDF and highest CP were in early cut silages (E-CT). The starch content in compound feed varied between 414-463g kg^{-1}DM and the starch content in the silages was not analyzed.

Table 7 Chemical composition (g kg^{-1}DM) of feed samples

	DM	Ash	CP	NDF	NDFom	ADF	Starch	EE
DRER	894	63	197	189	188	71	448	31
AUMD	891	71	192	153	152	54	414	40
AUNA	878	68	215	178	178	55	445	37
AUAB	881	73	202	166	166	57	463	36
E-CT	838*	77	227	337	333	193	-	41
M-CT	873*	68	166	480	477	272	-	35
L-CT	915*	58	102	640	639	360	-	33

*DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; NDFom: ash corrected NDF; ADF: acid detergent fiber; EE: ether extract *Silage dry matter showed here was the pre-dried dry matter content*

4.2 Prediction of nutrient digestibility using *in sacco* method

The result of parameter estimations and effective dry degradability (EDMD) of each nutrient are presented in Table 8. The significant ($P < 0.0001$) differences were observed for the immediately degradable fraction (A), potential degradable part (B) and the fractional degradation rate (C) among the compound feeds. The soluble part (assuming the immediately degradable part close to soluble part) of NDF in our result was set to zero. The highest content of soluble fractions in DM (40.6%) and starch (42.9%) were observed

in the compound feed DRER. The AUNA and AUAB contain lower soluble fractions than AUMD and DRER, especially in starch, only 6-7%. The pelleted form of AUMD revealed the lowest value in soluble fraction, rate of digestion and the effective degradability. However, relatively higher content of potential degradable parts was observed in DM (73%), CP (73.7%) and starch (103.7%).

Table 8 Nutrients degradation value and the parameter estimation

	DRER	AUMD	AUNA	AUAB	AUMD (pellet)	Root MSE	P
DM							
A	40.6^{a}	34.6^{b}	25.4^{c}	24.8^{c}	17.4^{d}	0.840	<.0001
B	50.3^{d}	56.1^{c}	62.6^{b}	63.4^{b}	73.0^{a}	1.243	<.0001
C	14.2^{d}	20.8^{b}	23.7^{a}	24.3^{a}	17.4^{c}	1.488	<.0001
EDMD $_{(0.05)}$	77.0^{b}	79.5^{a}	76.7^{b}	76.7^{b}	73.8^{c}	0.525	<.0001
CP							
A	33.8^{c}	39.4^{a}	36.5^{b}	33.1^{c}	19.5^{d}	1.752	<.0001
B	62.8^{b}	55.8^{d}	56.7^{d}	60.3^{c}	73.7^{a}	1.980	<.0001
C	7.5^{b}	8.5^{b}	12.2^{a}	11.7^{a}	12.2^{a}	1.471	<.0001
EDMD $_{(0.08)}$	63.5^{c}	67.7^{b}	69.8^{a}	68.0^{b}	63.2^{c}	1.400	<.0001
Starch							
A	42.9^{a}	16.8^{b}	7.1^{c}	6.1^{d}	-4.3^{e}	0.939	<.0001
B	55.0^{e}	82.5^{d}	92.1^{c}	93.0^{b}	103.7^{a}	1.108	<.0001
C	32.0^{c}	33.7^{c}	43.2^{b}	48.9^{a}	24.0^{d}	4.798	<.0001
EDMD $_{(0.05)}$	89.5^{a}	88.5^{b}	89.2^{a}	89.5^{a}	81.2^{c}	0.712	<.0001
NDF							
B	72.7^{a}	66.0^{b}	64.4^{cd}	65.1^{bc}	64.0^{d}	1.244	<.0001
C	6.7^{d}	11.3^{b}	12.3^{ab}	13.0^{a}	8.1^{c}	1.698	<.0001
EDMD $_{(0.03)}$	50.2^{b}	52.0^{a}	51.7^{a}	52.3^{a}	46.6^{c}	1.158	<.0001

A: immediately degraded dry matter (%); B: potentially degraded dry matter (%); C: fractional rate of degradation of b; EDMD: effective dry matter degradability (%) at different passage rate (5% for starch and dry matter, 8% for protein, 3% for NDF); statistical difference at P < 0.05; immediately degradable part of NDF was not calculated

There was a significant ($P < 0.0001$) difference among the feeds on the EDMD of nutrients (Table 8). The DM digestibility varied between 73.8-79.5% and for CP, starch

and NDF varied between 63.2-69.8%, 81.2-89.5%, and 46.6-52.3% respectively. The AUMD, in contrast with standard feed DRER has higher DM and NDF degradability ($P < 0.0001$). However, no significant difference was observed in the DM and starch digestibility of AUNA and AUAB compared with DRER. Moreover, no difference was observed in the NDF digestibility among the feeds AUMD, AUNA and AUAB, showing the highest EDMD (NDF) value. The AUNA showed the highest CP digestibility, while the DRER and pelleted AUMD showed the lowest value in EDMD of crude protein. The intact pellet form of the AUMD showed the lowest degradation value among all other groups.

4.3 Model fitting and degradation profile

Our *in sacco* data fitted the model of Ørskov & McDonald (1979), and the degradation profile are showed in Fig.6. The digestibility was increased with elongation of incubation hours. The intact pellet form of AUMD presented the slowest degradation profile of all feeds. Overall degradation profile of all nutrients among all compound feeds (except for pellet form) were similar, especially after 16h.

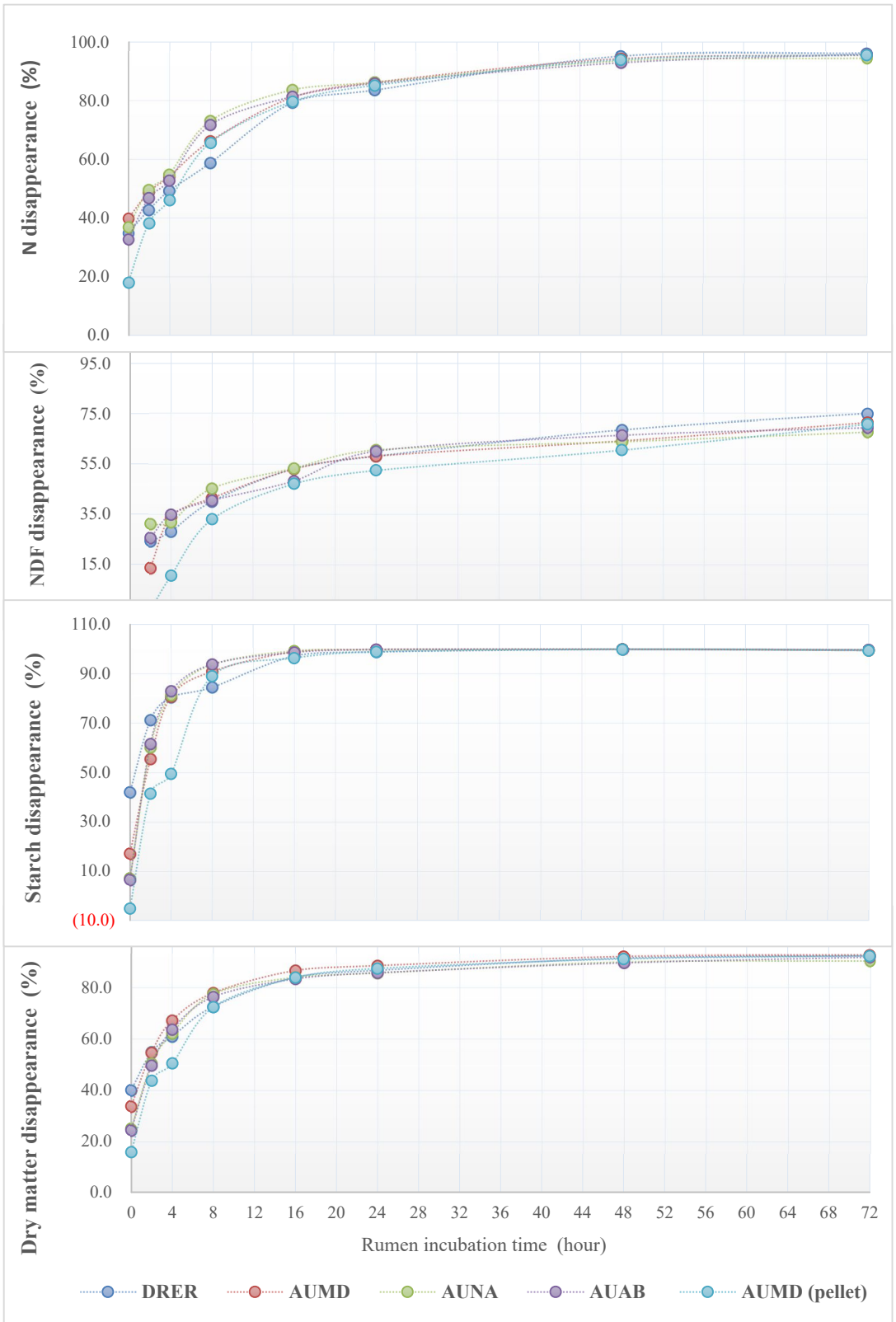

Fig.6 The degradation profile over time

4.4 *In vitro* gas production using Ankom RF

The GP profiles of all pure feeds and mixed rations are described in Figure 7. All compound feeds showed similar GP. The AUAB had a slightly higher cumulative GP after approx. 16 hours, while the AUNA appeared to have numerically lower GP. The GP profile of silages ranked in the order of early cut, middle cut, and late cut from highest to lowest cumulative GP. The GP for mixed rations, clear ranking gas production profile was observed reflecting that of the grass silages. Overall, the compound feeds mixed with early cut silages showed numerically high GP, with late cut silages mixed with compound feeds having slightly lowest GP. These differences are presented below (section 4.5) with parameter estimate.

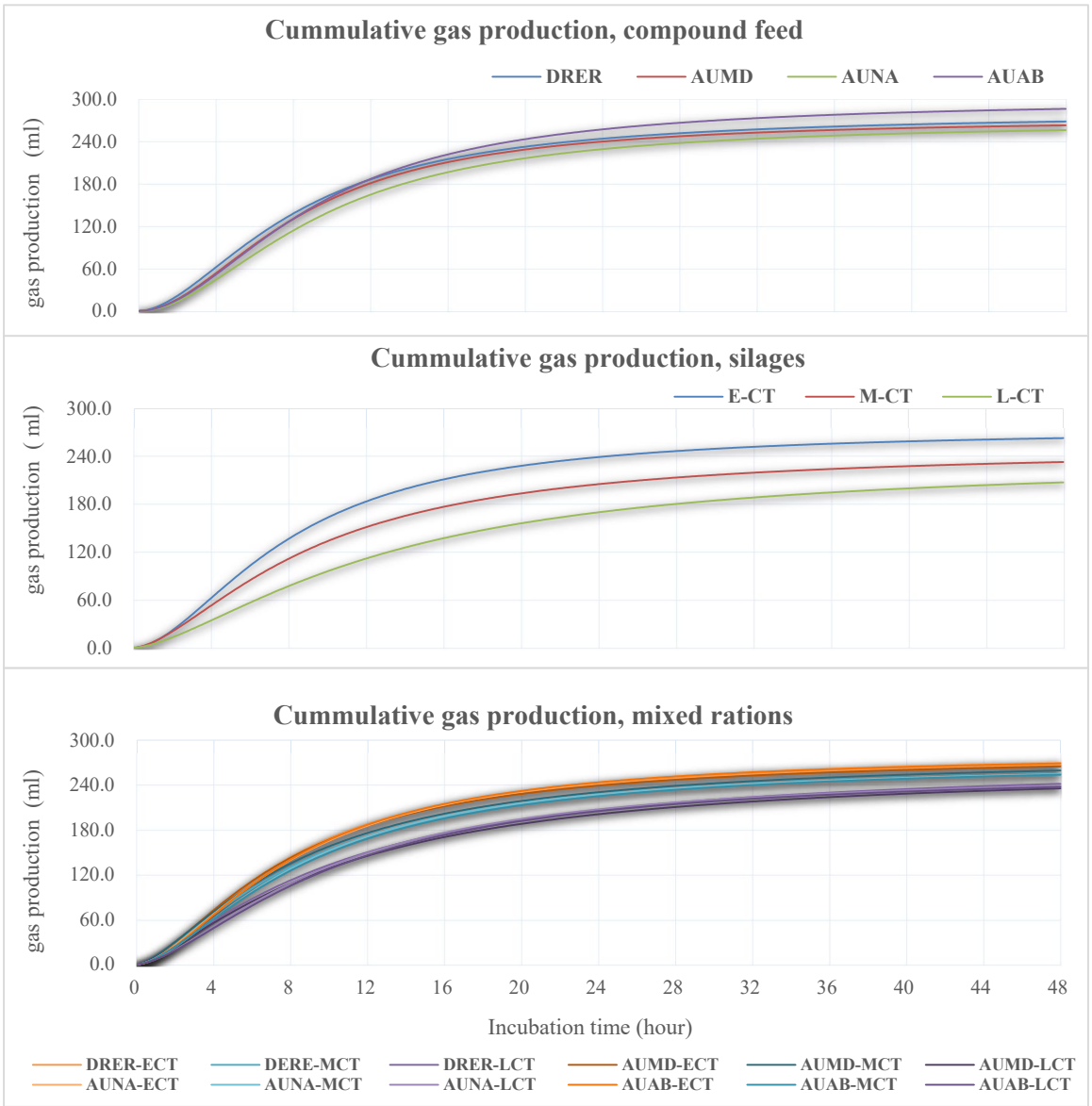

Fig.7 gas production profile of different feeds

4.5 Kinetic parameter estimations in *in vitro* gas production

The result of *in vitro* gas production parameter estimates and the fermented dry matter, expressed here as DMD (%), after 48h are presented in Table 9. There were no significant differences in asymptotic gas production among all compound feeds. AUNA may tend to have lower asymptotic gas production compared with others. DRER and AUMD have the higher dry matter degradation value (83.6% and 84.8 %) than others. The lowest DMD (69.1%) was observed in feed AUAB but tend to have a higher amount of gas production. For the silages, the total gas production and dry matter degradation was higher in early cut silages and much lower for late cut silages. In addition, the early cut silages revealed the highest dry matter digestibility (77.3%), while the DMD for the middle cut silages and late cut silages was 68.5% and 53.2%, respectively.

Table 9 The parameter estimation, pH measurement and fermented dry matter in compound feed and silages

Feed	A	B	C	pH	DMD (%)
DRER	280.5	8.1^{c}	1.8	6.4	86.3^{a}
AUMD	272.9	8.4^{bc}	1.9	6.4	84.8^{a}
AUNA	268.2	9.4^{a}	1.9	6.4	76.7^{b}
AUAB	299.3	9.1^{ab}	1.9	6.4	69.1^{c}
Root MSE	18.406	0.478	0.193	0.031	3.391
P	0.073	0.002	0.116	0.341	<.0001
E-CT	274.7^{a}	8.0^{c}	1.7^{a}	6.3^{c}	77.3^{a}
M-CT	250.2^{ab}	9.2^{b}	1.6^{b}	6.4^{b}	68.5^{b}
L-CT	235.5^{b}	12.8^{a}	1.5^{b}	6.4^{a}	53.2^{c}
Root MSE	20.359	0.552	0.076	0.042	4.556
P	0.038	<.0001	0.000	0.000	<.0001

A, asymptotic gas production (ml g-1 OM); B, the time of incubation where half of a produced (h); C, the shape of the curve; statistical difference at P < 0.05; DMD, the fermented dry matter after 48h; pH endpoint measurement

The parameter estimations and dry matter degradability of mixed ration are presented in Table 10. Similarly, there were no significant differences in asymptotic gas production among all mixed rations. However, for the DMD value, DRER-ECT and AUMD-ECT showed the highest degradation value 82% and 79.3%, respectively, followed by AUAB-ECT, DRER-MCT, AUMD-MCT AUNA-MCT AUAB-MCT and AUNA-ECT.

The significantly lowest degradation value was observed in the compound feed mixed with late cut silages, varied between 65.4% and 67.9%. The highest pH measurement was observed in the compound feed mixed with late cut silages, while the lowest measurement was observed for mixed diet with early cut silages.

Table 10 The parameter estimation, pH measurement and fermented dry matter in mixed rations

Feed	A	B	C	pH	DMD
DRER-ECT	281.0	7.8^{c}	1.6^{a}	6.3^{c}	82.0^{a}
DRER-MCT	274.4	8.5^{b}	1.5^{b}	6.4^{b}	72.7^{b}
DRER-LCT	270.4	10.2^{a}	1.4^{b}	6.4^{a}	65.4^{c}
AUMD-ECT	280.2	7.8^{c}	1.6^{a}	6.3^{c}	79.3^{a}
AUMD-MCT	279.2	8.4^{bc}	1.5^{b}	6.4^{b}	71.0^{b}
AUMD-LCT	263.4	10.3^{a}	1.4^{b}	6.4^{a}	65.4^{c}
AUNA-ECT	284.9	8.2^{b}	1.6^{a}	6.4^{b}	70.9^{bc}
AUNA-MCT	270.2	8.5^{b}	1.6^{a}	6.4^{b}	71.7^{b}
AUNA-LCT	259.1	9.9^{a}	1.6^{a}	6.4^{a}	67.9^{c}
AUAB-ECT	282.2	8.0^{bc}	1.7^{a}	6.4^{b}	79.1^{ab}
AUAB-MCT	271.2	8.8^{b}	1.6^{a}	6.4^{b}	71.5^{b}
AUAB-LCT	258.3	10.2^{a}	1.6^{a}	6.4^{a}	68.8^{c}
Root MSE	16.320	0.382	0.080	0.014	3.841
P	0.497	<.0001	<.0001	<.0001	<.0001

A, asymptotic gas production (ml per g DM); B, the time of incubation where half of the asymptotic gas production is achieved (h); C, the shape of the curve; statistical difference at P < 0.05; DMD, the fermented dry matter after 48h; pH endpoint measurement

4.6 Description of total gas production at different time points

The total GP after 12, 24, 36 and 48h are presented in Table 11, showing that the total GP were increased with incubation hours. The late cut silages and their mixed rations showed lowest total gas production, while the highest amount of gas production was observed in the feed DRER-ECT and AUAB.

Table 11 Description of the total gas production at different time intervals

Feed	GP12 (ml)	GP24 (ml)	GP36 (ml)	GP48 (ml)
AUAB	187.3	257.6	278.3	286.7
DRER	186.7	244.4	261.6	268.8
AUMD	181.4	240.3	256.8	263.3
AUNA	165.1	229.6	248.8	256.5
E-CT	183.7	239.3	256.0	263.1
M-CT	151.4	205.3	224.3	233.1
L-CT	112.0	170.0	194.9	207.5

DRER-ECT	187.6	241.5	258.9	266.6
DRER-MCT	171.1	225.8	245.5	254.8
DRER-LCT	150.1	206.6	229.8	241.7
AUAB-ECT	186.9	243.5	261.2	268.9
AUAB-MCT	168.3	225.1	244.7	253.8
AUAB-LCT	145.9	205.0	227.0	237.4
AUMD-ECT	185.7	239.2	256.9	264.9
AUMD-MCT	175.9	230.4	250.0	259.4
AUMD-LCT	145.2	200.9	223.8	235.4
AUNA-ECT	185.7	243.1	261.7	269.9
AUNA-MCT	171.2	227.3	246.1	254.6
AUNA-LCT	149.2	207.4	228.8	238.9
Mean	**145.6**	**203.5**	**225.3**	**235.8**
Minimum	**112.0**	**170.0**	**194.9**	**207.5**
Maximum	**187.6**	**257.6**	**278.3**	**286.7**

GP 12 / 24/ 36/ 48 are gas production at 12, 24 ,36 and 48 hours.

4.7 Estimation of OMD and ME

The calculated OMD and ME at different time points (24h and 48h) are shown in Table 12. For overall description, the OMD and ME value of all feeds were increased with incubation hours, with approx. 4-5% of increasement observed in the organic matter digestibility. For comparison, the lowest OMD and ME observed in the late cut silages and late cut silage mixed rations. However, AUNA-LCT showed better nutritional values than other late silage groups. The compound feed AUAB revealed the highest value of OMD and ME content, alternatively the lowest number observed in the late cut silages (L-CT).

Table 12 The calculated organic matter digestibility and metabolizable energy among all feeds

Feed	**OMD_{24} (%)**	**OMD_{48} (%)**	**ME_{24} (MJ/kg DM)**	**ME_{48} (MJ/kg DM)**
AUAB	70.2	75.4	11.8	12.3
DRER	67.6	71.9	11.6	12.0
AUMD	66.7	70.8	11.6	11.9
AUNA	65.8	70.6	11.4	11.8
E-CT	68.2	72.4	11.4	11.8
M-CT	59.3	64.2	10.8	11.2
L-CT	50.1	56.7	10.2	10.7
AUNA-ECT	68.5	73.3	11.6	12.0
AUNA-MCT	64.4	69.3	11.3	11.7
AUNA-LCT	59.5	65.2	10.9	11.4
AUAB-ECT	68.3	72.8	11.6	11.9

AUAB-MCT	63.7	68.8	11.2	11.7
AUAB-LCT	58.8	64.6	10.9	11.4
DRER-ECT	67.7	72.2	11.5	11.9
DRER-MCT	63.7	68.9	11.2	11.7
DRER-LCT	58.9	65.2	10.9	11.4
AUMD-ECT	67.2	71.8	11.5	11.9
AUMD-MCT	64.4	69.6	11.3	11.8
AUMD-LCT	57.8	64.0	10.8	11.3
Mean	**63.7**	**68.8**	**11.2**	**11.7**
Minimum	**50.1**	**56.7**	**10.2**	**10.7**
Maximum	**70.2**	**75.4**	**11.8**	**12.3**

OMD 24/48, ME 24/48 are organic matter digestibility and metabolizable energy calculation based on gas production at 24h and 48 h respectively

Furthermore, we observed increase of OMD (approx. 4-5%) and ME (around 0.4-0.5 MJ) value in M-CT-concentrate mixture group, compared with M-CT silage tested alone. The L-CT silages group showed the biggest improvements when mixed with concentrates, where the OMD and ME increased by approx. 8-9% and 0.6-0.7 MJ respectively, in contrast to L-CT tested separately.

4.8 Fractional rate of gas production

The fractional rate of gas production profile is shown in Figure 8. For compound feeds, the overall profile was similar among all feeds. However, feed AUNA and AUAB revealed the slightly slower rate before 12 hours incubation. For silages, the result was significantly different among three different quality silages, the early cut silages have the highest value. For mixed rations, the slowest fractional rate was observed in the late cut silages mixed with compound feeds and the difference was significant before 12 hours. The diet AUMD-LCT revealed the lowest fractional rate, especially after 4 hours

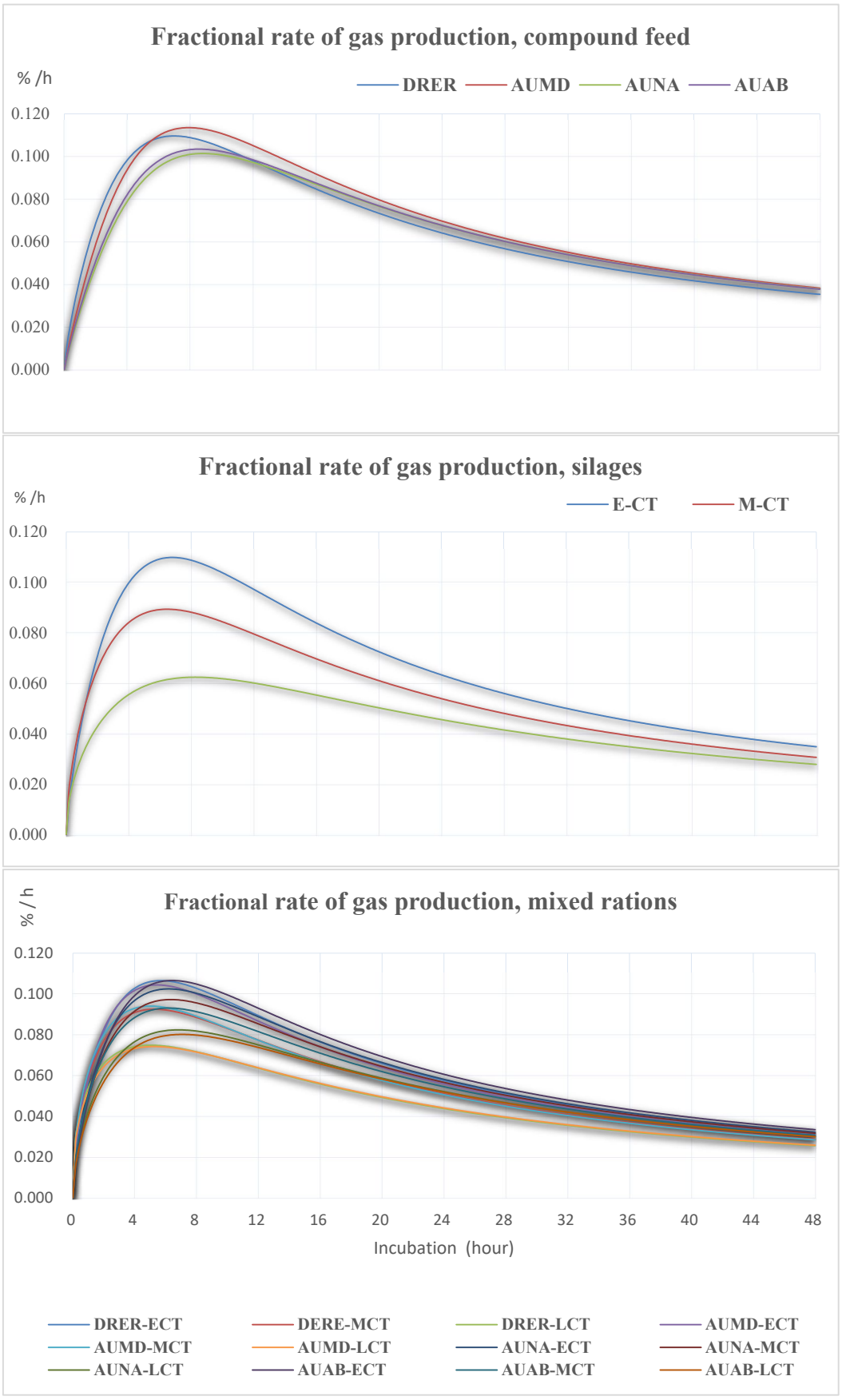

Fig.8 Fractional rate of gas production of all feeds

5 Discussion

The primary objective of this study was to compare the degradation characteristics and predicted digestibility of alkaline grain diet based on a high level of Norwegian ingredients with standard soy-based concentrate. Furthermore, the effects of mixing different formulations of compound feed with different qualities of grass/clover silage were tested. Overall, the results indicated that the alkaline grain diet compared with the standard diet have better or similar DM digestibility and gas production profile. The calculated OMD of the middle cut and late cut silages improved when mixed with concentrates. Hence, the possible reasons and the effects of the Alka-treatment will be discussed below.

5.1 The role of grain and alkaline grain

Grain fermentability determines the feeding value of the diets for ruminants by affecting volatile acids production, ruminal pH, cellulolytic activity, energy, and microbial protein supply (Archimède et al., 1997). Grains are usually fed to dairy cattle to increase the energy density to support high production requirements. However, when cows are fed with high amount of rapidly digestible starches such as barley in the mixed diet, negative associative effects were often observed and these effects reduce the feed utilization efficiency (Owens et al., 1986). Besides, cows may experience subacute rumen acidosis (Nikkhah, 2012). However, the digestion patterns or the adverse effects can be minimized by the grain processing or chemical pre-treatments, improving the use of efficiency of grains in the forage-grain mixed diets (Dixon & Stockdale, 1999; Humer & Zebeli, 2017). For example, the whole barley is less digestible for large ruminants, because of the grain coat. They usually rolled, ground, steam-flaked or pelleted prior to feeding (Nikkhah, 2012). However, external processing will increase the rate and extent of digestion in the rumen at the same time may cause a risk of suboptimal condition as mentioned above. Hence, some researchers found using high pressure heat-treatment or adding water prior to the rolling process to increase the moisture content and reduce too fine particle , therefore

reduce the rate of digestion and possibilities of rumen acidosis during fermentation peak (Anderson & Schroeder, 1999; Ljøkjel et al., 2003; Owens et al., 1997) furthermore increase ruminal pH, nutrients digestibility and feed efficiency.

In addition, numerous studies found increased straw digestibility with alkali treatment and frequent positive results were observed in the whole barley treated with sodium hydroxide solutions (Ørskov & Greenhalgh, 1977). The optimal rumen pH is balanced by the volatile fatty acids removal, and the buffer solution secreted into the rumen; besides, some dietary exogenous buffering feedstuffs may cause buffer activity (Allen, 1997; Dijkstra et al., 2012). Treatment of grain with urea might improve buffering capacity and enhance the crude protein content of these starchy grains as observed in our result, which agrees with other studies(McNiven et al., 1995; Miron et al., 1997). Furthermore, in the alkaline diets, little ruminal pH fluctuations have been found (McNiven et al., 1995).

In addition, the alkaline treatment, AUMD diet with high inclusion of the barley, did not affect the digestibility of the concentrate feed compared with the standard soy-based concentrate feed. No significant differences were found in the dry matter digestibility and starch digestibility among feed AUNA, AUAB and DRER using *in sacco* work. Alternatively, the AUMD compared with DRER showed a higher DMD. Furthermore, higher digestibility of CP and NDF were observed in the contrast group (AUMD, AUAB, AUNA) compared with the control diet DRER. Three possible reasons may explain this: Firstly, there is lower NDF and ADF contents in the contrast group compared with control diets. It is well known that the NDF digestibility (NDFD) negatively correlates with the feed NDF concentration; the higher NDF content may cause the lower NDFD (Collins et al., 2017; Volden, 2011). Secondly, alkaline grain increased the available energy and available N at the same time corresponding to the importance of synchronization of energy yielding substrate and N content as an important factor for increased efficiency of microbial protein synthesis and feed utilization (Nikkhah, 2012; Van Vuuren et al., 1989) . Last but not least, the alkaline treatment not only provide a satisfactory environment for feed digestion but also may enhance the digestibility of the NDF, by dissolving the linkage between the lignin and polysaccharides (Anderson et al., 1981; Chen et al., 2013; Ørskov & Greenhalgh, 1977).

The lowest nutrients digestibility and slower degradation rate (especially before 24 hours *in sacco* incubation) were revealed in the pellet form of the AUMD, The reason could be that the feeds in its pelleted form is less accessible for the microbes; usually the microbes need some time to attach and attack the feed through the bag pores as explained by (Stern et al., 1997). In addition, a negative value found in the soluble fraction of the pelleted AUMD, which was not expected and not plausible, is possible because that the soluble fraction is assuming particle loss after zero hour washing, hence little particle loss after 35 minutes washing of pellet, but the pellet may reabsorb water from surroundings after washing and drying.

For *in vitro* gas production, we observed similar results on gas production profiles, degradation values (DRER and AUMD) and endpoint pH level among all concentrates. As we know, gas production is mainly from ruminal degradation of carbohydrates, which reflects the extent of the fermentation and substrate digestibility (López et al., 1998; Pell & Schofield, 1993). Hence, it appears that the control diet and the contrast group have similar fermentation patterns and degradation ranking, at least which is true for feed DRER and AUMD.

5.2 The effect of silage maturity

As expected, the early cut silage group showed the numerally higher gas production, gas production rate, and dry matter digestibility in our result, which was proven by (Menke et al., 1979) the total amount of gas production decrease with the enhancing maturity(Menke et al., 1979). The cell wall content in the forages increases with the aging state (Collins et al., 2017) and, this change causes reduction in the gas production rate as well as feed digestibility (De Visser et al., 1990). The early cut silage contains a relatively higher readily fermentable substrates and less cell wall contents, in contrast to late cut silages. The soluble fraction and rapidly fermentable substrate positively correlated with the gas production rate (Beuvink, 1993). In addition, the availability of the substrate affecting the microbial growth and gas production rate; the more substrate available the higher gas production rate (Beuvink, 1993). Furthermore, in our gas production system, relatively

higher pH was observed in the late cut silages, which may be due to less available fast fermenting sugars in the late cut silages representing less proportion of the propionic acid production (De Visser et al., 1990).

5.3 Associative effects and digestibility of concentrate-forage diet

Grains or concentrates are usually easily digestible and have high energy density. Hence, a linear increasement of the digestibility of mixed diets are expected when graded levels of the concentrate feeds are added in the diets. However, the digestibility and energy level of the mixed diet increases quite slowly compared to if they are fed separately (Dixon & Stockdale, 1999). These results are explained by the associative effects, including digestive and metabolic interactions, which affect the digestibility and metabolizable energy supply (Dixon & Stockdale, 1999). Concentrates added to the roughage diets have both positive and negative effects: the positive associative effects are seen as the ME is greater than when they fed alone while, the negative associative is when ME is less than expected.

The M-CT group and L-CT group were ranked in descending order of high quality silage mixtures to low quality silage mixtures. In addition, depending on the gas production data, the calculated OMD and ME increased approx. 4-5% and 0.4-0.5MJ in middle cut silage mixtures compared with M-CT group tested separately (Table 12). While the difference between late cut silage mixtures and L-CT group was even bigger, approx. 8-9% for OMD. and 0.6-0.7 unit for ME, respectively.

The reason could be explained that the concentrate supplement is supplying additional nitrogen and easily fermentable carbohydrates to increase microbes for digesting forages as observed by (Ørskov, 1999). In addition, as well known, the cell wall components covered by the lignin resisting enzymatic digestion, however the alkali treatment on grains dissolved the hemicellulose and lignin (Jackson, 1977) and the digestibility of the cell wall component increased by the alkali treatment breaks linkages between lignin and polysaccharides (Humer & Zebeli, 2017). This effect has a beneficial effect for fiber digestion as observed in our result, which also has proven by (Anderson et al., 1981)

5.4 *In sacco vs. in vitro* gas production in this study

The nylon bag technique and *in vitro* gas production were proven to be reliable techniques to predict the *in vivo* digestibility, energy supply and microbial synthesis, feed intake and animal performance(Dijkstra et al., 2005; Khazaal et al., 1993; Krishnamoorthy et al., 1991; Ørskov & Reid, 1989). Comparative analysis of *in sacco* and *in vitro* data from our results were difficult and less meaningful, since only the 4 concentrate feeds were tested in the *in sacco* method. However, these four types of concentrate feeds were presented the same ranking of kinetic patterns in these two trials, with a slight difference in the gas production profile. The big difference is in nylon bag technique assumes to have a soluble fraction and potential degradable part , and the fractional rate of the digestion is rate of the slowly degradable part. Whereas for the *in vitro*, the rate of the gas production was based on a single degradation pool.

6 Conclusion

As expected, the alkaline technique creates the opportunities of high inclusion of local grain in the diet without negative effect on the dry matter digestibility, gas production profile, and possible energy supply. Urea pre-treatment increased the crude protein content of starchy grain and showed better or similar DMD value as standard concentrate diet (containing high proportion of imported soya) in *in sacco* study. In addition, this benefit was also found in *in vitro* gas production work; although there was no significant difference in total GP among all concentrates feed, feed AUNA tend to have a lower amount of GP as well as rate of GP. Furthermore, positive associate effects were found in a high proportion of alkaline grain mix with different quality silages. The cumulative GP and fractional rate of GP of middle cut and late cut silage mixtures were higher than they examined separately.

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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