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Department of Animal and Aquaculture Sciences
Faculty of Biosciences
Supervisor: Egil Prestløkken

Evaluation of feeding value of cereals and legumes for ruminants using in vitro gas production

Vurdering av fôrverdi av korn og belgvekster til drøvtyggere ved bruk av in vitro gassproduksjon

Ke Chao
Master in Feed Manufacturing Technology
Faculty of Biosciences

Declaration

I, Ke Chao, declare that this thesis is a result of my research investigations and findings. Resources of information other than my own have been acknowledged and a reference list has been appended. This thesis has not been previously submitted to any other university for award of any type of academic degree.

Signature…….

Date……02.06. 2020……..

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Ke Chao

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List of Abbreviations

g	gram
kg	kilogram
^{o}C	degree Celsius
mm	millimeter
min	minute
mmol	millimole
h	hour
ml	milliliter
L	liter
OMD	organic matter digestibility
ME	metabolizable energy
GP	gas produciton
DM	dry matter content.
OM	organic matter content
CP	crude protein content
aNDFom	ash corrected neutral detergent fiber

Summary

This thesis consists of a literature background and laboratory experiment. The literature review contains knowledge of raw ingredients, ruminal digestion of nutrients and feed evaluation methods of ruminants. Feed quality crucially effect either milk yield or growth rate of ruminants. Both in vivo and in situ methods are largely limited on feed evaluation. Therefore, the purpose of experiment was to characterize different feed ingredients and explore the relationship between chemical composition and feed degradation using in vitro gas production technique.

Nine different feeds and (or) ingredients (total mixed ration, barley, wheat, maize, oats, peas, horse bean, pea starch and horse bean starch) were milled to 1 mm particle size. The experiment was designed in 2 batches * 9 feed samples * three modules for each feed. Rumen fluid was collected from two non-lactating dairy cows with rumen cannula. Feed samples were incubated in buffered rumen fluid using in vitro gas production techniques. Gas production kinetics for 48 h incubation was measured and organic matter digestibility and metabolizable energy were calculated based on gas production and chemical composition of the feeds/ingredients.

In general results on gas production kinetics and estimated energy value consistent to previous references and the hypothesis for the experiment. Result demonstrates that starch in cereals and legume starch showed better degradability than in whole legumes. Maize had highest gas production but lowest gas production rate. In contrast, oats showed the most rapid fermentation but lowest gas production. There was a positive ($P<0.001$) correlation between starch content and in vitro gas production. Strong negative correlations occurred between aNDFom ($P<0.001$) content and gas production. Starch and CP level was positively correlated ($P<0.05$) with OMD and ME. Starch and aNDFom was positively correlated to gas production rate, whereas CP showed negative correlation ($P<0.05$) to gas production rate.

In vitro gas production technique can be used as an alternative of in situ technique to evaluate feed quality and predict energy value. The chemical composition and nutrients interaction of feeds highly effect nutrient degradation and energy value.

Kay words: In vitro gas production, chemical composition, rumen digestion, feed value.

Sammendrag

Denne avhandling består av en litteraturdel og egne undersøkelser. Litteraturdelen tar for seg råvarevurdering, fordøyelse av næringsstoffer og metoder for fôrmiddelvurdering til drøvtyggere. Fôrkvaliteten påvirker melkeyting og vekst hos drøvtyggere. Både in vivo og in situ metoder har sine begrensninger i vurdering av fôrverdi til drøvtyggere. Formålet med egne undersøkelser var å vurdere forskjellige fôrtyper med hensyn på fôrverdi ut fra kjemisk sammensetning og nedbrytning av fôr ved hjelp av in vitro gassproduksjonsteknikk.

Ni ulike fôrtyper og/eller fôrprøver (blandet fôrrasjon, bygg, hvete, mais, havre, erter, åkerbønner, ertestivelse og åkerbønnestivelse) ble malt på 1 mm sold. Disse ble undersøkt med in vitro gassproduksjon i 2 batcher med tre gjentak for hver fôrtype. Vomvæske ble tatt fra to ikke-lakterende kyr via ei vomkanyle og blandet med buffer. Fôrprøvene ble inkubert i bufret vomvæske og in vitro gassproduksjon ble målt over 48 timer. Gassproduksjonskinetikk for de 48 timene, og fordøyelighet av organisk stoff (OMD), ble beregnet. Omsettelig energi (ME) ble beregnet basert på gassproduksjon og kjemisk sammensetning av fôrprøvene.

Generelt er resultatene for gassproduksjonskinetikk og beregnet energiverdi i samsvar med tidligere forsøk og hypotesene for forsøket. Resultatene viste at stivelse i korn og stivelse fra belgvekstene hadde høyere nedbrytning i vom enn erter og åkerbønner. Mais hadde høyest gassproduksjon, men lavest gassproduksjonshastighet. Motsatt hadde havre høyest gassproduksjonshastighet, men lavest gassproduksjon. Det var positiv korrelasjon mellom stivelsesinnhold og in vitro gassproduksjon, mens det var en sterke negativ korrelasjon mellom aNDFom ($P<0.001$) og in vitro gassproduksjon. Innholdet av stivelse og råprotein (CP) var positivt korrelert ($P<0,05$) med OMD og ME. Stivelse og aNDFom var positivt korrelert, mens CP var negativt korrelert ($P<0,05$) med gassproduksjonshastigheten.

Konklusjonene fra forsøket er at in vitro gassproduksjon er et alternativ til in situ nedbrytning for å vurdere fôrkvalitet og forutsi energiverdi av fôr, og at kjemisk sammensetningen og samspill mellom næringsstoff i sterk grad påvirker nedbrytningen og energiverdien av fôr.

Nøkkelord: In vitro gassproduksjon, kjemisk sammensetning, vomfordøyelse, fôrverdi.

1. Introduction

Ruminant production both milk yield and growth are significantly influenced by feed quality which is mainly reflected in voluntary intake and extent of digestion (Getachew, DePeters et al. 2005). Dynamics of nutrient digestion in the rumen are one major determinant of utilization of feedstuffs by ruminants. Therefore, evaluation of digestibility and feed value of feed stuffs in rumen is necessary. In addition, it is also important from an environmental perspective, to reduce excretion of undigested feed nutrients, as well as emission of green-house gases. In developed countries, animal production systems are facing greater challenge of reducing environmental degradation associated with metabolic wastes in feces, urine and gases (Getachew, DePeters et al. 2005)..

Cereals represent the primary energy source in ruminant diets due to its cost-effective and high content of starch (Gozho and Mutsvangwa 2008). Legumes and its by-product are commonly used as a crucial protein sources in beef and dairy cow because of high available protein and complex amino acid combination. Nutritive value of feed depends on adequate nutrients with respect to chemical composition and degradability. It is important to find correct feeding value of these cereals and legumes in order to increase utilization and obtain a nutritionally balanced ration to the animals. Therefore, in order to complete feed evaluation, an effective tool is needed that results digestion in animals reflectively. In vivo technology as a traditional technology of evaluating feed nutritive value and digestibility is time-consuming, laborious, too costly and unsuitable for large-scale feed evaluation (Coelho, Hembry et al. 1988). In situ technique is not suited due to substrates with small particles are easily escaping through bags, which brings a very limited in situ data on site of starch degradation. Alternatively, in vitro gas production could be a method to evaluate the feed with convenient operation, less cost and allowing large number of samples to be evaluated simultaneously. In rumen, the starch is fermented to short chain fatty acids (acetate, propionate and butyrate) (Beuvink and Spoelstra 1992) which can be absorbed by rumen to provide energy for ruminant. Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation (Wolin, 1960). The contribution of fat to gas

production is negligible (Wolin 1960). Based on rumen digestion mechanism, in vitro gas production technique combined with gas production from rumen fermentation and chemical composition of feed to assess kinetics of fermentation, effects of secondary constituent and predict digestibility of feed.

The objective of this study was to investigate: (1) Feasibility of using in vitro gas production technology to evaluate feed degradation by gas production characteristics. (2) Variation of gas production kinetics among different samples (cereals vs legumes; legumes vs extracted starch from legumes; maize vs oats). (3) Estimation of organic matter digestibility and metabolizable energy based on gas production and chemical composition of feed. (4) Relationship between chemical composition of feed, gas production and feed energy value.

2. Theoretical literature review

2.1 Cereal grains

The term ‘cereal’ is given to the members of the Gramineae that are cultivated for their seeds (McDonald 2011). In general, nine species are determined: wheat (Triticum), rye (Secale), barley (Hordeum), oat (Avena), rice (Oryza), millet (Pennisetum), corn (Zea), sorghum (Sorghum), and triticale which is a hybrid of wheat and rye. For over a decade, cereal grain production worldwide has exceeded two billion tons, with growth ration around 2–3% annually. Almost 50% of cereal production is consumed as food, with another 35–40% used as feed and the remainder in other industrial utilization which includes production of starch and cereal-based by-products and alcoholic beverages. Cereal grains consist three major portions, the endosperm and embryo and bran. The different cereal crops can be easily recognized by their grains or flowering heads. For instance, wheat, rye and maize grains consist of the seed enclosed in a fruit coat (the pericarp) which are referred to ‘naked’ caryopses (kernels) (McDonald 2011). In barley and oats, the kernels are enclosed in husks formed by the fusing of the glumes (palea and lemma) and are referred to as ‘covered’ caryopses (Figure 1).

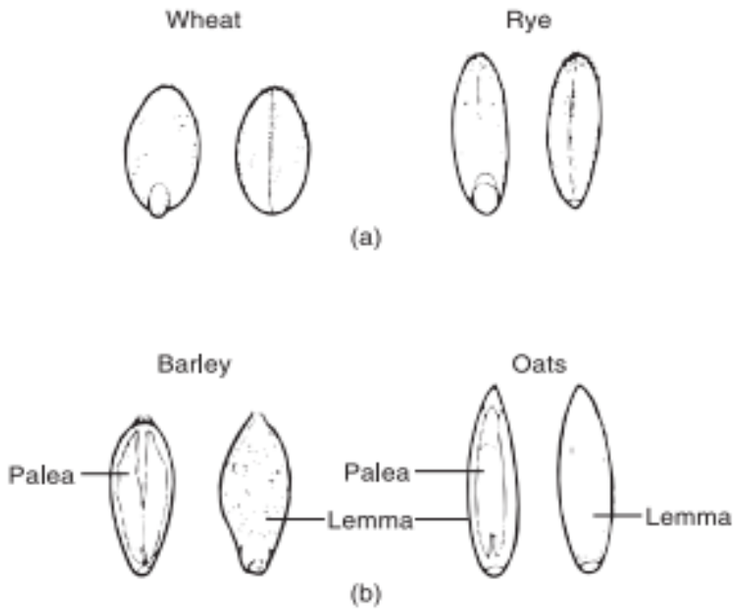


Figure 1. Kernels: (a) ‘naked’ kernels, (b) ‘covered’ kernels. (Finch, Samuel et al. 2014)

Composition and nutritive value of grain cereals

In feed evaluation and formulations, it is important to know the chemical composition of different feed ingredients. Starch is the major component stored in cereal grains, which is composed by two types of polysaccharides: 25% amylose and 75% amylopectin respectively. Chemical composition can vary due to many factors, including genotypes, environmental factors and cultivars (Watson and Ramstad 1987). Chemical composition of some of the cereal grains is shown in Table 1.

Table 1. Average chemical composition of some cereals on dry matter basis.

Grains	DM(g/kg)	CF[3]	Ash	ADF[4]	NDF[2]	CP[1]	Starch
Barley	860	53	26	64	201	115	599
Wheat	860	26	21	30	124	124	701
Maize	860	24	13	28	117	98	717
Oats	860	105	33	149	290	109	482
Sorghum	860	21	27	57	107	108	745

[1] Crude protein.

[2] Neutral detergent fiber; acid detergent fiber.

[3] Crude fiber.

[4] Acid detergent fiber.

Data of crude protein is from MAFF 1990 UKTables. Rest of data are from book chapter (McDonald 2011).

As shown in the table 1, wheat, sorghum and maize have the highest content of starch, followed by millet, barley and oats. Crude protein content is highest in wheat and lowest in maize, there is no significant differences in other ingredients. In addition, oats contain highest CF and NDF content, barley in the follow, wheat and maize contain relatively low content of these components.

Description of starch granule in grains

In cereals and other higher plants, starch granules are formed in amyloplasts. Each amyloplast contains one starch granule. The starches from widely in size and shape among different grains (Delcour and Hoseney 2010) (Table 2).

Table 2. Properties of starches granule from different cereals.

Grains	Granule shape	Granule size[1] (μm)
Barley	Round	20-25
	Elliptical	2-6
Wheat	Lenticular	20-35
	Round	2-10
Oat	Polyhedral	3-10
Maize	Round	15
Sorghum	Round	25

[1] Data is adapted from (Lineback 1984)

Wheat and barley have two shapes and sizes of starch granule. For barley, the size of spherical granules larger than elliptical granules with granule size 20-25 μm and 2-6 μm respectively. For wheat starch, the large, lenticular granules are sized 20-35 μm, the small, spherical granules are sized 2–10 μm. The starch granules of maize and sorghum both have spherical shape and quite same size 15 μm in maize and 25μm in sorghum. The starch granules of oats are small with 3-10 μm and shaping polyhedral (Delcour and Hoseney 2010).

Starch granules are mainly consisting three polymers, α-glucan which composed amylose, amylopectin, and (1→3,1→4)-β-glucan. The α-glucans amylose and amylopectin (Figure 3) are main components of starch granules, accounting for 30 and 70% (w w^{-1}) respectively, which is accumulated by MacGregor and Fincher (MacGregor and Fincher 1993). The (1→3,1→4)-β-glucans are the main components of cell walls in the starchy endosperm of barley grains, accounting for about 70% (w w^{-1}) of those walls (Fincher1975)

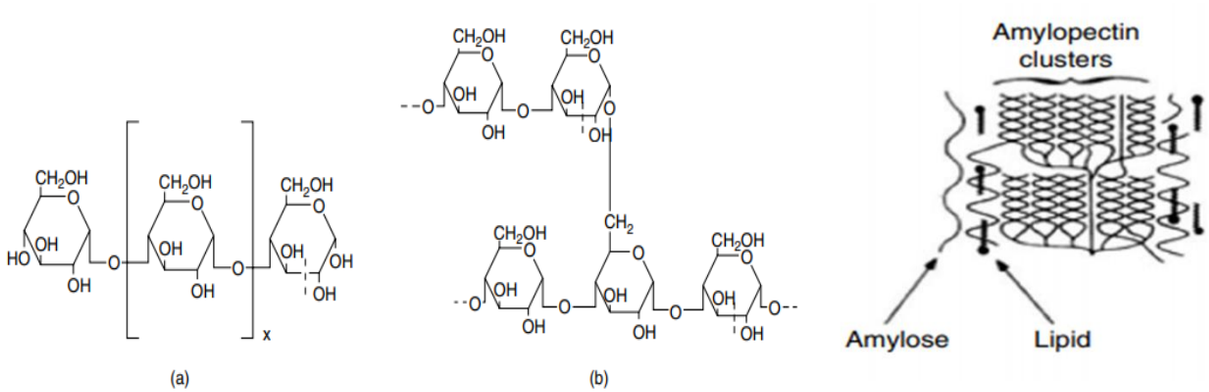


Figure 2. Schematic diagram of (a) amylose; and (b) amylopectin and crystal structures of the starch polymers. (Trafford and Fincher 2014)

In cereal, the endosperm cells are packed with starch granules in a protein matrix. Maize protein occurs in the endosperm as a matrix protein and discrete protein bodies which are composed mainly of a prolamin called “zein”. (Delcour and Hoseney 2010). The endosperm of maize contains about 44% zein protein which has been proved with negative correlation to ruminal starch degradability (Philippeau, Landry et al. 2000). The distribution of protein in oats is different comparing in the other cereals, the prolamin of oats constitute only 10–15% of the total protein. The prolamin of barley and wheat make up about 40% of the protein but lower zein protein than maize due to their proteins are relatively high in lysine. The zein and cross-linked zein fractions are detected very low in lysine (Delcour and Hoseney 2010).

2.1.1 Barley.

Barley (Hordeum vulgare L) is one of the oldest cultivated plants, and according to statistics (FAOSTAT, 2017), 146.3 million tons barley is annually cultivated worldwide. In terms of cereal production quantity, barley ranks fourth in the world, following maize, rice and wheat (Yangcheng, Gong et al. 2016). In many parts of the world, particularly in Norway, barley has been primarily used as the main energy source in the diets of ruminants due to its suitability for growth in the northern Norway.

Structure of barley

Barley consists of the surrounding husk – formed from the lemma (dorsal husk or hull) and palea (ventral husk or hull), pericarp, testa, aleurone layer, endosperm and germ or embryo. The longitudinal section of mature barley is shown in Fig. 3.

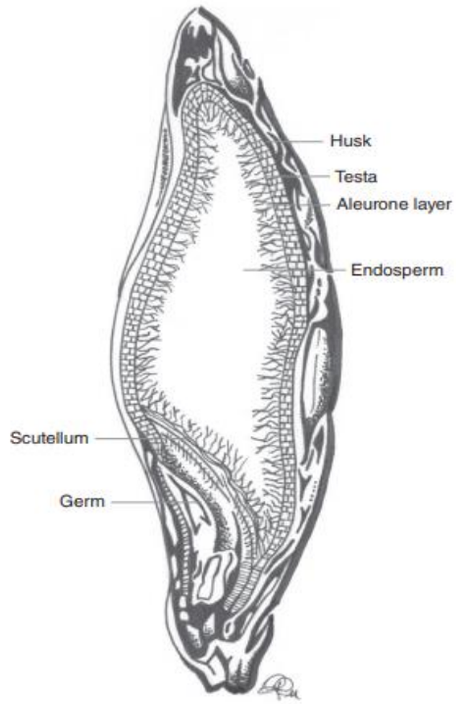


Figure 3. Structure of mature barley grain from (Arendt and Zannini 2013)

The starch content in barley ranges 62 to 77% of total grain dry weight (Bhatty and Rossnagel 1998). Husk and pericarp consist of cellulose, hemicellulose, lignin and lignan which is the major constituents of insoluble fiber. In the aleurone layer, the endosperm consists of protein-rich cells that containing enzymes involved in endosperm reserve mobilization(Jadhav, Lutz et al. 1998). In addition, other constituents, such as fats, polyphenols and pigmental materials, are deposited in the stable protein structure of this layer.

Composition of barley

Starch is the most abundant component in barley, consist approximately 68% of dry matter. The non-starch polysaccharides (NSP) in barley include cell wall components such as pentosans, β-glucan (3.6 to 9%), cellulose, fructan and glucomannan (MORRISON, TESTER et al. 1993). Protein content ranges around 10% to 11%, the amino acid composition of barley protein is quite same to the other cereal grains. Total fiber content ranges 11% to 34%, including 3 to 20% soluble dietary fiber (Cho 2001).

2.1.2 Maize

Maize (Zea mays), also well known as corn, is grown throughout the world, and over 1250 million tons of corn is cultivated on approximately 185 million/ha of land worldwide (FAOSTAT, 2017). Maize has the largest annual production and accounts for around three quarters of the annual coarse-grain production. Most of its production is used as feed. Maize exist in a variety of types and colors, commonly yellow color which contains a pigment, cryptoxanthin, a precursor of vitamin A. Maize grain is composed of endosperm approximately 80%, 10 % germ, 5 to 6 % pericarp, and (0.8%–1.0%) tip cap (Figure 4). The pericarp is outer layer of maize, characterized by high crude fiber content, mainly consisting of hemicellulose, cellulose, and lignin. In endosperms, large number of cells, each packed with starch granules embedded in a continuous matrix of protein (Singh, Singh et al. 2019). Furthermore, the cell walls consist of NSP (non-starch polysaccharides), including β-glucan and arabinoxylan, proteins, and phenolic acids. The germ is composed of the embryo, is characterized by high fat and protein content respectively 33% and 18%, and low starch content approximately 8% (Singh, Singh et al. 2019).

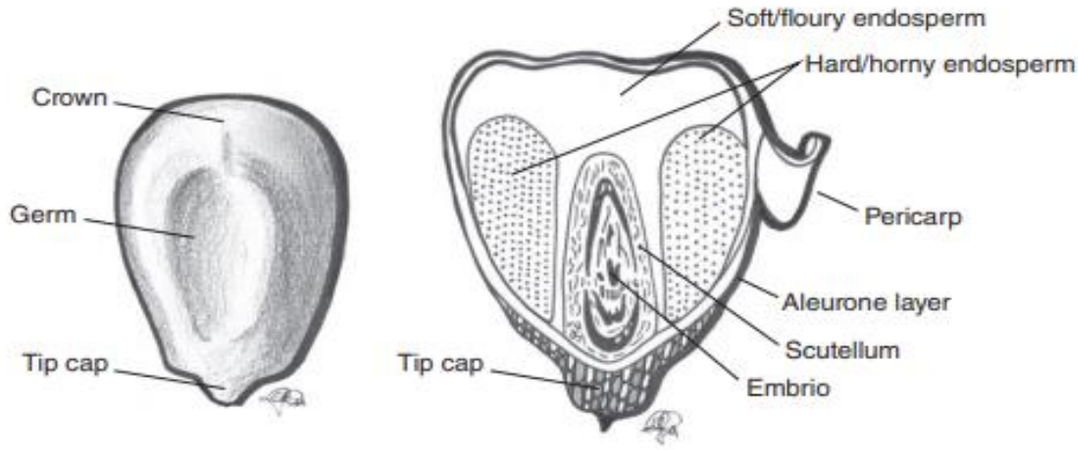


Figure 4. A maize kernel in longitudinal cross-section, showing its component parts (Corke 2015)

Composition of Maize

Starch is the most abundant constitution in maize, contributed 74.4% to 76.8% of maize on a dry matter basis (Odjo, Béra et al. 2018). The second abundant component of maize is protein, which

differs in content according to different section, for example, the grains of waxy maize contained higher (11.03%) protein content than normal maize (8.05%–8.62%) and flint-dent (8.5%–8.7%) maize (Odjo, Béra et al. 2018). Thereafter, quantity of 4–5 % lipid, 1–3 % sugar, and 1–4 % ash is followed in the maize kernel.

2.1.3 Oats

Oat (Avena) is ranked the fifth most economically important cereal, over 23.2 million tons of corn is cultivated worldwide (FAOSTAT, 2017). Oat is primarily used as feed for ruminant and horses due to its large content of soluble fiber, meanwhile, human consumption is increasing in recent years. The same as barley, a hull covers the oat caryopsis (Figure 5) and represents 30% to 40% of the total grain weight, mostly consist cellulose and hemicellulose.

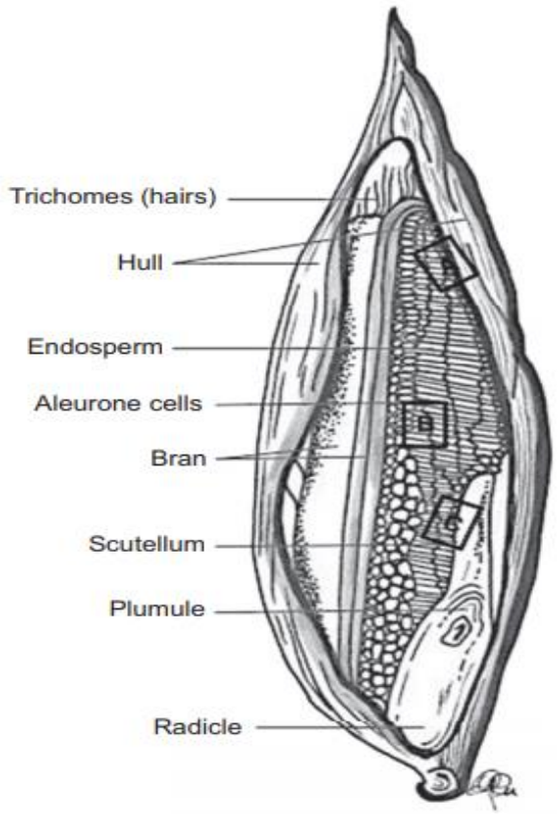


Figure 5. Morphology of the oat kernel (Arendt and Zannini 2013)

The caryopsis is composed of 38% to 40% bran, 3% germ, and 58% to 60% starchy endosperm (Lásztity 1998). Chemically, oat bran comprises 67.9 % carbohydrates, 15–22 % dietary fiber protein, 10.4 % β-glucan, 8.6 % fat which leads oats as a good lipid resource. The outer layer contains mainly protein, beta-glucan, and phenolic compounds. Inside of the endosperm consists of dominant quantity of proteins, starch, and β-glucan, whereas the germ contains mainly lipids and proteins (Lásztity 1998).

Composition of oat

Starch content ranges from 43% to 53% on a whole grain basis, which is dramatically lower than other grains. Because of the highly fibrous hull content (Frølich and Nyman 1988), whole oat has high fiber concentration, ranging from 20% to 37% in various samples. The protein concentration of oat is relatively high in comparison with other cereal grains, ranging 12% to 20 percent due to various of whole oat or hull-less samples. In contrast, oats contain much higher levels of lipids than other cereals (over two-fold) which is distributed throughout the endosperm and germ, therefore, oats are excellent source of lipids and unsaturated fatty acids (Brown, Alexander et al. 1966)

2.1.4 Wheat

Wheat (Triticum) represents one of the most important field crops in the world, originated from Syria to Kashmir, and southwards to Ethiopia. Over 854.9 million tons of wheat is cultivated worldwide (FAOSTAT, 2017). Wheat is the major grains in the diets of about a half of the world's population and therefore has an important impact on their nutritional quality. In Europe, wheat is also used as the energy source of feeding animals, it is mainly cultivated in the south of European countries. The main characteristic of the wheat kernel is oval shape of the embryo. Pericarps consist several layers, including the outer epidermis, hypodermis, cross cells, tube cells, seed coat and nucellar tissue (Delcour and Hoseney 2010) as shown in Figure 6.

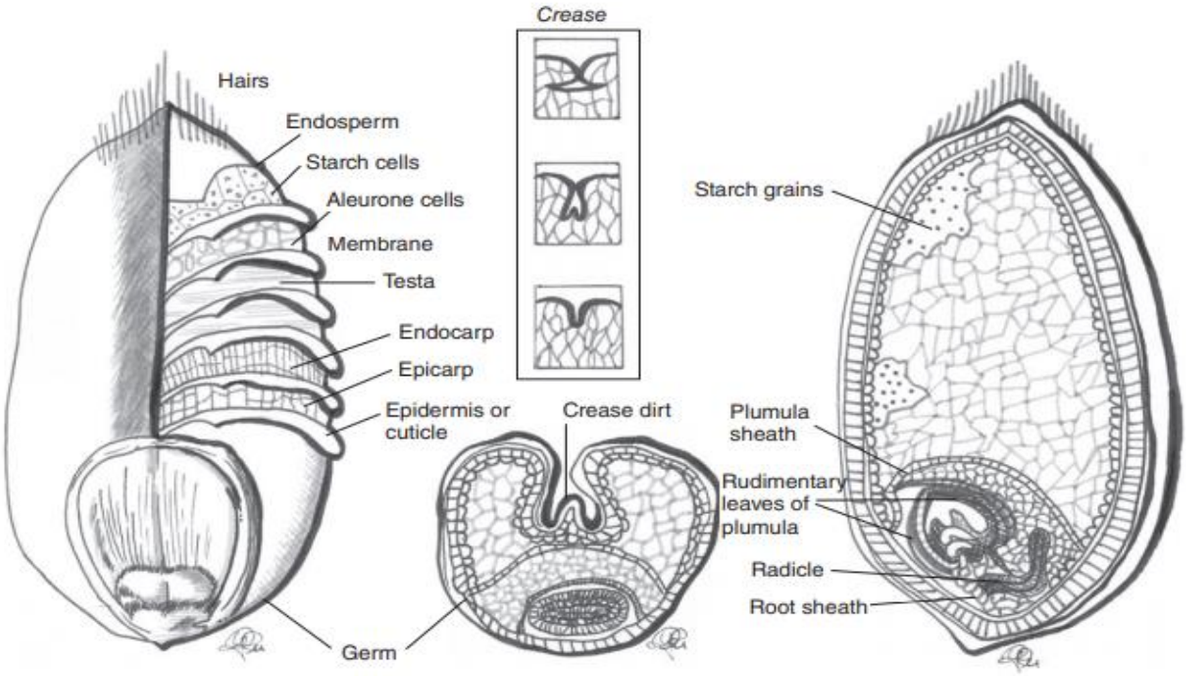


Figure 6. Longitudinal and cross-sections of wheat kernel. (Arendt and Zannini 2013)

The wheat germ ranges up 2.5–3.5 % of the kernel and locates at the lower dorsal side of the caryopsis, which is relatively high in protein (25 %), sugar (18 %) mainly sucrose and raffinose, and 5% ash (Delcour and Hoseney 2010). The starchy endosperm cell is composed by 75 % polysaccharide, 20 % 1–3,1–4 β-glucans, 7 % β-glucomannan and 2 % cellulose (Bacic and Stone, 1980).

Composition of wheat

Wheat consist similar starch and NDF content as maize, which ranges 70% to 75% and 10% to 13% respectively. Generally, the wheat protein content ranges from 6 % to more than 16% (Delcour and Hoseney 2010).

2.2 Pulses

The term legume encompasses more than 13,000 different species, legumes or pulses are members in Leguminosae family. Legumes play a dominant role in the diets of humans and

livestock worldwide, which is the second most important food source after cereal grains. The widely grown species are soybeans, peanuts, dry beans, peas, broad beans, chickpeas, and lentils. Since the forbiddance of animal proteins in ruminant concentrates in Europe, suppling rumen undegradable proteins from plant proteins is getting more important for lactation cows. Because of the high protein content and relatively high starch and oil content, legumes are globally used in animal industry particularly for ruminant. Prior to consumption, pulse seeds undergo several processing methods include dehulling, soaking, boiling, pressure cooking as well as germination (Eyaru, Shrestha et al. 2009).

Legume seed structure

Legumes seed coat vary in size, shape, color, and thickness among the different species. However, basic structure of seed has no significant differences. Mature legume seeds contain three major components: the seed coat (testa), the embryo, and the endosperm. In contrast of grain cereals have dominant portion of endosperm, most of legume seeds, however, have small portion of endosperm at maturity, as the cotyledons of the embryo, where the energy is stored for growth of embryo. Thus, the cotyledons provide the great majority of the nutritional components, with fiber and protein, of which a significant portion is found in the seed coat (Salunkhe and Kadam 1989). The structure of a typical legume seed, soybean seed, is shown in Figure 7.

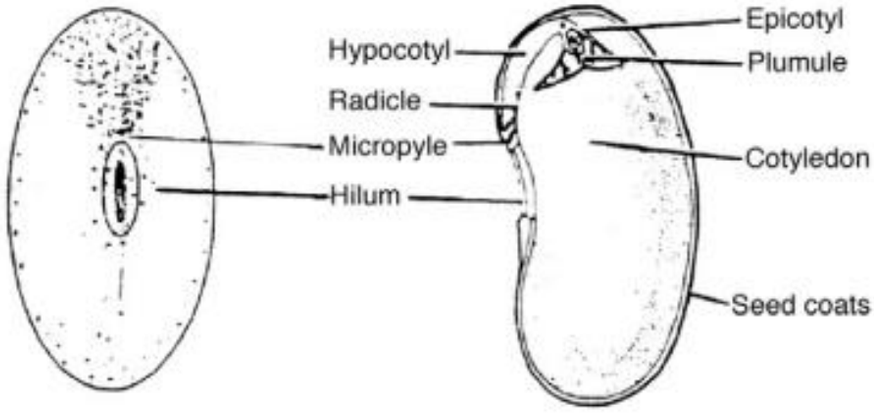


Figure 7. Structure of soya bean seed. From (Liu 1997)

The protein content of the legume ranges from 19.30% to 26.12% of the edible portion, particularly with some soybean seed varieties containing much than 50% protein (Salunkhe and

Kadam 1989). Carbohydrate as another main nutritional compound, generally contain about 60–65%, slightly lower than cereals (70–80%). The primary storage carbohydrate is starch, which constitutes a major fraction (8% to 36%) of the total carbohydrates (Reddy, Pierson et al. 1984). Thereafter, dietary fiber constitutes as the second important fraction of carbohydrate with range 14% to 28% in most of pulses. Typical chemical composition of some pulses is shown in Table 3

Table 3. Composition[5] of peas, horse bean, black chickpea blue lupin and vetch in dry matter basis.

Grains	DM (g kg^{-1})	CF[3]	Ash	ADF[4]	NDF[2]	CP[1]	Starch
Pea	901	43	25	73	101	201	325
Horse bean	900	62	31	112	216	236	287
Black chickpea	880	91	27	121	169	205	382
Blue lupin	900	16.1	26	229	269	291	80
Vetch	898	50	34	75	217	230	435

[1] Crude protein.

[2] Neutral detergent fiber; acid detergent fiber.

[3] Crude fiber.

[4] Acid detergent fiber.

[5] Data from (Grela, Kiczorowska et al. 2017) (Abreu and Bruno-Soares 1998).

2.2.1 Peas

Pea (Pisum sativum L.) is one of the oldest domesticated crops and is currently grown worldwide. Production of dry pea ranged 17.6 to 23.4 million tons/ha (FAOSTAT 2013). Peas are classified in categories, in which field peas, market peas and dried peas, mainly providing forage for animal feed, nutritional source for human consumption and animal concentrated feed respectively. Peas are recognized as a low-cost, readily available source of protein, carbohydrates, and minerals. LCA studies showed benefit of replacing soybean by European locally produced pea in ruminant feed, resulting in a 15% reduction of GHG carbon footprint of milk (Sasu-Boakye, Cederberg et al. 2014)

Composition of peas.

Protein content in peas vary depending on different species and growing environment, ranging

from 13.7% to 30.7% in seed DM (Hood-Niefer, Warkentin et al. 2012). Starch and fiber are major components of peas, 46 and 20 % of seed DM in average, respectively (Tzitzikas, Vincken et al. 2006).

2.2.2 Horse bean

Horse bean (Vicia faba L.) is an important food legume that distributed in more than 55 countries. Vicia faba is well known as its common name such as faba bean and horse bean or field bean. Production of horse bean worldwide is 4.56 million tons of dry grains (FAOSTAT, 2012). Horse beans form an important part of the diet of people in developing countries. In Europe, the horse beans are mainly used for animal feeding. It can be used in compound feeds in rations for cattle, sheep, pigs and poultry. The immature crop can be harvested for silage or hay. Horse bean silage makes an excellent feed for cattle and sheep (Adsule and Akpapunam 1996).

Composition of horse bean

Horse bean seeds have a relatively high protein content (26.2%). The major part of protein is characterized as globulin which is further differentiated into legumin and vicilin fractions (Adsule and Akpapunam 1996). Starch is the dominant component of seed carbohydrates. Other components was reported by Cerning (Cerning 1975), in which 41% starch, 10% sugars, 8% crude fibre and 1.5% lignin in bean seeds respectively.

2.3 Pulses by-product technology

Traditionally, pulses are used in a whole or split form. In recent years, in order to produce various product of pulses, pulses industry focused on the fractionation to produce various food ingredients. Two methods are commonly used to process pulse seeds into different fractions including pulse proteins, fibers and starches (Hoover, Hughes et al. 2010).

For both methods, before the procedure, whole seeds of pulses are dehulled. In the wet method, the dehulled pulse seeds are soaked and then milled under a moist state, then, the protein in the suspension can be extracted and concentrated using either alkaline extraction-isoelectric precipitation, acid extraction, water extraction, salt extraction and ultrafiltration methods (Boye,

Zare et al. 2010). Thereafter, the starch will remain in the suspension, purifying by washing again to remove protein residual and filtrating through a pore size screen to remove fiber residue, which is followed by recovering and drying to obtain isolated starch in the end. In the dry method, the dehulled pulse seeds are milled to obtain a pulse flour, which is further separated using air classification technology based on their different densities and particle sizes, in which the protein-rich flour is finer and lighter than starch-rich flour (Boye, Zare et al. 2010). In comparison of these two methods, air classification technology is more widely used worldwide because of lower investment of process.

2.4 Physiology of ruminant digestion

Ruminants are evolved a special digestive system that involves microflora fermentation of food before digesta through their own digestion enzymes, which is adaptable to digest plant fiber. The stomach of the ruminant is divided into four compartments as shown in the figure 8.

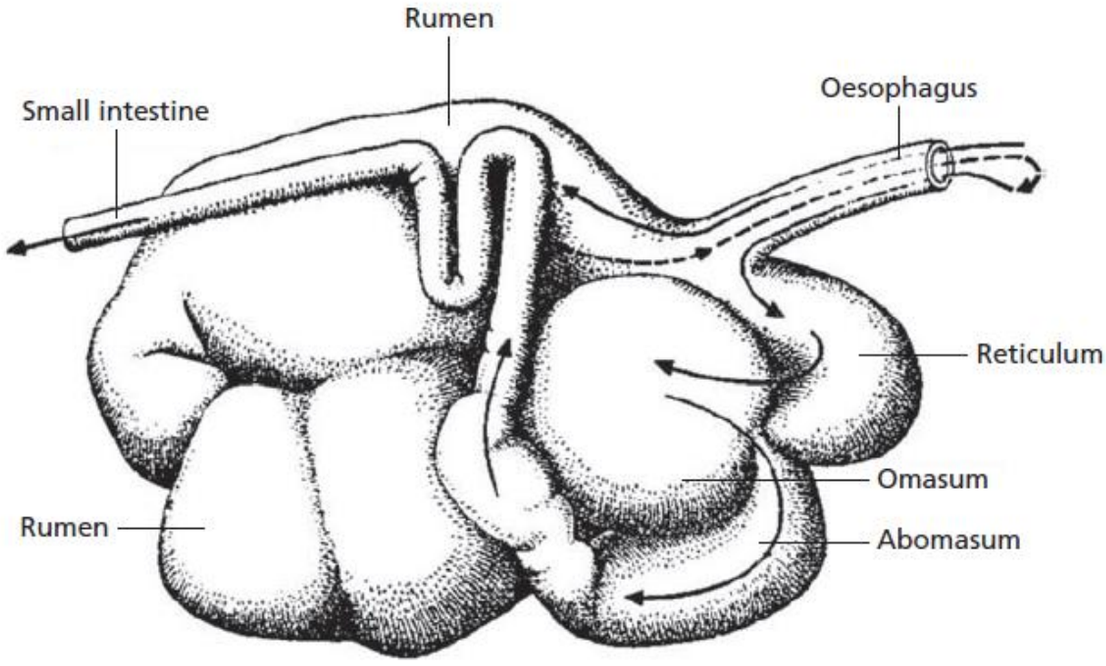


Figure 8. Pictorial representation of the rumen, reticulum, omasum abomasum of ruminant. Source from Annison E F and Lewis D (Annison and Lewis 1959)

The fermentation activity in the rumen by microbes produce volatile fatty acids and particularly butyric acid from the fermented cereals. Fermentation process encourages the formation of papilla, small tissues, that increases the surface area of digestive gut for absorbing nutrients. Hence, a combination of fibrous and starchy food can induce the development of the rumen and weaning process(McDonald 2011).

Rumen is a watery organ which contains 850-930g kg^{-1} of water in average (McDonald 2011), while it often exists in two phase: lower liquid phase, where finer substrates and food particles are suspended, and an upper phase, in which a layer of drier and coarse solid material. The main characteristic of ruminant is rumination action, due to the content of rumen is continually mixed by the rhythmic contraction of its walls, during rumination, material at the anterior end is turned back to the mouth through esophagus by a wave of contraction (McDonald 2011). In reticulum, a continuous culture system for anaerobic bacteria is provided. It has notably two species, protozoa and fungi. In rumen, bacterial and co-enzymes hydrolysis α 1 - 4 and α 1 - 6 bonds of amylose and amylopectin, particularly, protozoa play a role in the process of ingesting and digesting starch granules. Mendoza (Mendoza, Britton et al. 1993) proved it by eliminating protozoa from the rumen of sheep, the extent of ruminal starch digestion was greater than comparison group was observed. Furthermore, the hyphae of fungi may play an important role in bacterial attachment by creating lesions in the surface of plant tissue (McAllister, Bae et al. 1994). When digesta enter the rumen, the content of rumen is partially fermented to volatile fatty acids which provides 50% to 80% metabolism energy to host animal, microbial cells, methane and carbon dioxide. The gases are lost by eructation and the volatile fatty acids are absorbed by rumen wall. The combination of microbial cells and undegraded food components pass to the abomasum and small intestine where they are digested by enzymes secreted from the host animal. As digesta enter the large intestine where is a second phase of microbial digestion, the volatile fatty acids are absorbed, but microbial cells are excreted with undigested food components in faeces.

Like other constantly culture systems, the rumen requires a homeostatic environment. The acids

produced with the aid of fermentation are theoretically succeed of lowering pH of rumen liquid to 3.8, but the pH is commonly maintained at 5.5 - 6.5. Both phosphate and bicarbonate contained inside the saliva have buffer function. In addition, the temperature of rumen liquor stays near to body temperature of animals (38-42C°) (McDonald 2011).

2.4.1 Digestion of Carbohydrate

Carbohydrate is the main energy source of ruminant. The diet of ruminant contains considerable quantities of cellulose, hemicellulose, starch and water-soluble carbohydrates that are mainly formed as fructans. Hemicellulose, cellulose and pectins are considered as fibrous carbohydrates (FC), whereas fructans and starch are non-fibrous carbohydrates (NFC). At the early stage of herbage, each kilogram of dry matter approximately contains 400 g cellulose and hemicellulose, and 200 g of water-soluble carbohydrates. The β-linked carbohydrates are associated with lignin that consist approximately 20-120 g kg^{-1} DM. The breakage of carbohydrates in the rumen can be divided into two stages as shown in the (figure 9)

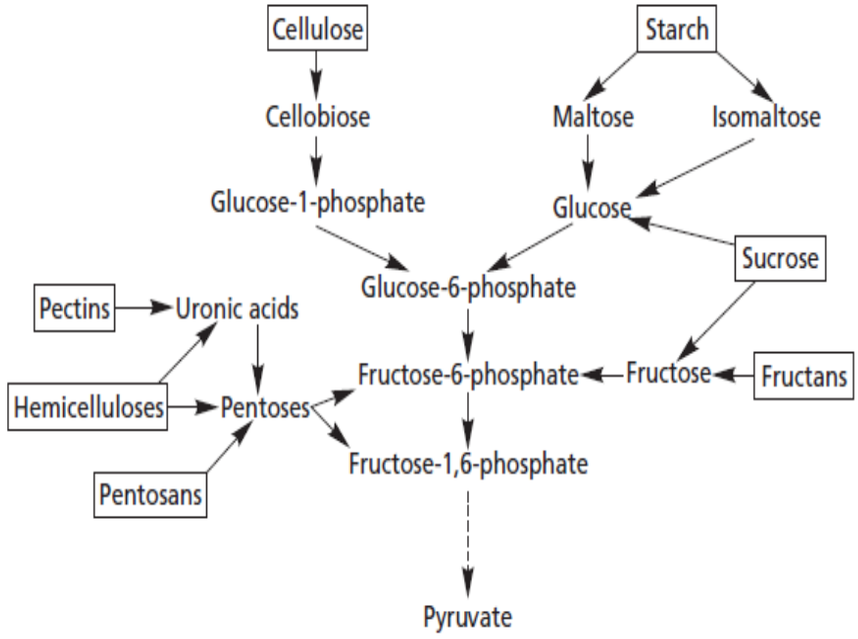


Figure 9. The conversion line of carbohydrate to pyruvate (McDonald 2011).

The first one is that the breakdown from complex carbohydrates to simple sugars. Cellulose is

decomposed by β - 1,4 - glucosidases to cellobiose which is then converted to glucose or during an action of phosphorylase to glucose-1-phosphate. Starch and dextrin are first converted to maltose and isomaltose by amylases and then converting to glucose or glucose-1-phosphate by maltase, maltose phosphorylases or 1,6 - glucosidases. Consequently, the extent of ruminal starch degradability has important implications in protein nutrition of the ruminant. When oats, barley or wheat are fed to ruminants as whole or crushed grain, at least 90% of the starch is fermented in the rumen, while maize is different due to a slower rate of starch digestion, up to 40% of corn starch can be found to escape fermentation in the rumen (Ørskov, Fraser et al. 1969) The crucial step of bacterial digestion is particle attachment of feed, approximately three - fourths of fiber, protein, and starch digestion is accomplished by bacteria that are attached to feed particles(McAllister, Bae et al. 1994). By the digestion of sucrose, fructose is produced together with glucose. The major production of hemicellulose breakage are pentoses which are attacked by enzymes of the β - 1,4 linkages to give xylose and uronic acids and then be converted to xylose. Pectins and xylose are also able to produce uronic acids, by either pectin hydrolyzing to pectic acid then convert to uronic acids or hydrolysis of the xylans. At the end of first stage, pyruvate is the final product.

For the second stage, pyruvate as a key that originate the pathways that link the conversion of pyruvate to the end production of rumen carbohydrate digestion, which are acetic, butyric acids (VFA), carbon dioxide and methane (Figure 10).

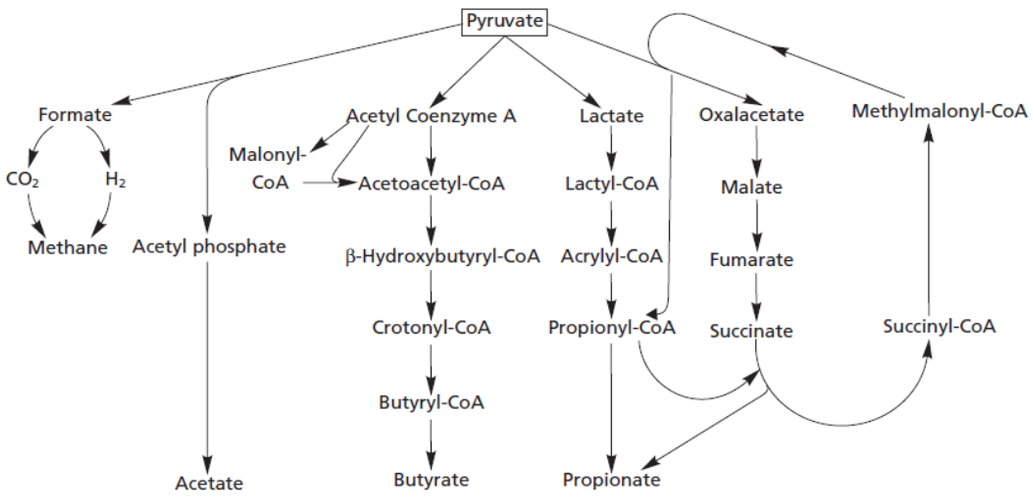


Figure 10. Conversion line of pyruvate to volatile fatty acids in the rumen (McDonald 2011).

2.4.2 Ruminal digestion of protein

Figure 11 illustrates the pathway of protein digestion in rumen. From the top, microorganisms (proteolytic organisms such as protozoa) hydrolyze food protein into peptides and amino acids. Whereas some amino acids are further deaminated and converting to organic aids, ammonia and CO_2, for example, valine is converted to iso-butyric acid. The produced ammonia, small peptides and amino acids by proteolytic bacteria and protozoa are utilized to synthesis microbial proteins which is broken down to produce nitrogen (N_2). Another fraction along with dietary undegradable protein is digested and absorbed in the abomasum and the small intestine. In

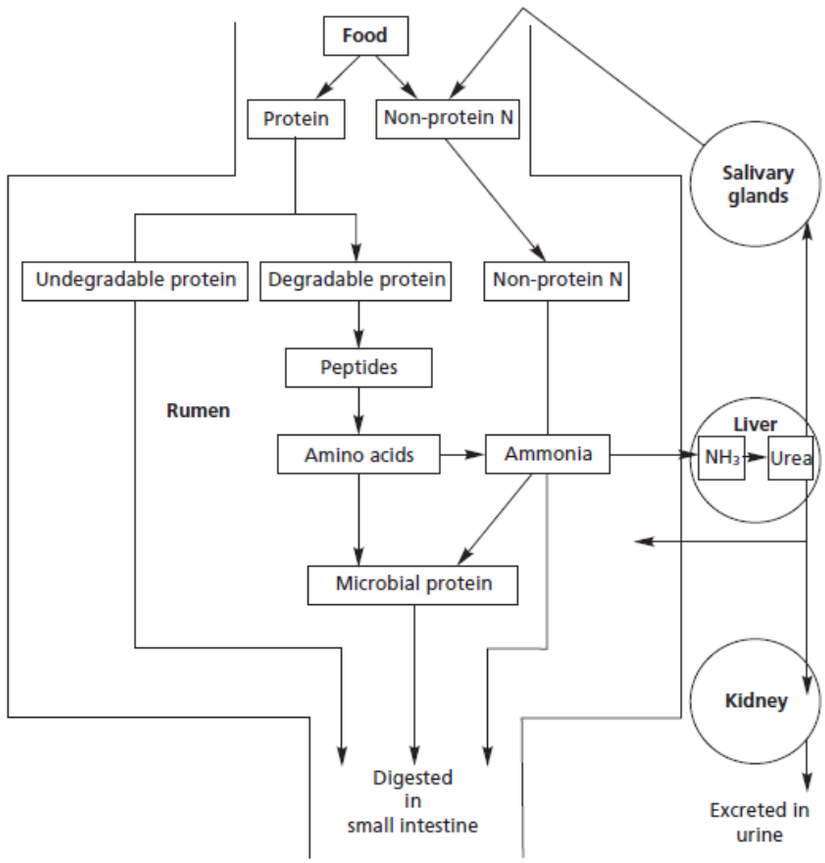


Figure 11. Digestion and metabolism of N-compounds in rumen (McDonald 2011).

addition, ammonia plays a key role in balancing microbial degradation and protein digestion.

When intake of protein is low which consequence lower concentration of ammonia and slow growth rate of rumen organisms. The breakdown of carbohydrate will be decreased. In converse, if ammonia content is too high to exceed optimal concentration, consequently, it will be absorbed pass through rumen wall into blood and converted to urea by carrying to the liver. Some portion of urea return to rumen through rumen wall or saliva, however, most of it will waste in urine.

2.5 Methods for measuring nutrients digestion in ruminants.

2.5.1 In vivo technology

In vivo measurement requires that animals are surgically prepared with cannula in rumen, by using suitable markers for measuring flow rate of digesta and difference between microbial and dietary nutrients flowing to the small intestine (Stern and Satter 1982) to estimate the accurate value. However, animal in vivo experiments are suffering ethic problem of animal right, treating experiments animals with human attitude is needed. Meanwhile, in vivo measurement of rumen digestibility is time-consuming and labor-intensive. Hence, inexpensive, simple-procedure and reliable alternative measurements are needed. As alternatives, in situ and in vitro procedure have been developed in recent years, including in situ, in vitro and in vitro gas production.

2.5.2 In situ technology

In situ technique is a relatively low-cost and simple-operation method compared to in vivo method. Technically, the principle of this method is that suspending bags that contain different feedstuff in the rumen and measuring substrates disappearance at various time (Michalet-Doreau and Ould-Bah 1992) to estimate feed digestibility after calculation. In situ dacron bags has been most commonly used for estimating protein degradability in rumen. Dewhurst et al (Dewhurst, Hepper et al. 1995) suggest that the dacron bag technique exists uncertainties about measuring carbohydrates which contain considerable levels of water-soluble materials, or small particles, which are easily pass through from bags before it is fermented. A comparison between dacron bags and in vitro procedure shows a certain overestimation of OM degradation in using dacron bags. The disadvantage of macron bags is tested due to feed digesta with small particle is easily escaping through the macron bags, which results inaccurate parameters.

2.5.3 In vitro true digestibility technology

In vitro procedure was initiated by Tilley and Terry (Tilley and Terry 1963) method which initially for predicting digestibility and as a selection tool for improving the nutritional quality of forages. By adding ruminal fluid in buffer solution in the sealed container, a simulated rumen environment can be modelled with low labor work and less harmful of animals. In earlier years, decrease in pH is one of major concern when using the in vitro technique to measure fiber digestibility because sensitivity of pH is much higher of cellulolytic bacteria than amylolytic species (Therion, Kistner et al. 1982). Fortunately, this issue has been solved by Grant and Mertens (Grant and Mertens 1992). An in vitro buffering system capable of pH control between 5.8 and 6.8 that has been successfully developed to measure fiber digestion in vitro (Grant and Mertens 1992). When using in vitro techniques, maintenance of anaerobic condition is a challenge. Grant and Mertens (Grant and Mertens 1992) tested that purging tubes with carbon dioxide but not gassing continuously resulted in a 56% increase in lag time for NDF digestion and a 69% decrease in rate of NDF digestion. Another approach of in vitro procedure was tested by Nsahlai and Umunna (Nsahlai and Umunna 1996) that sheep feces been used as inoculum to determine in vitro digestibility and a coefficient of correlation of 0.88 between DM digestibility obtained using reconstituted feces and DM digestibility obtained using ruminal fluid has been found. This modification presented a hypothesis that ruminally cannulated animals probably are not required in some extent.

2.5.4 In vitro gas production

In vitro gas production technique is used to estimate the organic matter degradability of ruminant feedstuffs. In this method, the kinetics of feedstuff degradation can be determined from the volume of gas produced during fermentation, the amount of gas released directly as a product of the fermentation and indirectly from buffered rumen fluid after incubation (Beuvink, Spoelstra et al. 1992) (Pell and Schofield 1993). In vitro gas methods primarily measure digestion of soluble and insoluble carbohydrates (Menke 1988), and the amount of gas produced from a feed on incubation reflects production of volatile fatty acids (VFA), which are a major source of

energy for ruminants. Gas arises directly from microbial degradation of feeds, and indirectly from buffering of acids generated as a result of fermentation. The final pH of the incubated medium can be measured to estimate the degree of fermentation and increase in volatile fatty acids. Upon incubation, substrates are partly solubilized. The soluble components are rapidly fermentable after incubation. Substrates and their components can be resistant to these processes to different extents, resulting in substantially different gas production profiles. About half of the gas is carbon-dioxide arising from neutralization acids of buffer, and the mixture of methane and carbon-dioxide arising from fermentation of carbohydrates and proteins to volatile fatty acids. Mathematical description of gas production profiles allows analysis of data and comparison of substrates or fermentation environment characteristics and can provide useful information concerning the fermentability of soluble and slowly fermentable components of the substrate (Groot, Cone et al. 1996). One advantage of this methods is that it can be applied to large numbers of food samples, especially if recorded automatically. Another advantage is that it is able to account both soluble and insoluble substrates, which is over the traditional gravimetric method (Pell and Schofield 1993). Disadvantage is that gas production only assumes rumen fermentation of total gas production kinetics but not adaptable for detecting individual constituent.

2.6 Construction of hypotheses

In this study, eight different starch rich concentrates sources and one total mixed ration are chosen to estimate degradation kinetics, in which barley, oats and wheat are main source of animal feed in Europe, maize represents as the most abundant energy source mainly cultivated in America. Peas and horse bean and their by-product pea starch and horse bean starch are main protein sources of ruminants. In terms of chemical composition of cereals and pulses, a variation among different species is predicted, which somehow influence degradation of nutrients in the rumen. In order to improve feeding and herd efficiency, the evaluation of feedstuffs for digestibility, and efficiency of suppling nutrients is necessary for ruminants. In this study, in vitro gas production technology was chosen in due to its advantages such as cost-wise, less laboratory work and convenience to accommodate many samples.

Therefore, the hypotheses of this experiment were: (1) In vitro gas production technology is adoptable to evaluate feed degradation by gas production characteristics. (2) As compared gas production parameters, legume extracted by-products will be fermented more rapid and produce more gases than whole legumes. (3) Maize will produce highest quantity of gases, but it will be the slowest gas producer. In contrast, oats will be fermented most fast but produce lowest content of gas production. (4) Chemical composition of feed is correlating to gas production and feed value.

3. Material and methods

3.1 Experimental facilities and feeding of animals for collection of rumen fluid

Experiment was conducted at metabolism unit and experimental animals were selected for collection of rumen fluid in NMBU Ås Gård. Contributors of rumen fluid are two non-lactating dairy cows which are all equipped with flexible rumen canula. Cows were fed at maintenance level and following Nordic standardized diet consists 67% hay and straw and 33% concentrate. Daily ration was divided into two meals of equal size with an adaptation period of 14 days (Norfor).

3.2 Experimental feed samples

The following nine feedstuffs were examined: total mixed ration, barley, wheat, maize, peas, pea starch, horse beans, horse beans starch and oats. Total mixed ration is mixer of other samples and small content of silage. Pea starch and horse bean starch were obtained according to processing air classification technique on peas and horse bean. All feedstuffs were grinded with particle size of 1mm by grinding and sieving.

3.3 Chemical analysis of samples

The DM (dry matter) (103℃) and ash content were determined using ISO 5984 method (550℃ for a minimum of 4 h). The ash corrected NDF (aDNFom) content was analyzed according to Filter Bag technique (for A200 and A200I), 2017, NDF Method, Method 6 (Arrival Technology). Nitrogen content was analyzed by Kjeldahl-N Method 2001.11 (AOAC, 2002). CP was estimated as N × 6.25. Starch content was analyzed based on AACC Method 76-13-01 (Total Starch Assay Procedure - Megazyme amyloglucosidase / alpha-amylase method), and that corresponds to AOAC method 996.11 (-Starch (Total) in Cereal Products). All the analysis was done in LabTek (NMBU).

3.4 In vitro gas production experimental design

The experiment was designed in 2 batches × 9 feed samples × three modules for each

feed. Inoculum fluid was collected from two non-lactation cows (as described above). The in vitro gas production procedure followed Ankom gas production manual (ANKOMRF Gas Production System, 2018).

Preparation of samples

Samples were prepared for incubation as follows:

1. Approximately 1 g DM of each feed was weighed into 250 ml ANKOM bottle in triplicates
2. The bottles were sealed with aluminium paper and placed at a 39C incubator overnight.

Preparation of buffer solution

The buffer solution is prepared according to Goering and Van Soest buffer solution formula in Table 4.

Table 4. Formula[1] of making Goering – Van Soest buffer solutions

Solutions	Formula of making solutions
Resaruzin 0.1% (w v^{-1}) solution:	Dissolve 0.1 g resaruzin into 100 ml H20
In vitro micromineral solution:	13.2g CaCl2·2 H2O + 10.0 g MnCl2·4H20 + 1.0 g CoCl2·6 H2O + 8.0 g FeCl3·6 H2O (Dilute in 100ml distilled water)
In vitro macro-mineral solution:	5.7 g Na2HPO4 anhydrous + 6.2 g KH2PO4 anhydrous + 0.6 g MgSO4·7 H2O Bring volume to 1 L using Distilled Water
In vitro buffer solution:	3 g NH4HCO3 + 35 g NaHCO3 Bring volume to 1 L using Distilled Water

[1] Sources is from (Goering and Van Soest 1970)

Before buffer solution was prepared, all glassware was maintained at 39°C. Resaruzin 0.1% (w v^{-1}) solution, in vitro buffer solution, in vitro micromineral solution and in vitro macro-mineral solution were prepared using distilled water throughout based on formula in the table 3. The final buffer solution was prepared, per litre solution, by the following steps: 250mL in vitro buffer solution; 250 mL in vitro macro-mineral solution; 1.25 mL of resazurin (0.1% solution);

and 125 μL in vitro micromineral solution and bringing the total volume to 1000 mL with distilled water. The solution was continuously flushed with CO_2 under stirring at 39^0C for about 2 hours with final addition of a reducing solution. The final reduced solution is colorless (Figure 12)

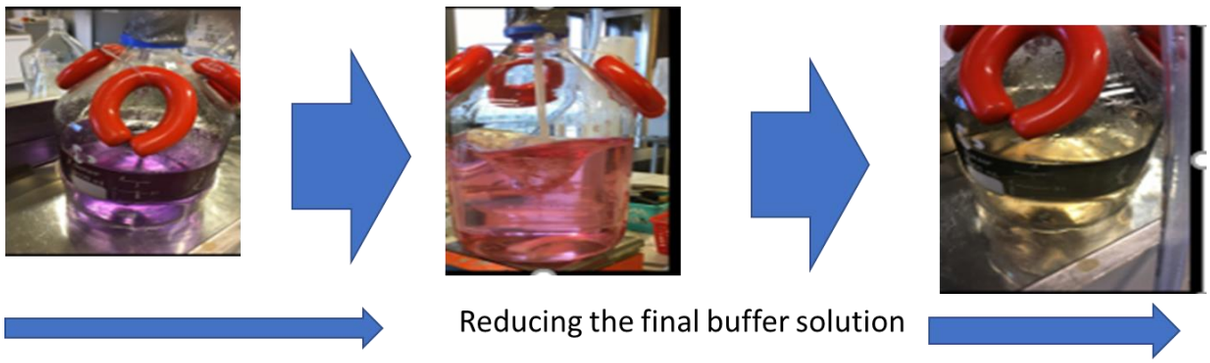


Figure 12. Colour change of final reduced buffer solution

About 66.7 mL of the reduced buffer solution is injected to each ANKOM GP bottles and were left in the incubator (390C) to equilibrate until the rumen inoculum is prepared. reducing solution.

Preparation of rumen inoculum

Rumen inoculum was prepared when the buffer was equilibrating. About 2 L thermo bottles was preheated by filling with 39°C water until collection of rumen inoculum. Approximately 600 to 1,000 ml of rumen inoculum was removed each cow and placed in thermos by using the appropriate collection procedures. The time required for all the operations was less than 30 minutes.

Incubation

The rumen inoculum from the thermoses was removed into the glass bottle through 200 μm Nitex clothe to eliminate big feed particles. The inoculum was gently mixed in a water-bath and about 33.3 mL of the rumen inoculum was dispensed into the Ankom bottles, making the final volume (buffer + inoculum solution) 100 ml. Thereafter, the samples were incubated at 39℃ for 48 h, continuous shaking using Gyro-shaker (Figure 13) with continuous gas pressure and temperature recording from individual modules using automated system (ANKOM RF GP

system).

Figure 13. ANKOM GP modules under incubation at 39^0C

3.5 Calculations and statistical analysis

3.5.1 In vitro gas production

During the incubation, the pressure in module headspace changes, with respect to the atmospheric pressure measured at the start of the incubation (Po), were transmitted via a radio frequency to a PC with a settled frequency of 1 minute. Gas in the headspace of the bottles was automatically released by opening a closed valve when a threshold pressure variation of +0.75 psi was reached.

The gas pressure measured during incubation can be converted to moles of gas produced using the 'ideal' gas law, and then converted to milliliters (ml) of gas produced using Avogadro's law. The ideal gas law: $n = p\ (V \times RT^{-1})$. At atmospheric pressure measured in psi (1 psi = 6.894757293 kilopascal) 1 mole will occupy 22.4 L at 273.15°K and 101.325 kPa (standard conditions). Therefore, gas measured in moles can be converted to gas measured in ml as follows

Avogadro's law: gas production (ml) = n × 22.4 × 1000; where n = gas produced in moles (mol), p = pressure in kilopascal (kPa), V = head-space volume in the Glass Bottle in Liters (L), T = temperature in Kelvin (K), R = gas constant (8.314472 $L \cdot kPa \cdot K^{-1} \cdot mol^{-1}$).

3.5.2 Estimation of organic matter digestibility and metabolizable energy

The in vitro organic matter digestibility (OMD) and metabolizable energy (ME) contents of the samples were calculated from the net 24 h gas volume (200ml g^{-1} OM), crude protein and ash contents according to the following equation (Menke 1988): OMD (%) = 14:88 + 0:889×GP + 0.45×CP + 0:0651×XA; ME = 2.43 + 0.1206 × GP(24h) + 0.0069 CP (Krishnamoorthy, Soller et al. 1995); where GP = net gas volume at 48 h or 24 h fermentation (200ml g^{-1} OM); CP = crude protein content (g /100 g DM) and XA = ash content (g 100 g^{-1} DM).

3.5.3 In vitro gas production kinetics

Analysis of variation of gas production parameters were processed in NLIN procedure in SAS (SAS 9.4), based on the model (Groot, Cone et al. 1996): $G = A/ (1+B^C/ t^C)$; $R= Ct^{C-1} / B^C + t^C$; where G (ml g^{-1} OM) represents the amount of gas produced per gram of organic matter (OM) incubated, at time t after incubation. R (h^{-1}) represents the relatively fractional rate of substrate digestion at time t after incubation. A (ml g^{-1} OM) represents the asymptotic gas production. B (h) is the time after incubation at which half of the asymptotic amount of gas (A) has been formed, and C is a constant determining the sharpness of the profile. Rmax (maximum rate of gas production) is obtained when the microbial population is big enough such that it no longer limits the fermentation process of the substrate and digestion is not reduced by chemical or structural barriers of the potentially digestible material at this point (Groot, Cone et al. 1996). Tmax (time when reach the maximum gas production rate) is obtained where the R reached the maximum rate. Statistical analysis was performed using SAS (SAS 9.4). The analysis of variance in GLM procedure was used to test the effect of ingredient type on the parameter estimates (A, B, C), OMD and ME estimates. GLM correlation procedure was used to indicate the relationship between chemical composition and gas production characteristic and energy value.

4. Results

4.1 Chemical composition of samples

Chemical composition of the substrates assayed in the study is shown in Table 5. The content of dry matter varied from 874 g kg^{-1} in wheat to 907.2 g kg^{-1} in pea starch. The highest content of CP was observed in horse bean (296.7 g kg^{-1}), followed by peas (225.8 g kg^{-1}), pulses by-products (182.5 g kg^{-1} in horse bean starch and 141 g kg^{-1} in pea starch respectively). Cereal based feeds contain relatively low content of CP, respectively wheat (158.3 g kg^{-1}) that observed highest CP in cereal, followed by oats (127.5 g kg^{-1}), barley (109.8 g kg^{-1}) and maize (91.1 g kg^{-1}) contains lowest CP. In contrast, cereal based feeds were mostly observed higher content of fiber (aNDFom) than pulses and pulses by-products, however, maize exceptionally contains relatively low aNDFom content. Two pulses by-product, pea starch and horse beans starch were observed as extremely low content of aNDFom. For starch content, the greatest content was observed in maize (642.5 g kg^{-1}), in following pea starch (636.6 g kg^{-1}) and horse bean starch (621.9 g kg^{-1}), followed by cereal grains, wheat (570.9 g kg^{-1}), barley (541.5 g kg^{-1}) and oats (419.4 g kg^{-1}). Pulses contains lowest starch content, peas (315.5g kg^{-1}) and horse bean (308.4g kg^{-1}).

Table 5. Chemical composition* of experimental feed examples (g kg^{-1} DM)

Feed ingredient	DM[1] (g kg^{1})	Ash	OM[2]	aNDFom[4]	CP[3]	Starch
Total mixed ration	916.0	62.0	938.0	391.1	166.7	211.2
Barley	879.4	22.6	977.4	209.0	109.8	541.5
Wheat	874.9	18.3	981.7	142.1	158.3	570.9
Maize	882.4	14.5	985.5	93.2	91.1	642.5
Peas	885.8	28.7	971.3	138.3	225.8	315.5
Pea starch	907.2	18.7	981.3	18.0	141.0	636.6
Horse beans	878.2	32.6	967.4	128.8	296.7	308.4
Horse beans starch	900.8	20.5	979.5	17.4	182.5	621.9
Oats	880.6	21.3	978.7	359.6	127.5	419.4

[1] Dry matter content.

[2] OM: organic matter content.

[3] CP: Crude protein content.

[4] aNDFom: ash corrected neutral detergent fiber.

*Composition is based on deuplicate analysis for each feed ingredient

4.2 In vitro gas production kinetics and GLM contrast among feed samples

Gas production parameters and GLM contrast of feed samples after incubated in buffered rumen fluid is presented in Table 6. All parameters were found different among substrates (P <0.001). It can be observed that maize (332.34 ml g^{-1}) showed highest asymptotic gas production A value, with following horse bean starch, barley, pea starch, peas, horse bean and oats (221.46 ml g^{-1}) formed the lowest A value in a significant difference to others (P < 0.001). The parameter B is observed that oats (6.99 h) took shortest time to reach half of the asymptotic gas volume, with ranking pea starch, horse bean starch, barley, wheat, peas, total mixed ration and maize (12 h) is shown the longest time to reach half of A parameter. A good variation was obtained of pH among samples (P < 0.001). The value of pH was significantly lower in maize, pea starch and wheat. In contrast, higher pH was observed in horse bean, oats and peas. Pea starch and horse bean at 24 h incubation showed highest gas production 291.58 ml g-1 OM and 288.64 ml g-1 OM respectively. Peas, barley, maize and wheat in followed with gas production from 277.94 ml g-1 OM to 270.53 ml g-1 OM degressively. Oats and total mixed ration both showed the extreme low gas production with quantity 204.53 ml g-1 OM and 203.81 respectively. At 48 h incubation, maize showed highest gas production 316.32 ml g-1 OM, followed by pea starch, peas, horse bean starch, barley, wheat, horse bean, total mixed ration and lowest gas production was observed in oats with only 217.03 ml g-1 OM.

Table 6. In vitro gas production parameters and GLM contrast of feed samples after incubated in buffered rumen fluid

Feed ingredient	A[1]	B[2] (h)	C[3]	pH	GP[4] 24	GP[5]48
Total mixed ration	271.2^{d}	10.8^{b}	1.39^{c}	6.40^{b}	203.81	240.84
Barley	309.7^{b}	9.13^{d}	2.16^{b}	6.17^{d}	275.53	301.34
Wheat	304.0^{b}	9.31^{d}	2.21^{ab}	6.10^{d}	270.53	296.08
Maize	332.3^{a}	12.0^{a}	2.15^{b}	6.01^{e}	271.49	316.32
Peas	314.0^{b}	9.95^{c}	2.32^{ab}	6.37^{b}	277.94	306.04
Pea starch	312.33^{b}	7.99^{e}	2.40^{a}	6.09^{de}	291.58	308.18
Horse beans	286.77^{c}	11.5^{a}	2.07^{b}	6.53^{a}	235.35	272.56
Horse beans starch	309.47^{b}	8.72^{d}	2.60^{a}	6.23^{c}	288.64	305.82
Oats	221.46^{e}	6.98^{f}	2.02^{b}	6.43^{b}	204.53	217.03
S. E	5.79	0.215	0.078	0.025	4.23	5.38
Significance	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

GLM contrast among cereals and legumes

Peas vs Pea starch	0.822	<0.001	0.440	<0.001	0.081	0.330
Horse beans vs Horse bean starch	0.006	<0.001	<0.001	<0.001	<0.001	<0.001
Cereals[6] vs Maize	<0.001	<0.001	0.845	<0.001	<0.001	<0.001
Wheat and Barley vs Maize	0.003	<0.001	0.685	<0.001	0.745	0.037
Cereals vs legumes[7]	0.070	<0.001	0.369	<0.001	0.210	0.200
Legumes vs legume starch[8]	0.058	<0.001	<0.001	<0.001	<0.001	<0.001
Legumes vs Maize	<.001	<0.001	0.604	<0.001	0.006	<0.001
Cereals vs legume starch	<0.001	<0.001	<0.001	0.407	<0.001	<0.001

[1] Asymptotic gas production (ml g^{-1} OM).
[2] Time to half of the asymptote (h).
[3] Constant determining the sharpness of the curve.
[4] Gas production at 24 h (ml g^{-1} OM).
[5] Gas production at 48 h (ml g^{-1} OM).
[6] Barley, wheat, oats and maize
[7] Peas and horse bean
[8] Pea starch and horse bean starch

There was a significant variation ($P<0.001$) in gas production parameter B and pH from all samples. In terms of A parameters and gas production, horse bean starch was significantly different ($P<0.001$) from horse bean starch, but no distinctly difference from pea starch and peas. Maize showed a significant difference from others. There was no difference of gas production from cereals (barley, wheat, oats, maize) and legumes (peas and horse bean).

Gas production profiles (Figure 14) and fractional rate (Figure 15) are fitted the mono-phasic model of (Groot, Cone et al. 1996). In the pattern of fermentation, oats prior increased gas production up to 6 hours, followed by pea starch, horse bean starch, barley, wheat and peas, maize and horse bean are observed with slow production curve in contrast to others. In the range of incubation time 6 h to 12 h, gas production curve of oats reached inflection point and met the maximum production rate (Rm) 0.145 h^{-1} from 7 h and production curve slowed down afterwards distinctively. Thereafter, pea starch reached inflection point with Rm 0.152 h^{-1} at 9.17 h, followed wheat with Rm 0.119 at 9.67 h, barley have same Rm (0.119) as wheat at 10.17 h, horse bean starch with Rm 0.153 h^{-1} at 10.50 h, peas with Rm at 11.33 h and horse bean with Rm 0.09 h^{-1} at 11.83 h respectively. In the range of incubation time 12 h to 24 h, maize started to reach inflection point at 12.67 h with Rm 0.09 h^{-1}. Gas production curve slowed down was observed

in all feeds. In the range of incubation time 24 h to 48 h, gas production curve slowed down further until incubation was finished.

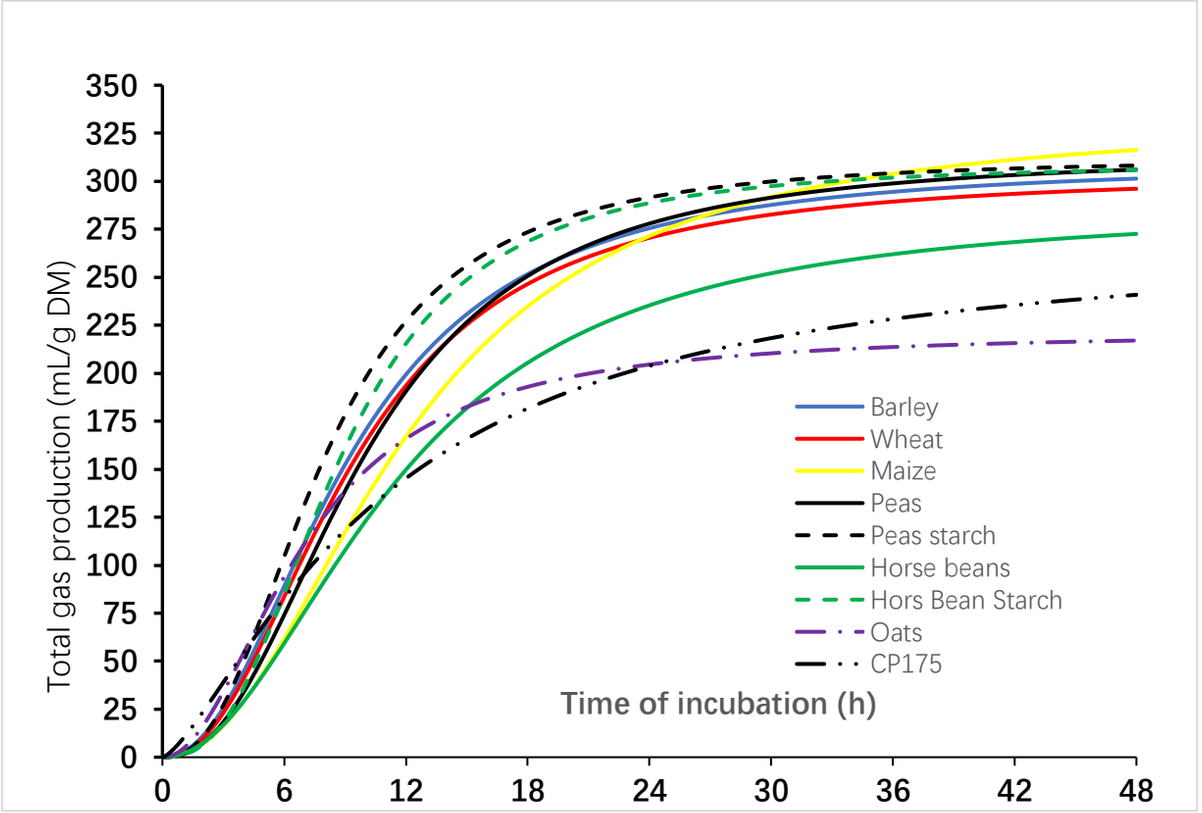


Figure 14. Cumulative gas production within 48 hours of in vitro incubation.

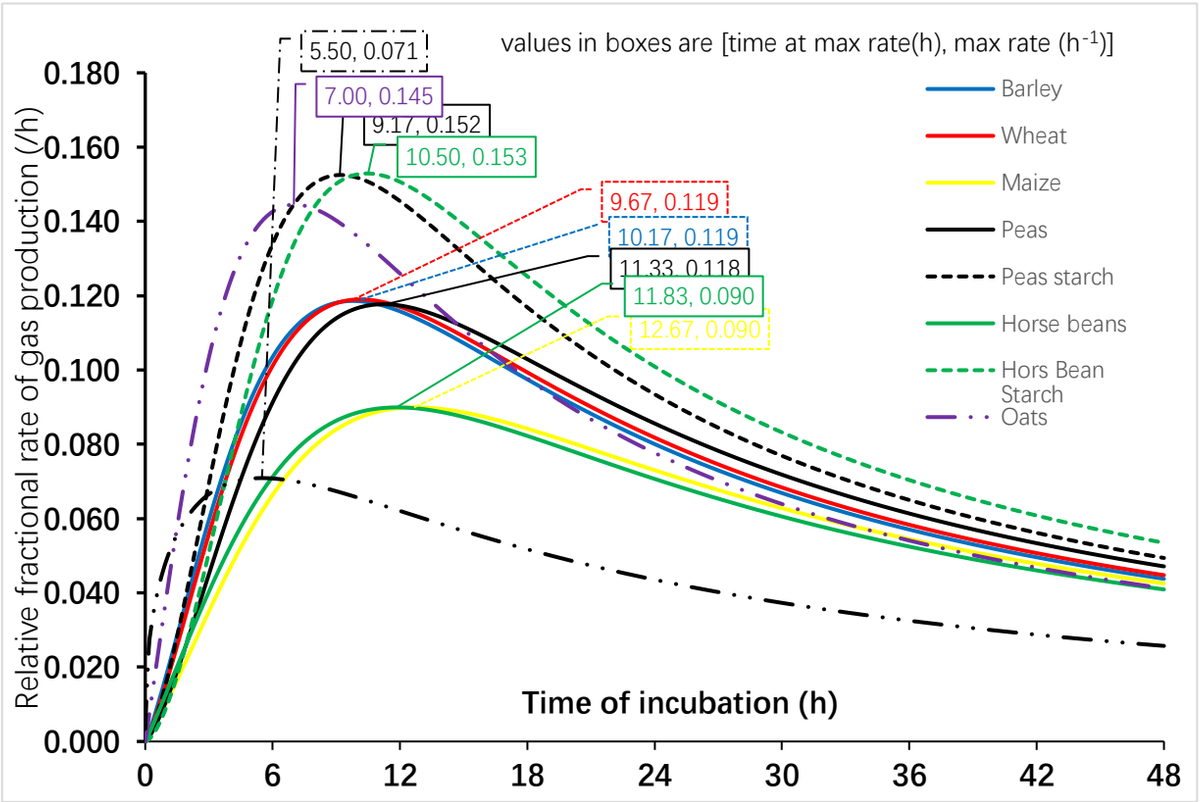


Figure 15. Relative fractional rate of gas production within 48 hours of in vitro incubation. Values in boxes are hours before max rate of gas production (h) and the fractional rate of gas production at that time (h^{-1}).

4.3 Estimated energy value and GLM contrast among feed samples

Variation in OMD and ME and GLM contrast among feed samples are shown in Table 7. OMD and ME ranged from 59.57% to 79.28% and 8.06 MJ/kg to 10.65 MJ/kg, respectively There was a significant ($P < 0.001$) difference among samples in OMD and ME values. The highest OMD value was reported in peas, followed horse bean starch, horse bean, pea starch, wheat, maize and oats. The rank of ME among feed samples were peas, horse bean starch, pea starch, horse bean, maize and wheat (same value), barley and oats, which was consistent to OMD ranking.

Table 7. Estimated energy value and GLM contrast among different feedstuff.

Feed	OMD[1] (%)	ME[2] (MJ kg^{-1})
Barley	73.8^{b}	9.73^{bc}
Horse bean starch	78.0^{a}	10.5^{a}
Horse bean	76.9^{ab}	10.1^{b}
Maize	74.6^{ab}	9.60^{c}
Oats	59.6^{c}	8.06^{d}
Peas	79.3^{a}	10.7^{a}
Pea starch	76.5^{ab}	10.3^{ab}
Wheat	75.2^{ab}	9.88^{b}
S. E	0.84	0.01
Significance	<0.001	<0.001
GLM contrast among cereals and legumes		
Peas vs Pea starch	0.02	0.01
Horse beans vs Horse bean starch	0.35	0.01
Cereals[3] vs Maize	<0.001	0.01
Wheat and Barley vs Maize	0.86	0.04
Legumes[4] vs others	<0.001	<.001
Cereals vs legumes	<0.001	<.001
Legumes vs Maize	0.001	<.001

[1] Organic matter digestibility

[2] Metabolizable energy

[3] Barley, wheat, oats and maize

[4] Peas and horse bean

After GLM contrast procedure, there was a distinctive variation in OMD, ME from all samples. In terms of OMD, there were differences (P<0.05) in between peas and pea starch but no distinctive differences between horse beans and horse bean starch. A significant (P<0.001) difference was shown in legumes from others. Maize showed a difference (P<0.001) from cereals, but no difference with contrasting of maize to wheat and barley, which indicated that maize significantly different from oats. For ME, all samples showed a certain difference from others.

4.4 Relationship between chemical constituents and fermentation products

Overall relationships between chemical composition and gas production characterizes are shown in table (Table 8). The starch was positively correlated with parameter A (P<0.05), 24h and 48 h (P<0.001) gas production (Figure 16). Crude protein was observed no strong correlation of gas production. Remarkably, NDF was observed negatively correlated (P<0.001) with A, OMD, ME, 24h (Figure 17) and 48h gas production with significant difference (P<0.001). Starch and CP level was positively correlated (P<0.05) with OMD and ME. The aNDFom positively correlated (Figure 18) to gas production rate, whereas CP showed negative correlation (P<0.05) to gas production rate (Figure 19).

Table 8. Correlation (r) between chemical composition (g kg^{-1} DM), in vitro gas production characteristics, gas production at 24 h and 48 h, organic matter digestibility (OMD), metabolizable energy (ME)

	Gas production parameters		Gas production		Energy value	
	A	B	24 h	48h	OMD	ME
Starch	0.38	-0.13	0.449	0.48	0.31	0.32
P	0.01*	0.35	<0.001**	<0.001**	<0.05*	<0.05*
CP	-0.07	0.24	-0.097	-0.12	0.37	0.44
P	0.61	0.05*	0.491	0.38	<0.05*	0.001**
aNDFom	-0.68	-0.30	-0.729	-0.75	-0.76	-0.77
P	<0.001**	0.03*	<0.001**	<0.001**	<0.001**	<0.001**

* means P<0.05.

** means P<0.001.

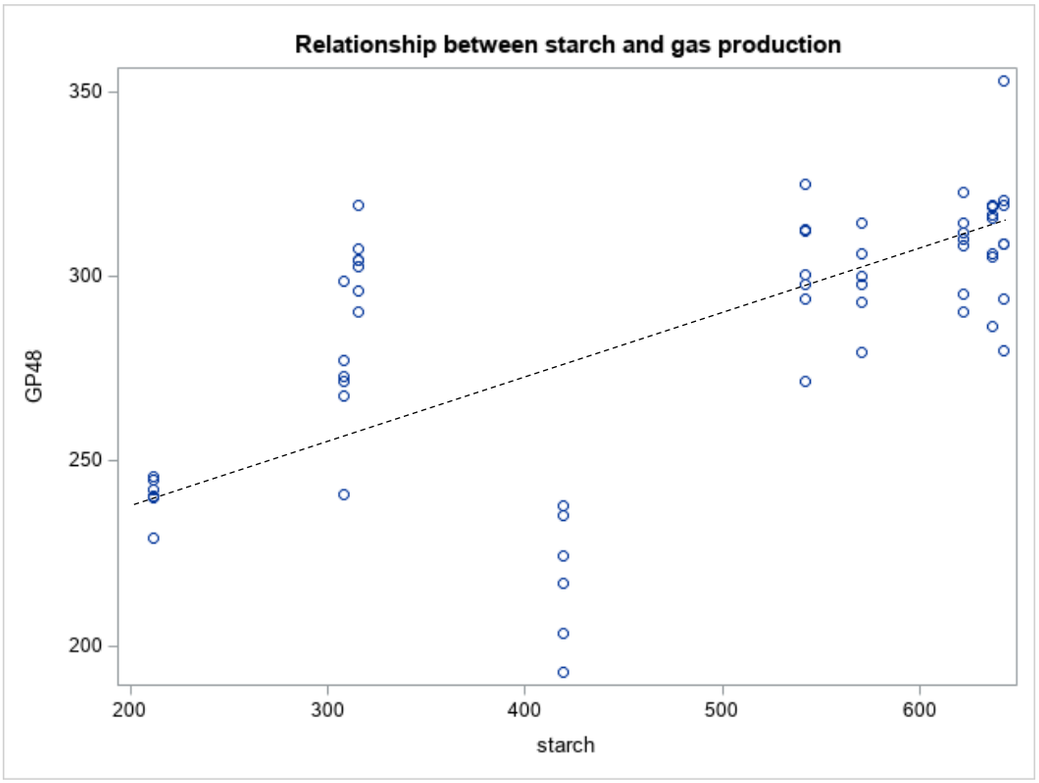


Figure 16. Plot of relationship between starch content and gas production in feed samples

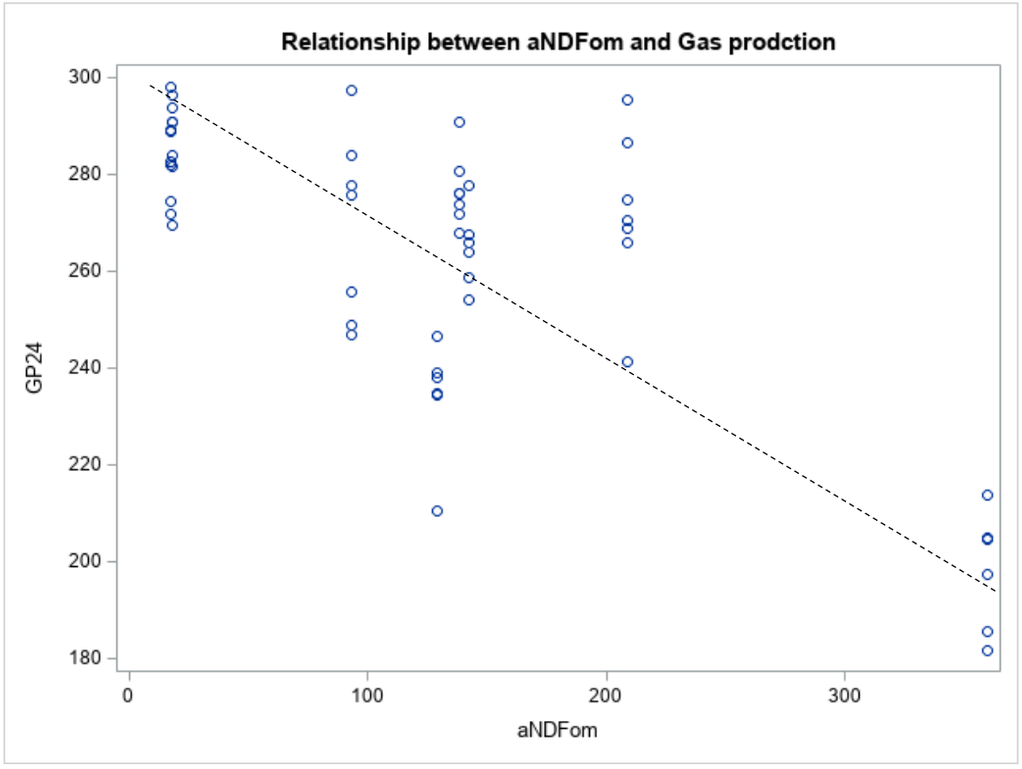


Figure 17. Plot of relationship between aNDFom content and gas production in feed samples

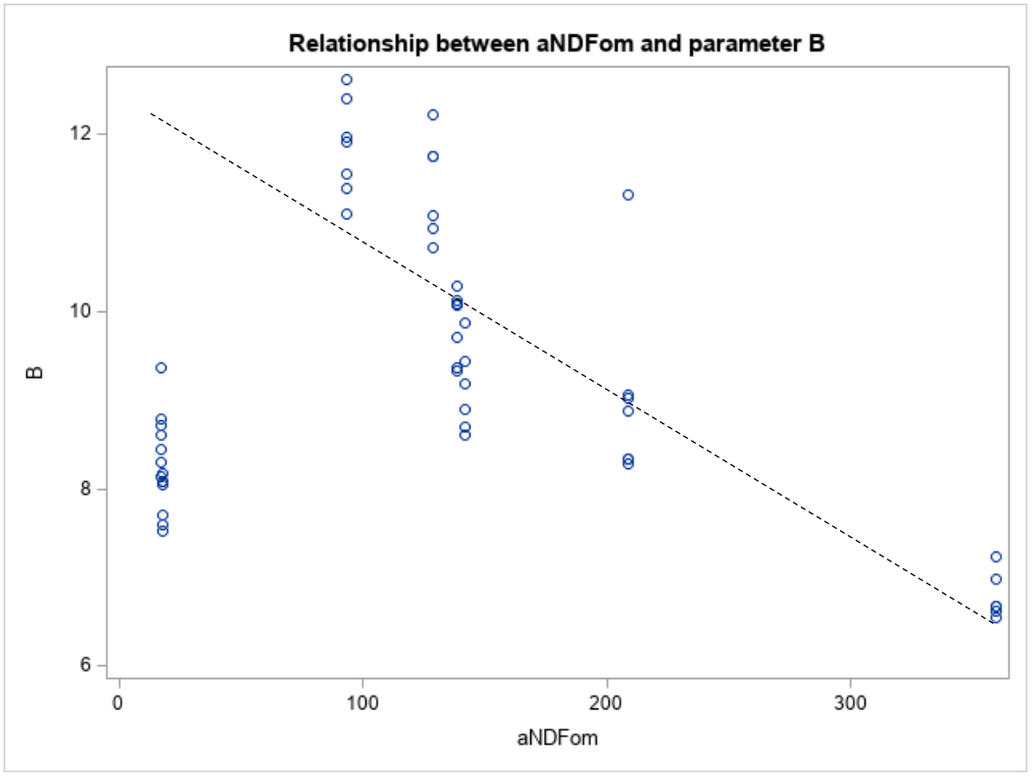


Figure 18. Plot of relationship between aNDFom content and parameter B (GP rate)

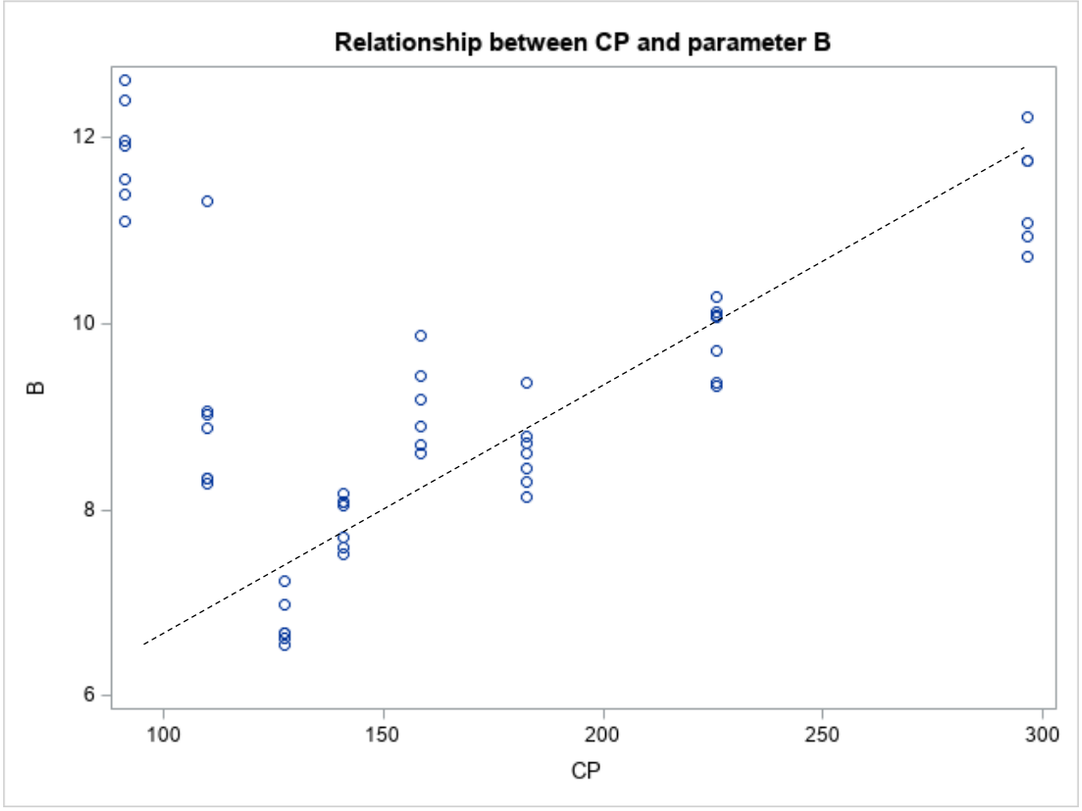


Figure 19. Plot of relationship between CP content and parameter B (GP rate)

5. Discussion

5.1 In sacco technique vs In vitro gas production

In sacco dacron bag technique is most commonly used to estimate microbial protein and carbohydrate degradation in the rumen. Advantages of this methods are easily reproducible, less time consuming and requires minimal apparatus. However, Dewhurst et al (Dewhurst, Hepper et al. 1995) claimed that in sacco method is not adaptable with samples contain pure starch and small particles because of the high proportion of water-soluble materials that can escape through the bag in the rumen before fermented, consequently, an overestimated substrates degradation will occur. In vitro gas production method presents an advantage over in sacco in which it eliminates interference of small particles for both soluble and insoluble substrates (Pell and Schofield 1993). Therefore, in vitro gas production technique is more often used as an alternative to predict the rate and extent of digestion of feedstuffs. In vitro gas production is promising significant economy in labor and time. However, in vitro gas production method is not perfect, the in total gas production kinetics and parameters are not able to detect differences in individual substrate digestion (Grant and Mertens 1992). Thus, starch and dry matter degradation parameters derived from in situ studies and gas production studies may not reflect each other correctly. As an example, in situ techniques over predicted effective degradability for barley and wheat while under predicted degradability for slowly fermented grains such as maize (Offner and Sauvant 2004). Therefore, in vitro methods may be more suitable than in situ methods for obtaining digestion rate data. If individual digestibility is needed, further measurement such as residual collection and analysis are required to describe individual nutrients (starch, protein, fiber) degradation.

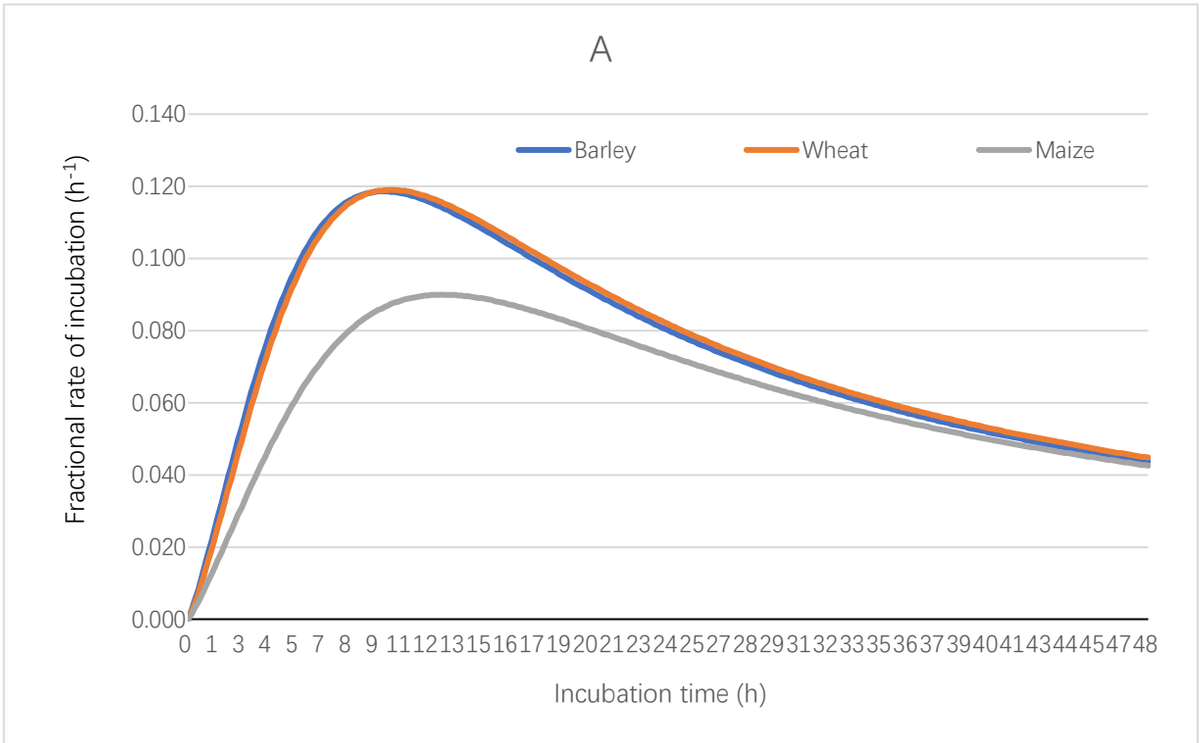
5.2 In vitro gas production performance of different feedstuffs

Gas production from different feed ingredient depends on its chemical composition. In our study, all examples are starch rich grains with variety of chemical composition. It has been proved that the relationship between gas production and starch degradation was linear (Chai, Van Gelder et

al. 2004). Therefore, the kinetic analysis of gas production from incubation is expected to directly provide information based on the predominant carbohydrate fraction (mainly starch and small portion of fibrous constituent). Furthermore, Chen et al (Chen, Pell et al. 1999) showed that most of the variation in the shape of the gas production and fractional rate curves could be attributed to starch fermentation. The extent of starch degradation is dependent on its structure. Variation of shape and size of starch granules, amylose and amylopectin content, interaction between starch granules and protein matrix, are factors affecting degradation characteristics for each plant species (Offner, Bach et al. 2003). Degradation of protein in rumen produces has been documented with relatively lower gas production compare to carbohydrates (Makkar, Blümmel et al. 1995). In our study, during the 48 hours incubation, a good variation of GP and GP rate was formed among the different feeds.

5.2.1 In vitro gas production in maize, barley and wheat

Carbohydrate degradation take a large extent to affect gas production performance. After incubation, maize was detected the slowest fermentation speed of fermentation comparing to barley and wheat (P<.0001) (Figure 20). It can be explained that starch granules of barley and



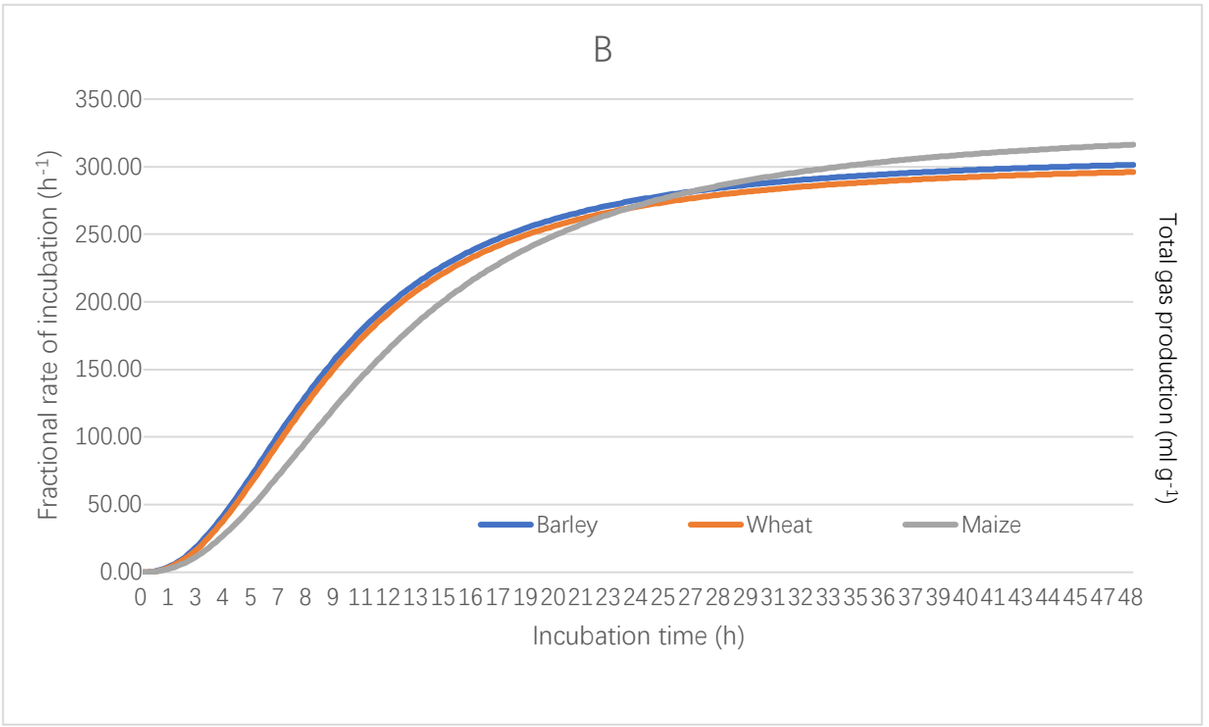


Figure 20. Cumulated profile of fractional rate (A) and total gas production (B) of maize, barley and wheat

wheat are loosely associated with the protein matrix (Evers and Bechtel 1988). In contrast, the endosperm of maize contains two portions, the floury and the horny endosperm. The starch associated with protein matrix in the floury endosperm has no differences compare to barley and wheat, but in the horny endosperm, starch granules are tightly embedded in the protein matrix (Hoseney 1986). On the other hand, starch granules in barley and wheat endosperms have a relatively small particle size. Consequently, the smaller starch granules have a larger surface area available for microbial and enzymatic starch hydrolysis which results in rapid degradation (McAllister and Cheng 1996). Furthermore, zein proteins, which exist in maize has been proved with negative correlation to ruminal starch degradability (Philippeau, Landry et al. 2000). Slow fermentation rate of maize indicates that reduced starch degradation and thereby increased escape from the rumen, which consequently decrease digestibility of starch in small intestine. It can be explained that efficiency of post ruminal starch digestion decreases with increased starch passage into the small intestine (Nocek and Tamminga 1991). In this study, the ranking of

fractional rate of gas production was wheat, consistent with previous study on in situ starch degradation rates (Herrera-Saldana, Huber et al. 1990) and in vitro gas production kinetics study of Lanzas (Lanzas, Fox et al. 2007). It indicates that gas production technique is applicative and somehow providing valid degradation curve of fractional rate. In terms of total gas production curve, starch fermentation contributes major proportion of gas production (Lanzas, Fox et al. 2007). Total gas production of maize is significantly higher ($P<0.001$) than wheat and barley due to its higher starch content.

5.2.2 In vitro gas production in cereals and legumes

The result confirms the hypothesis that starchy cereals are more rapid fermented than legumes. A significant difference ($P<0.001$) of gas growth rate between most of cereals and legumes is shown in Figure 21. It can be explained that cereals contain higher starch content than legumes. On the other hand, cereals grains contain higher aDNFom content than legumes. In this study, aNDFom showed a significant positive correlation to gas production rate, which helps cereals are more rapidly fermented at the beginning of incubation than legumes. Furthermore, crude

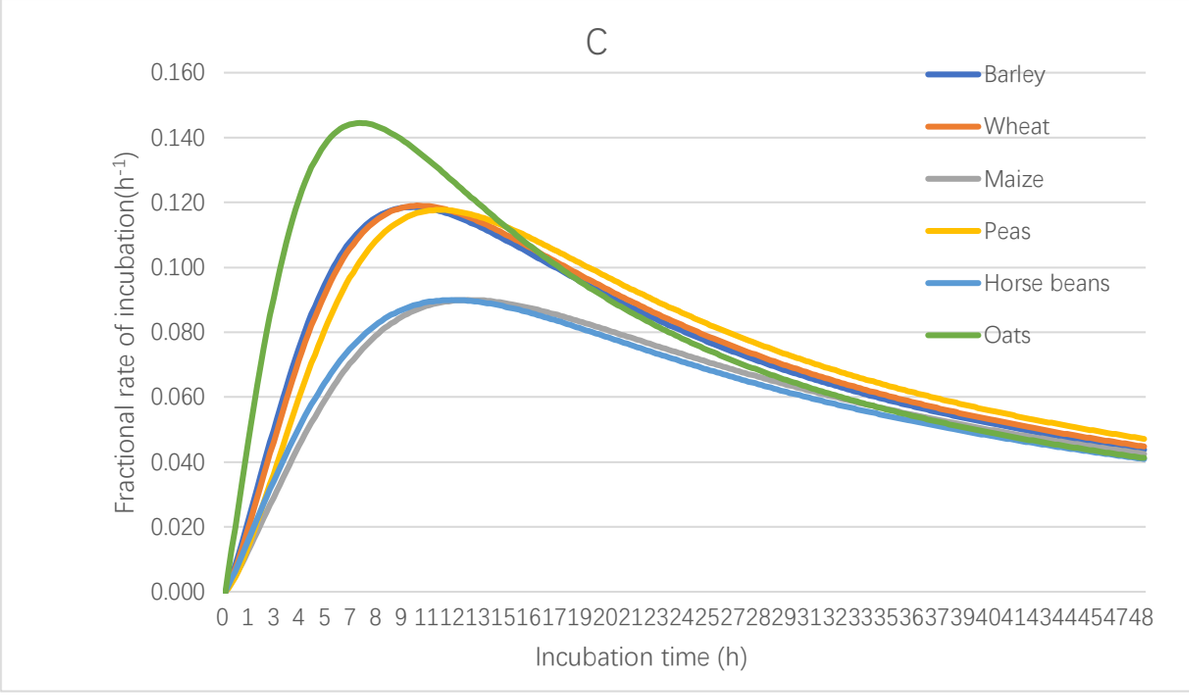


Figure 21. Cumulated profile of fractional rate © and total gas production (D) of cereals and legumes

protein content is high in legume, a negative correlation ($P<0.005$) between CP and gas production rate was analyzed in the result. The rate of gas production formed the most rapid in oats. The primary cause is probably that oats starch is easily fermented during ruminal digestion due to its small particle size (3-10 μm) of starch granules (Lineback 1984). Small starch granules have a larger surface area available for microbial and enzymatic starch hydrolysis which results in rapid degradation. Furthermore, unlikely to maize, starch granules in oats has a loosely bond with protein matrix, which positively facilitate fermentation.

5.2.3 In vitro gas production of legumes and starch-extracted legumes

Based on result of GLM contrast procedure and in vitro gas production kinetics profiles, pea

starch and horse bean starch have more rapid gas production than peas and horse bean, which is consistent to our hypothesis (Figure 22).

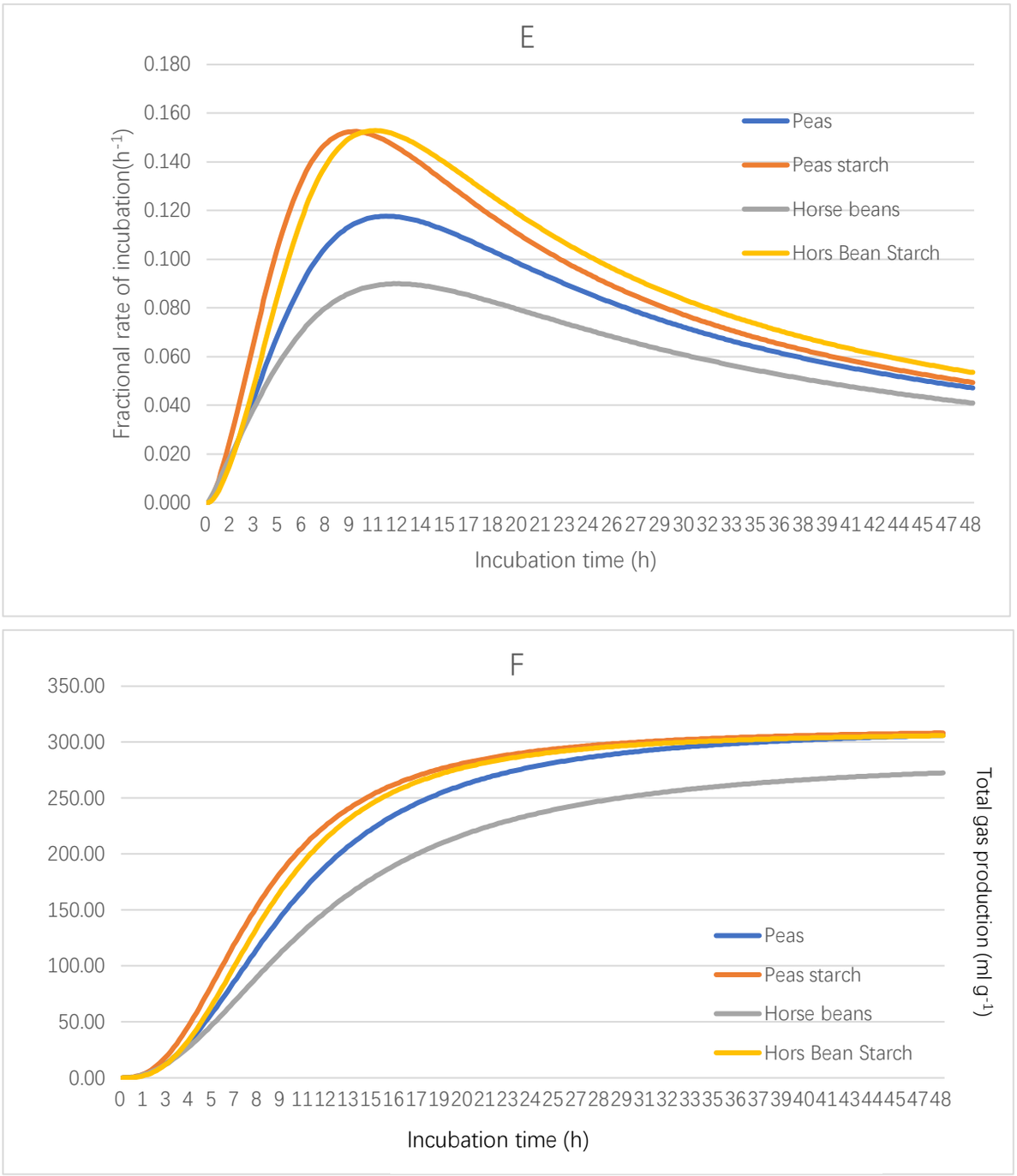


Figure 22. Cumulated profile of fractional rate (E) and total gas production (F) between Peas, pea starch, horse bean and horse bean starch.

The primary explanation is that pea starch and horse bean starch contain higher ratio of starch: protein than peas and horse beans. Starch content is positively correlated to gas production rate. It might can be explained that higher starch contents caused an increase in amylolytic bacteria populations in the buffered rumen fluid, enabling higher levels of starch degradation (Chai, Van Gelder et al. 2004). Furthermore, protein content is higher in peas and horse bean starch than pea starch and horse bean starch. Previous study of Getachew reported the negative correlation between crude protein and gas production rate which is consistent to our result ($P<.005$) (Getachew, Robinson et al. 2004). Hence, pea starch and horse bean starch are more rapidly fermented than whole legumes.

Antinutrients factors might be the secondary cause that slow down the fermentation in peas and horse beans. Tannins and phenolic component would decrease gas production because of a portion of methanogens is attached to protozoa (Vogels, Hoppe et al. 1980) which is the major microbial population that involved in carbohydrate degradation. Current study has shown that peas has higher gas production and more rapid gas production rate compared to horse beans (Abreu and Bruno-Soares 1998), which is consistent within our results. The factor lead to this consequence might be both two antinutrients are higher in horse bean than peas, peas would be less interrupted and more rapid fermented than horse bean, which is shown in the gas production rate curve. In terms of gas production, horse bean starch is expectedly higher than horse bean ($P<.001$) because of higher content of starch. However, peas and pea starch show no difference of gas production each other, which can be primarily explained that peas contain high amount of readily fermentable starch, simple sugars and oligomeric carbohydrates (Adamidou, Nengas et al. 2011) which significantly promotes gas production. On the other hand, differences in proportions of soluble and insoluble non-starch polysaccharides might have had an additional effect on gas production (Adamidou, Nengas et al. 2011). According to Cone et al. (1997) and Groot et al. (1996) the major part of initial gas production is caused by fermentation of the water-soluble components, which also indicates peas probably has high ratio of water-soluble components.

5.3 Relationship between chemical composition and fermentation products

The correlation analysis revealed a strong correlation between gas production at 24h, 48h, A parameter and starch content. It can be explained that carbohydrate fractions directly affect the fermentation kinetics, where starch promotes a more intense and rapid fermentation. Conversely, structural carbohydrates cause a slower and less extent of fermentation. Poor correlation between rate of gas production and starch is formed in this study, which is consistent with the previous report of Naahlai (Nsahlai, Siaw et al. 1994). However, there was a strong negative correlation between aNDFom concentration and gas production ($P<.001$), which is in agreement with previous studies giving negative effects of NDF and lignin on gas production (Van Soest 1982) (Makkar, Singh et al. 1989). Larbi et al (Larbi, Smith et al. 1998) documented that the negative correlation between CP and gas production was showed previously, which is not consistent in our result, where the CP content of grains was non-correlated with gas production but negatively correlated ($P<.005$) to gas production rate.

5.4 Estimated organic matter digestibility and metabolizable energy

The gas production technique is an alternative method with less expensive than in situ to estimate the nutritional value of feeds. Gas production technique has been recommended mainly as a tool for ranking feeds due to its poor repeatability (Hall and Mertens 2008). Instead of measuring disappearance of insoluble substrates in situ or in vitro, the gas production technique measures appearance of gas, which is generated from the fermented OM as well as gas which is released from the buffer in relation to the acid properties of the end-products of fermentation (Tagliapietra, Cattani et al. 2011). Therefore, despite a good correlation between the amounts of OM digested and gas produced is expected (Makar 2004), the degree of correlation is influenced by such as methodological factors (Nazarov and Ormiston 1986), differences in experimental protocol, feed and inoculum characteristics, which all alter release of gas from the medium. These factors could also influence repeatability and reproducibility of GP measurements.

The OMD values of the feeds were within the ranges reported by Menke and Steingass (Menke 1988) using the estimation methods. The value of OMD of peas and horse bean are lower than

previous study by Abreu et al.(Abreu and Bruno-Soares 1998). Despite the estimated value is lower than feed table in reference (Norfor), the ranking among cereals is within the right rank, for instance, oats had lowest OMD, legumes had higher OMD than cereals, which are comparable to Norfor table. The ME values of the feeds were within the ranges reported by Menke and Steingass (Menke 1988) using the estimation methods, where the ME values of various European feeds ranged from 4.5 to 15 MJ kg−1 DM. The estimated metabolizable energy (ME) is highly consistent of previous study which used the same equation and method (Getachew, Crovetto et al. 2002). However, ME is lower than data in Norfor for same grains, in which ME values for barley, wheat and oats were reported to be 13.2; 14.1; and 12.9 MJ kg-1 DM in Norfor compare to 9.73; 9.88 and 8.87 MJ/ kg in this essay. Same to OMD value, ME in this study ranking the right sequence based on Norfor table.

5.5 Relationship between chemical composition and energy value

Estimated energy value in gas production method is strongly correlated to gas production quantity. Therefore, the chemical compositions that correlate to gas production are also correlate to energy values. In this study, legumes showed higher OMD than cereals due to high CP content which positively correlated ($P<0.05$) to OMD. A highly positive correlation between starch and OMD and ME indicates that starch fermentation greatly contributes to OMD value. A strong negative correlation ($P<0.001$) between aNDFom and OMD was analyzed, which is consistent to previous study that Musco et al. (2016) reported negative significant correlations between content of NDF in grass and in vitro organic matter degradability ($r = -0.70$) and fermentation rate of GP ($r = -0.70$). Metabolizable energy is consistent to OMD, chemical compositions that correlate to OMD also has same relationship to ME.

6. Conclusion

Based on the results obtained from the present experiment, in vitro gas production can offer a feed fermentation kinetics in rumen fluid and using empirical equations to estimate the OMD and ME content of feeds. But, for individual substrates, fermentation kinetics can rarely reflect the individual degradation by gas production alone. The chemical composition and nutrients interaction of feeds effect nutrient degradation and energy value. Consistent to the hypothesis, starch-extracted legumes showed more rapid fermentation than legumes. Maize produced highest quantity of gases with slowest gas production rate. In converse, oats had lowest gas production and fastest fermentation. There was a strong correlation between chemical composition and gas production, which is also reflected on OMD and ME. Interaction between starch, NDF, and other polymers is highly affect to nutrient fermentation. It is difficult to conclude that starch degradability only depends on individual factors. Therefore, more detailed studies are needed to conduct to verify these findings.

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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

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Norway