Substrate Levels, Carbohydrate Degradation Rate and their Effects on Ruminal End-Product Formation

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Abstract

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New feed evaluation systems for ruminants, based on dynamic-mechanistic models and predictions of production responses to nutrients, demand accurate prediction of end-products formed during rumen fermentation. The aim of the present work was to investigate the effects of levels of different substrates and carbohydrate degradation rates upon ruminal end-product formation.

An *in vitro* method was introduced to evaluate feeds with respect to ruminal starch degradation. The method was based on incubation of feed samples in a buffered rumen fluid solution and a subsequent enzymatic analysis of remaining starch. Heat treatment generally increased rate or extent of starch degradation to glucose. Use of single time point determinations correlated reasonably well with degradation rate estimates and offers the possibility to reduce the cost of analysis.

Two experiments with a 2^3 factorial arrangement of treatments were performed with an *in vitro* rumen simulation technique (SIMCO) to investigate the direct effects and interactions of several dietary factors upon ruminal fermentation. Response in volatile fatty acid (VFA) proportions to starch level was dependent on starch and neutral detergent fibre (NDF) sources. Faster degradation rates of starch and NDF had a positive effect upon microbial N efficiency and the propionate proportion. Increasing the level of N improved microbial N efficiency and increased the proportion of propionate and butyrate at the expense of acetate. Increased feeding level resulted in a lower microbial N efficiency and a lower proportion of propionate. The results of the two *in vitro* experiments clearly point out that rumen VFA proportions and microbial N efficiency are not only affected by the level of different substrates fermented in the rumen but also their degradation rates.

Data from Nordic dairy cow experiments were analysed and modeled with respect to effects of feed characteristics upon rumen fermentation patterns. The final model differed relative to published VFA models by predicting a smaller effects of starch but large effects of silage lactate levels on propionate proportions.

To improve the prediction of end-product formation, better characterization of feeds are needed. Carbohydrate degradation rate and type of forage are factors that probably should be included in future models.

Keywords: dairy cattle, *in vitro* technique, modelling, microbial N efficiency, rumen fermentation, starch degradation, stochiometry, VFA.

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„*En hvatki er missagt er í frœðum þessum, þá er skylt at hafa þat heldr, er sannara reynist”*

Ari fróði Þorgilsson (1067-1148)

Contents

Appendix

Papers I - IV

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals (**I-IV**):

I. Sveinbjörnsson, J., Murphy, M. Udén, P. 2006. In vitro evaluation of starch degradation from feeds with or without heat-treatments. *Submitted.*

II. Sveinbjörnsson, J., Murphy, M., Udén, P. 2006. Effect of the proportions of neutral detergent fibre and starch, and their degradation rates, on in vitro ruminal fermentation. *Animal Feed Science and Technology. In Press.*

III. Sveinbjörnsson, J., Murphy, M., Udén, P. 2006. Effect of the level of dry matter and protein and degradation rate of starch on in vitro ruminal fermentation. *Animal Feed Science and Technology. In Press.*

IV. Sveinbjörnsson, J., Huhtanen, P. Udén, P. 2006. The Nordic dairy cow model Karoline – development of VFA sub-model. In: *Nutrient digestion and utilisation in farm animals: modelling approaches.* Edited by E. Kebreab, J. Dijkstra, A. Bannink, W. J. J. Gerrits & J. France. CAB International, Wallingford, UK, pp. 1-14. *In Press.*

List of abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BW	Body weight
cEE	concentrate ether extract
cNDF	Concentrate NDF
CP	Crude protein
DIP	Degradable intake protein
DM	Dry matter
EC	Adenylate energy charge
ESD	Effective starch degradation
fNDF	Forage NDF
INDF	Indigestible NDF
LA	Lactic acid
MRT	Mean retention time
NDF	Neutral detergent fibre
NSC	Non-structural carbohydrate
PDV	Portal drained viscera
Re	Rest fraction
RNA	Ribonucleic acid
VFA	Volatile fatty acids
WSC	Water soluble carbohydrates

Introduction

Cellulose, the most abundant carbohydrate in the world, and other fibre carbohydrate have no nutritional value for animals unless they are first metabolised by gut microbes. The ability of rumen microbes to convert fibre and simple nitrogen compounds to volatile fatty acids (VFA) and microbial cells, which can be utilised by the host, is one reason for the great evolutionary success of ruminants and for their domestication. Simple-stomached animals have also been domesticated and used for food production. This group of animals largely depends on direct enzymatic digestion of non-fibre carbohydrates and proteins. This results in a higher efficiency than through fermentation, which always involves considerable energy losses through heat and methane. However, large areas of land are naturally unsuitable for low-fibre food or feed production, whereas the production of fibrous feeds is often both efficient and sustainable. Therefore, it is essential to increase our knowledge of factors controlling the site of digestion of both fibrous and non-fibrous substrates in the ruminant gastrointestinal tract and the proportional redistribution of organic matter by rumen fermentation into VFA, microbial matter and methane. The subject of this thesis is within this fascinating area and some basic knowledge related to that subject will be reviewed shortly in the following sections.

Importance of VFA in ruminant metabolism

Absorbed VFA account for as much as half to over three-quarters of the metabolisable energy supply to the lactating dairy cow (Lomax & Baird, 1983; Reynolds & Huntington, 1988; Reynolds *et al.*, 1988; Casse, Rulquin & Huntington, 1994; De Visser *et al.*, 1998; Reynolds *et al.*, 2003; Kristensen, 2005). As opposed to monogastric animals, ruminants absorb only minor amounts of hexose sugars. Nevertheless, glucose is very important in ruminant metabolism, particularly in pregnant and lactating animals (Baldwin & Kim, 1993; Bell, 1993; Brockman, 1993). Even on high-concentrate diets, glucose absorption from the gut accounts for less than one-third of whole-body glucose turnover (Bergman, 1973; Van der Walt, Baird & Bergman, 1983). The portal drained viscera (PDV), which includes most of the alimentary tract, the pancreas and the spleen (Lindsay, 1993), utilises considerable quantities of glucose (Brockman, 1993). Rarely more than half of the starch digested in the small intestines is recovered as glucose in the portal vein (Kreikemeier & Harmon, 1995; Reynolds, Sutton & Beever, 1997; Reynolds *et al.*, 1998). Gluconeogenesis takes place in the liver (80–90%) and kidneys (10–20%) and provides most of the glucose used by the ruminant (Bergman, 1975). A major part of the glucose utilised by the lactating dairy cow is taken up by the mammary gland, mainly for production of lactose. Significant amounts of glucose are also used by the brain, uterus, and in lipid synthesis (Brockman, 1993; Danfær, Tetens & Agergaard, 1995). For a cow in the sixth month of pregnancy, yielding 25 kg of milk and gaining 0.5 kg body weight (BW) per day, gluconeogenesis must provide 2–2.5 kg/d of glucose, assuming that 0.1–

0.2 kg is absorbed from the digestive tract, according to estimations by Danfær, Tetens & Agergaard (1995).

Propionate is the major precursor of glucose in the fed state, and spares other possible glucose precursors like amino acids, glycerol and lactate for synthetic functions. According to data summarised by Danfær, Tetens & Agergaard (1995), propionate generally provides 50 to 60% and lactate 10 to 20% of total glucose flux rate. Glycerol contributes little except when the cow is in a severe negative energy balance. Estimates of the contribution of amino acids to glucose synthesis vary from 1 to 50% of glucose flux rate. It was estimated that this difference corresponded to an almost twofold difference in total dietary protein requirements for cows yielding 30 kg milk per day. As feed protein is expensive compared to carbohydrates, knowledge that can help minimise the direct or indirect use of dietary protein for glucose production in the ruminant is very valuable.

Acetate is the major source of energy to the ruminant. According to simulation studies by Baldwin & Kim (1993) using the dairy cow model presented by Baldwin, France & Gill (1987), a cow in early lactation yielding 25 to 30 kg milk/d, oxidises approx. 55 to 65% of the acetate. The rest is used for lipogenesis in the mammary gland and adipose tissue. According to the same simulations, acetate, propionate and butyrate absorbed from the digestive tract accounted for approximately 0.3, 0.2 and 0.1 of the total absorbed energy, respectively. Butyrate is mainly converted to ketone-bodies (3-OH-butyrate), which like acetate, are used both for oxidation and lipogenesis. The ruminal epithelium metabolises around three-fourths of the absorbed butyrate to 3-OH-butyrate (Kristensen, Danfær & Agergaard, 1998; Kristensen, 2005). During fasting, fatty acids from lipolysis may replace acetate and butyrate as energy sources (Brockman, 1993).

Substrates fermented to VFA within the rumen

The major fermentation substrates are dietary carbohydrates, mainly cellulose, hemicellulose, pectin, starch and soluble sugars. On diets high in ruminally-degraded protein, a large amount of amino acids resulting from protein hydrolysis are deaminated and then converted to VFA. Lipids contribute little to rumen VFA production, as they are normally a very small proportion of the diet and only the glycerol and galactose moieties are fermented (France & Siddons, 1993). Fermented feeds, like silages, may contain considerable quantities of fermentation end-products such as organic acids and alcohols with lactate usually the dominant end-product if the silage fermentation has been successful. The animal absorbs these fermentation products but they contribute little (lactic acid) or nothing (VFA) as energy sources for rumen microbes (Chamberlain, 1987).

Feed polysaccharides are first degraded by microbes to monosaccharides, primarily hexoses and pentoses, before they are absorbed and metabolised to VFA, methane and ATP. Differences in ruminal carbohydrate degradation can largely be explained by the different functions of the carbohydrates within the plant. The two main types of feed polysaccharides are: plant storage polysaccharides like starch

and fructans, and structural polysaccharides like cellulose and hemicellulose, which constitute the major part of the plant cell wall. Fructans and simple sugars like glucose, fructose and sucrose are rapidly and fully degraded in the rumen. The starch and cell wall polysaccharides are more complex with respect to rumen degradation, and this will be dealt with in the following sections.

Competition between the rates of degradation (k_d) and passage (k_p) from the rumen determines the extent of degradation of a feed component in the rumen. It is often calculated as the ratio of $k_d/(k_p+k_d)$, whereas the fraction escaping rumen digestion can be calculated as $k_p/(k_p+k_d)$ (Van Soest, 1994). This calculation assumes a single and completely mixed rumen compartment. More advanced models have been presented by France *et al.* (1990), Mertens (1993) Van Soest (1994) and others, accounting also for factors like lag times, distinct substrate pools with different degradation rates, *etc*. However, these models only seek to describe digestion kinetics and often cannot be used in continuous models, as every meal then has to be modelled.

Properties of starch with respect to rumen degradation

Starch is primarily composed of two structurally distinct α-linked polymers of glucose, *i.e.* amylose and amylopectin. Amylose is an essentially unbranched polymer of α-1,4-linked D-glucose units, with a chain length of several hundred units. The more abundant component of starch, amylopectin, is a much larger polymer of D-glucose units linearly chained by α-1,4-linkages but with α-1,6 branch points every 20 to 25 glucose units (Rooney & Plugfelder, 1986). The percentage of branching in amylopectin varies from 4.0 to 5.5 compared to only 0.2 to 1.0 for amylose (Santacruz, 2004). Normal cereal starches contain 20 –to 35% amylose, but cultivars are available with very high amylose contents (high-amylose maize, 40 –to 80% amylose) and waxy cereal starches with almost no amylose (Rooney & Plugfelder, 1986; Chesson & Forsberg, 1988; Mills, France & Dijkstra, 1999). Amylose and amylopectin molecules are held together by hydrogen bonds, forming highly organised starch granules (Rooney & Plugfelder, 1986). Granules from tubers are generally large and spherical, those from cereals small and polyhedric, but legume starch granules are kidney shaped (Gallant *et al.*, 1992).

Starch granules have both crystalline and amorphous areas. The crystalline region, primarily composed of amylopectin, is resistant to water entry and enzyme attack. The amorphous region (gel phase) is less dense and richer in amylose than the crystalline area but includes also the α-1,6 branch points of amylopectin. Water moves freely through it and amylase attack begins in this region, while the hydrolysis of the crystalline region occurs more slowly (Rooney & Plugfelder, 1986). It is generally accepted (Santacruz, 2004) that a radial arrangement of the amylopectin glucan chains within the starch granules is responsible for the semi-crystalline nature of starch, as mentioned above.

X-ray diffraction analysis of native starches yields two types of spectral patterns, the A- and B-type, which points to two kinds of crystalline structures. The A-type pattern is seen in cereal starches, but tuber starches and amylose-rich starches have the B-type pattern. B-type starches hold more water than A-type starches. Legume starches yield an intermediate (C-type) pattern, which appears to be a mixture of A and B type patterns rather than a distinct crystalline structure (Annison & Topping, 1994).

Starch granules are insoluble in cold water and swell reversibly as they take up water during gradual heating. Root and tuber starches swell more than cereal starches (Rooney & Plugfelder, 1986). The starch granules undergo gelatinization, or irreversible loss of the native structure, when sufficient energy is applied to break the intermolecular hydrogen bonds (Zobel, 1988). The granules absorb water, swell, exude part of the amylose and become more susceptible to enzyme degradation during gelatinization. The gelatinization starts in the amorphous regions, but penetration of heat and moisture into the crystalline regions is slower. The amorphous areas are plasticized by water, providing sufficient mobility for the crystallites to melt. More heat or mechanical energy is required for this to occur when water is limiting. Mechanical gelatinization of starch may occur during milling and grinding of cereals and dilute alkali and acid promote gelatinization of starch with or without heat. Heat, moisture, mechanical and chemical treatments are often combined when processing starchy materials (Rooney & Plugfelder, 1986).

Retrogradation is the re-association of starch molecules separated during gelatinization and results in liberation of bound water from the gel. It may in that sense be viewed as the opposite of gelatinization, but the retrograded starch does not have the semi-crystalline character of the native starch. Retrogradation can result in starch forms that are resistant to enzyme attack (Rooney & Plugfelder, 1986; Mills, France & Dijkstra, 1999), but does not seem to restrict ruminal degradation as much as digestion by non-ruminants (Chesson & Forsberg, 1988).

Cereal starches are normally more easily degraded than root and tuber starches with legume starches as intermediates. Heat treatments improve degradability of the poorly digestible starches in most cases (Offner, Bach & Sauvant, 2003). Interactions with proteins can reduce the susceptibility of both native and processed starch to enzyme hydrolysis. In the vitreous endosperm of maize and sorghum the starch granules are completely embedded in a protein matrix and degrade more slowly than in the more floury endosperm of barley, wheat and other cereal starches. Treatments that hydrolyse the protein matrix give rise to increased rate of starch degradation as the surface area of starch in contact with enzymes increases (Rooney & Plugfelder, 1986).

Extracellular microbial enzymes are responsible for ruminal starch hydrolysis. The α-amylase acts randomly and therefore degrades both amylose and the linear chains of amylopectin, whereas β-amylase removes maltose units from the non-reducing ends of all starch chains. After approximately 50% of the amylopectin has been degraded to maltose by β-amylase, a residue known as a β-limit dextrin is

left, which is protected from further attack by the 6-linked branch points. For complete hydrolysis of amylopectin, enzymes are required such as glucamylase that act on the α-1,6-linkages. Maltases, maltose phosphorylases, or α-1,6-glucosidases, hydrolyse maltose and isomaltose to produce glucose or glucose-1-phosphate (Chesson & Forsberg, 1988; Mills, France & Dijkstra, 1999).

Properties of cell walls with respect to rumen degradation

Cellulose and hemicellulose are the two major components of plant cell walls and are available for ruminal fermentation. Lignin, another important component of mature cell walls, is not only itself resistant to microbial digestion, but also reduces the overall digestibility of cellulose and hemicellulose, owing to the spatial arrangement of lignin within the plant. Pectic substances are also sometimes regarded as a distinct class of cell wall polysaccharides, although the distinction between the pectic and hemicellulosic polymers is not always clear. Hemicellulose polymers are those not extracted by hot water or chelating agents but which are soluble in alkali or acid detergents (Van Soest, 1994).

Hydrolysis of cell walls results in the release of monosaccharide residues. Between 70 to 90% are accounted for by rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and uronic acid, which is defined as the sum of galacturonic acid and glucuronic acid or its 4-O-derivatives. Pectic substances are a complex of polysaccharides based on chains of α-1,4-linked galacturonic acid units, but include monosaccharide residues like rhamnose, galactose and arabinose (Chesson & Forsberg, 1988). The composition of hemicellulose is somewhat variable between mono- and dicotyledons and between primary and secondary cell walls, but in all cases β-1,4-linkages are the most common. Grass hemicellulose consists mainly of a xylan (β-1,4-xylopyranoside) backbone with various side chains. The simple sugar components of hemicellulose are arabinose, xylose, mannose, galactose and glucuronic acids (Van Soest, 1994). Cellulose consists of linear chains of β-1,4-linked glucose units that aggregate, forming microscopically visible fibrils where the individual glucan chains are extensively cross-linked by hydrogen bonding (Chesson & Forsberg, 1988). These microfibrils are considerably more resistant to chemical or enzymatic attack than the free glucan chains (Krassig, 1985). The cell walls of monocotyledons have a much higher proportion of cellulose but lower proportions of hemicellulose and pectic substances than dicotyledons (Chesson & Forsberg, 1988).

Degradation of cell walls within the rumen is initiated by the attachment of rumen microbes to feed particles. Major bacterial species attaching to particles are the cellulolytic bacteria *Ruminococcus albus*, *R. flavefaciens* and *Fiberobacter succinogenes*. Rumen fungi and protozoa also colonize and degrade plant fragments to differing degrees (Akin, 1986). Only small proportions (less than 15%) of rumen cellulase, hemicellulase and glycosidase activities are found in cell-free cultures. Bacteria with high glycosidase activity are loosely bound to plant materials, whereas the hemicellulolytic and cellulolytic bacteria are more firmly attached (Williams & Strachan, 1984). A detailed account of the enzymes

involved in microbial degradation of cell walls in the rumen will not be given here.

There is a large variation in the susceptibility of cell walls towards ruminal degradation. The surface of plant particles is effectively protected from microbial attack by epicuticular waxes. Invading microbes have to rely on natural openings such as stomata or broken edges of the feed particles. The fine structure of the polysaccharides, such as the crystallinity of cellulose, seems to have a relatively minor effect on cell wall degradability compared to the effects of lignification. Although lignin is the major cell wall component limiting digestion of cell wall polysaccharides in the rumen, lignin concentration alone is only partially responsible for variations in cell wall digestibility. Lignin seems to exert its negative effects on cell wall polysaccharide digestibility by shielding the polysaccharides from enzymatic hydrolysis. Cross-linkage of lignin and cell wall polysaccharides by ferulic acid bridges may be a prerequisite for these effects to occur (Jung & Allen, 1995). About 20 different types of linkages have been identified within the lignin macromolecule, compared to only the glycosidic linkage in the polysaccharides (Chesson & Forsberg, 1988). Depending largely upon lignification, cellulose digestibility in ruminants can vary from 0 to 100%. Other inhibitors than lignification include silicification and cutinisation. The intrinsic properties of cellulose itself also seem to play some role, as unlignified cellulose fractions from different sources show widely diverse rates of digestion. Hemicellulose digestibility is closely related to that of cellulose, as both are negatively related to lignification. However, hemicellulose is the most complex and most closely attached to lignin of the plant polysaccharides (Van Soest, 1994).

General aspects of rumen microbial metabolism

Dietary carbohydrates are degraded to hexoses and pentoses as discussed in the previous sections. Pentoses proceed mainly through the transketolase and transaldolase reactions of the pentose cycle, which yield hexose and triose phosphate. As the majority of the carbohydrates are in the form of hexose, they are metabolised to pyruvate, almost exclusively by the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (France & Siddons, 1993). The EMP pathway is the most common pathway of hexose metabolism in both aerobic and anaerobic microorganisms (Gottschalk, 1979). This is advantageous to anaerobic bacteria, because it maximises the yield of ATP (Russell & Wallace, 1988). Pyruvate proceeds to acetate or butyrate with acetyl-CoA as an intermediate. Propionate is formed mainly via succinate (randomising pathway) but an alternative pathway (direct reductive pathway) involves acrylate (Russell & Wallace, 1988; France & Siddons, 1993). These reactions can be summarised as follows (Wolin, 1960):

hexose → 2 pyruvate + 4H
pyruvate → acetate + CO_2 + 2H
2 pyruvate → butyrate + 2 CO_2
pyruvate + 4 H → propionate + H_2O
CO_2 + 8H → methane + 2 H_2O

The reducing equivalents (H), formed during glycolysis, results in NAD being converted into NADH. A subsequent re-oxidation of this NADH is then required for the fermentation to continue (Russell & Wallace, 1988). As propionate is a hydrogen sink but the formation of acetate (and butyrate) involves a release of hydrogen, an increased acetate:propionate (A:P) ratio increases the need for release of hydrogen through methane.

According to Russell and Wallace (1988), the fermentation of one hexose to acetate, propionate or butyrate, yields 4, 4 and 3 ATP, respectively, assuming that propionate is produced by the randomising pathway via succinate. The acrylate pathway only yields 2 ATP per hexose, as does the fermentation to lactate, ethanol or valerate.

The ATP produced by rumen fermentation is required for microbial growth. Bauchop and Elsden (1960) found a correlation between the amount of ATP which could be derived from catabolism, and the yield of cell mass (Y_{ATP}, g/mol ATP). The values ranged from 8.3 to 12.5 with an average of 10.5 from studies of several bacterial species. Later work has shown a much greater range (Stouthammer & Bettenhaussen, 1973) and that many rumen bacteria have Y_{ATP} above 20 (Russell & Wallace, 1988). Approximately two-thirds of the ATP is needed for polymerisation reactions, and transport is also a significant energy cost (15 to 27% of the total). Energy costs for synthesising different cell constituents differ and are, for example, threefold greater for protein than for polysaccharide (Stouthammer, 1973).

Russell (1983) studied the growth of *Prevotella* (formerly *Bacteriodes*) *ruminicola* on a medium with ammonia as the sole nitrogen source. He found that Y_{ATP} increased when protein hydrolysate was added, and that little glucose was used as carbon source. Maeng and Baldwin (1976a; 1976b) found that the yield of microbial protein per mole ATP was higher when the nitrogen came both from amino acids and ammonia, rather than only from one of these sources. The stimulatory effects of amino acids upon microbial growth appeared to be related closely to the amounts of starch remaining in the rumen, suggesting that the growth of amylolytic bacteria is especially stimulated by amino acids (Maeng & Baldwin, 1976a). Maeng *et al.* (1976) found that the specific growth rate of rumen microbes as well as Y_{ATP} was highest when 25% of urea-N was replaced by amino acid-N in the incubation medium. A very important concept was shown by Stouthammer and Bettenhaussen (1973), that lower proportions of ATP is used for maintenance at high specific growth rates, and consequently, that Y_{ATP} is improved.

Differences in the proportional redistribution of organic matter into microbial protein and different end-products are considerable with respect to available nutrients for absorption by the animal. Beever (1993), using relationships provided by Baldwin, Lucas & Carbrera (1970), gave examples of three different diets; high forage, high cereal and high molasses, stimulating acetate, propionate and butyrate production, respectively (Table 1).

Acetate-induced fermentation is the most efficient for the microbes, as it results in the greatest amount of ATP produced per mole hexose. The amount of VFA energy to the animal is greatest with propionate induced fermentation. That is also the type of fermentation pattern that is most efficient in transferring carbohydrate energy to useful fermentation end-products (VFA + ATP), in line with lesser methane production.

Table 1. End-product formation and energetic efficiency resulting from fermentation of hexose by different pathways (Beever, 1993).

	High forage	High cereal	High molasses
Moles end-product from 1 mole hexose:			
Acetate	1.34	0.90	0.94
Propionate	0.45	0.70	0.40
Butyrate	0.11	0.20	0.33
Methane	0.61	0.38	0.54
ATP	4.62	4.38	4.50
Total VFA	1.90	1.80	1.67
Energetic efficiency:			
VFA energy (MJ/mol hexose)	2.109	2.306	2.163
VFA as proportion of hexose energy	0.73	0.80	0.75
VFA + ATP as prop. of hexose energy	0.85	0.92	0.87

Rumen microbial populations and their substrate preferences

The following section is based on a summary by Theodorou & France (1993), where original references can be found. Major bacterial species involved in degradation of cellulose within the rumen are *Fiberobacter succinogenes*, *Ruminococcus albus*, *R. flavefaciens* and *Eubacterium cellulosolvens*. Hemicellulose is also degraded by some of the cellulolytic microbes and also by other bacteria such as *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*. Protozoa as well as *B. fibrisolvens* and *Lachnospira multiparus* degrade pectin. Some cellulolytic bacteria, like certain strains of *F. succinogenes,* are also amylolytic. However, the principal amylolytic species, such as *B. amylophylus*, *Selenomonas ruminantium* and *Streptococcus bovis* have limited capacity to utilise other polysaccharides than starch. These microbes, and soluble sugar utilisers like *Megasphaera elsdenii*, compete with many other microbes for these rapidly degrading substrates. Most rumen bacteria, except for the main cellulolytic bacteria, are to some extent proteolytic. However, *B. amylophylus*, *B. fibrisolvens*, *P. ruminicola* and the proteolytic butyrivibrios are probably the major proteolytic species in the rumen. Most species of rumen bacteria and fungi, but only a few protozoal species, produce cellular nitrogen compounds from ammonia. Some ruminal bacteria greatly rely on products of primary fermentation. As an example, *Veillonella parvuvla*, *M. elsdenii* and *S. ruminantium*, convert lactate and succinate to acetate and propionate. *Methanobrevibacter ruminantium* and *Methanosarcina barkeri* utilise H_2 and CO_2 or formate, acetate, methylamine and methanol for methane production.

Protozoa are able to degrade all major plant constituents, although much of their energy and nitrogen requirements are obtained by phagocytosis of other microbes.

The entodiniomorphid ciliates, such as *Epidinium* can engulf plant fragments for subsequent digestion. Holotrich protozoa like *Dasytricha* and *Isotricha* metabolise soluble sugars but also produce cellulases for degradation of plant biomass polymers (Hobson & Wallace, 1982; Theodorou & France, 1993).

Rumen concentrations *vs.* absorption of VFA

In the analytical approach to rumen fermentation modelling, advanced by Koong *et al.* (1975) and Murphy, Baldwin & Koong (1982), the amount of individual VFA available for absorption is predicted from the amount of different substrates fermented in the rumen, multiplied by the estimated fermentation coefficients for these substrates. The coefficient estimates are largely from rumen VFA concentrations (Murphy, Baldwin & Koong, 1982) rather than absolute VFA production rates. It is assumed that VFA proportions in the rumen at any time reflect the proportions in which they are absorbed. Data on rumen VFA concentrations are commonly collected in digestion studies. They are relatively easy to obtain, although representative sampling is particularly important with respect to the heterogeneous nature of ruminal contents and dynamics of fermentation (Corley *et al.*, 1999). The concentration of individual VFA in the rumen depends on the balance between the net rate of production and the rate of removal from the rumen (Bergman, 1990). The net VFA production rates in the rumen correspond to the amounts removed by absorption and passage (France and Siddons, 1993). The gross (total) production rates can be defined as the total amount of acid entering the VFA carbon pool per time unit. The differences between net and total production rates in the rumen is partly due to interconversions between individual VFA, particularly acetate and butyrate (France and Siddons, 1993), but also because of carbon exchange between VFA and other metabolites or products of microbial metabolism (Kristensen, 2001). In the subsequent sections of this thesis, "VFA production" will be used for net VFA production, unless otherwise stated.

The fermentation pattern measured as rumen concentrations would reflect the pattern of VFA production if all VFA were absorbed at the same fractional rate. Early results indicated that this might be true for forage-type diets, but less reliable for concentrate diets (Leng & Brett, 1966; Esdale, Broderick & Satter, 1968; Sharp, Johnson & Owens, 1982). It is now accepted (Bergman, 1990; Dijkstra *et al.*, 1992; Lopez *et al.*, 2003) that, especially at low rumen pH, the fractional rate of VFA absorption from the rumen increases with increasing chain length, i.e. in the order acetate<propionate<butyrate. Experimental work of Dijkstra *et al.* (1993), where VFA solutions were introduced into the washed rumen of dairy cows, demonstrated that an increase in initial rumen pH from 4.5 to 7.2 decreased fractional absorption rates of acetate, propionate and butyrate from 0.35, 0.67 and 0.85 to 0.21, 0.35 and 0.28, respectively. According to these estimates, a better agreement between rumen concentration and production of VFA on roughage than concentrate type of diets could now be explained by smaller differences in absorption rates between individual VFA in high-pH roughage diets.

Dijkstra *et al.* (1993) also found that initial concentrations of the individual VFA affected their fractional absorption rates differently. Butyrate was unaffected while propionate, and especially acetate, was absorbed at a lower fractional rate with increased initial concentrations. This indicated that saturation kinetics were involved in VFA absorption. Lopez *et al.* (2003) infused VFA continuously into the rumen of sheep. The effects of the level of VFA infusion on fractional absorption rates were insignificant for acetate, but propionate and butyrate were absorbed at significantly higher fractional rates at higher levels of infusion. The effects of VFA infusion level on absolute VFA absorption rates were highly significant and positive for all VFA, "confirming that VFA absorption in the rumen is mainly a concentration dependent process" (Lopez *et al.*, 2003, p. 2609).

Dijkstra *et al.* (1993) established equations describing VFA absorption from their work, accounting for the effects of pH and ruminal VFA concentration. These equations predicted unrealistically low absorption rate of acetate relative to propionate and butyrate, compared to *in vivo* results (Bannink *et al.*, 2000). Kristensen and Harmon (2004a) reported that fractional absorption of acetate was 0.56 in a study where ruminal VFA concentrations and pH (6.5 ± 0.1) were relatively stable during incubation of VFA buffers, which were continuously agitated by gassing. In the study of Dijkstra *et al.* (1993), the fractional absorption rate of acetate was 0.33 for a treatment with an initial pH of 6.3. However, pH increased during the measurement period, data were corrected for liquid passage from the rumen, and buffers were not continuously gassed. These differences in experimental conditions could explain the differences in observed fractional absorption rates in the two studies.

Although many studies show that fractional absorption rates of acetate, propionate and butyrate are not very different at ruminal pH above 6.3 (Dijkstra *et al.*, 1993; Lopez *et al*., 2003; Kristensen & Harmon, 2004a & b), it seems that differences in fractional absorption rates between individual acids are important on rapidly fermenting diets. VFA are absorbed from the rumen both as anions and as undissociated acids (Kramer *et al.*, 1996; Gäbel & Sehested, 1997). According to the Henderson-Hasselbach equation, about 99% of the VFA are on the anionic form at pH 6.8 but 76% at pH 5.3 (Lopez *et al.*, 2003). The undissociated acids are very lipid-soluble and may therefore permeate biological membranes more easily than in the anionic form. This means that if absorption of VFA would occur only as passive diffusion of undissociated acids, the absorption rate should follow the concentration of the undissociated acids according to the Henderson-Hasselbach equation. Both *in vivo* and *in vitro* experimental results (review by Gäbel & Sehested, 1997) have shown a much smaller effect of pH on VFA transport than would be expected by this theory. The current view is therefore that there is considerable absorption of VFA from the rumen in the anionic form. The mechanism appears to be coupled to the exchange of VFA anions, and probably also Cl^- with HCO_3^-. This exchange, together with disappearance of undissociated VFA, helps to keep intraruminal pH within physiological limits (Gäbel & Sehested, 1997).

In vivo methods for measuring VFA production

VFA concentration patterns in the rumen do not fully reflect the absorption of individual acids, as discussed above. Direct measurements of VFA absorption, in combination with digestion studies on animals consuming normal diets, would, hopefully aid in establishing more realistic VFA stochiometry. It is not an easy task, however, to measure VFA absorption. A variety of non-tracer methods were used in early attempts to quantify rumen VFA production (Warner, 1964; Hungate, 1966). These include (France & Siddons, 1993):

a) Perturbation of the steady state: where the rate of production of an acid (or total VFA) in steady state is calculated from the change in its ruminal concentration when the acid is infused.

b) Portal-arterial difference in VFA concentration: here VFA concentration is measured in arterial blood entering the rumen (carotid artery) and in venous blood draining the rumen (the portal vein). The difference in concentrations provides a measure of the amount of VFA entering the blood from the rumen, if the rate of blood flow is known. This method was used by Barcroft, McAnally & Phillipson (1944) to demonstrate that VFA from rumen fermentation are absorbed and utilised by the animal. However, this method does not account for the fact that individual VFA are metabolised to a different degree in the portal drained viscera (PDV) on their way to the portal vein (Bergman, 1990; Kristensen, 2005).

c) Methane production: methane production has been used to obtain indirect estimates of VFA production. The estimated methane production is multiplied by the ratio of individual or total VFA to the methane produced, as determined by measurements or stochiometrical calculations.

The non-tracer methods were later superseded by isotopic tracer techniques (Bergman *et al.*, 1965; Weller *et al.*, 1967; Morant, Ridley & Sutton, 1978; Armentano & Young, 1983). The tracer methods use compartmental analysis to interpret isotope dilution data, either by adopting a single (total VFA) or a three-pool scheme (acetate, propionate, and butyrate). It can, however, be extended to any number of pools. Steady-state conditions are normally assumed and the label is constantly infused. Theoretically, it can be adapted for non-steady state conditions and single injection of label (France & Siddons, 1993). Contrary to the non-tracer methods, multi-isotopic methods account for major inter-conversions between individual VFA (Leng & Brett, 1966). However, acetate also exchanges carbon with other metabolites than VFA in the rumen (Kristensen, 2001), which poses severe limitations on the isotope dilution techniques with regard to acetate absorption. Also, this technique does not account for VFA production in the large intestines.

Due to these and other limitations of the tracer methods, portal absorption studies based on the multi-catheterized cow (Huntington, Reynolds & Stroud, 1989) have received increased attention. One reason has been that, contrary to earlier assumptions (Bergman & Wolff, 1971), ruminal epithelium metabolises

none or small amounts of acetate and propionate absorbed from the rumen (Kristensen *et al.*, 2000). These observations were made based on experiments where VFA were infused to temporarily washed rumens of multi-catheterized sheep. It was found that acetate and isobutyrate where fully accounted for in the portal vein and only 5 to 10% of the propionate was apparently metabolised during absorption. This was in agreement with other *in vivo* studies that showed that only a small percentage of propionate is metabolised to lactate during absorption (Weigand, Young & McGilliard, 1972; Weekes and Webster, 1975). This revealed that rumen microbes metabolise significant fractions of the acetate, propionate and isobutyrate produced according to isotope dilution studies (Kristensen, 2001; 2005). There is an extensive metabolism of butyrate and valerate in the PDV. Typical recoveries of butyrate are 25% or less in the portal vein from functional and washed rumens of sheep (Kristensen, 2005).

Practical application of the methodology based on the multi-catheterized cow requires that portal-drained visceral metabolism of arterially supplied metabolites are accounted for (Kristensen *et al.*, 1996; Kristensen, Pierzynowski & Danfær, 2000), as well as the metabolism by rumen epithelia of butyrate and long-chained VFA (Kristensen, 2001).

In vitro methods for measuring VFA production

As discussed above, methodological problems related to *in vivo* measurements of ruminal VFA production are partially caused by the complexity of the VFA metabolism in the animal. *In vitro* methods, where rumen conditions are simulated have therefore been used to estimate the VFA, gas and microbial protein production from different diets. Quantitative measurements are easier to make *in vitro* because VFA are not absorbed. When *in vivo* and *in vitro* results are compared, it must be kept in mind that what happens *in vivo* is reality, whereas *in vitro* is only a simulation. How close *in vitro* simulations are to reality is not yet known.

In vitro rumen systems can be sub-divided into batch and continuous culture types. In a batch culture there is only one initial supply of substrate. As fermentation goes on, the concentration of substrate decreases but that of fermentation end-products rises. In the continuous culture, substrate is added and end-products removed over an extended culture period, in order to simulate more closely the conditions in the rumen (e.g. Ewart, 1974; Nocek, 1988).

Ewart (1974) and Czerkawski (1976) have presented the most important aspects of the criteria that have to be met when designing an artificial rumen for the study of rumen microbial metabolism. Temperature, redox potential, pH and osmotic pressure have to be kept within the same range as in the rumen. Substrate and culture flow must be easy to control, as well as stirring or mixing of the culture. Replication must be possible. Measurements, sampling and removal of products must be easy and reproducible. It is an advantage if the system is able to maintain normal microbial population, including protozoa, and a stable production of total

and individual volatile fatty acids. However, the balance between simplicity and the perfect simulation can be difficult to obtain. The choice of system must therefore depend on the purpose of the experiment. For example, for rapidly fermented substances, much information can be gathered from short (2 to 8 h) batch culture incubations. This can be done with a much simpler apparatus than long-term simulations that are used to study more complex aspects of rumen function.

Czerkawski (1976) divided artificial rumens into three main types; the bulk incubation type, the continuous flow type and the semi-permeable type. Each of these types can be either open or closed. The bulk type artificial rumen is a batch culture system, whereas the other two types are designed for continuous cultures. An example of the bulk type is the apparatus used by Czerkawski and Breckenridge (1969). In the continuous flow type, the nutrients are mixed with buffer solution and pumped continuously into the reaction vessel. The reaction mixture is either pumped out or allowed to overflow. Steady state is achieved when the dilution rate is in balance with the growth rate of the microorganisms (Czerkawski, 1976). In a semi-permeable artificial rumen, fermentation end-products are removed continuously by dialysis (Rufener, Nelson & Wolin, 1963; Abe & Kumeno, 1973; Nakamura & Kurihara, 1978). It is possible to design a unit such that the reaction mixture is diluted continuously. That design simulates physiological conditions quite closely but is very complex and difficult to operate (Czerkawski, 1976).

The theory of continuous culture says that microbial growth is limited by only one substrate, but is also controlled by dilution rate to some extent. Applying the theory of continuous culture requires that substrate flow and dilution rate remain constant and are within specified limits, and that the volume of the culture is constant. An apparatus that enables all this is a chemostat (Ewart, 1974).

The rumen is analogous to the continuous culture in many aspects, but there is also some divergence between the systems. Hungate (1966) particularly pointed out the non-linear kinetics of the rumen processes as being the reverse of the chemostat, *i.e.* the rumen is "cyclic" rather than the "steady-state" of the chemostat. Many *in vitro* systems recognize this and use periodic feeding. Sometimes these systems are referred to as continuous, even though they do not operate at a steady state, but because the culture can be maintained over an extended period (Ewart, 1974). The terms semi-continuous culture (*e.g.* Nocek, 1988) and repeated fed batch culture (RFB) (Theodorou & France, 1993) are also used for these types of systems.

Outflow rates of the liquid and solid phases normally differ in the rumen. *In vitro* systems without differential outflow rates can result in low protozoal numbers and low digestibilities of the solid substrates (Miettinen & Setälä, 1989). Outflow rates of solids and liquids can be separated by placing feeds into nylon bags that are removed at determined intervals (Weller & Pilgrim, 1974; Czerkawski & Breckenridge, 1977) or by filtering the outflow (Hoover, Crooker & Sniffen, 1976). Systems with removal of end-products by dialysis (Rufener,

Nelson & Wolin, 1963; Abe & Kumeno, 1973; Nakamura & Kurihara, 1978) have also maintained protozoa successfully. Slow-growing microbes are maintained by selective particle retention in the rumen. *In vitro* systems have been developed that attempt to simulate rumen metabolism more closely by simulating rumen particle retention (Teather & Sauer, 1988; Fuchigami, Senshu & Horiguchi, 1989; Murphy & Lindgren, 1998).

Aims of the thesis

The general aim of the thesis was to increase the knowledge on the effects of levels of different substrates and carbohydrate degradation rates upon ruminal end-product formation. The specific objectives were to:

a) establish an *in vitro* method for measuring rates of ruminal starch degradation and apply it to different feedstuffs, with and without heat treatments (Paper **I**).

b) investigate the direct effects and interactions of levels and degradation rates of carbohydrates (starch and NDF), protein availability and DM level upon ruminal fermentation using an *in vitro* rumen simulation technique (Papers **II** and **III**).

c) analyse data from Nordic dairy cow experiments with respect to effects of feed characteristics upon rumen fermentation pattern with the objective to develop a sub-model for rumen VFA production for use in the Nordic dairy cow simulation model Karoline (Paper **IV**).

Materials and methods

The methodology used is briefly summarised below, but details of experimental procedures, chemical and statistical analysis are described in the four papers upon which this thesis is based. The *in vitro* experiments reported in Papers **I, II** and **III** were conducted at Kungsängen Research Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden. The *in vivo* data analysed in Paper **IV** was collected from dairy cow digestion studies in Finland, Denmark, Norway and Sweden.

Measurements of ruminal starch degradation *in vitro* (Paper I)

An *in vitro* method was used in four studies to evaluate rate and extent of ruminal starch degradation from various feeds with or without heat treatments. The method was based on incubation of feed samples with buffered rumen fluid solutions and subsequent enzymatic analysis of remaining starch. Starch was considered degraded when hydrolysed to at least glucose.

In all studies, effects of different heat treatments upon starch degradation were examined. The most detailed investigation of heat treatments was made in Study 3. Here, the effects of both autoclaving time (15, 30, 60 min) and temperature (115, 130 and 145 °C) upon starch degradation rates in peas were tested. Other feeds tested in the four studies were potatoes, potato starch, barley, wheat and maize. In Study 4, VFA production after fermentation for 8 hours was measured together with starch degradation.

The amount of feed fermented was adjusted for its starch content, such that approximately 75 to 80 mg of starch was incubated, resulting in a total sample size of 160 mg or less for feeds containing at least 50% starch. Each feed sample was incubated with 7 ml rumen fluid and 14 ml *in vitro* media (Goering & Van Soest, 1970) in 50 ml polypropylene centrifuge tubes. Rumen fluid was obtained from a cannulated lactating cow fed a diet of 550 g/kg DM grass silage and 450 g/kg DM concentrates.

The *in vitro* system was used both for single time-point determinations of remaining starch and for estimating fractional degradation rates (k_d; /h). Statistical analyses were performed with the GLM ANOVA procedure of MINITAB 11 (Minitab, Inc., PA., USA) and for estimating fractional degradation rates (k_d), the standard least-squares minimisation procedure in Table-Curve™ (Jandel Scientific, San Rafael, CA, USA) was used to fit the model:

$S = 1 – \exp\text{-}k_d \times t;$

where k_d is the fractional rate of starch degradation per hour and t is the incubation time.

Simulations of rumen digestion by the SIMCO system (Papers II & III)

The SIMCO *in vitro* rumen simulation system used in the experiments reported in Papers **II** and **III** was a modification of the system presented and evaluated by Murphy & Lindgren (1998). Details of the SIMCO version used are presented in Paper **II**. The culture was maintained over a 10-day period, but the SIMCO system, as operated in these experiments, would be defined as a semi-continuous (Nocek, 1988) or a repeated-fed batch (RFB) culture (Theodorou & France, 1993). It recognised the non-linear kinetics of rumen processes and periodic feeding and intermittent buffer inflow and digesta outflow was used. The construction of the system and the routines used were aimed at obtaining normal (ruminal) stratification of vessel contents. This was done in order to maintain protozoa and other slow-growing microbes by selective particle retention, as occurs in the rumen. However, the daily routines also had to accommodate what would be manageable during normal working hours.

The feeding routine was the same for both experiments (Papers **II** and **III**), *i.e.*, the fermenters were fed four equal portions of solid feed at 8.00 h, 10.00 h, 14.00 h and 16.00 h. In the first experiment (Paper **II**), 0.3 g DM/vessel/d of NH3-N was

fed continuously as part of the buffer media. In the second experiment (Paper **III**), variable amounts of both NH3-N and peptone (casein hydrolysate) were included in the buffer media. The buffer was pumped in at three different rates, in connection with the daily feeding schedule. This was done in order to simulate the diurnal saliva flow and pH pattern. Digesta was removed through a glass tube from the bottom, mainly from the liquid phase. The removal of digesta followed a similar schedule in both experiments, except that in Paper **III**, removal was done six times (from 8.00 h to 18.00 h), compared to five times daily (8.00 h to 16.00 h) in Paper **II**. This resulted in a somewhat smaller diurnal fluctuation in vessel volume in the experiment of Paper **III**. Average vessel volume was in the range 1100 to 1150 ml/d and the total daily buffer flow varied between 1800 to 2000 ml/d, resulting in a liquid dilution rate close to 0.07/h or 1.7 turnovers/d in both studies. During digesta removal, the mixing of digesta was maintained at a slow rate to ensure that the liquid fraction was homogeneously mixed but stratification of vessel contents would still exist. An exception to this (both in Papers **II** and **III**), was emptying at 8.00 h, when the digesta mixing rate was increased until vessel contents were completely homogenized. This was done, based on previous experience, to prevent an excessive accumulation of indigestible material in the fermenters.

Table 2. A schematic representation of experimental treatments in SIMCO experiments. See Papers **II** and **III** for details

Paper **II**

		NDF source	
		Fast	Slow
Starch source	Fast	300 g starch/kg DM 600 g starch/kg DM	300 g starch/kg DM 600 g starch/kg DM
	Slow	300 g starch/kg DM 600 g starch/kg DM	300 g starch/kg DM 600 g starch/kg DM

Paper **III**

		N level	
		Low (N1)	High (N2)
Starch source	Fast	20 g DM 40 g DM	20 g DM 40 g DM
	Slow	20 g DM 40 g DM	20 g DM 40 g DM

Both SIMCO experiments (Papers **II** and **III**) had a 2^3 factorial arrangement of treatments, allowing for simple investigations of interactions as well as main effects of treatment factors. There were two levels of each of three treatment factors, *i.e.* a total of eight treatment combinations ($2 \times 2 \times 2$). Each of the eight treatments was replicated twice within two separate runs, giving a total of 16 observations. The GLM ANOVA procedure of MINITAB 11 (Minitab, Inc., PA., USA) was used in analyses of the data as described in the papers in more detail. Table 1 is a schematic explanation of the experimental treatments. Two types of potato starch were tested in both experiments; a raw, slow-degrading and a

cooked, fast-degrading, based on earlier degradation measurements (Paper **I**). The slow-degrading NDF source tested in the first SIMCO experiment (Paper **II**), was a late-cut Timothy, and the fast-degrading NDF source was from early-cut Meadow grass. A mixture of these two NDF sources was used in the second SIMCO experiment (Paper **III**).

Standard chemical procedures were followed for analysis of feed and digesta in the SIMCO experiments, as described in Papers **II** and **III**.

Analysis of *in vivo* rumen fermentation data (Paper IV)

A database was formed, including 29 dairy cow digestion studies with a total of 107 experimental treatments, of which 51 were from experiments carried out in Finland, 33 in Denmark, 12 in Norway and 11 in Sweden. The database included treatment means on feed intake, cow body weight (BW), feed composition and molar proportions of individual VFA (acetate, propionate, and butyrate) observed in the rumen. Information on the contents of ash, forage NDF (fNDF), concentrate NDF (cNDF), starch (St) and crude protein (CP) was recorded for all diets. Lactic acid (LA) and VFA had been measured in most silages, but had to be estimated from table values in a few cases. A rest fraction (Re) was defined as:

Total DM – ash – fNDF – cNDF – St – CP – LA - VFA.

Substrate proportions truly digested in the rumen were based on rumen digestion studies, when available, but in other cases rumen digestion coefficients were either obtained from *in sacco* or *in vitro* data or estimated from total tract digestibilities. It was assumed that LA and Re fractions were completely digested in the rumen.

The database was used to:

a) evaluate published fermentation coefficients; here only a subset of the database was used.

b) carry out multiple regression analysis (by Genstat 6.1, Lawes Agricultural Trust©, 2002) to investigate relationships between dietary factors and the molar proportions of the three major VFA.

c) establish, based on information gained by the multiple regression analysis, a stochiometrical model accounting for the most important dietary effects upon rumen VFA pattern. For this purpose the Solver tool (Fylstra *et al.*, 1998) in Microsoft® Excel was used, which employs the generalized reduced gradient (GRG2) non-linear optimisation code (Lasdon *et al.*, 1978).

All models fitted are explained in detail in Paper **IV**.

Results

Starch degradation *in vitro* – effects of heat treatments (Paper I)

In the four *in vitro* studies presented, heat treatments were shown to increase rates or extent of starch degradation. In Study 1, about one-quarter of the starch in cooked potatoes remained after 5 h of fermentation compared to one-half in raw potatoes. A considerable proportion of the remaining starch was in the soluble form. In Study 2, cooking increased starch degradation rate in isolated potato starch (from 0.038 to 0.197 h^{-1}), and native starch in barley and wheat had similar rates of degradation (0.117 and 0.109 h^{-1}, respectively).

In Study 3, autoclaving for only 15 min did not result in significant changes in degradation rates. When peas were autoclaved for 30 min, only the highest temperature (145 °C) significantly increased the degradation rate of the pea starch compared to the control (0.175 *vs.* 0.110 h^{-1}). When autoclaving for 60 min, both 130 and 145 °C increased the degradation rate (0.211 and 0.193 h^{-1}, *vs.* 0.110 h^{-1} for the control). Autoclaving at 115 °C did in no case change starch degradation rates in peas. In Study 4, the ratio of starch degraded by 8 h incubation was increased ($P<0.001$) by heat treatments of pure potato starch (0.155 *vs.* 0.870), peas (0.491 *vs.* 0.815), barley (0.686 *vs.* 0.913) and maize (0.351 *vs.* 0.498). Measurements of volatile fatty acid production in the fermentation tubes showed a lower ($P<0.001$) acetate:propionate ratio for the faster fermenting, heat-treated feeds. The regression of the acetate:propionate ratio upon 8-h degradability showed an $R^2 = 0.85$:

Acetate:propionate ratio = 1.77 - 0.926 * 8-h degradability ($P<0.001$)

Treatment effects upon *in vitro* ruminal digestion, fermentation and microbial protein production in SIMCO (Papers II & III)

Starch digestibility was not affected by treatment factors other than type and level of starch, in the two experiments. The starch digestion coefficient was in the range 0.95 to 1.00 in both experiments, except at the highest starch levels in Paper **II**. At these levels, digestibility of raw starch was 0.83 to 0.90. The raw potato starch was visually accumulating during the feeding hours (not reported in the papers) and this could also be judged from the diurnal pH pattern. Before feeding in the morning, however, most starch had disappeared. The digestibility of NDF differed considerably between the two types of hay (0.32 and 0.57 for Timothy and Meadow grass, respectively), used in Paper **II**. In addition, NDF digestibility was greatly depressed by increasing starch level, especially in the case of the rapidly-degrading cooked starch. In the second study (Paper **III**), NDF digestibility was 0.46 in treatments with raw starch compared with 0.36 in treatments with cooked starch.

VFA efficiency was in most cases in the range 0.40 to 0.50 g VFA/g organic matter truly digested (OMTD). However, in the former study (Paper **II**), the higher starch level resulted in a significantly lower VFA efficiency, *i.e.* 0.36 compared to 0.51 g VFA/g OMTD at the lower starch level. It is possible that some lactic acid was produced but not accounted for.

In both SIMCO experiments, increased degradation rate of starch resulted in an increase in the proportion of propionate at the expense of acetate (as well as butyrate in Paper **II**). The proportion of other VFA (i-butyrate, n-valerate, and i-valerate) were also higher for cooked *vs*. raw starch in Paper **II**, but not in Paper **III**. In Paper **II**, interactions between treatments demonstrated that the response in VFA pattern to starch level was dependent on starch and NDF sources. The only significant main effects of hay type (Paper **II**) on VFA proportions were on butyrate, which was higher for Meadow grass hay than for Timothy (168 *vs*. 127 mmol/mol total VFA). There were significant interactions between hay type and starch level (Paper **II**) upon acetate and butyrate proportions and a three-way interaction (hay type × starch type × starch level) upon acetate and butyrate proportions.

Faster degradation rates of starch (Papers **II** and **III**) and NDF (Paper **II**) had positive effects upon microbial N efficiency, whereas starch level (Paper **II**) did not affect this parameter uniformly. However, interactions of forage and starch source reported in Paper **II** showed that when a rapidly degrading Meadow grass hay partly replaces starch, the efficiency of microbial protein production increases. Increasing the level of N improved microbial N efficiency in Paper **III**, and increased the proportion of propionate and butyrate at the expense of acetate. Increased feeding level resulted in a lower microbial N efficiency and a lower proportion of propionate. Furthermore, interactions between starch degradation rate and N level upon VFA pattern were apparent.

Analysis of *in vivo* rumen fermentation data (Paper IV)

Through analysis of the Nordic database, both by a multiple regression and a stochiometrical approach, it was possible to relate rumen VFA pattern to six individual substrate classes and also to correct for the effects of feeding level (FL) and concentrate ether extract level (cEE). According to this analysis, approximately 80% of fNDF and cNDF is fermented to acetate. About half of the lactate (LA) is fermented to propionate, and the rest fraction gives a higher than average proportion of both propionate and butyrate. The proportion of starch in the diet had very small effects on VFA pattern, but increases in FL and cEE resulted in higher propionate and lower acetate and butyrate levels. The root mean squared prediction error (RMSPE) for the stochiometrical model with corrections for FL and cEE was 20.8, 14.4 and 16.8 mmol/mol total VFA for acetate, propionate and butyrate, respectively, when fitted to the whole database (107 treatments). This combined model is presently used as the VFA-sub-model in Karoline (Danfær *et al.*, 2006). A further examination of the prediction errors of this model showed that some of the unexplained variance could be related to

different effects of individual substrates upon VFA composition, depending on their concentration in the diet.

General discussion

Methods for predicting ruminal starch degradation

New systems for feed evaluation based on dynamic-mechanistic models and predictions of production responses to nutrients (*e.g.* Danfær *et al.,* 2006) require data on starch degradation characteristics. The lack of clear progress in this area is limited by: a) difficulties in obtaining reliable *in vivo* reference data, including technical difficulties in duodenal starch flow measurements; b) limitations of alternative laboratory techniques (Huhtanen & Sveinbjörnsson, 2006, *in press*.).

The *in sacco* procedure is the most commonly used method for determining feed degradation in the rumen (Nocek, 1988; Nocek & Tamminga, 1991). Recently, Offner, Bach & Sauvant (2003) built and analysed a database on *in sacco* starch degradation measurements from 302 experimental observations containing 22 untreated raw materials, some of which had been subjected to physical or chemical treatments. Table values were reported for rates of starch degradation and effective starch degradability in the rumen for the different feedstuffs, corrected for laboratory effects, which where considerable and highly significant. Following up this work, Offner & Sauvant (2004) collected a database with 316 observations of starch digestibility in growing and lactating cattle, equipped with ruminal and duodenal cannulas. In line with earlier studies (*e.g.* Nocek & Tamminga, 1991), it was shown that *in vivo* ruminal starch digestion, when predicted by *in sacco* data, was generally underestimated for slowly degrading feedstuffs but overestimated for those degrading rapidly. Recent studies designed to compare *in sacco* and *in vivo* starch degradation confirm this (Tothi *et al.*, 2003; Hindle *et al.*, 2005). Possible reasons for discrepancies between *in sacco* and *in vivo* data are; loss of small starch granules from the bags (Nocek, 1988), no particle size reduction within the bags (Ewing & Johnson, 1987) and differences in microbial activity within and outside the bags (Nocek, 1988; Noziere & Michalet-Doreau, 1996). These methodological problems may affect the starch sources differently, depending on their rate of degradation.

The data presented in Paper **I** does not allow detailed comparison with alternative methods for measuring ruminal starch degradation. However, *in vitro* starch disappearance rate was 20 to 40% higher than reported by Cone and co-workers (Cone *et al.*, 1989; Cone & Vlot, 1990; Cone, 1991) for peas, wheat, barley and maize. Calculated effective starch degradabilities also seemed to be closer to the *in vivo* values published by Offner & Sauvant (2004) than those of Cone and co-workers. During the selection and adaptation of the method used in Paper **I**, the main goal was the ability of the method to evaluate the intrinsic characteristics of the feed. Starch degradation should not be limited by the *in vitro* system itself. Therefore, an emphasis was put on creating a substrate-limited system, with optimal conditions for ruminal microbial activity regarding pH,

anaerobiosis, redox potential, microbial numbers, essential nutrients, *etc.* (Mertens, 1993). The results obtained with the system so far indicate a good progress in this respect and that the system is capable of ranking feeds with respect to starch degradation rates and/or extent of degradation at a single time-point. For a further comparison of methods for measuring ruminal starch degradation, including the method presented in Paper **I**, see Huhtanen & Sveinbjörnsson (2006, *in press*).

When effective rumen starch degradation is calculated, passage rates are as important as degradation rates. Rate errors will bias effective degradation more at low digestion rate relative to passage rate. Calculations are most often based on fixed passage rates, or are functions of DM intake or other factors (*e.g*. Offner & Sauvant, 2004). Studies based on rumen evacuations (Tothi, *et al.*, 2003) have revealed that the assumption of fixed first-order fractional rate of starch passage is an oversimplification, and that different starches behave quite differently in this respect. This is an additional and probably quite important explanation for discrepancies between digestibilities measured *in vivo vs*. estimated from *in sacco* or *in vitro* degradations.

Effects of heat treatments upon ruminal starch degradation

In vitro results showed substantial positive effects of heat treatments upon starch degradation. These effects were greatest for pure potato starch, which was the basis for our choice of raw and cooked potato starch, representing slow and fast degrading starches, respectively, in the experiments presented in Papers **II** and **III**. Rather intensive heat treatments are needed to increase the rate of pea starch degradation, judged from the results of different autoclaving times and temperatures reported in Paper **I**. This is also indicated by *in sacco* results as discussed in Paper **I**. Among the factors that can explain the variable outcome of processing on starch degradation are the opposite effects of starch gelatinisation and retrogradation (Goelema *et al.*, 1999). Offner & Sauvant (2004) reported that the *in sacco* technique overestimates digestion of rapidly degrading starches, such as in barley. Increases in barley starch degradation from expansion were detected *in vitro* (Paper **I**) and *in vivo*, but not *in sacco* (Tothi *et al.*, 2003). Actually, the potential for increases in starch degradation *in sacco* was limited, considering a range of effective starch degradation (ESD) from 0.930 to 0.960 for untreated barley, which was an overestimation relative to the *in vivo* values (Tothi *et al.*, 2003).

Microbial N efficiency in the SIMCO *in vitro* system

The microbial N efficiency (mg microbial N/g OMTD) was generally low in our SIMCO studies (Papers **II** and **III**), compared to *in vivo* values (Stern & Hoover, 1979; Doreau & Ferlay, 1995; Shingfield, 2000). This was the case in two other studies using the same system (Murphy & Lindgren, 1998; Udén, 2006). The SIMCO system is designed to simulate selective particle retention by allowing stratification of vessel contents into a floating (top), suspended (middle) and

sedimented (bottom) layers. The floating layer in the top mimics the low-density fibre particles found in the upper layer of the rumen. By emptying the SIMCO vessels mainly from the middle and bottom layers, the liquid retention time was kept within a normal range.

However, the solid retention time is not easily controlled in the SIMCO system, as it is largely determined by the transport of digesta from the top to the middle and bottom layers. The fibre particles float as long as they are large enough and produce gas in sufficient quantities to maintain buoyancy (Sutherland, 1988). Udén (2006) found that the mean retention time (MRT) of indigestible NDF (INDFom) had a strong negative correlation with the amount of INDFom fed. Furthermore, microbial efficiency decreased with increasing MRT of INDFom. The feeding level in SIMCO, as used in the four studies reported (Murphy & Lindgren, 1998; Udén, 2006; Papers **I** & **II**), was less than one-tenth of the feeding level in a dairy cow, assuming a rumen liquid volume of 60 l. Therefore, Udén (2006) concluded that the low microbial efficiency in the SIMCO system was caused by long solids retention times, resulting from low feeding levels. A negative effect of increased feeding level on microbial efficiency observed in Paper **III** contrasts with this assumption. However, based on other parameters measured there (protozoa ratings, propionate levels), it was suggested (Paper **III**) that this could be caused by sub-optimal environments for slower growing microbes at the lower feeding level, resulting in a possible dominance of faster growing, amylolytic species.

More rapidly fermenting starch (Papers **II** & **III**) and fibre (Paper **II**) resulted in higher microbial N efficiency *vs.* the slower ones, but increases in starch levels did not result in a similar positive response. Furthermore, partial replacement of starch with rapidly degrading forage increased microbial N efficiency. This demonstrated that the proportion of substrate incorporated into microbial matter is not only affected by total substrate availability, but also by substrate differences. In Paper **III**, increased N levels (mainly amino-N) led to increased microbial N efficiency, especially with rapidly degrading starch at the higher feeding levels. In addition, a higher microbial N efficiency was observed in Paper **II** with rapidly *vs.* slowly degrading starch and forage fibre. This should be considered in relation to the generally high level of amino-N and total-N in all diets. These findings are in accordance with results from other *in vitro* (Van Kessel & Russel, 1996) and *in vivo* (Soto *et al.*, 1994; Chikunya *et al.,* 1996) studies, and suggest that amino-N only stimulates microbial growth when the energy source permits a fast growth rate.

VFA efficiency in the SIMCO *in vitro* system

The VFA production accounted for, on average, 0.44 and 0.47 of OM truly digested in Papers **II** and **III**, respectively, and the only significant treatment differences were between the two starch levels in Paper **II**. The average VFA efficiency was lower than observed by Udén (2006) with the SIMCO system (0.54 on average), and considerably lower than indicated by theoretical calculations

(*e.g.*, Beever, 1993). Comparison with *in vivo* data is difficult, due to difficulties in measuring actual VFA production rates, as discussed earlier. It is likely that lactic acid was produced in some treatments (not measured), which could explain the low efficiencies found with the high starch levels. Udén (2006) found a positive correlation between solid retention time and total VFA flow from the SIMCO system. In that study, as well as the study of Murphy & Lindgren (1998), treatment effects on VFA and microbial N efficiencies compensated each other in some cases, which should be expected (Beever, 1993). However, in Papers **II** and **III**, significant treatment differences in microbial N efficiency were found, but VFA efficiency was less affected by experimental treatments. Using fixed ratios to estimate microbial N from measured RNA and the production of lactic acid or other end-products not measured in the digesta may explain the apparent lack of effects observed.

Ruminal fermentation pattern - *in vivo* and *in vitro*

The analysis of *in vivo* data from the Nordic database presented in Paper **IV** established relationships between digested substrates and rumen VFA pattern, which, in some respects, differ from other estimations of stochiometric coefficients (Murphy, Baldwin & Koong, 1982; Bannink *et al.*, 2000; Bannink *et al.*, 2006). The main differences were; a) weak effects of starch on ruminal propionate ratios and, b) that silage lactate was included as a separate substrate pool, mainly converted to propionate. The average dietary starch content in the Nordic database (Paper **IV**, Table 1.1) was 166.2 g/kg DM, and of the 107 diets in the database, only 40 contained more than 200 g starch/kg DM and only 12 diets contained more than 250 g starch/kg DM. Although this reflects the dietary situation for many dairy cows in the Nordic countries, it is a low level of starch compared to what some other analyses of starch effect upon VFA pattern are based on. For example, in the most recent analysis (Bannink *et al.*, 2006), starch was on average 250 and 270 g/kg DM for roughage and concentrate diets, respectively.

Average observed VFA molar proportions in the Nordic database (Paper **IV**) were 669, 193 and 138 mmol/mol VFA for acetate, propionate and butyrate, respectively. Mills, France & Dijkstra (1999) summarised rumen fermentation data from 22 studies with lactating dairy cows from the literature where different starch sources were fed to dairy cows. Diets with barley yielded rumen acetate, propionate and butyrate in the proportions 574, 278 and 105 mmol/mol VFA whereas for maize the proportions were 620, 228 and 118 mmol/mol VFA, respectively. Sorghum and wheat yielded average VFA patterns close to those for maize. The average starch proportion in the diets was around 300 g/kg DM both for barley and maize, but the ruminally-digested starch was on average 4.50 kg/d for barley diets compared to 3.75 kg/d for maize diets. The average ruminally digested starch in the Nordic database (Paper **IV**) was 2.57 kg/d. In the analysis of Bannink *et al.* (2006), ruminally digested starch was on average 2.64 and 3.11 kg/d for roughage and concentrate diets, respectively. The proportion of propionate in VFA from starch according to their analysis was 0.22 and 0.31 for

the respective diets. From these comparisons, it seems relevant to conclude that negative effects of starch on the acetate:propionate ratio increases with increased level of ruminally digested starch. It is unclear if this is caused purely by an increased amount of degraded starch, irrespective of starch source, or by starch degradation rate *per se*. This question has not been answered by *in vivo* results as, in most cases, similar amounts of the different starch sources were fed, resulting in higher levels of ruminally-fermented starch for the faster degrading starch sources. A further complication is the possibility of variable passage rates of the different starch sources *in vivo* (Tothi *et al.*, 2003).

The *in vitro* rumen studies presented in this thesis, especially in Paper **II**, were designed with these limitations of *in vivo* studies in mind. Similar amounts of the fast and slow degrading starch sources were degraded each day, but at very different rates. The results clearly showed that an increased degradation rate of starch, rather than the total amount of starch degraded, results in increased propionate (Papers **II** and **III**) and butyrate (Paper **III**) proportions, at the expense of acetate. The propionate levels were higher than normally seen *in vivo*, but the levels of starch were high compared to most *in vivo* studies. In fact, *in vitro* rumen studies with unusually high levels of non-structural carbohydrates (Stokes *et al.*, 1991b), or at a low pH (Yang, Beauchemin & Vedres, 2002), have demonstrated even higher propionate proportions (>400 mmol/mol VFA) than found in Papers **II** and **III**. Brown *et al*. (2002) observed higher propionate and lower acetate proportions with an *in vitro* gas production system relative to *in vivo* observations for eight forage diets. Given that the fractional absorption rate of VFA increases with increasing chain length (Bergman, 1990; Dijkstra *et al.*, 1992), dietary factors affecting butyrate and propionate production should give relatively higher responses *in vitro* where no absorption occurs compared to *in vivo*.

With this in mind, it seems that the SIMCO VFA patterns (Papers **II** and **III**) were within the expected range. However, there are considerable differences in tolerance of microbial species to *in vitro* conditions, which may result in differences in microbial metabolism *in vitro* and *in vivo*. Some *in vitro* systems are practically defaunated, and even though the SIMCO system seems quite efficient in maintaining protozoa (Murphy & Lindgren, 1998), protozoa scores declined during the 10-day incubations. This was most apparent at the higher starch levels in Paper **II**, indicating that the high starch levels were unfavourable to the protozoa. Rumen protozoa mainly produce acetate and butyrate, as reviewed by Jouany, Demeyer & Grain (1988). Low levels of protozoa might therefore be part of the explanation for high propionate levels in the SIMCO studies (Papers **II** and **III**).

Direct comparisons of *in vivo* and *in vitro* results from long-term rumen simulations are rare. The *in vitro* study of Stokes *et al.* (1991b), where altogether 13 combinations of non-structural carbohydrate (NSC) and degradable intake protein (DIP) levels were compared, had a companion *in vivo* study (Stokes *et al.*, 1991a), where three combinations of NSC and DIP levels were tested. Increased level of NSC resulted in decreased acetate, but increased propionate and butyrate

proportions *in vitro*. The *in vivo* effects were similar but smaller and not statistically significant, probably because of fewer observations.

The direct effects and interactions of level *vs.* rate of starch degradation, forage type, N level and feeding level on VFA pattern *in vitro* are discussed in more detail in Papers **II** and **III**. In the following sections, however, these results will be considered in relation with other information relevant to rumen fermentation modelling.

The "one-bug model"

Modelling rumen digestive processes must necessarily deal with the partitioning of carbon and nitrogen into volatile fatty acids, microbial protein, fermentation gases, ammonia and undigested dietary protein, starch, fibre, *etc*. Knowledge of the control mechanisms is of central importance for the application of mechanistic models as tools in ruminant feed evaluation. Dynamic-mechanistic rumen models, where this has been attempted have been published (Dijkstra, *et al.*, 1992; Dijkstra, 1994; Baldwin, 1995; Lescoat & Sauvant, 1995; Danfær *et al.*, 2006). The Cornell Net Carbohydrate and Protein System (CNCPS) probably has the most mechanistic approach among the current static feed evaluation systems used in the advisory services (Fox *et al.*, 2004).

Reichl & Baldwin (1976) adapted equations of a pathway balance model to simulate the growth requirements and metabolism of eight rumen microbial groups. This model was non-unique and during sequential solutions the rumen microflora was simplified considerably. The conclusion from this work was that the knowledge of rumen microbial functions and interactions was inadequate for simulating the rumen ecology. This led to the adoption of the "one-bug model", which accepts the concept that the balance of microbial populations in the rumen and their products of fermentation are largely determined by diet composition. The implementation of this approach required that end-products formed from each chemical entity in the diet was empirically defined, since the microbes fermenting each chemical entity were not defined in the model (Baldwin, 1995, p. 231). This resulted in the formulation of an analytical model presented by Koong *et al.* (1975). Based on this approach, Murphy, Baldwin & Koong (1982) presented stochiometrical relationships, later updated by Murphy, Baldwin & Ulyatt (1986), where different VFA patterns were related to soluble carbohydrates, starch, hemicellulose, cellulose and protein, for roughage and concentrate type of diets.

The rumen models mentioned earlier have in most cases relied on these stochiometrical relationships. Unfortunately, model predictions of individual VFA fractions have been poor (Neal, Dijkstra & Gill, 1992; Pitt *et al.*, 1996, Bannink, De Visser & Van Vuuren, 1997; Dijkstra & Bannink, 2000). Bannink *et al.* (1997) tested different hypothesis regarding the inaccuracy of VFA pattern prediction, using the model of Dijkstra (1994). They concluded that in order to improve the model in these aspects, it would be most important to devote further work on the representation of VFA absorption kinetics and VFA coefficients of fermentation

stochiometry. Bannink *et al.* (2000) further developed these aspects. A new set of VFA coefficients was evaluated and a model was developed which includes VFA metabolism by stomach epithelia. Simulations made by this model (Bannink *et al.*, 2000) resulted in lower net portal recoveries of propionate in particular, than indicated by recent data (review by Kristensen, 2005, see Introduction).

Feed - microbial interactions in the dynamic rumen ecosystem

In Paper **IV**, a limited evaluation is reported of the fermentation coefficients presented by Bannink *et al.* (2000) and those derived by Murphy, Baldwin & Koong (1982). Neither of these coefficient sets performed satisfactorily in predicting VFA proportions based on feed information from a Nordic database. Consequently, the analysis of Nordic dairy cow data (Paper **IV**) demonstrated substrate effects on VFA proportions that were different from what has been previously published. It is therefore logical to consider in which aspects the Nordic diets are unique. Specific dietary aspects will be discussed below, in relation to their possible effects on the rumen ecosystem and its end-product formation. Here, explanations will be provided why fermentation models based on fixed proportions of substrates into end-products do not predict rumen metabolism accurately.

In Paper **IV**, there was no propionate increase in response to increased starch levels. Even at high concentrate levels, only moderate propionate levels were observed in Nordic dairy cow experiments (Van Gylswyk & Murphy, 1993; Volden, 1999; Murphy, Åkerlind & Holtenius, 2000; Mydland, 2005). *In vitro* VFA responses were mostly dependent on the sources of starch and forage (Paper **II**). Dairy cows are often fed diets based on grass, especially on restrictively fermented grass silage in the Nordic countries. It has been pointed out (*e.g.* Murphy, Khalili & Huhtanen, 1993; Huhtanen, 1998) that the rumen fermentation patterns, resulting from these diets, are characterised by relatively low proportions of propionate (<200 mmol/mol VFA). Increases in the proportion of barley-based concentrates have only minor effects on propionate proportions, whereas increases in the proportion of butyrate at the expense of acetate commonly occur (review by Huhtanen, 1998). This may be explained by increases in protozoa population with the increasing barley-based supplementation (Chamberlain, Thomas & Anderson, 1983), as protozoa mainly produce acetate and butyrate (Jouany, Demeyer & Grain, 1988).

Jaakkola & Huhtanen (1993) confirmed this theory by finding increases in the protozoa population for grass silage and grass hay diets when the proportion of barley-based concentrate was increased (250, 500 and 750 g concentrate DM/kg total DM). The proportion of propionate decreased slightly with both forages when the amount of concentrate was increased from the low to the intermediate level, but increased from 157 to 170 mmol/mol VFA with silage and from 157 to 188 mmol/mol VFA with hay-based diets, going from the intermediate to the highest level. The butyrate proportion increased and acetate proportion decreased with increasing concentrate proportion with both forages. The increases in

protozoa populations with concentrate levels were similar for silage- and hay-based diets. Therefore, it is possible that a higher propionate proportion for the hay-based diets at the highest concentrate level was caused by some differences in bacterial populations.

Friggens *et al.* (1998) analysed the effects of various supplements on VFA patterns for diets based on grass silage. They found that increased starch levels had positive influences on butyrate rather than on propionate levels, like in Paper **IV**. On grass silage diets, rumen fermentation pattern is greatly influenced (see below) by the extent of silage fermentation (Huhtanen, 1998) and these effects often override the effects of supplementation (Keady & Mayne, 2001; Shingfield, Jaakkola & Huhtanen, 2002). According to the analysis of the Nordic database (Paper **IV**), more than half of the lactic acid from silage is converted to propionate, and the remainder is nearly equally divided between acetate and butyrate. Similar proportions were found by Jaakkola & Huhtanen (1992) from rumen infusion of lactate. A strong positive relationship between silage lactic acid concentration and propionate proportion in rumen VFA was found by Van Vuuren, Huhtanen & Dulphy (1995) in a review of cattle data.

Feeding silages high in lactate may ensure a daily supply of lactate that maintains a large population of lactate fermenting bacteria in the rumen. It has also been suggested that protozoa make an important contribution to lactate fermentation in the rumen (Chamberlain, Thomas & Anderson, 1983), which aids in preventing a decrease in ruminal pH (Williams & Coleman, 1988). A quantitative meta-analysis on the effect of defaunation of the rumen (Eugene, Archimede & Sauvant, 2004) demonstrated that with diets containing less than 500 g/kg DM of concentrate, pH increased after defaunation. However, the inverse was true with higher levels of concentrate, and is in line with the results referred to earlier (Williams & Coleman, 1988). Lactic acid utilisation by protozoa and their ability to store starch granules and other carbohydrates, thereby delaying the fermentation process could explain this phenomena (Eugene, Archimede & Sauvant, 2004). Holotrichs and entodiniomorphid ciliates, are inhibited by propionic acid (Kobayashi & Itabashi, 1986), which could partly explain the elimination of protozoa with diets high in starch (Eadie & Mann, 1970). The positive influences of concentrate ether extract on propionate proportion found in the analysis in Paper **IV** may be the result of partial defaunation by lipids. Tesfa (1993) found that rapeseed oil supplementation decreased the protozoal population and increased the proportion of propionate at the expense of acetate and butyrate. The differences in VFA pattern between the two feeding levels in the *in vitro* study of Paper **III**, may also have been related to less favourable conditions for protozoa at the lower feeding level, due to less distinct stratification of vessel contents, as discussed in Paper **III**.

In addition to the "protozoa effects" on VFA pattern, differences in bacterial populations and metabolism of individual species would be expected to partially explain the lack of universal applicability of the "one-bug model." The requirements for specific growth factors, maintenance energy requirements, maximum growth rates and ability to alter metabolism are amongst the factors

determining which bacterial species dominate on different diets (Murphy, 1990). *P. ruminicola* is the dominant bacteria in the rumen on many diets, often followed by *B. fibrisolvens* (Stewart & Bryant, 1988). Van Gylswyk, Wejdemar & Kulander (1992) suggested that the reason for the general predominance of these two species is that they (particularly *P. ruminicola*) only require low concentrations of certain growth factors for attaining high growth rates. Another explanation of the predominance of *P. ruminicola* and *B. fibrisolvens* is that these species utilise a wide range of substrates, including starch, cellulose (*i.e., B. fibrisolvens*), hemicellulose and various products from plant cell wall degradation by other species. *B. fibrisolvens* is the main butyrate-producing bacteria in the rumen (Russell & Wallace, 1988), but produces also acetate. *P. ruminicola* produces mainly acetate, although some strains can produce propionate through the acrylate pathway (Stewart & Bryant, 1988).

S. ruminantium also occurs in quite high proportions in the rumen, especially when high amounts of cereal grains are fed. This species does not degrade cellulose or hemicellulose, but utilises the intermediary products, such as cellodextrins and xylo-oligosaccharides, and can therefore be quite abundant also on high-fibre diets. It is thought to be the most important propionate-producing bacteria *in vivo*, although on high-concentrate diets the contribution of *M. elsdenii* can be quite important (Russel & Wallace, 1988). *S. ruminantium* uses the randomising pathway (Paynter & Elsden, 1970) but *M. elsdenii*, the acrylate pathway (Ladd &Walker, 1965). Van Gylswyk & Murphy (1993) found that when roughage was changed from hay (or hay plus straw) to silage, the proportion of *P. ruminicola* increased. They suggested that this was caused by lower concentrations of certain growth factors in the silage diets. They found *B. fibrisolvens* in lower numbers, but more effective cellulose digesters, *i.e.*, *R. albus* and *R. flavefaciens* were in higher numbers on silage than hay diets, indicating greater cellulolytic activity on the silage diets. *Fibrobacter* (formerly *Bacteriodes*) *succinogenes* is an important cellulolytic bacterium in the rumen when roughages with low digestibility are fed (van Gylswyk & van der Toorn, 1986). However, this species was barely detected in the rumen of cows fed grass silage (van Gylswyk, 1990; van Gylswyk & Murphy, 1993). The main end-products of *F. succinogenes* are acetate and succinate. Succinate is converted to propionate by other species, like *S. ruminantium*. Low numbers of *F. succinogenes* could therefore be one of the explanations for low propionate levels when grass silage-based diets are fed.

The “one-bug model”, based on stochiometrical relationships (Koong *et al.*, 1975; Murphy, Baldwin & Koong, 1982), relies on fixed stochiometric relationships for individual substrate classes. Although there is a tendency for “fibre-, starch- and sugar- fermenting bacteria” to produce acetate, propionate and butyrate, respectively, this is an oversimplification. Results from pure cultures have shown that cellulolytic bacteria like *F. succinogenes* and *R. flavefaciens* produce mostly succinate, an intermediate that is ultimately converted to propionate (Lana, Russell & Van Amburgh, 1998). In addition, most starch-fermenting bacteria can produce large amounts of acetate (Hungate, 1966). Furthermore, as briefly mentioned above, many of the important bacterial species

in the rumen rely greatly upon crossfeeding. Crossfeeding involves the utilisation of products from polysaccharide and protein degradation, as well as of intermediary metabolites, such as succinate (Murphy, 1990).

Increased dietary concentrations of water soluble carbohydrates (WSC) increase the proportion of butyrate at the expense of acetate *in vivo*, with little or no effect on the propionate proportion, according to published stochiometrical coefficients (Bannink *et al.*, 2000; Murphy, Baldwin & Koong, 1982) and regression equations (Friggens *et al.*, 1998). In the analysis of the Nordic database in Paper **IV**, WSC were included in the "rest fraction (Re)," together with pectins and other organic feed components not accounted for by analysis of NDF, starch, crude protein, ether extract and organic acids. The analysis showed a positive relationship between the rest fraction and butyrate and propionate proportions at the expense of acetate. Pectin in the rest fraction might to some extent explain the relatively high propionate proportion derived from that fraction. Pectin-rich feeds, like un-molassed sugar beet pulp, have been observed to decrease the proportion of butyrate and with a tendency to increase propionate (Huhtanen, 1988; Murphy, Khalili & Huhtanen, 1993). Therefore, further separation of the rest fraction might improve the prediction of rumen VFA pattern.

Attempts to refine the "one-bug model"

The above discussion should give some ideas about why stochiometrical models that only relate fermentation pattern to different substrate classes do not have universal applicability. It seems that in order to improve the VFA predictions over a wide range of diets, the properties of the different microbial groups present in the rumen must somehow be accounted for. Dijkstra (1994) presented a model with three microbial groups, *i.e.* protozoa, amylolytic and cellulolytic bacteria. Nagorcka, Gordon & Dynes (2000) developed a new set of fermentation coefficients, dependent not only on substrate, but also on different fermentation patterns, which characterise these microbial groups. Considerable improvements in predicting VFA proportions were achieved when Nagorcka, Gordon & Dynes (2000) incorporated these new fermentation coefficients into the model of Dijkstra (1994). These efforts therefore demonstrated that there is a scope for improving the prediction of VFA proportions in the rumen by accounting for differences between microbial groups in the rumen.

From a modelling point of view, the level of aggregation is an important factor and should be decided with respect to the overall purpose of the model (*e.g.* Baldwin, 1995). Should species' differences be accounted for by representing different microbial groups? If so, is the above mentioned three-microbial group representation adequate? Should the group of amylolytic bacteria be divided further to account for the differences in end-products between individual bacteria? These are examples of questions that can be raised. It was discussed earlier, however, that the original reason for the development of the "one-bug model' was that a model representing eight different microbial groups failed to yield unique solutions (Reichl & Baldwin, 1976).

The fermentation coefficients derived by Nagorcka, Gordon & Dynes (2000) were based on estimates of the stochiometry of individual bacterial genera or protozoa type. These were combined, somewhat arbitrarily, in proportion with the contribution of the individuals to the function of each of three microbial groups. However, fitting this kind of model by statistical iteration procedures seems impossible without more information on rumen microbial pools than normally are available from dairy cow digestion studies. If the intention is to develop more universal rumen fermentation models, a very wide range of such data is needed. This leads to the consideration if there is a scope for better utilizing the data that already exists to improve the prediction of rumen VFA proportions. How can crossfeeding between different species be accounted for? Are there any generalisations that can be made to relate the predictions of rumen VFA pattern not only to digested substrates but also to the most important characteristics of the rumen microflora? Some theories or considerations in this respect will be mentioned here.

One important aspect is the energy availability within the microbial cells. A measure of that is the adenylate energy charge (EC) = (ATP + 0.5 ADP) / (ATP + ADP + AMP), that was first postulated by Atkinson (1968) where AMP, ADP and ATP are adenosine mono-, di- and tri-phosphates, respectively. Higher EC means greater ability of bacteria to maintain a highly-charged state. Different bacterial species have their own characteristic EC, which they strive to maintain. This means that ATP-utilizing reactions occur in a fixed relation to the ATP-producing reactions. Degradation of feedstuffs in the rumen is therefore dependent on the ability of the rumen environment to support bacterial growth (Murphy, 1990).

Erfle, Mahadevan & Sauer (1981) demonstrated that diets with high contents of rapidly available energy gave rise to high ATP contents of cells, and an increased energy charge. However, as discussed above and by Sauvant & van Milgen (1995) cell ATP contents as well as EC appear to be bound by maximum limits. Bacterial species that digest starch generally have higher ATP concentrations than cellulose digesting species (Forsberg & Lam, 1977; Hobson & Summers, 1972). Sauvant & van Milgen (1995) postulated that the partition of carbon between different fermentation pathways is closely linked with the control of ATP production and its use for microbial growth. The cyclic nature of the rumen environment plays an important role here. Usually, microbial growth in the rumen is energy-limited (Russell & Strobel, 1993). In such a situation, it is likely that fermentation pathways that yield maximum ATP per unit substrate will dominate, which favours acetate production (Russell & Wallace, 1988). At times, such as after a meal of rapidly degrading carbohydrates, excess carbon will be available. Then, metabolic routes providing less ATP per carbon of VFA are favoured. Faster growing species (Russell & Baldwin, 1978) such as *S. bovis* and *S. ruminantium* may increase dramatically, and produce lactate in spite of a very low ATP yield per mole hexose fermented (Russell & Baldwin, 1978; Russell & Baldwin, 1979). However, because of their fast growth these species are able to outgrow the slower growing acetate producers by producing more ATP per unit time, in spite of less ATP produced per unit substrate (Hungate, 1979; Russell & Wallace, 1988). *M.*

elsdenii ferments lactate to propionate by the acrylate pathway and has been estimated to metabolise on average 0.74 of the lactate produced in the rumen (Counotte *et al.*, 1981; Counotte *et al.*, 1983). Mydland (2005), found that populations of both the lactate producer *S. bovis* and the lactate utilizer *M. elsdenii* increased as a result of increased intake of rumen digestible carbohydrates. However, a great variation between individual cows was observed. Interestingly, the maximum intake of concentrate for the individual cows in the experiment had a strong positive correlation with the ratio of *M. elsdenii*:*S. bovis*, at times of sub-acute rumen acidosis.

It is also important to consider that the redox status associated with the production of VFA carbon varies between individual VFA. Propionate is an effective H sink, whereas acetate is an H source. A shift in the VFA profile towards propionate at the expense of acetate, and eventually butyrate can therefore be seen as a temporary adaptation to energy and hydrogen excess (Sauvant & van Milgen 1995). Sauvant & van Milgen (1995) suggested that the regulatory influences of energy and redox status could be included in a model as a way to renew the empirical stochiometric equations of Murphy, Baldwin & Koong (1982). It is unclear, however, how this concept should be implemented in a rumen model. The fractional rate of substrate degradation in the rumen might be a key variable in this respect. In combination with rate of feed (substrate) intake, it determines the quantity of substrate(s) available to rumen microbial fermentation per unit of time, and the likely "load" of energy and hydrogen in the system. Multiple regression analysis of a large database of rumen digestion studies, mainly with US diets, demonstrated that including substrate degradation rates as explanatory variables improved predictions of VFA proportions considerably compared to when only substrate concentrations were included (Beauchemin *et al.*, 1997). This is in line with the *in vitro* results presented in Paper **II** and **III**, which pointed out interactions between availabilities of different substrates (starch, fibre, protein) in this respect.

It can be questioned if the "one-bug" stochiometrical approach is theoretically sounder than multiple regression models relating VFA proportions to relevant feed information (Friggens *et al.*, 1998; Beauchemin *et al.*, 1997; Paper **IV**). Both rely on empirical relationships between feed and end-products formed, but assume only one microbial group representing all the known metabolic functions. The multiple regression model seems to have more flexibility in encompassing new variables such as; degradation rates, effects of non-fermentable feed components (Paper **IV**), interactions between different substrates, *etc*. However, in Paper **IV**, a stochiometrical model was also presented with corrections for feeding level and ether extract from concentrates. The model with these corrections is currently used as the VFA sub-model in the Nordic dairy cow simulation model Karoline (Danfær *et al*., 2006) and, if needed, other factors could be added to the model. One candidate could be starch degradation rates, investigated in Papers **II** and **III**. However, in the Nordic database used in Paper **IV**, most of the starch was from the same source (barley), so solving for starch degradation rate was not realistic. Introducing new "correction" factors into a model, originally based entirely on substrate preferences, must be done with care. It can be argued that introducing

new variables into a model will always improve the fit and one could end up with a totally meaningless model that has a perfect fit. “If the concepts which comprise the model are valid and adequate, computed results reflect reality. If not, computed results reveal inadequacies in current knowledge.” (Baldwin, 1995, p. 25).

Fermentation models representing different microbial groups would be theoretically sounder than multiple regression models but the difficulties with these types of model have already been discussed. Attempts have been made to introduce effects of microbial population differences into the “one-bug model” by fitting different fermentation coefficients for roughage *vs*. concentrate diets (Murphy, Baldwin & Koong, 1982; Murphy, Baldwin & Ulyatt, 1986; Bannink *et al*., 2006). This seems to represent some of the variations in microbial metabolism among diets. For example, a much larger proportion of starch was fermented to propionate on concentrate *vs.* roughage diets. Results presented in Paper **IV** and the ideas presented above indicate that fitting fermentation coefficients for an increased number of diet types could be worth trying. Roughage diets could be classified by the conservation method (hay, silage) and plant type (grass, legume, whole-crop, *etc*.). Concentrates could be differentiated based on their degradation rates, for example. An analysis of this kind would require a large dataset representing a wide range of diets.

Conclusions

- An *in vitro* method for measuring starch degradation was presented. The method considers starch degraded after hydrolysis to glucose.
- Heat treatment generally increased rate of starch degradation *in vitro*.
- *In vitro* simulations of rumen fermentation (SIMCO system) are useful for understanding the controls of fermentation, especially if considered in relation to *in vivo* data and basic rumen microbiology.
- Starch affects rumen VFA pattern differently depending on the level of starch in the diet and the nature of the forage consumed, according to *in vitro* and *in vivo* results.
- Positive effects of starch upon propionate proportion and microbial N efficiency *in vitro* were dependent upon starch degradation rate and protein availability.
- Negative effects on the rumen protozoal population are likely to increase the positive effects of starch on the propionate proportion.
- Current fermentation models based almost entirely on substrate preferences oversimplify the rumen ecosystem.
- To improve VFA prediction, rumen models must represent better the different functions of rumen microbes, such as crossfeeding and the ability of different species to alter their metabolism.
- Using a large database representing a wide variety of diets, fermentation coefficients could be fitted to several different types of diets in order to better capture differences in the rumen ecosystem.

Samantekt

Lífið á jörðinni byggir á því að plöntur og sumar bakteríur umbreyta koldíoxíði og vatni í lífræn efni og súrefni (og um leið sólarorku í efnaorku) með ljóstillífun. Stór hluti þeirra lífrænu efna sem plöntur mynda við ljóstillífunina eru kolvetni. Með tilliti til hlutverka í plöntunum má setja kolvetnin í þrjá flokka: 1) efnaskiptakolvetni, svo sem glúkósi og frúktósi; 2) forðakolvetni, svo sem sterkja (í korni), súkrósi (í sykurrófum) og frúktanar (í grösum); og 3) frumuveggjakolvetni, svo sem pektín, hemisellulósi og sellulósi.

Flokkunin með tilliti til þarfa dýrsins er eilítið önnur. Einsykrur, svo sem glúkósa, geta skepnur sogað beint upp úr meltingarfærum til notkunar í efnaskiptum sínum. Tvísykrur (t.d. súkrósa) og fjölsykrur á borð við sterkju og frúktana þarf að melta, þ.e. sundra niður í einsykrur, áður en þær eru uppsogaðar. Það gerist fyrir tilstilli ensíma sem er að finna í maga einmaga dýra og smáþörmum jórturdýra. Frumuveggjakolvetnin (tréni), skilgreind skv. efnagreiningaraðferð sem NDF (Neutral Detergent Fiber) eru hins vegar ekki meltanleg með ensímum meltingarfæranna. Þessi kolvetni eru gríðarlega mikilvæg í lífríkinu, sellulósi er t.d. það kolvetni sem mest er til af á jörðinni. Næringarframboð mannkyninu til handa væri því mun minna ef ekki nyti við örvera í vömb jórturdýra sem gerja þessi kolvetni. Vambargerjunin gefur af sér:

1) orku sem nýtist örverum til vaxtar – þessar örverur eru svo meltar í smáþörmum og gefa þar af sér stóran hluta þess próteins sem jórturdýrið hefur úr að moða í efnaskiptum sínum; 2) rokgjarnar fitusýrur (volatile fatty acids=VFA) einkum edikssýru, própíonsýru og smjörsýru, sem eru uppsogaðar af jórturdýrinu og gefa sem nemur 50-70% af þeirri orku sem það notar í efnaskiptum; 3) gas, einkum metan.

Fyrir tilstilli örveranna verður því annars ómeltanlegt tréni aðgengilegt jórturdýrinu til framleiðslu mjólkur, kjöts og annarra afurða. Þetta sambýli jórturdýra og örvera og sá hagur sem af því er fyrir mannkyn, er meginástæðan fyrir því hversu mikilli útbreiðslu jórturdýr (nautgripir, sauðfé, geitur, o.s.frv.) hafa náð í villtri náttúru og sem húsdýr. Lauslega er áætlað að jórturdýr framleiði um þriðjung þess kjöts og nánast alla þá mjólk sem mannkynið neytir.

Auk trénisins gerja vambarörverurnar m.a. sykrur og stóran hluta sterkju og próteins í fóðrinu, en hluti þessara efna er þó meltur í smáþörmum. Framboð einstakra efna til uppsogunar hefur afgerandi áhrif á magn og samsetningu afurða. Prótein, bæði beint úr fóðri og örveruprótein notast til mjólkurpróteinframleiðslu og vöðvauppbyggingar (kjöt). Edikssýra og smjörsýra nýtast til framleiðslu á mjólkurfitu og líkamsfitu. Glúkósi er nauðsynlegur til framleiðslu á mjólkursykri og þar með mjólk en kýr sem framleiðir 25 kg af mjólk á dag þarf til þess 2-2,5 kg af glúkósa. Ekki eru líkur til að nema 0,1-0,2 kg af glúkósa sogist upp frá meltingarfærunum, við meltingu á sterkju í smáþörmum. Restin verður til við glúkósanýmyndun í lifur, þar af gjarnan um helmingur úr própíonsýru. Glúkósanýmyndun úr próteini (amínósýrum) getur verið frá nánast engu og upp í

allt að helming af glúkósaþörfinni. Reiknað hefur verið út að þessi mismunur samsvari um tvöföldun á próteinþörfum hjá kúm í meðalnyt. Prótein er dýrt og bæði óhagkvæmt og óumhverfisvænt að nota það til glúkósaframleiðslu. Til þess að stýra sem best fóðrun gripanna, m.t.t. magns og samsetningar afurða, er nauðsynlegt að skilja sem best þá þætti sem ráða því hve stór hluti einstakra fóðurefna gerjast í vömbinni, hve mikið myndast af örverupróteini, gasi, edikssýru, própíonsýru og smjörsýru. Rannsóknirnar sem gerðar voru í þessu verkefni snerust um að auka þann skilning. Nánar tiltekið um að rannsaka áhrif af hlutföllum einstakra fóðurefna og gerjunarhraða kolvetna á gerjunarmynstur í vömb.

Sett var upp rannsóknastofuaðferð (*in vitro*) til að mæla gerjunarhraða sterkju. Aðferðin byggir á að fóðursýnum er komið fyrir í tilraunaglösum ásamt vambarvökva og stuðpúðalausn (buffer) undir loftfirrðum aðstæðum og við eðlilegan „vambarhita“ (39-40°C). Gerjun er svo stöðvuð á tilteknum tímapunktum og fundið út með ensímatískum aðferðum hve mikil sterkja er ógerjuð á hverjum tímapunkti. Gögn fyrir mismunandi tímapunkta hjá hverri fóðurtegund eru notuð til að reikna út gerjunarhraða, þ.e. hve hátt hlutfall sterkjunnar sem til staðar er gerjast á hverri klukkustund. Með því að mæla gerjunarhlutfall eftir aðeins einn tímapunkt, t.d. 8 tíma, má einnig fá allgóða hugmynd um gerjunareiginleika fóðursins á tiltölulega ódýran hátt. Fóðurtegundir sem höfðu fengið mismunandi hitameðferðir voru mældar. Hitameðferðir juku almennt gerjunarhraða sterkju.

Gerðar voru tvær tilraunir með gervivömbum (*in vitro*). Tilraunaaðstaðan var 8 gervivambir, hver samsett úr 2,5 lítra glerhólk sem vambarvökvi var settur inn í á fyrsta degi. Stuðpúðalausn var stöðugt dælt inn í vambirnar og þær fóðraðar fjórum sinnum á dag. Innihaldinu var hringdælt til að líkja eftir hreyfingum vambarinnar en jafnframt var tappað af innihaldinu 5-6 sinnum á dag. Hver keyrsla stóð í 10 daga, og hvor tilraun samanstóð af tveimur eins keyrslum.

Báðar tilraunir voru skipulagðar sem 2^3 þáttatilraunir. Í báðum tilraunum var einn þátturinn sterkjugerð, og var þar um að ræða annars vegar hæggerjanlega, hráa kartöflusterkju, og hins vegar hraðgerjanlega, soðna kartöflusterkju. Hinir tveir þættirnir voru: a) í fyrri tilrauninni: sterkjuhlutfall: þ.e. 30 eða 60%; og gerð gróffóðurs, þ.e. hraðgerjanlegt, snemmslegið vallarsveifgras eða hæggerjanlegt, síðslegið vallarfoxgras; b) í seinni tilrauninni: prótein (N) magn í fóðri og fóðrunarstig (lágt eða hátt).

Fóðrunarmeðferðirnar og ýmsar víxlverkanir þeirra á milli sýndu ýmis mjög athyglisverð áhrif á gerjunarmynstur og örverupróteinframleiðslu. Aukinn gerjunarhraði sterkju leiddi af sér hækkað hlutfall própíonsýru á kostnað edikssýru og (í fyrri tilrauninni) smjörsýru. Áhrif sterkjuhlutfalls á gerjunarmynstur voru hins vegar háð gerjunarhraða sterkjunnar og gróffóðurgerðinni. Aukinn gerjunarhraði, bæði á sterkju og gróffóðri, hafði jákvæð áhrif á örverupróteinframleiðslu. Þegar hluta sterkjunnar var skipt út fyrir hraðgerjanlegt gróffóður, jókst sömuleiðis virkni örverupróteinframleiðslunnar. Aukið magn af próteini í fóðri leiddi til aukinnar örverupróteinframleiðslu og hækkaðs hlutfalls

própíonsýru og smjörsýru á kostnað edikssýru. Hækkað fóðrunarstig lækkaði própíonsýruhlutfall og minnkaði virkni örverupróteinframleiðslu. Ennfremur var gerjunarmynstur háð víxlhrifum á milli próteinmagns í fóðri og gerjunarhraða sterkju. Niðurstöður þessara tveggja tilrauna sýna að gerjunarmynstur og örverupróteinframleiðsla í vömb er ekki eingöngu háð magni einstakra fóðurefna sem gerjast í vömb heldur einnig gerjunarhraða þessara efna.

Gögn úr norrænum mjólkurkúatilraunum voru greind með tilliti til áhrifa fóðrunarþátta á gerjunarmynstur í vömb, með það að markmiði að þróa undirlíkan af vambargerjun til nota í hinu samnorræna hermilíkani af mjólkurkú, Karólínu. Það markmið náðist, en helstu atriði sem þessi greining leiddi í ljós að væru öðruvísi skv. þessum norrænu gögnum heldur en ýmsum öðrum, voru: mjög veik áhrif sterkjumagns í fóðri á fitusýruhlutföll, og jafnframt var mjólkursýra í votheyi sett inn sem sérstök breyta, en um helmingur mjólkursýrunnar verður að própíonsýru í vömb. Einnig leiða hækkað fóðrunarstig og aukið magn fitu úr kjarnfóðri til hækkunar á hlutfalli própíonsýru á kostnað edikssýru og smjörsýru, skv. líkaninu.

Líkön af vambargerjun sem gera ráð fyrir föstum hlutföllum gerjunarafurða úr hverju tilteknu fóðurefni, einfalda vistkerfi vambarinnar of mikið. Til að bæta árangur í að spá fyrir um niðurstöður vambargerjunar út frá fóðurupplýsingum, þurfa slíkar upplýsingar að vera ítarlegri og tengjast betur hugsanlegum áhrifum á örveruflóru vambarinnar. Gerjunarhraði kolvetna og gerð gróffóðurs (verkunaraðferð, grastegund, sláttutími) eru meðal þeirra þátta sem skynsamlegt virðist að taka tillit til.

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