Domestic protein feeds in dairy production

Potential of rapeseed feeds and red clover

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Abstract

The purpose of the four studies that form the basis for this thesis was to extend the knowledge about how to utilize the domestic protein feeds in Swedish dairy production. To optimize the use of nitrogen (N) from both concentrates and forages into milk product, and thereby improving the profitability and the resilience of the domestic dairy farms.

In a study where soybean meal was replaced with solvent-extracted, heat-moisture-treated rapeseed meal, milk yield and milk protein yield increased and nitrogen use efficiency was higher with rapeseed meal supplementation. Methane yield (g CH_4/kg dry matter intake) and methane intensity (g CH_4/kg energy-corrected milk) decreased quadratically with increased dietary crude protein concentration.

Increasing the proportion of red clover silage in the diet did not reduce the need for protein supplementation. While the cows were still responsive to protein supplementation as heat-moisture-treated rapeseed expeller, incremental protein intake from red clover did not increase feed intake or milk yield. The changes observed with increased red clover silage intake were attributed to decreased nitrogen use efficiency and increased milk urea nitrogen. Increased protein supplementation decreased methane yield.

Replacing crimped barley with solvent-extracted, heat-moisture-treated rapeseed meal to increase dietary crude protein concentration was associated with greater total neutral detergent fibre digestibility and potentially digestible detergent fibre digestibility. The digestion rate of potentially digestible detergent fibre also increased. Omasal flow of non-ammonia nitrogen increased with increased protein supplementation, but simultaneously the flow of microbial non-ammonia nitrogen and microbial efficiency tended to decrease, with the calculated proportion of milk protein originating from rumen microbial protein showing a marked decline.

Omasal crude protein flow and milk protein yield were positively related to *in vitro*-estimated utilisable crude protein flow at the duodenum. Moreover, there was a strong correlation between estimated utilisable crude protein (g/kg DM) at 16, 20 and 24 hours of incubation, but results at 16 hours resulted in the best mixed model fit.

Overall, these findings show that with grass silage-based diets Swedish dairy cows could theoretically maintain or even increase milk yield when fed only domestic rapeseed meal as protein feed.

Keywords: ruminant, protein supplement, nitrogen efficiency, rapeseed, legume

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Just keep swimming
Dory, in Finding Nemo

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Gidlund, H., Hetta, M., Krizsan, S. J., Lemosquet, S., Huhtanen, P. (2015). Effects of soybean meal or canola meal on milk production and methane emissions in lactating dairy cows fed grass silage-based diets. *Journal of Dairy Science*, 98, pp. 8093-8106.

II Gidlund, H., Hetta, M., Huhtanen, P. (2017). Milk production and methane emissions from dairy cows fed a low or high proportion of red clover silage and an incremental level of rapeseed expeller. *Livestock Science*, 197, pp. 73-81.

III Krizsan, S. J., Gidlund, H., Fatehi, F., Huhtanen, P. Effect of supplementation of heat-treated canola meal on ruminal nutrient metabolism in lactating dairy cows (submitted).

IV Gidlund, H., Vaga, M., Ahvenjärvi, S., Rinne, M., Ramin, M., Huhtanen, P. Predicting omasal flow of non-ammonia N and milk protein yield from in vitro determined utilizable crude protein at the duodenum (manuscript).

Papers I-II are reproduced with the permission of the publishers.

The contribution of Helena Gidlund to the papers included in this thesis was as follows:

I Planned the study together with the co-authors, collected, prepared and analysed samples, processed the data and was responsible for writing the manuscript.

II Planned the study together with the co-authors, collected, prepared and analysed samples, processed the data and was responsible for writing the manuscript.

III Worked jointly with the co-authors on: planning the study, daily maintenance in the barn, collecting, preparing and analysing samples and processing the data. Was responsible for writing parts of the manuscript.

IV Planned the study together with the co-authors, performed the laboratory analysis, processed the data and was responsible for writing the manuscript.

Abbreviations

AA	Amino acids
CP	Crude protein
DMI	Dry matter intake
ECM	Energy corrected milk
iNDF	Indigestible neutral detergent fibre
MCP	Microbial crude protein
MP	Metabolisable protein
MPY	Milk protein yield
MUN	Milk urea nitrogen
N	Nitrogen
NAN	Non-ammonia N
NANMN	Non-ammonia, non-microbial N
NDF	Neutral detergent fibre
NH_3	Ammonia
OM	Organic matter
OMD	Organic matter digestibility
pdNDF	Potentially digestible neutral detergent fibre
RDP	Rumen degradable protein
RSM	Rapeseed meal
RUP	Rumen undegradable protein
SBM	Soybean meal
tRSM	Solvent-extracted, heat-moisture-treated rapeseed meal
uCP	Utilisable crude protein
uCP16	Utilisable crude protein flow at 16 hour of in vitro incubation
uCP20	Utilisable crude protein flow at 20 hour of in vitro incubation
uCP_{24}	Utilisable crude protein flow at 24 hour of in vitro incubation

1 Introduction

1.1 Global and domestic demand for protein foods and feeds

According to FAO (2011) estimates, global demand for milk and meat will increase by 58 and 73%, respectively, between 2010 and 2050. The demand will be driven by growth in the global population from 7.2 billion in 2013 to 9.6 billion in 2050, and this population growth will mainly take place in developing countries (FAO, 2013). Increased animal production to meet the increasing demand for milk and meat will require increased feed production, with a projected increase in production of protein cake and meals reported by FAO (2004).

In Sweden, domestic production of protein feeds has been increasing during the past 16 years, as shown in Figure 1 (Statistics Sweden, 2016). Simultaneously, imports of the major protein feeds soybean and rapeseed have decreased (FAO, 2017), although they are still quantitatively of great importance for Swedish animal production. In Sweden, the trend for increased production of protein feeds cannot be attributed to population growth, but rather to economic and other social considerations. One of the reasons could be because the price of imported protein feeds has been fluctuating. Another is that there is currently a national debate concerning Swedish self-sufficiency, feeding human-edible food as animal feed and importation of feeds produced in countries with less strict environmental regulations.

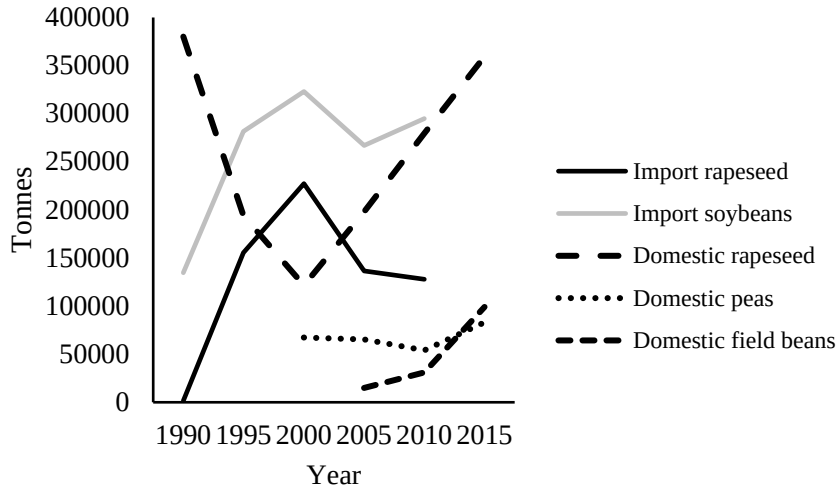

Figure 1. Imports to Sweden of rapeseed and rapeseed cake (dark solid lines) and soybeans and soybean cake (light grey solid lines), 1990-2013 (Source: FAO, 2017). Domestic production of rapeseed (1990-2015), peas (2000-2015) and field beans (2005-2015) is also shown (dashed lines) (source: Statistics Sweden, 2016).

The crops that can be grown as animal feed in Sweden, apart from high quality forage, are rapeseed, field beans and peas. Lupin, linseed and domestic soybean are also grown in small quantities and processed in Sweden. In 2015, production of peas and field beans occupied 48 375 ha and rapeseed production occupied 94 525 ha (Statistics Sweden, 2016). The acreage of pulse crops has been steadily increasing in recent decades, with small declines in some years, while that of rapeseed has fluctuated between 75 000 and 167 000 during the past 25 years (Statistics Sweden, 2016). Potential domestic production of peas and field beans is estimated to be 50 000-100 000 ha each, with a maximum of 150 000 ha in total, and potential rapeseed production is estimated to be 150 000-175 000 ha. (Gustafsson *et al*., 2013). This shows that it would be practically possible to replace the amount of imported protein feeds with domestic protein feeds, if protein quality and economic conditions are not taken into consideration.

1.2 Role of the dairy cow as a protein food producer

Ever since humans first domesticated cattle thousands of years ago, these animals have been important for human survival, due to their ability to digest fibrous feeds and convert them into meat, milk and draught power. With population growth and industrialisation, demand for dairy and meat products has

increased. Through advances in animal breeding and feed quality, the average milk yield per cow in Sweden increased from 3190 to 8634 kg milk per year between 1961 and 2014 (FAO, 2017). The advances in feed quality mainly derive from improved digestibility of ley crops and increased use of concentrate, mainly grains and by-products from human food production. However, many concentrate feedstuffs (*e.g.* grains and soybean) could be consumed directly by humans or compete for land with crops used for human consumption, which creates an ethical dilemma concerning how resources are used within dairy production (and animal production as a whole). However, the improved protein quality of the output should be considered in the balance between inputs of human-edible products and output of animal products for food (FAO, 2004).

1.3 Ruminant nitrogen metabolism

Dairy cows can survive and even produce milk without dietary amino acids (AA), using nitrogen (N) only in the form of urea and ammonium salts (Virtanen, 1966). This reveals the unique ability of the ruminant to produce microbial protein from non-protein N (Forbes and France, 1993; Van Soest, 1994).

Rumen microbes (bacteria, protozoa and fungi) ferment dietary carbohydrates and rumen-degradable protein (RDP) (Forbes and France, 1993). The microbes are thereby provided with energy and building blocks for their own maintenance and for synthesis of novel microbial protein, in a synchronised process. All 10 AA considered essential for humans can be synthesised by rumen microbes from dietary and endogenous N fractions. Nitrogen that is not bound in microbes or does not follow the digesta to the small intestine is absorbed as ammonia (NH_3) through the rumen wall to the blood and converted to urea in the liver. This urea is recycled to the rumen mainly via saliva, through the rumen wall, or excreted in the urine. The recycling of urea to the rumen reduces the N requirement and prolongs survival when the animal is exposed to a low-protein diet.

Rumen-undegradable protein (RUP) and rumen microbial protein follow the digesta flow to the small intestine. Together with small amounts of endogenous crude protein, RUP and microbial crude protein (MCP) make up metabolisable protein (MP), which is hydrolysed by intestinal digestive enzymes to peptides and AA. These AA become available to the animal via absorption through the intestinal mucosa. The AA that are absorbed in the small intestine are a function of the AA pattern of MP and intestinal digestibility.

1.4 Nitrogen emission from dairy cattle

Nitrogen emissions from dairy production are an economic and environmental concern, because NH_3 is lost to the atmosphere and can contaminate surface waters and groundwater (Tamminga, 1992; Van Horn *et al*., 1994). The major fractions of N in manure are in the form of NH_3 and organic N, and derive from undigested dietary protein, microbial protein and endogenous N in faeces, and from urea in urine. The major source of NH_3 in manure is urea from urine (Van Horn *et al*., 1994). The N in urine is mainly in the form of urea, which is rapidly converted to NH_3 in the presence of the enzyme urease. There is much urease present in faeces, and hence urinary urea starts converting to NH_3 when it hits the floor in the dairy barn. If the environment is acidic, NH_3 reacts with hydrogen and form the ammonium cation (NH_4^+), which is non-gaseous, preventing volatilisation of NH_3 to the atmosphere. However, dairy cow manure has a pH above 7 and thus large amounts of NH_3 are volatilised to the atmosphere. Between 50-75% of manure N can be lost, mostly through volatilisation of urinary NH_3 (Van Horn *et al*., 1994). Ammonia can also be converted via the nitrification process to nitrate (NO_3^-), which can leach to surface waters and groundwater (Van Horn *et al*., 1994). Loss of manure NH_3 also occurs though denitrification, where anaerobic bacteria convert nitrate to nitrous oxide (NO_2), the most potent greenhouse gas, and further to N gas (N_2).

Increasing dietary crude protein (CP) concentration is known to increase milk production, but also decreases N use efficiency, leading to greater losses of N via manure. Kauffman and St-Pierre (2001) showed that urinary N increased with increasing N intake, while faeces N remained fairly constant. Broderick (2003) found that increasing the dietary CP concentration from 15.1 to 18.4 % decreased N use efficiency from 31 to 25 %. Simultaneously, urinary N increased from 23 to 35 % of dietary N and faecal N decreased from 45 to 41 % of dietary N. Hence, decreasing the dietary CP concentration and feeding the dairy cow according to actual requirements would decrease N excretion (Paul *et al*., 1998). Furthermore, the excreted N would be less susceptible to be lost to the air and via leaching.

1.5 Techniques for estimating protein value

The challenge with protein nutrition in dairy animals is to estimate and provide a diet containing enough RDP of the right quality to maximise microbial synthesis, and simultaneously provide RUP of high intestinal digestibility to optimise the profile and amounts of AA (NRC, 2001). Moreover, this must be achieved without overfeeding of N, which would lower the profitability and cause unnecessary losses of N to the environment.

Dietary protein consists of fractions with differing rumen degradability, from instantly degraded to undegradable (McDonald *et al*., 2002). The degradability depends on protein source, protein solubility, level of proteolytic activity in the rumen, retention time, rumen pH, microbial species and surface area of the proteins to which microbes can attach (Broderick *et al*., 1991; Forbes and France, 1993; McDonald *et al*., 2002). To estimate this complex rumen digestion process, different *in vivo*, *in situ* and *in vitro* techniques have been developed.

1.5.1 *In vivo*

In vivo measurement of rumen protein degradability and microbial protein yield requires cows with cannulas in the digestive tract, and the use of markers for estimating digesta flow and microbial flow is unavoidable (McDonald *et al*., 2002). Therefore *in vivo* studies are very expensive and labour-intensive.

In vivo methods for measuring nutrient flows are limited to using cannulas located in the omasum, abomasum and duodenum, with different positive and negative effects (Harmon and Richards, 1997). One difference between sampling sites is the amount of endogenous contribution to the N flow. This causes errors in estimation of RUP with *in vivo* methods. Sampling from the omasum, instead of the abomasum or duodenum, could possibly decrease this error. Huhtanen *et al*. (1997a) developed a sampling technique, later modified by Ahvenjärvi *et al*. (2000), whereby omasal digesta was sampled through a ruminal cannula. They found that organic matter (OM) flow was lower into the omasum than the duodenum, probably because of endogenous OM secretions into the abomasum, and neutral detergent fibre (NDF) digestion in the omasum. Additional benefits with performing omasal sampling though a rumen cannula are that ruminally cannulated animals are easier to keep than animals cannulated in other sections along the digestive tract and that sampling in the omasal canal enables better measurement of soluble non-ammonia N flow from the rumen compared with duodenal sampling, since abomasal degradation of microbial N not create a bias (Ahvenjärvi *et al*., 2000).

Total digesta collection would give the most reliable results when measuring the quantity and composition of digesta flow, but this method is terminal for the animal and hence has its limitations in practice. Earlier, re-entry cannulas were used to perform total collection (Forbes and France, 1993). This method was found to change digesta flow and the use of digesta markers was necessary. Preparing animals with simple cannulas and estimating digesta flow by analysing a marker in the digesta has proven to be a reliable and animal-friendly procedure. Faichney (1975) developed the double-marker system, where the ideal marker is indigestible, does not influence digestion or passage and has

similar flow characteristics as (or is associated with) the fraction to mark, and the method used to analyse the marker in the digesta must be specific and sensitive.

Flow marker systems consider that the digesta consists of liquid and particle phases, which have different flow characteristics, and each phase corresponds to an individual marker (France and Siddons, 1986). Another consideration is that the digesta samples must be representative. A sample is considered representative when it has the same chemical composition as the total digesta flowing past the cannula in a steady-state period. This mainly means during a whole feeding cycle, and therefore sampling usually takes place during 24 hours or during one 12-hour feeding cycle when the animals are fed twice daily with equal portions at 12-hour intervals. However, it is very difficult to get a representative sample through the cannula. This problem can be overcome by assuming that digesta flow occurs in independent phases and performing sampling that is representative of each phase. One method is based on estimation of individual phase flows and another is based on reconstitution of digesta to obtain the composition of the representative digesta. The unrepresentative digesta sampling and digesta reconstitution approach does not require representative digesta sampling or the different phases to be isolated from the sample (France and Siddons, 1986). Furthermore, it is sufficient that the markers associate mainly to their specific phase, rather than associating exclusively with just one phase.

The triple-marker method is a refinement of the Faichney (1975) double-marker method and includes three separate phases: liquid, small particles and large particles, to which three individual markers are associated (France and Siddons, 1986). Ahvenjärvi *et al.* (2003) found that double-marker systems based on ytterbium (Yb), in combination with chromium (Cr) or indigestible NDF (iNDF) have sufficient reliability for determination of OM, N and NDF flow. However, they suggest that using cobalt (Co) in a triple-marker system would further improve the accuracy of estimation of total digesta flow. Their tests on use of a single-marker method indicated that the sample was not representative of true digesta (Ahvenjärvi *et al.,* 2003).

1.5.2 *In situ*

Protein evaluation systems requires simple methods to determine rumen protein degradability, and the *in situ* method has become the most common (Nozière and Michalet-Doreau, 2000). A small amount of feed sample is placed in a nylon bag with a pore size of about 30-50 µm. The bag is suspended in the rumen for incubation, following which the degradability is calculated from nutrient

disappearance from the bag. This is a cheap method compared with *in vivo* determinations, but ruminally cannulated animals are required.

The disadvantages of the *in situ* method are low repeatability and lack of reproducibility in ring tests (Madsen and Hvelplund, 1994). The future success of this technique depends on the ability to standardise the procedure. However, this has proven difficult, although the sources of variation are well known and include the characteristics of bags and samples, the necessary exchanges between the bag and the rumen environment, and the assumptions made on degradation kinetics (Nozière and Michalet-Doreau, 2000).

One major problem with the *in situ* method is that disappearance from the bag is considered synonymous with degradability, although it is known that small particles and soluble fractions can leave the rumen without being degraded. Furthermore, the population of microbes within the bag is less dense than that in the rumen digesta, indicating a risk of decreased microbial digestion in the bag (Meyer and Mackie, 1986). Moreover, undegraded feed protein can be contaminated with microbial protein, resulting in underestimation of the degradability of the feed protein (Nocek and Grant, 1987). There have been attempts to deal with these errors, *e.g*. Hvelplund and Weisbjerg (2000) developed an equation to correct for particle losses from the bags by assuming similar degradation characteristics for particles lost from the bags and those remaining in the bags.

1.5.3 *In vitro*

There are several different types of *in vitro* methods for determining rumen protein degradability and microbial protein synthesis. Protein solubility in buffer solution shows a good correlation with protein solubility in enzymatic solution and the prediction error is small within feed types, but is poor over a range of feeds (McDonald *et al*., 2002).

The development of *in vitro* incubation systems using rumen fluid, with the possibility to measure NH_3 concentration and gas production (Menke *et al*., 1979), has expanded the capability for determining rumen protein degradation. However, since the system relies on measurement of NH_3 concentration, there is a problem in separating the simultaneous processes of feed protein degradation by microbes in the rumen fluid and microbial synthesis. There have been various attempts to separate these two actions. For example, Raab *et al*. (1983) developed a system based on NH_3-N release and gas production in which a graded level of carbohydrates was used to determine NH_3-N release at zero gas production, when no fermentable carbohydrates were available and no microbial

synthesis would occur. Broderick (1987) added chloramphenicol with hydrazine to inhibit microbial synthesis.

The *in vitro* incubation system was further developed by Edmunds *et al.* (2012) to a system without the need for separating RUP from MCP. Using a modified Hohenheim gas test (Steingaß *et al*., 2001) involving modification of the Raab *et al*. (1983) method to the standard Hohenheim gas test (Menke and Steingaß, 1988), they measured the NH_3 concentration after incubation of feed with rumen fluid. The concentration of non-ammonia-N (NAN) was used to calculate utilisable CP (uCP), which corresponds to MCP and RUP. The method seems to be simple and have high repeatability, and it is claimed that MP can be estimated using the same procedures as in current protein evaluation systems for AA content, endogenous fractions and digestibility (Edmunds *et al.*, 2012).

1.6 Treatment of protein feeds

In theory, reducing rumen protein degradability by various treatments or by using feeds with naturally high RUP is a strategy that can be applied to increase the supply of dietary AA to the small intestine. It is speculated that this increased supply of AA to the cow would increase milk production.

Within the area of dairy cow nutrition, there has been great emphasis in recent decades on dietary protein escaping digestion in the rumen and manipulation of rumen conditions and feeds to increase this process (Van Soest, 1994). The focus on RUP arose from the assumption that microbial output was a function of rumen-fermentable matter at relatively constant efficiency, which would make it favourable to protect less soluble protein from rumen fermentation. However, protein solubility is also a very important factor for microbial efficiency (Van Soest, 1994). This raises questions as to whether protein solubility is a more important factor for rumen microbial yield or for increased provision of dietary AA in the lower digestive tract.

The easiest method to reduce the protein degradability of the diet is to include feeds with naturally low rumen degradability (Tamminga, 1979). Other methods include different types of heat treatment or chemical treatments (aldehydes, tannins, volatile fatty acids). With chemical treatment, the aim is to create a modification arising from linkages between the chemical and dietary protein. These linkages should be resistant to degradation in the rumen, but broken in the acidic environment of the stomach and further hydrolysed by the digestive enzymes in the small intestine. However, there is a risk of creating linkages that are totally resistant to breakdown and hence of increasing the indigestible N fraction. Furthermore, treatments to increase RUP can actually destroy some AA. For example, heat treatment of rapeseed meal (RSM) decreases the

concentration of lysine (Moshtaghi Nia and Ingalls, 1995; Dakowski *et al*., 1996), while xylose treatment of soybean meal (SBM) decreased the relative contribution of lysine and arginine, by 17 and 7 %, respectively, in a study by Harstad and Prestløkken (2000).

Heat treatment causes coagulation or denaturation of the protein, which reduces the solubility and accessibility of the protein to rumen microbes, but does not create the linkages formed in chemical treatments (Van Soest, 1994). However, if the heat treatment is too extreme, the dietary N can become totally indigestible due to the Maillard reaction, which creates lignin-like matrices between amino groups and carbohydrates (carbonyls and dehydroreductones). The first step of the Maillard reaction can be reversible, but with the total Maillard reaction the polymerisation between protein and carbohydrate is permanent.

Other research has challenged the theory of improved milk production with an increased level of RUP in the diet. For example, Santos *et al*. (1998) found increased milk production in only 17% of comparisons between supplementation of SBM and feed sources high in RUP like heat- and chemically treated soybean meal, maize gluten meal, distiller's grains, brewer's grains, blood and bone meal, feather meal or mixtures of these. Similarly, Ipharraguerre and Clark (2005) found very small differences (-2.5 to +2.8%) in milk yield between feeding solvent-extracted SBM and supplements high in RUP. In addition, in a meta-analysis of large datasets, ruminal CP degradability, estimated according to NRC (2001), was not an important factor for predictions of milk protein yield (MPY) or N use efficiency (Huhtanen and Hristov, 2009).

2 Objectives

The purpose of the studies that form the basis for this thesis was to extend the knowledge about how to utilize the domestic protein feeds in Swedish dairy production. To optimize the use of N from both concentrates and forages into milk product, and thereby improving the profitability and the resilience of the domestic dairy farms. More specific objectives were to:

- Compare production responses and methane emissions from grass silage-based diets for dairy cows supplemented with increased levels of SBM or solvent-extracted, heat-moisture-treated RSM in grass silage-based diets to dairy cows
- Study the possibility of reducing protein supplementation by increasing the proportion of red clover silage in the diet of dairy cows
- Study ruminal N metabolism and incorporation of N into milk protein when feeding incremental levels of solvent-extracted, heat-moisture-treated RSM to dairy cows.
- Study the relationship between estimated uCP at the duodenum measured *in vitro* and omasal flow of NAN using samples from previous flow studies.

3 Materials and Methods

The animal studies (Papers I, II, and III) were conducted at Röbäcksdalen Research Centre (Swedish University of Agricultural Sciences, Umeå, Sweden) using the experimental herd of Nordic Red dairy cows. The *in vitro* study (Paper IV) was conducted at the analytical laboratory at the Department of Agricultural Research for Northern Sweden (Swedish University of Agricultural Sciences, Umeå, Sweden). The omasal flow studies that provided data used in Paper IV were performed at the Department of Agricultural Research for Northern Sweden (Swedish University of Agricultural Sciences, Umeå, Sweden) and at Natural Resources Institute Finland (Luke).

3.1 Paper I and Paper II

3.1.1 Experimental design

The studies described in Papers I and II were conducted using a cyclic change-over design with two replicates of two blocks according to a 2 × 4 factorial arrangement with periods of 21 days (Davis and Hall, 1969). In Paper I, the factorial arrangement consisted of two protein supplements (SBM and treated RSM) and four levels of dietary CP concentration, while in Paper II the factorial arrangement included two different grass and red clover silage mixtures and four levels of dietary CP concentration (incremental replacement of barley with heat-moisture-treated rapeseed expeller). The study in Paper I ran for four periods over 21 days each, while that in Paper II ran for three periods with the same period length.

3.1.2 Animals and treatments

The Nordic Red cows were kept in a loose housing system and milked twice daily, at 06.00 and 15.00 h. They were fed *ad libitum* with a total mixed ration and feed intake was registered automatically. In Paper I, 28 lactating cows with an initial milk yield of 35±6.6 kg/day were blocked according to parity and milk yield, and randomly allocated to seven dietary treatments. These treatments consisted of one control diet without additional protein supplement, and six diets supplemented with either SBM or solvent-extracted, heat-moisture-treated RSM to reach three incremental levels of inclusion. In Paper II, 32 lactating cows with initial milk yield of 32±6.9 kg/day were blocked according to parity and milk yield and randomly allocated to eight dietary treatments. These treatments consisted of two control diets with either a high or low level of red clover, and six diets with either a high or low red clover silage inclusion together with three incremental levels of heat-moisture-treated rapeseed expeller.

3.1.3 Feeds

Crimped barley and a premix were the basic concentrates fed in both studies. In Paper I, the forage was a 50:50 mixture of two different cuts of primary growth grass silage. Additional concentrates fed were SBM and tRSM (ExPro-00SF, AArhusKarlshamn Ltd., Malmö, Sweden). In Paper II the forage consisted of a regrowth grass-red clover silage that was mixed with either low (30 %) or high (70 %) inclusion of a red clover silage mix. The red clover silage mix consisted of 50:50 primary- and regrowth pure red clover silage. The protein supplement fed was heat-moisture-treated rapeseed expeller (Öpex, Mildola Ltd, Espoo, Finland). According to the manufacturer, this product is permitted for use in organic feed production, while solvent extraction of protein feeds is not permitted.

3.1.4 Measurements

Measurements were performed in the same way in Papers I and II, and data for statistical analysis were limited to days 15-21 in each period. Individual feed intake was registered daily with roughage intake control feeders (Insentec B.V. Markness, the Netherlands). Milk yield was recorded for all milkings with gravimetric milk recorders (SAC, S.A. Christensen and Co. Ltd., Kolding, Denmark). All cows were weighed after morning milking on days 19-21. Milk samples for analysis of composition were collected during four consecutive milking occasions and morning milking samples were pooled, as were evening samples. Emissions of methane (CH_4) and carbon dioxide (CO_2) were measured

in a head chamber system (Greenfeed system, C-Lock Inc., Rapid City, SD), which the animals were encouraged to visit by concentrate feeding. In Paper I, gaseous emissions were only recorded in the last two periods, when the Greenfeed system was working. Furthermore, in Paper I blood sampling was performed on 14 cows after morning milking on day 19, for plasma analysis of AA. Faeces sampling was also performed in Paper I, again in the last two periods. This was to determine digestibility in connection with the measurements of gaseous emissions.

3.1.5 Statistical analysis

The data were analysed with the mixed model procedure in SAS (SAS Inc. 2002-2003, release 9.3; SAS Inc., Cary, USA) using the statistical model:

$$Y_{ijkl} = \mu + B_i + C_j(B_i) + P_k + T_l + \varepsilon_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the mean for all observations, B_i is the effect of block i, $C_j(B_i)$ is the effect of cow j within block i, P_k is the effect of period k, T_l is the effect of treatment l, and $\varepsilon_{ijkl} \sim N(0,\sigma^2_e)$ is the random residual error.

The contrasts used in Paper I were comparison of tRSM and SBM, linear and quadratic effect of dietary CP concentration, and interaction between protein supplement and linear and quadratic effects of dietary CP concentration.

The contrasts used in Paper II were comparison of low or high inclusion of red clover silage, linear and quadratic effects of dietary CP concentration, and interaction between the proportion of red clover silage and linear and quadratic effects of CP concentration.

3.2 Paper III

3.2.1 Experimental design, animals, treatments, and feeds

The study described in Paper III was conducted using a Latin square design including four dietary treatments and four experimental periods of 21 days. Four ruminally cannulated Nordic Red cows with initial milk yield of 29±9.1 kg/day were used. The cows were housed in tie stalls, fed manually twice per day and walked to the milking parlour at 06.00 and 18.00 h.

The treatments consisted of one control diet without an additional protein supplement and three diets supplemented with incremental levels of tRSM (ExProo-00SF, AarhusKarlshamn Ltd., Malmö, Sweden). In addition, crimped barley and a mineral and vitamin mix were fed. The forage was a grass and red clover silage prepared from primary growth and regrowth.

3.2.2 Measurements

Two rumen evacuations were performed to decide rumen pool size and digestion kinetics. Digesta flow to the omasum was determined using the omasal sampling technique devised by Huhtanen *et al*. (1997a), and modified by Ahvenjärvi *et al.* (2000). The flow of OM, NDF and N was estimated with the triple-marker method according to France and Siddons (1986), using the markers chromium-EDTA, ytterbium-acetate and iNDF for the fluid phase, small particles and large particles, respectively. Microbial N flow was determined using ^{15}N as a marker. To study the incorporation of dietary N and microbial N into milk protein, the milk was analysed for ^{15}N enrichment.

3.2.3 Statistical analysis

The data were analysed with the general linear model in SAS (SAS Inc. 2002-2003, Release 9.2; SAS Inst. Inc., Cary, USA), using the statistical model:

$$Y_{ijk} = \mu + C_i + P_j + D_k + \varepsilon_{ijk},$$

where Y_{ijk} is the dependent variable and μ is the mean for all observations, C_i is the effect of cow i, P_j is the effect of period j, D_k is the effect of diet k, and $\varepsilon_{ijk} \sim N(0, \sigma^2_e)$ is the random residual error. Contrasts were included for evaluation of linear and quadratic responses to dietary CP concentration.

3.3 Paper IV

3.3.1 Experimental design and diets

The samples from eight *in vivo* omasal flow studies with 34 diets were incubated in an *in vitro* system. The diets were constructed from ingredient samples in the same proportions as used in flow studies. Each diet was randomly distributed within and between four *in vitro* runs. In each run, two blanks were incubated. The forage consisted of different cuts of grass and red clover silage, pure grass silage and pure red clover silage, and mixes of these. The concentrates studied

were barley, oat, RSM, rapeseed expeller, soybean expeller, pea, field bean and blue lupine.

3.3.2 *In vitro* procedure

The *in vitro* procedure followed that of Hetta *et al*. (2003), with some modifications. Rumen fluid was collected from three lactating dairy cows after morning milking. The fluid was pre-incubated with a carbohydrate mixture to reduce background NH_3 by stimulating microbial uptake of NH_3. After pre-incubation, the rumen fluid was mixed (20:80 v/v) with a buffered mineral solution (Menke and Steingaß, 1988) under constant stirring and flushing with CO_2. The buffer solution was modified to contain 38 g $NaHCO_3$ and 1 g $(NH_4)HCO_3$ with addition of distilled water to 1000 mL.

The liquid phase was sampled (0.4 mL) for NH_3-N measurements at 0.5, 4, 8, 12, 24, and 30 hours of incubation, according to Karlsson *et al*. (2009), and stored at -20 ºC until further analysis. The NH_3-N in the dilution was analysed with a continuous flow analyser (AutoAnalyzer 3 HR, SEAL Analytical Ldt, UK).

3.3.3 Calculations

Utilisable CP was calculated according to the equation by Edmunds et al. (2012):

$$uCP\ (g/kg\ DM)\ = \frac{\left(NH_{3blank} + N_{sample} - NH_3N_{sample}\right)}{weight\ (mg\ DM)\ \times 6.25\ \times 1000}$$

To estimate the concentration of uCP at time point 16, 20, and 24, natural logarithm of uCP (g/kg DM) at time points 0.5, 4, 8, 12, 24, and 30 h of incubation was plotted against time. An exponential function was used to calculate uCP concentration at estimated time points 16, 20, and 24 h.

Estimated supply of uCP_x at 16, 20, and 24 hours of incubation (uCP_{16}, uCP_{20}, uCP_{24}) was calculated as:

$$uCP_x\ flow\ (kg/day) = estimated\ uCP_x\ (g/kg\ DM)\ \times 1000\ \times DMI\ (kg)$$

3.3.4 Statistical analysis

The data were analysed with the SAS programme (2003, release 9.3, SAS Institute Inc., Cary, USA). Least square means of the uCP concentration (g/kg DM) between runs were calculated with the statistical model:

$$Y_i = \mu + D_i + R_j + B_k + e_{ijk}$$

where Y_i is the dependent variable, μ is the mean of all observations, D_i is the effect of diet i, R_j is the effect of run j, B_k is the effect of bottle k, and $e_{ijk} \sim N(0, \sigma_e^2)$ is the random residual error.

Some of the variation in *in vivo* data is related to differences between studies, and this variation must be excluded in order to obtain an accurate relationship with *in vitro* data. Therefore the relationship between the *in vitro* and *in vivo* data was evaluated with the MIXED procedure in SAS as:

$$Y = \mu + B_1X_{1ij} + b_0 + b_1X_{ij} + B_2X_{2ij} + e_{ij},$$

where Y is the dependent variable, μ, B_1X_{1ij}, and B_2X_{2ij} are the fixed part of the model, $b_0 + b_1X_{ij}$ and e_{ij} are the random parts of the model, i = 1…8 studies, and j = 1…n_i values.

The fit of the model was compared according to Akaike's information criterion (AIC). The model with the smallest AIC value was assumed to be the most correct.

The relationship between *in vitro* and *in vivo* data was also evaluated with simple regression:

$$Y_i = \mu + B_1X_{1ij} + B_2X_{2ij} + e_{ij},$$

where Y is the dependent variable, μ, B_1X_{1ij}, and B_2X_{2ij} are the fixed part of the model, and e_{ij} are the random parts of the model, i = 1…8 studies, and j = 1…n_i values.

4 Results

4.1 Paper I

Dry matter intake (DMI) was higher with tRSM diets compared with the SBM diets. The intake of NDF increased more with increased dietary CP concentration when tRSM was fed, as did the intake of MP. Increased dietary CP did not affect DMI, but increased the intake of CP from 3.1 to 4.3 kg/day. No effects on apparent digestibility were found between treatments.

Milk yield increased with the tRSM diets compared with the SBM diets, and milk yield increased more with increased dietary CP concentration when feeding tRSM diets compared with SBM diets. Increased dietary CP concentration increased yield of milk, energy-corrected milk (ECM), milk protein and lactose.

Increased dietary CP concentration decreased N use efficiency more when the cows were fed the SBM diets than the tRSM diets, and milk urea N (MUN) increased more with SBM than tRSM when dietary CP concentration was increased.

There were no differences in plasma concentrations of histidine, methionine or lysine between tRSM and SBM diets, while SBM diets increased the concentrations of phenylalanine and proline. Increased dietary CP concentration increased plasma concentrations of branched-chain AA, essential AA, total AA, and several individual AA.

There were no differences in total CH_4 emissions between treatments. Methane yield (g/kg DMI) decreased quadratically, and the lowest emissions occurred with diets with a medium dietary CP concentration. The CH_4 intensity (g/kg ECM) decreased more with tRSM diets than SBM diets when dietary CP concentration increased.

4.2 Paper II

The regrowth red clover silage was of poor quality, as evidenced by a high concentration of butyric acid and NH_3-N (4.3 g/kg DM and 120 g/kg N, respectively). The grass silage and primary growth red clover silage were of good quality.

Increasing the proportion of red clover silage had no effect on DMI, but increased CP intake, and decreased the intake of metabolisable energy, NDF and potentially digestible NDF (pdNDF). Increased dietary CP concentration increased DMI and also intake of all other nutrients analysed.

The production responses to increased dietary CP concentration differed depending on whether the supplementation was made with red clover silage or rapeseed expeller. Forage treatment had no effect on milk yield or ECM yield. A high proportion of red clover silage decreased milk protein concentration. Increasing the dietary CP concentration by supplementation with rapeseed expeller quadratically increased the yield of milk, milk protein and lactose. The highest milk yield was observed with diets with a medium level of protein supplementation. Milk fat and lactose concentration decreased with increased dietary CP concentration.

The N use efficiency decreased with high proportion of red clover silage in the diet, while the concentration of MUN milk urea nitrogen increased. Similar responses were seen for N use efficiency and MUN concentration with increased dietary CP concentration.

Total emissions of CH_4 and CO_2 did not differ between forage treatments, but the CH_4 yield tended to be higher with a high proportion of red clover silage in the diet. Increased dietary CP concentration decreased the yield of both gases, and the decrease in CH_4 yield was found to be greater with a low red clover silage proportion. With low red clover silage proportion, the CH_4 yield declined with increased dietary CP concentration, but with high red clover silage proportion the lowest CH_4 yield was observed with low dietary CP concentration. The CH_4 intensity was numerically lowest with the low dietary CP concentration, but the difference was not statistically significant.

4.3 Paper III

Dry matter intake increased with increasing dietary CP concentration, as did intake of OM, NDF, pdNDF and N. Milk yield increased with increasing dietary CP concentration, while N use efficiency decreased.

The pH and NH_3 concentration in the rumen decreased with increasing dietary CP concentration. There were no changes in total volatile fatty acids

concentration, but the molar proportion of propionate increased and butyrate decreased in the rumen with increasing dietary CP concentration.

The rumen pool size of iNDF increased with increasing dietary CP concentration and the digestion rate of pdNDF also increased, while the passage rate of pdNDF tended to decrease. The NDF turnover time also decreased with increasing dietary CP concentration.

Increasing dietary CP concentration did not influence omasal flow of OM. The omasal flow of NDF tended to increase with increasing dietary CP concentration, but the flow of pdNDF did not differ. The ruminal true digestibility of OM tended to decrease, while the total tract digestibility of both NDF and pdNDF increased, with increasing dietary CP concentration.

Omasal flow of NAN and non-ammonia, non-microbial N (NANMN) increased with increasing dietary CP concentration, while microbial NAN decreased numerically despite increased feed intake. The ruminal true NAN digestibility (ruminal CP degradability) decreased with increasing dietary CP concentration, while the total tract apparent N digestibility increased. The microbial efficiency tended to decrease with increasing dietary CP concentration.

The ^{15}N enrichment of milk protein N, ruminal NAN and rumen microbial N decreased with increased dietary CP concentration. The estimated proportion of milk protein N originating from rumen microbial N decreased with increasing dietary CP concentration, while the estimated proportion of milk N originating from dietary protein increased. The treatments tested did not affect the proportion of milk protein originating from either ruminal NAN or body N turnover. Recovery of the ^{15}N infused in milk protein N decreased, and the proportion of ^{15}N that was absorbed as NH_3-N in the reticulorumen increased with increased dietary CP concentration.

4.4 Paper IV

The *in vitro* estimated uCP supply at the duodenum after 16 hours of incubation resulted in the best prediction of omasal CP flow and MPY compared with 20 and 24 hours of incubation, as indicated by smaller residual mean square error and Akaike's information criterion. The correlation between uCP concentration determined at different time points was strong (R^2 = 0.94-0.99).

The most important finding was a relationship between uCP supply and omasal CP flow. The mixed model regression gave a slope of less than 1 (0.77) and a positive intercept (0.40), both of which were significant ($P < 0.01$). The

coefficient of determination (0.87) indicated a reasonable relationship between the predicted and observed factors.

The residual (difference between omasal CP flow and calculated uCP supply) was negatively related to OMD and the ratio rumen NDF digestibility/total NDF digestibility, and tended to be influenced by dietary CP concentration when analysed with the fixed regression model. Analysis with mixed model regression indicated that the residual was negatively related to DMI, but not to the other factors tested.

Residual analysis with the fixed model revealed that omasal CP flow (g/kg DMI) – uCP supply (g/kg DM) estimated at 16 h of incubation was positively related to DMI, negatively related to OMD and the ratio of rumen NDF digestibility/total NDF digestibility, and tended to be positively related to dietary CP concentration. Residual analysis of mixed model regression showed that adjusted omasal CP flow (g/kg DMI) – uCP concentration (g/kg DM) estimated at 16 h of incubation, was not associated with any other factor.

The quadratic uCP mixed model predicted MPY better than the quadratic omasal CP flow, based on smaller Akaike's information criterion and adjusted residual mean square error. There was no difference in model fit between time points 16, 20 and 24 hours of *in vitro* incubation.

5 Discussion

5.1 Replacing soybean meal with rapeseed meal

Lactating dairy cows have been found to be more responsive to supplementation with RSM compared with SBM when fed diets based on grass silage (Shingfield *et al*., 2003; Vanhatalo *et al*., 2003; Paper I). However, Murphy *et al*. (1985) found no difference in milk yield (18.3 and 18.2 kg/day) or MPY (574 and 562 g/day) on supplementing grass silage with SBM or RSM. More recently, Broderick *et al*. (2015) found numerically higher production responses (39.3 and 38.4 kg milk/day) with RSM compared with SBM supplementation of diets based on lucerne and maize silage. Furthermore, superiority of rapeseed feeds over SBM has been pointed out in two meta-analyses. Huhtanen *et al*. (2011) found that the marginal increase in milk yield and MPY with increasing CP concentration was greater with RSM (3.4 kg milk and 136 g milk protein per kg increase in CP intake) than SBM (2.1 kg/kg and 98 g/kg, respectively). Later, Martineau *et al*. (2013) found that milk yield and MPY increased when RSM replaced SBM, maize gluten meal, cottonseed meal, distiller's product or a mixture of protein feeds. It has also been shown that replacing SBM with RSM clearly decreases milk urea N, which is reflected in greater N use efficiency (Shingfield *et al*., 2003; Vanhatalo *et al*., 2003; Paper I).

The response in terms of MPY (g/day) found in six different studies comparing rapeseed and soybean supplementation is summarised in Figure 2. Most studies show a greater response in MPY with rapeseed supplementation, as indicated by the fact that most data points are located above the line y = x. Furthermore, the slope is slightly greater than 1.0 (1.09), indicating that with higher production, dairy cows respond somewhat better to rapeseed than soybean supplementation. One possible explanation for this could be that the lower-yielding cows in the study by Tuori (1999) were supplemented with

rapeseed containing more glucosinolates than the modern varieties fed in later studies. Glucosinolates have been found to possibly decrease DMI (Tuori, 1992), while in a study on growing cattle, Aronen and Vanhatalo (1992) suggested that the effect of RSM on live weight gain was more related to the concentration of glucosinolates than to protein degradability.

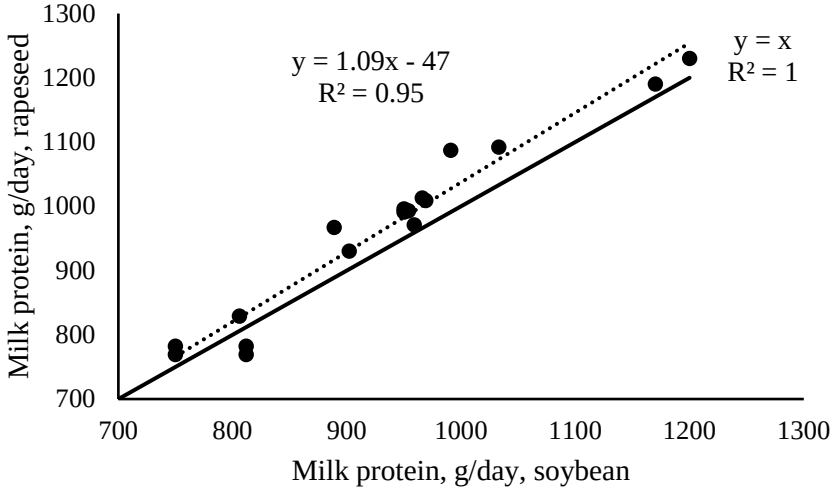

Figure 2. Direct comparisons (n=17) of milk protein yield (g/day) resulting from supplementation with rapeseed feeds and soybean feeds. The dashed line represents the actual relationship found in direct comparisons, while the solid line represents y = x (R^2 = 1.00) (sources: Tuori, 1992; Shingfield *et al*., 2003; Vanhatalo *et al*., 2003; Broderick *et al*., 2015; Rinne *et al*., 2015; Paper I).

The positive production responses with rapeseed feeds could be expected to originate from increased DMI. This was confirmed in a meta-analysis where RSM supplementation was found to increase DMI more than SBM (Huhtanen *et al*., 2011), but has been difficult to show in production studies due to lack of statistical power. In Paper I, supplementation with tRSM tended to increase DMI compared with SBM supplementation. Similarly, Shingfield *et al*. (2003) found only small numerical differences in DMI between diets supplemented with rapeseed expeller or SBM. Other studies have found that DMI increases with supplementation with rapeseed cake and RSM compared with SBM (Vanhatalo *et al*., 2003), and with solvent-extracted RSM compared with solvent-extracted SBM (Broderick *et al*., 2015).

Although it is sometimes difficult to obtain significant differences in production studies, direct comparisons between diets supplemented with either

rapeseed or soybean feeds indicate that increased DMI also results in increased yield of energy-corrected milk (Figure 3).

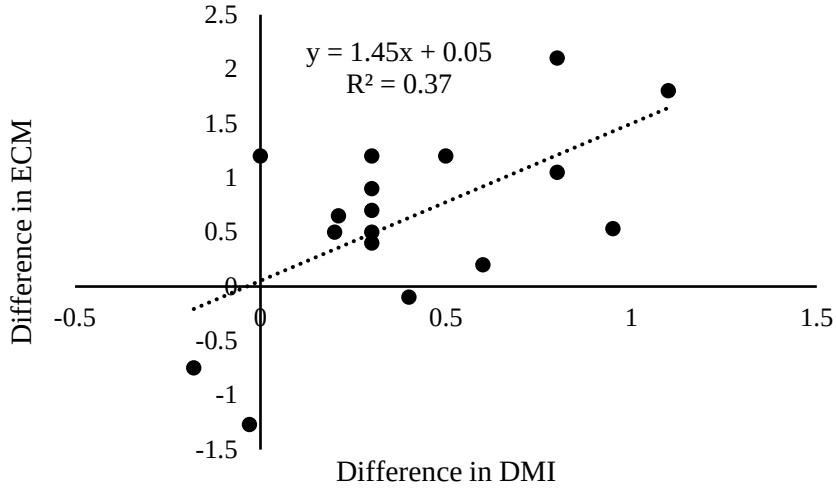

Figure 3. Difference in dry matter intake (DMI) (kg/day, x-axis) and energy-corrected milk (ECM) yield (kg/day) between diets (n=17) in studies comparing supplementation with rapeseed and soybean feeds (sources: Tuori, 1992; Shingfield *et al*., 2003; Vanhatalo *et al*., 2003; Broderick *et al*., 2015; Rinne *et al*., 2015; Paper I).

The reasons why rapeseed feeds increase DMI are not clear. An increased rate of cell wall digestion has been suggested as one important reason why protein feeds in general appear to increase DMI (Oldham, 1984). However, digestibility does not seem to be an important factor explaining the difference in DMI between RSM or SBM supplementation. Supplementation with RSM could be expected to decrease digestibility, since the concentration of iNDF is greater (Paper I). However, in Paper I there was no difference in apparent total tract digestibility between diets supplemented with treated RSM or SBM. Similarly, Shingfield *et al.* (2003) found no differences in digestibility between these supplements but, interestingly, found an interaction between protein supplementation and dietary CP concentration. With increasing CP concentration, DM and OM digestibility decreased slightly with diets supplemented with rapeseed expeller and increased with diets supplemented with SBM (Shingfield *et al.*, 2003). This could be expected from the higher intrinsic digestibility of SBM compared with RSM. In a more recent study,

Rinne *et al*. (2015) also found a significant interaction between protein source and dietary CP concentration for total tract OM digestibility, with greater increases with soybean expeller than rapeseed expeller, but found no differences in pdNDF digestibility. However, Brito and Broderick (2007) found greater digestibility of NDF and hemicellulose with diets supplemented with RSM than with solvent-extracted SBM or cottonseed meal, which they attributed to the higher digestibility of rapeseed fibre compared with fibre from the other protein supplements. They also found that solvent-extracted SBM and RSM had similar acid detergent fibre digestibility. In the rumen evacuation study described in Paper III, increased supplementation tRSM had no effect on rumen NDF pool size but increased the digestion rate of pdNDF, suggesting that improved digestion rate could be at least partly involved in the DMI responses observed with protein supplementation. However, milk yield was low in Paper III and rumen fill was not likely to have limited intake.

Total tract N digestibility was not different between diets supplemented with rapeseed expeller or SBM in the study by Shingfield *et al*. (2003). However, N digestibility increased more with SBM than with rapeseed expeller when the dietary CP concentration was increased. Rinne *et al*. (2015) found increased total apparent N digestibility with soybean expeller compared with rapeseed expeller, but no differences in apparent ruminal or true ruminal N digestibility, or rumen NH_3-N concentration, between the supplements. Moreover, they did not find any differences between soybean expeller and rapeseed expeller in terms of N intake or the flow of NAN, microbial N and NANMN. Similarly, Brito *et al*. (2007) found no differences between supplementation with solvent-extracted SBM or RSM. Thus improved digestibility of DM, OM, fibre or dietary N is clearly not the explanation for the higher DMI with RSM than SBM supplementation.

It has been suggested by Huhtanen *et al*. (2011) that increased DMI and increased production work together, creating a 'pull effect' where an increased and/or more balanced supply of AA increases production. This increase in production creates an increase in energy demand, resulting in increased DMI by the cow. This has been demonstrated in studies comparing feed sources of true protein providing different AA patterns. For example, Chamberlain *et al*. (1992) found that fish meal increased DMI more than supplementation with feather meal, while Shingfield *et al.* (2001) found that rapeseed expeller had a similar effect to wheat gluten meal. In terms of being an equivalent source of AA for milk protein synthesis, feather meal lacks histidine and methionine, and wheat gluten lacks lysine and methionine, compared with RSM. Chamberlain *et al.* (1992) pointed out the importance of the AA composition of RUP, while Shingfield *et al.* (2001) suggested that the increased ECM yield observed with rapeseed expeller was partly due to increased tissue AA availability. Similarly,

reduced plasma concentration of lysine and methionine was suggested to have contributed to decreased DMI and milk yield when supplementing formic acid-treated grass silage-based diets with field beans compared to RSM (Puhakka *et al*., 2016). Furthermore, they suggested decreased ratio of AA supply to energy at tissue level as the reason for a significant decrease in silage DMI feeding high dietary CP concentration.

Both RSM supplementation (Ahvenjärvi *et al*., 1999; Rinne *et al*., 2015; Paper III) and increasing the proportion of red clover silage in the forage (Dewhurst *et al*., 2003a; Vanhatalo *et al*., 2009) have been shown to increase NAN flow from the rumen. Furthermore, red clover silage and RSM supplementation increase *in vitro* uCP supply (Paper IV). The different intake responses to increased CP supplementation between RSM and red clover silage in Paper II confirm the suggestion that better AA composition is the main factor behind the improved milk yield with RSM supplementation. Further support is provided by Vanhatalo *et al*. (2009), who found that red clover silage increased the omasal flow of histidine, suggesting improved AA supply, but the plasma histidine concentration was unchanged and the methionine concentration decreased. This is contradictory to findings in studies infusing histidine (Kim *et al*., 2001) or supplementing with RSM (Rinne *et al*., 2015), where an increased concentration of histidine in the digesta also increased plasma histidine concentration. Reference could also be made to previous studies evaluating diets containing grass silage treated with formic acid-based additive or bacterial inoculant and postruminal addition of casein (Huhtanen *et al*., 1997b; Miettinen and Huhtanen, 1997). The restrictively fermented silage (formic acid-treated) in those studies increased microbial protein synthesis and the plasma concentration of lysine and branched-chain AA in a similar way to casein infusions. These factors imply that the production response should be positive, but infusion of casein still gave a much greater response in plasma histidine concentration and MPY. This indicates not only the varied responses of different feed rations in terms of the AA profile supplied to the small intestine, but also that the AA profile which is absorbed can differ from that provided with the digesta, and hence impact upon the production response.

The AA composition of (MP) absorbed in the small intestine acts as the basis for milk protein synthesis and the basal diet determines which AA will be limiting. With grass silage-based diets, histidine is the first limiting AA (Kim *et al*., 1999; Vanhatalo *et al*., 1999). Replacing grass silage with red clover silage limits the supply of methionine (Vanhatalo *et al*., 2009), while methionine and lysine are well-recognised as the first limiting AA with maize silage-based diets (Schwab *et al*., 1996).

Rapeseed meal have a somewhat higher content of histidine than SBM (NRC, 2001), which is one explanation for the higher production with RSM supplementation of grass silage-based diets (Shingfield *et al*., 2003). Histidine is probably also the first limiting AA in microbial protein (Fraser *et al*., 1991). According to NRC (2001), the concentration of histidine and methionine in microbial protein is 4-4.3% and 4.9-5.2% of total essential AA, respectively. Rapeseed meal contains 6.6 and 4.4 % of histidine and methionine, respectively, and SBM contains 6.1 and 3.2 %, respectively. In comparison, in milk protein the histidine concentration is 5.5% of total essential AA and the methionine concentration is also 5.5%. A meta-analysis by Martineau *et al*. (2014) concluded that RSM increases the plasma concentration of essential AA, including histidine, lysine and methionine, compared with other protein supplements such as maize gluten meal, cottonseed meal, distiller's products or a mixture of more than one protein source, including SBM.

5.2 Treatment of rapeseed meal to improve the protein value

Unexpectedly, the response to tRSM in Paper I was smaller than the response to heat-moisture-treated rapeseed expeller reported by Shingfield *et al*. (2003), although the increase in total CP intake was greater in Paper I. The difference in response could be due to the different pre-treatments of the protein feedstuffs. For example, Tuori (1992) found that heat-moisture treatment tended to reduce the concentration of AA more in RSM than SBM, with the histidine concentration in particular being reduced by 20% in the treated RSM. Direct comparisons of MPY in studies examining supplementation with treated or untreated rapeseed feeds indicate a lack of difference in production responses due to treatment (the slope is similar to 1) (Figure 4). This is consistent with findings in the meta-analysis by Huhtanen *et al.* (2011), which was based on production responses to incremental CP intake and also included indirect comparisons and also found no differences in marginal milk protein responses between supplementation with regular and treated RSM (136 and 133 g milk protein per kg increase in CP intake, respectively). Furthermore, Huhtanen and Hristov (2009) claim that the role of dietary CP degradability as a determining factor for MPY is overvalued the protein evaluation systems in NRC (2001). Thus it appears that treatment to increase the concentration of RUP in rapeseed feeds in order to increase the production response is a less important option. This is possibly because the benefits of increased RUP could be lost due to reduced microbial protein synthesis in the rumen (Paper III). The results in Paper IV

indicated that the uCP method can overestimate the effect of heat treatment on protein flow to the small intestine.

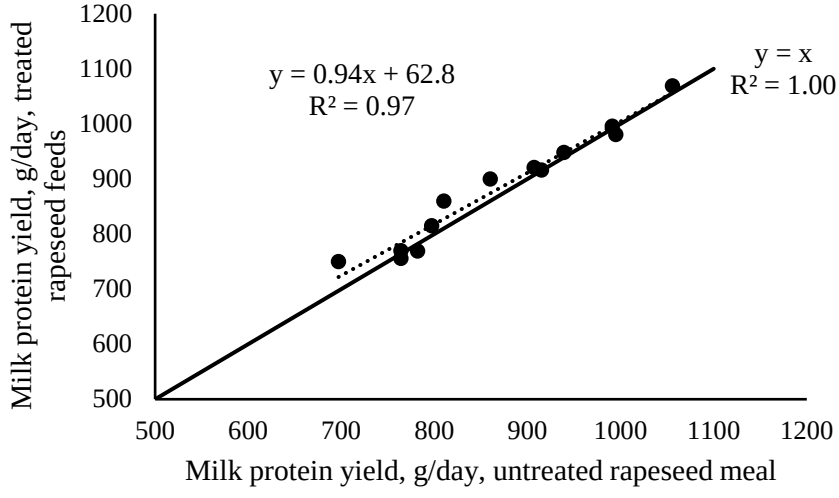

Figure 4. Direct comparison of milk protein yield resulting from supplementation with treated or untreated rapeseed meal in *in vivo* studies. The dashed line represents the actual relationship found in direct comparisons, while the solid line represents y = x (R^2 = 1.00) (sources: Bertilsson *et al*., 1990; Tuori, 1992; Bertilsson *et al*., 1994; Huhtanen and Heikkilä, 1996; Rinne *et al*., 1999; Vanhatalo *et al*., 2003).

The reasons why increased concentration of RUP due to treatment not increase the protein value could be because of reduced intestinal digestibility. Huhtanen et al. (2011) demonstrated that treated RSM had lower true CP digestibility calculated according to the Lucas test. Furthermore, the lack of increased protein value could be due to that treatments can destroy AA (Harstad and Prestløkken, 2000). Tuori (1992) showed that heat-moisture treatment decreased most AA, and especially lysine and histidine was reduced by over 15%. Further, Broderick et al. (2010) suggested with an analysis of omasal flow data that the protein evaluation system of NRC (2001), which is based on in situ determination of protein degradability, overestimated the difference in RUP supply. The expected, but often absent production response due to treatment of protein feeds can be speculated to be caused by that the total supply of AA to the small intestine does not increase as predicted and/or that the treatment cause

some change so the protein receive a less optimal AA profile for absorption in the small intestine.

In Paper IV the *in vitro* uCP ranked the diets precisely in terms of the omasal CP flow in kg/d or in g/kg DMI, but the slopes varied between studies. In two studies the slopes were considerable below 1.0, which indicates that the uCP method over-predicted the differences observed in vivo. In two other studies grain was replaced with untreated RSM. With these studies the slope between uCP supply and omasal CP flow was closer to 1.0, and with these studies the observed increase in CP flow to the omasal canal was 89 and 111% of the increase in the uCP supply.

The reason why increasing the concentration of RUP does not increase the protein value has been suggested to be related to reduced microbial protein synthesis and reduced intestinal digestibility (Huhtanen *et al*., 2011). Increasing the proportion of RUP in the diet results in a decreased proportion of RDP. If RDP is fed below the microbial requirements for maximum growth, this will affect microbial synthesis and ruminal digestion (Clark *et al*., 1992). Reynal and Broderick (2005) found that dietary RDP concentration had a positive linear effect on MPY and microbial NAN flow at the omasum, and that maximal microbial protein production occurred at an RDP of 12.3% of DM. In Paper III, supplementation with tRSM showed a tendency to decrease microbial flow to the duodenum and to reduce microbial efficiency, while dietary NAN flow increased. Similarly, in the study by Rinne *et al*. (2015), an increased proportion of rapeseed expeller or soybean expeller decreased the microbial efficiency.

Surprisingly, Ahvenjärvi *et al.* (1999) found no differences in MPY or in omasal flow of dietary NAN and microbial NAN between supplementation with RSM or heat-moisture-treated rapeseed expeller, despite differing effective protein degradability between these feedstuffs (82 and 64 %, respectively, according to Rinne *et al*. (1999)). Moreover, Ahvenjärvi *et al.* (1999) found no differences in either microbial NAN flow at the omasum or microbial efficiency when comparing RDP supplementation from urea with RDP from RSM or heat-moisture-treated rapeseed expeller. Huhtanen *et al*. (2011) suggested that the high concentrations of free AA and peptides in the formic acid-treated silage used by Ahvenjärvi *et al*. (1999) satisfied microbial requirements that a basal diet of maize silage, lucerne silage and maize grains used by Broderick and Reynal (2009) could not.

Broderick and Reynal (2009) formulated four diets to contain the same amount of RDP and RUP by increasing the amount of lignosulphonate-treated SBM and urea at the expense of solvent-extracted SBM, hence decreasing the RDP from true protein. They found that replacing RDP from SBM with RDP from urea increased the excretion of urea-N in urine, and the concentration of

MUN and ruminal NH_3-N. Furthermore, replacing true protein with non-protein N from urea decreased MPY, which those authors attributed to the causal factors of decreased omasal flow of microbial NAN, microbial efficiency and omasal flow of AA (Broderick & Reynal, 2009). A decreasing effect of RUP on microbial protein outflow from the rumen has also been indicated in two meta-analyses (Santos *et al.*, 1998; Ipharraguerre and Clark, 2005). In addition, the German feed protein evaluation system (GfE, 2001) has been shown to give better predictions of MPY than the NRC (2001) approach (Schwab *et al*., 2005). The German model calculates the contribution of microbial MP from metabolisable intake and dietary MP using constant degradability and digestibility values for RUP, while the NRC model estimates the degradability and digestibility of RUP using *in situ* methodology.

The reason why the increased concentration of RUP due to treatment did not increase the protein value could be because of reduced intestinal digestibility. Huhtanen *et al*. (2011) demonstrated that treated RSM has lower true CP digestibility, calculated according to the Lucas test. Furthermore, the lack of increased protein value could be due to the treatment destroying AA (Harstad and Prestløkken, 2000). Tuori (1992) showed that heat-moisture treatment decreases the concentrations of most AA, with lysine and histidine concentration in particular being reduced by over 15%. Furthermore, following an analysis of omasal flow data, Broderick *et al*. (2010) suggested that the NRC (2001) protein evaluation system, which is based on *in situ* determination of protein degradability, overestimates the difference in RUP supply. The expected, but often absent, production response due to treatment of protein feeds can be speculated to be caused by the total supply of AA to the small intestine not increasing as predicted and/or by the treatment causing some change so the protein has a less optimal AA profile for absorption in the small intestine.

5.3 Production response to increased proportion of red clover silage in the forage

Several studies have shown that including red clover silage in the diet of dairy cows has the potential to increase DMI and milk production in comparison with grass silage inclusion (Dewhurst *et al*., 2003b; Moorby *et al*., 2009; Halmemies-Beauchet-Filleau *et al*., 2014). Since red clover silage increases the omasal flow of dietary and total NAN compared with grass silage (Dewhurst *et al*., 2003a; Vanhatalo *et al*., 2009), it could be speculated to have a protein supplement saving effect. Due to the known effect of increased omasal NAN flow with red clover silage in the diet (Vanhatalo *et al*., 2009), the AA supply could be expected to increase and this would increase DMI by prompting the ‘pull effect’.

However, this hypothesis was not verified by Paper II. Moreover, a previous study by Dewhurst *et al*. (2003b) did not indicate that red clover silage inclusion decreased the need for concentrate when aiming to reach the same production level.

In Paper II, there was no protein supplement saving effect with a higher proportion of red clover silage in the diet, although the cows were still responsive to protein supplementation from RSM. However, there were also no production differences between a low or high proportion of red clover silage in the diet, despite the lower total intake of metabolisable energy with diets with a high inclusion of clover. This can indicate either improved efficiency of metabolisable energy utilisation and/or allocation of energy between milk production and body tissues. In Paper II, lower digestibility and poor fermentation quality of one of the red clover silages appeared to have suppressed the positive effects on forage DM intake that are usually seen with red clover silage inclusion.

It is not clear why increased red clover silage inclusion does not reduce the need for protein supplementation in the dairy cow diet. Vanhatalo *et al*. (2009) suggested that the lower concentration of methionine in omasal digesta and in plasma limits milk production with red clover silage-based diets compared with grass silage-based diets. They also suggested that the methionine in red clover silage has decreased bioavailability and was the explanation for the decreased milk protein concentration they observed with red clover silage inclusion in the diet. However, omasal flow of methionine (g/day) was numerically greater with red clover silage diets compared with grass silage diets (Vanhatalo *et al*., 2009). In a companion study (Kuoppala *et al*., 2009), higher concentrations of lignin and iNDF in red clover silage compared with grass silage were reflected in higher intake of iNDF and lower intake of pdNDF with red clover silage-based diets. Despite this, the digestion rate of pdNDF was increased with red clover silage-based diets. In addition, the rumen pool size of NDF and pdNDF was smaller with red clover silage diets, and hence should not limit DMI (Kuoppala *et al*., 2009). Furthermore, delaying the harvest decreased the digestion rate of pdNDF for grass silage-based diets, but increased it for red clover silage-based diets. This difference was reflected in the DMI response, where delayed harvest decreased DMI with grass silage-based diets but increased DMI with red clover silage-based diets. (Kuoppala *et al*., 2009). Similarly, Huhtanen *et al*. (2007) demonstrated that red clover silage had positive effects on DMI when the concentration of digestible organic matter in silage DM was similar.

A review by Steinshamn (2010) confirmed that red clover silage increased DMI and milk production compared with grass silage. This was possibly due to differences in NDF fractions and in the structure of the fibres. In legumes, lignin

is mainly present in the xylem (the plant transport system), while the other parts of the legume (which lack lignin) are almost completely digestible (Wilson and Kennedy, 1996). Grasses differ in structure from legumes since they contain lignin in all plant tissues except the transport system (phloem). This would explain the faster digestion of pdNDF with red clover silage, since although the lignin concentration is higher in red clover it is mainly present in a small part of the plant, rendering only that part undigestible (Steinshamn, 2010).

5.4 Production and environmental impact of increased dietary crude protein concentration

It is well known that increasing dietary CP concentration increases milk production, but also decreases N use efficiency. This was verified in Papers I, II and III. In all three studies, the highest CP level was too high relative to animal requirements. Although there was a statistically significant positive linear effect on MPY with increasing dietary CP concentration, the highest CP concentration caused a numerical decrease in yield within the production studies (Papers I and II).

Papers I and II both showed that it is possible to decrease CH_4 yield (g CH_4/kg DMI) with increasing dietary CP concentration. The main reason for this is probably that ruminal fermentation of protein produces less CH_4 than fermentation of carbohydrates. Bannink *et al*. (2006) and Sveinbjörnsson *et al.* (2006) showed by stoichiometric calculations that fermentation of protein produces 30-50% less CH_4 than fermentation of carbohydrates. Another likely reason is that increased DMI due to increased dietary CP concentration could reduce the CH_4 yield (Yan *et al*., 2000). Paper III provided further explanations for decreased CH_4 yield with increased dietary CP concentration: i) The low rumen degradability of the tRSM reduced the amount of substrate fermented in the rumen; ii) the proportion of propionate in rumen volatile fatty acids increased; and iii) the tendency for decreased efficiency of microbial protein synthesis could indicate a decreased contribution from microbes as a hydrogen sink.

Methane intensity can be expected to be improved (numerical decrease) with optimal protein supplementation, partly due to small decreases in CH_4 yield and partly due to increased milk yield. Therefore, increased dietary CP concentration could have a lowering effect on the environmental impact, due to decreased CH_4 yield. Unfortunately, that gain would be at the expense of increased N losses via manure due to decreased N use efficiency (Broderick, 2003; Papers I-III). The N use efficiency varies within and between production systems. Gerber *et al*. (2014) reported N use efficiency in milk production of between 15 and 35 %,

which is high in comparison with beef production (4-8%; 17-24% in Schiavon *et al.*, 2012), low in comparison with poultry production (25-62%), and quite similar to that in pig production (10-44%). When the N use efficiency for all diets tested in Papers I, II and III was plotted against the dietary CP concentration (Figure 5), there was a clear correlation in all three studies (R^2 = 0.95-0.98). The negative slope in the diagram reflects the negative effect of increased dietary CP concentration on N use efficiency. The slope indicates a decrease in N use efficiency of between 1.3 and 1.7 g per g/kg DM increase in CP. This is similar to the range of 1.2-1.4 g per g/kg DM increase in CP found in a meta-analysis comparing datasets from North American and northern Europe (Huhtanen and Hristov, 2009). In Paper II, the highest N use efficiency was found with the lowest inclusions of red clover silage, *i.e*. the control diet and the diet with low rapeseed expeller supplementation (34 and 33 %, respectively). The lowest N efficiency in Paper I was found with the highest SBM supplementation (23%). The concentration of MUN increased when N use efficiency decreased, and was a good indicator for ranking diets according to their N use efficiency.

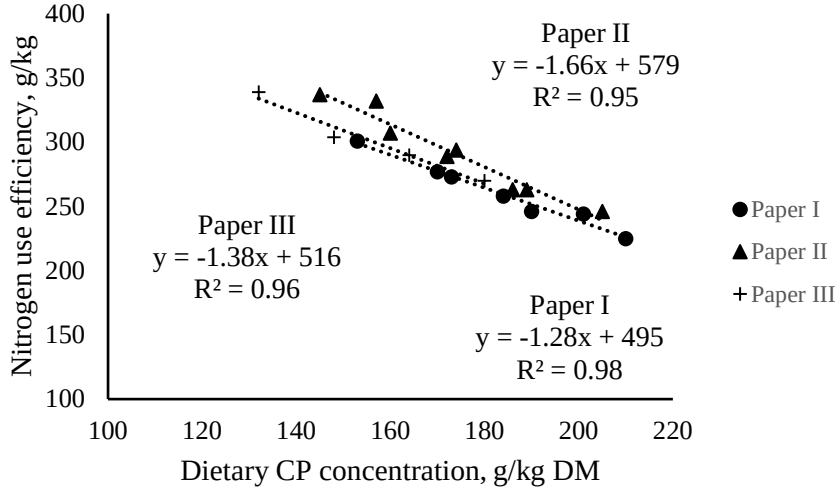

Figure 5. Nitrogen use efficiency (g milk N/kg N intake) as a function of dietary crude protein (CP) concentration (g/kg dry matter) for all diets included in Papers I, II and III.

Each animal production system should aim for the highest efficiency possible within economic restrictions. The advantage of dairy production is that the feed intake of a dairy cow consists of a large proportion of forage, which is non-edible to humans. In Sweden, this forage mainly comes from leys containing grass and clover, which can be grown throughout almost the entire country and could be

considered the driving force of Swedish dairy production. Paper II demonstrated the implications of feeding a high proportion of red clover silage in the diet in terms of decreased N use efficiency. However, another advantage of legumes is the reduced requirement for N fertilisers and, especially in organic systems, lower efficiency in milk production may not be a great relative disadvantage provided that manure is effectively utilised in feed production systems. To improve the N use efficiency within Swedish dairy production even further, protein feed supplementation of the dairy cow diets has to be optimised to complement the forage, with good knowledge of the expected production response. Castillo *et al*. (2001) suggest that manipulating the ratio of degradable protein in the rumen and the energy that is available to the microbes gives the best pre-conditions for the most efficient N use.

5.5 Potential for using *in vitro*-determined utilisable crude protein at the duodenum to predict milk production responses

Most of the current feed protein evaluation systems rely on *in vivo* (microbial MP) and *in situ* (dietary MP) estimations of feed values. These estimations have different problems and contribute with different methodological error and variation. Therefore, estimation of protein feed value using *in vitro* methods would be more suitable for routine analysis. They can overcome some of the incorrect assumptions that are made using the *in situ* method; that soluble protein is completely degraded in the rumen, that small particles that escape from the bag without being degraded are assumed as degraded, and that rumen degradability is under-estimated using the *in situ* method due to microbial contamination of the undegraded protein residues in the bag.

The *in vitro* method developed by Edmunds *et al*. (2012) for estimating uCP supply at the duodenum offers a procedure were measurements of RUP and microbial protein are performed simultaneously. This is the advantage of the method, but could also be a disadvantage since microbial and feed N cannot be separated. Another risk with the *in vitro* method using rumen fluid is that the microbial community might be impaired at the start of incubation.

In the work presented in this thesis the uCP method predicted the ranking of diets well according to the CP flow in the omasum when DMI and diet composition varied within practical ranges. The slope of the relationship between uCP supply and omasal CP flow was close to 1.0 (fixed regression model) or 0.77 (mixed model), indicating that the uCP model estimated quantitative differences reasonably well. Although, there was a negative mean bias (uCP supply > omasal CP flow). This bias is probably due to

methodological inaccuracies influencing the calculation of uCP concentration. Two possible explanations for this can be suggested: (i) immediate uptake of NH_3-N from the incubation medium to the bacterial intracellular pools and/or (ii) that NH_3-N production from the blank constituents (microbes and nutrients in the rumen fluid) starts earlier and is greater without substrate.

A further concern was that the uCP approach tended to overestimate the omasal CP flow with treated protein supplements, but not with untreated. Unfortunately, the treated and untreated samples were not from the same *in vivo* studies.

Milk protein yield was predicted reasonably well when the mixed model was used, as it compares uCP and MPY within the same study. The best model fit was found with inclusion of quadratic uCP. The adjusted residual mean square error was 30.2 g/day in that case, which is slightly higher than the 31.0 g/day found with a quadratic regression model of MPY from omasal CP flow (Huhtanen *et al*., 2010). A slightly larger adjusted residual mean square error for milk protein yield (47 g/d) was reported by Firkins *et al*. (2006) for a model based on DMI and dietary concentrations of RDP and RUP. In addition, prediction of MPY has been found to be better when using a constant value for efficient protein degradability, instead of an empirically determined value (Tuori *et al*., 1998). This questions the accuracy of empirically determined values and indicates the importance of evaluating a method that determines protein value against *in vivo* production responses in order to be relevant.

5.6 Options for supplying protein to dairy cows

In earlier research and in Paper I, there was a clear indication that SBM in the diet of dairy cows can be replaced with RSM without any decrease in production. Based on the assumption that the main oilseed included in feed mixes produced in Sweden (apart from rapeseed) is soybean, then dairy and beef cattle in Sweden were fed about 45 000 tonnes of soybean in 2015, which was about 40% of the total amount of soybean used in feed mixtures for animals in Sweden (Statistics Sweden, 2016). These 45 000 tonnes of soybean in the diet of dairy and beef cattle could in practice be replaced with rapeseed feedstuffs, but it can also be speculated that dairy cows are overfed with protein and that beef cattle can be reared without protein supplements (Huuskonen *et al*., 2013).

Zero ruminal N balance (omasal CP flow = CP intake) has been estimated to occur at a dietary CP concentration of 14.7% of DM and an RDP concentration of 10.6% of DM (Broderick *et al*., 2010). At higher concentrations, N absorption through the rumen wall will exceed the recirculation of N via saliva and back through the rumen wall and the N losses will markedly increase (Broderick *et*

al., 2010). In Sweden, the recommendation is that the average dairy cow diet should contain a CP concentration of about 16-17%. The dietary CP concentration in the diet of dairy cows could possibly be reduced overall, and it should be emphasised that there is no need for concentrate CP supplementation when the basal diet contains a grass silage with low (12%) compared with high (17%) CP concentration (Shingfield *et al*., 2001). Although the economic optimum for protein supplementation is a function of current milk and feed prices, dairy producers should ensure that microbial fermentation and growth are efficient in order to maximise microbial protein yield from the rumen, as this is effectively the least expensive source of protein (Forbes and France, 1993).

In addition to increasing the dietary CP concentration with rapeseed or soybean feedstuffs or increasing the proportion of red clover silage in the diet of dairy cows, there are several other practical strategies through which dairy cows can be supplied with protein to improve milk production. Interest in feeding peas and field beans and production of these crops have increased in the past decade, with the emphasis on the possibility of producing home-grown protein feed for dairy cows on-farm or on a neighbouring farm and of reducing protein feed costs. However, in production studies the results have been disappointing. For example, Puhakka *et al.* (2016) found that supplementation with field beans instead of RSM of diets based on formic acid-treated grass silage reduced silage intake and milk production. Moreover, the plasma concentrations of lysine and methionine decreased with field bean supplementation and this was suggested to be the limiting factor for increased milk production compared with diets supplemented with RSM. Similarly, Ramin *et al.* (2015) found decreased milk production when comparing rapeseed expeller and peas or field beans for supplementation of a grass silage-based diet. The rumen protein degradability of peas and field beans is high, but can be theoretically reduced by heat treatment, although Ramin *et al*. (2015) observed no positive effect of heat treatment of field beans on milk production. Heat treatment of barley, lupin and SBM has been found to decrease rumen *in situ* degradability (Mogensen *et al*., 2008) but, unlike in the study by Ramin *et al.* (2015), this was also reflected in increased production responses. The response to heat treatment of barley and lupin feedstuffs was a numerical increase in production, while treatment of SBM increased milk production significantly, by 1.5 kg ECM (Mogensen *et al*., 2008). Vaga *et al*. (2016) used an *in vitro* method for evaluating the quality of protein feeds by estimating uCP at the duodenum. They found that the concentration of uCP increased only marginally when grass silage and barley were replaced with peas or field beans in order to increase the dietary CP concentration from 130 to 180 g/kg DM, indicating high degradability of these feedstuffs. However, the estimated uCP at the duodenum increased markedly following heat treatment of

peas and field beans. The low concentrations of methionine in legume seeds, especially in field beans (0.6-0.8 g/100 g AA, compared with 1.8-1.9 and 2.5-2.6 g/100 g AA in RSM and milk, respectively, according to LUKE (2017) and NorFor (Volden, 2011) feed tables can become a limiting factor even when ruminal CP degradability is decreased by heat treatment.

The results of feeding legume grains have been disappointing to date. Furthermore, if the production of legume grains increases, this will be at the expense of grain production, since there is little extra land of top quality to take into use in Sweden (Gustafsson *et al*., 2013). However, Rondahl *et al*. (2007) suggested that use of whole-crop peas and oats in a mixture with grass-red clover silage can have a concentrate saving effect. Using whole crops containing mixtures of legumes and grains could provide good possibilities to manipulate the ratio of rumen-degradable protein and fermentable energy in the diet. Furthermore, in some Swedish cropping systems it might be more secure to use whole crops in the crop rotation instead of harvesting legumes as grains, especially if the production of legumes fits within the farm cropping system and is eligible according to the rules of green direct payments system. Under the current rules of green direct payments farmers receiving payments are required *e.g*. to diversify their range of crops and maintain permanent grasslands (Agriculture and Rural Development, European Commission), with the aim to reduce the negative effects of European agricultural industry on the environment and support the biodiversity of the agricultural landscape.

6 Conclusions

The main conclusion to be drawn from the data presented in this thesis is that supplementation of grass silage-based diets fed to lactating dairy cows with rapeseed meal can be considered better than supplementation with soybean meal. Hence, in theory, Swedish dairy cows could maintain, and possibly even increase, their milk yield when fed only domestic rapeseed meal as their protein feed. Today this is not feasible due to the high amounts of protein feed required in intensive dairy production systems and due to the fact that production of domestic rapeseed is a function of available land, crop rotation and price of rapeseed versus other crops.

Red clover in forage leys and in the diet of dairy cows has great potential as a sustainable protein feed due to its nitrogen-fixing ability. Unfortunately, the theory that a higher proportion of red clover silage in the diet can reduce the need for protein supplementation was not supported by the work of this thesis. Further research is required to understand why, and how to optimise the maturity of red clover and the total ration formulation to improve the protein value of red clover silage fed to lactating dairy cows.

Increased dietary CP concentration seemed to decrease enteric CH_4 yield and intensity in dairy cows, but mainly as an effect from increased DMI and milk yield. Thus increased feeding of protein decreases CH_4 emissions from dairy production, but must be considered a poor method for this purpose due to the associated effects of decreased efficiency of conversion of feed N into milk protein. This thesis also showed that N use efficiency is decreased on increasing the dietary CP concentration both with concentrates and forages, with the greatest feed N use efficiency being achieved with the lowest dietary CP concentration studied.

Replacing crimped barley with solvent-extracted, heat-moisture-treated rapeseed meal to increase the dietary crude protein concentration increased omasal flow of NAN, but simultaneously tended to decrease the flow of microbial NAN and microbial efficiency. Furthermore, the calculated proportion

of milk protein originating from rumen microbial protein decreased. Together with the decreased N use efficiency, this reflects a decrease in conversion of feed N into milk protein. Optimised use of feed N requires high rumen microbial efficiency and a supply of RUP with an AA profile suited for milk protein synthesis. Hence, in terms of optimum N use efficiency, feeding heat-treated protein feeds should be studied in more detail. This thesis confirmed suggestions that calculated microbial MP and RUP may not be additive when ruminal protein degradability is reduced by heat treatment.

The *in vitro* method of Edmunds *et al*. (2012) demonstrated good potential in predicting omasal CP flow and MPY from estimated uCP supply at the duodenum. Using this type of method to evaluate feed protein value may prove to be quicker, simpler and more accurate than *in situ* methods. Both omasal CP flow and MPY were positively related to the estimated supply of uCP at the duodenum. Furthermore, there was a strong correlation between estimated uCP concentrations (g/kg DM) at 16, 20 and 24 hours of incubation, but the values at 16 hours resulted in the best mixed model fit.

7 Future perspectives

The reason why a higher proportion of red clover silage in the diet increases the omasal flow of NAN, in a similar way to a protein supplement, but does not give the expected production response needs further investigation. This is particularly important because red clover is such a useful crop in the ley, with its N fixing ability, which can decrease the need for artificial fertiliser. Red clover has great potential to become an environmentally sustainable protein feed in dairy production if the limiting factors could be identified and diets optimised accordingly. Identifying how to manipulate the carbohydrates in the diet and the AA profile that reaches the duodenum could possibly increase the conversion of red clover N into milk protein.

Domestic field beans and peas have disappointing effects on milk production. These crops are legumes, fixing N from the air, and can decrease the need for fertilisers. However, they are more expensive to grow than cereals, give lower yield per unit area and yield can vary substantially from year to year. Ongoing work within plant breeding is improving these factors, but to be efficiently used in dairy production more research is needed on how to feed these alternative protein feeds, possibly by addition of rumen-protected AA.

Heat treatment of protein feeds to increase the protein value is common. However, care should be taken when heat-treating feeds as studies have shown that the chemical structure of different protein feeds can undergo different changes following a particular treatment. When heat-treating protein feeds, the purpose of the treatment must be considered. In theory, it should decrease rumen degradability and increase the amount of dietary AA absorbed in the small intestine and later used for increased milk protein yield. Additional research is needed to define the need for heat treatment of alternative protein feeds and further standardise the procedures used, as treatment often takes place directly on-farm.

Optimising protein feeding by using better protein evaluation systems is constantly in need of improvement. Improvements could be sought to increase

farm income and to optimise the use of nutrients, preferably simultaneously. Hence, in future research less focus should be on maximising milk yield and more on developing feed evaluation systems taking the whole farm (feeding, production, crop rotation, manure handling *etc*.) into the calculations.

The ability to screen diets quickly with correct evaluation of the milk protein production response could be achieved with the *in vitro* method for estimating uCP at the duodenum. However, more work is required to optimise the prediction of milk production using this method. Factors such as different feeds and diet formulations and animal traits such as DMI and production levels must be taken into consideration. In addition, it would be highly interesting to further investigate the role of the blank and its possible effect in creating a methodological error in calculations of uCP supply. In the near future, the method of estimating uCP at the duodenum should be analysed on a routine basis in production trials.

Popular science summary

The interest in using domestic protein feeds in Swedish milk production has increased. It is hoped that imports of protein feeds can be reduced, to the benefit of production in Sweden, partly because imported protein feeds are often produced under less strict regulations than in Sweden, particularly regarding working conditions and environmental impact. These factors are among those consumers are assumed to consider when purchasing dairy products. In addition, it would reduce the Swedish dairy industry's sensitivity to fluctuations in world market prices of imported feeds. There is also an opportunity for the individual dairy farm to save money by growing its own protein feed instead of importing it.

This thesis showed that completely replacing soybean meal in the dairy cow ration with rapeseed meal is practically possible and can even increase milk production. In other studies, feeding peas and field beans has unfortunately not produced the same positive milk response, while the theory that increasing the amount of red clover in the diet could reduce the need for protein feed was not confirmed in this thesis. Increasing the proportion of red clover silage in the diet gave similar milk yield, but decreased nitrogen use efficiency, which caused greater losses of nitrogen to the environment via manure and, in particular, urine. More research is needed to determine how the additional nitrogen in legumes can be utilised more effectively by dairy cows, as the use of legumes in pastures and crop rotations is important in fixing nitrogen from the air. This reduces the dependency on purchased nitrogen fertilisers which, like imported feeds, suffer from fluctuating world market prices.

Heat treatment of protein feeds to improve the protein value and increase milk yield appears to be an increasingly questionable practice. The heat treatment is likely to destroy essential amino acids and reduce digestibility in the rumen. It is known that the composition of amino acids in microbes present in the rumen, rather than in different types of feed protein, is optimal for the synthesis of milk protein. Lower rumen degradability of feed reduces

opportunities for microbes in the rumen to assimilate protein for growth and a reduction in microbial growth reduces the availability of the most optimal and economic protein. Furthermore, if the amount of amino acids originating from microbial protein is reduced, much higher demands are placed on the amino acid composition of the rumen undegradable feed protein reaching the small intestine, in order to sustain milk production.

Using methods based on analyses quickly performed in the laboratory for evaluating complete diets, and the amount of milk produced by dairy cows fed these diets, would be much better than performing expensive and time-consuming feeding trials in research facilities with lactating cows. Laboratory-based methods would make it possible to test more diets and get results more rapidly. This would contribute to faster progress in feed evaluation systems, which are important tools used by farmers for estimating what they should feed their cows for the best milk yield and economic outcome. The method tested in this thesis for measuring the utilisable crude protein supply at the duodenum shows great promise, but further research is required to verify it with different kinds of feed and cows with different production levels.

Populärvetenskaplig sammanfattning

Intresset för att använda inhemska proteinfodermedel inom svensk mjölkproduktion har ökat. Förhoppningen är att importen av proteinfoder kan minskas till fördel för produktion i Sverige. Dels för att proteinfodermedel som importerats ofta har producerats under mindre strikta regelverk än svenska, bland annat vad gäller arbetssituation och miljöpåverkan. Dessa faktorer är något som konsumenter anses basera sina köp av mejeriprodukter på. Dessutom är förhoppningen att det ska minska industrins känslighet för de fluktuerande världsmarknadspriser som de importerade fodermedlen innefattar. Att det finns en möjlighet för den enskilda mjölkgården att spara pengar på att odla eget proteinfoder istället för att importera det.

Att helt byta ut sojamjölet i mjölkkons foderstat mot rapsmjöl har inte bara visat sig fungera lika bra, utan det har till och med ökat mjölkproduktionen. I andra studier har utfodring med ärtor och åkerböna tyvärr inte visat samma positiva produktionsrespons och teorin att ökad mängd rödklöver i foderstaten skulle minska behovet av proteinfoder stärks inte av den här avhandlingen. Ökad proportion rödklöverensilage i foderstaten gav samma mjölkavkastning men minskad N effektivitet vilket orsakar större förluster av kväve till miljön via gödsel och främst urin. Mer forskning behövs för att utvärdera hur den stora extra mängden kväve i baljväxter ska kunna utnyttjas av kon på ett effektivt sätt eftersom användningen av baljväxter i vall och växtföljden är ett så viktigt inslag för att fixera kväve från luften. Detta flyttar beroendet från inköp av kväve via mineralgödsel, vilket liksom importerade fodermedel har varierande världsmarknadspriser.

Att förbättra proteinvärdet och öka mjölkavkastningen genom att värmebehandla proteinfodermedel är en metod som framträder alltmer tvivelaktig. Värmebehandlingen riskerar att förstöra essentiella aminosyror och minska smältbarheten i vålmmen. Vi vet att sammansättningen av aminosyror i mikroberna som finns i vålmmen är den mest optimala för syntes av mjölkprotein, jämfört med alla foderprotein. En lägre smältbarhet av protein i

vånmen minskar möjligheterna för mikroberna att tillgodogöra sig protein för tillväxt och en minskad mikrobtillväxt minskar tillgången på det mest optimala och ekonomiska proteinet. Dessutom, om mängden aminosyror från mikrobprotein minskar ställs mycket högre krav på aminosyrasammansättningen i det osmälta foderproteinet som når tunntarmen.

Att kunna utvärdera hela foderstater och hur mycket mjölk korna skulle avkasta från dessa genom analyser gjorda på laboratorier skulle vara optimalt. Istället för att utföra mycket dyra och tidskrävande utfodringsförsök i en försöksladugård med mjölkande kor. Det skulle göra det möjligt att testa fler foderstater och få resultat snabbare, vilket skulle bidra med snabbare framsteg i de foderutvärderingssystem som uppskattar vad bönderna bör fodra sina kor med för bästa avkastning och ekonomiska resultat. På metoden som mäter användbart protein i tunntarmen (utilizable crude protein at the duodenum) finns det stora förväntningar. Men ytterligare forskning krävs för att verifiera metoden med olika sorters foderstater och kor med varierande mjölkavkastning.

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