Final Report SLU EkoForsk Research Program

Optimization of protein supplementation in organic milk production

Pekka Huhtanen, Helena Gidlund and Mårten Hetta, Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences, Umeå

FOREWORD

This document is the final report of the project "Optimization of protein supplementation in organic milk production" financed by SLU EkoForsk research program during. After approved changes in the project plan two production studies with lactating dairy cows were conducted along with developments of in vitro techniques to evaluate feed protein values. We would like to thank everyone included in this project which made it possible, from financiers to laboratory personnel to barn staff. Also, a special thanks to our colleagues at the Natural Resources Institute Finland (Luke; former MTT Agrifood Research Finland) for sharing feed samples from several of their in vivo studies making our in vitro study possible.

Primary Silage vs. Regrowth Silage Production Study

The study was presented at Mjölkföretagardagarna in Umeå, 2014. A paper is being prepared for international publication during 2016-2017.

Domestic Protein Production Study

The study was presented at Jordbruksverkets FoU dag, 2014, and was also presented as a poster at the Nordic Feed Science Conference in Uppsala, 2015. A paper is being prepared for international publication during 2016.

Study of In Vitro Feed Protein Value

A paper is being prepared for international publication during 2017.

SAMMANFATTNING
Inom ekologisk mjölkproduktion är utfodring av protein en sorts flaskhals. Det är av stor vikt att utfodra grovfoder med utmärkt proteinvärde och kraftfoder som kompletterar grovfodret till så låg kostnad som möjligt. Inom ekologisk foderproduktion är det centralt att använda sig av baljväxter på grund av dessa växters förmåga att fixera kväve från atmosfären. Rödklöver är den vitt använda baljväxten i grovfoderproduktion och ärt och åkerböna är alternativ som kraftfoderkomplement.

Det här projektet siktar på att bestämma produktionsvärden och responsfunktioner för olika proteinkomplement inom ekologisk mjölkproduktion. Foderstaterna innehåller olika grovfoder och mängder av rödklöver, liksom olika tillskottsfoder vid ökande nivåer. Tillskottsfodren är alla möjliga att producera i Sverige.

Två produktionsstudier med mjölkande kor utfördes vid Röbäcksdalens forskningsstation i Umeå. Studierna visade ingen effekt på produktionsresultaten förutom lite lägre koncentration av mjölkprotein med ökande nivåer av rödklöver i foderstaten till mjölkkor. Dessutom fanns inga skillnader i produktionsresponser mellan utfodring av ärt, behandlad och obehandlad åkerböna eller rapsexpeller. Även om rapsexpeller ökade mjölkmängd och mjölkproteinkoncentrationen, så ökade det inte mängden energikorrigerad mjölk.

En in vitro studie undersökte sambandet mellan flödet av icke-ammoniakkväve vid bladmagen för 34 foderstater med koncentrationen av användbart råprotein (uCP) betämt in vitro från samma foderstater. Data från in vivo-studierna var insamlade from tidigare arbeten av vårt forskningsteam och från våra kollega forskningsteam vid Natural Resources Institute Finland (Luke; tidigare MTT Agrifood Research Finland). Foderstaterna var grovfoderbaserade och kompletterade med kraftfoder som kan produceras i Sverige. Genom att studera sambandet mellan produktionsresultaten från in vivo-studierna med uCP-koncentrationerna från in vitro-studien fann vi in vitro-metoden lovande för att utvärdera proteinvärdet av foderstater. Skattningsfelet för råprotein-flödet var ungefär 5 % av medel-råprotein-flödet när slumpmässig försökseffekt inte inkluderades och ungefär 10 % med fix-modell regressionsanalys. uCP skattade mängden mjölkproten mer exakt än råproteinflödet vid bladmagen. Det kan bli aktuellt i framtiden att använda uCP-metoden för att screena proteinvärden av foderstater och selektera de mest lovande alternativen för produktionsförsök.

SUMMARY
Within organic dairy production protein feeding is a kind of bottleneck. It is of great importance to feed forage with excellent protein value and concentrates that complements the forage to as low cost as possible. In organic feed production the use of legumes is crucial due to those plants ability to fixate nitrogen from the atmosphere. Red clover is the widely used legume in forage production, and pea and field beans are alternatives as concentrate supplements.

This project is aiming at determining production values and response functions to different protein supplements in organic dairy production. The different feeds are including different forages and levels of red clover inclusion, as well as supplementary feeds at increasing levels. The supplementary feeds are all possible to produce in Sweden.

Two production studies with lactating dairy cows were performed at Röbäcksdalen research station in Umeå. The studies showed that increasing level of red clover in the ration to dairy cows had no effect on production results except for a bit lower milk protein concentration. Also, there was no difference in production responses between feeding peas, treated and untreated field beans or rapeseed expeller. Although rapeseed expeller increased milk yield and milk protein concentration, it did not increase the yield of energy corrected milk compared to the other supplements.

An in vitro study investigated the relationship between in vivo non-ammonia N flow at the omasum for 34 diets with in vitro determined utilizable crude protein (uCP) concentration from the same diets. The data from in vivo studies were gathered from earlier work by our research team and from colleague research teams at Natural Resources Institute Finland (Luke; former MTT Agrifood Research Finland). The diets were forage based and supplemented with concentrates that can be produced in Sweden. Studying the relationship between the in vivo production results with the in vitro uCP concentrations we found the in vitro method to be promising for evaluating the protein value of diets. Prediction error of CP flow was about 5% of mean CP flow when random study effect was removed and about 10% with fixed model regression analysis. uCP predicted milk protein yield more precisely than omasal CP flow. It could make possible in the future to use uCP method for screening protein value of the diets and select the most promising alternatives for production experiments.

BACKGROUND

Livestock production has a major impact on the environment. The choice of livestock product in a diet can mitigate environmental impact (de Vries & de Boer, 2010). With the growing global population it is important to utilize local resources for human food production. In the northern latitudes forage-based milk production is one of the most efficient and sustainable systems to produce high quality human food from local natural resources. The large project involving organic milk production system at Öjebyn research farm in Northern Sweden demonstrated that inputs can be reduced while maintaining a high milk yield per cow and per ha. Conventional system (high input) and organic system (low input) were compared in a 12 year whole farm including 2 full crop rotations (e.g. Fagerberg et al., 1996).

In 2014 the value of organic food sold within Swedish commerce had a value of 12757 million SEK that was 45% higher than in 2013 (8802 million SEK) (SCB, 2014). In 2013 the market of organic food comprised 4.3% of the total food market in Sweden and had increased with 0.4% compared with 2012 (SCB, 2014). The same year organically produced milk, cheese and egg comprised 6.7% of the total milk, cheese and egg market. The consumer trend today is towards more natural products and a higher fat content in milk products is no longer a hinder to many consumers (KRAV, 2010). Nutritional quality is becoming increasingly important in food choices because of consumer awareness of the links between diet and health. Recent discoveries have identified a number of "bioactive" components in milk with many potential positive health effects (Bauman et al., 2006).

Protein feeding is somewhat a bottleneck in organic dairy production. Production of organic protein feeds such as rapeseed is difficult and feed prices are high. Legumes such as pea (Pisum sativum) and field bean (Vicia faba) are other alternative protein sources. They have two advantages: they can be locally produced and as leguminous plants they can fix atmospheric N. Ruminal crude protein (CP) degradability of pea protein is high (Hedqvist and Udén, 2006) that may result in a low efficiency of N utilization for milk protection. Omasal non-ammonia N (NAN) and feed N flow were less with pea compared with rapeseed meal containing diets in dairy cows (Ahvenjärvi et al., 2005, unpublished report). Compared to control diet without protein supplementation NAN flow per kg DM intake did not increase indicating that incremental protein from peas was lost as ammonia from the rumen. On the other hand, ruminal protein degradability of red clover silages is lower compared with grass silages as a result of polyphenoloxidase system (Vanhatalo et al., 2009). Therefore it is possible that protein characteristics of red clover and pea protein balance each other improving overall N efficiency. Pea has a high digestibility and energy concentration (high starch, low indigestible NDF) and is therefore an ideal substrate for microbial protein synthesis in the rumen. Recent meta-analysis demonstrated that the contribution of microbial protein to the total protein supply from the small intestine is greater and undegraded feed protein less than the current protein evaluation systems predict (Broderick et al. 2010). The analysis also indicated that rumen protein balances was obtained when dietary CP concentration was 14% and milk urea concentration 17 mg/100 ml, above which levels a large proportion of protein is lost as ammonia N that is converted to urea and excreted in urine. The effect of degradability on milk protein yield responses seemed to be overvalued based on

meta-analysis of about 1800 diets in milk production trials (Huhtanen and Hristov, 2009). Their analysis indicated that energy intake was the main factor influencing milk protein yield.

Protein feeding strategies have a great impact on sustainability of organic dairy production. Dietary protein concentration should be high enough to assure that feed intake, diet digestibility, and consequently energy supply and milk yield are not significantly compromised. Recent analysis of literature data (Huhtanen et al., 2008; Nousiainen et al., 2009) demonstrated that supplementary protein both increased forage intake and improved diet digestibility. Because of the high prices of organically grown protein feeds it is crucial to feed economically optimal level of protein, not maximizing yield.

We hypothesize that in organic milk production protein requirements can be met by locally produced feeds. Secondly, that the requirements of supplementary protein can be reduced by optimizing microbial protein synthesis with high quality forages and energy supplements. Thirdly, the economic optimum of protein feeding in organic milk production is below biological optimum.

The objectives are to determine productive values of different protein supplements in organic milk production (1), determine response functions to supplementary protein that allows optimizing the economy (2), and determine interactions between forage quality and protein (3) and to evaluate if the protein responses are related to production level of cows.

MATERIAL AND METHODS

Primary growth vs. Regrowth Silage Production Study

Design, Animals, and Experimental Feeds

Over three periods containing 21 days 32 lactating Swedish Red cows were fed 8 different diets (Table 1.) as a cyclic change-over design (Davis and Hall, 1969). The diets were fed as ad libitum TMR and consisted of 60% forage and 40% concentrate. The forage consisted of three different silages, a second cut grass silage, and two red clover silages (1st and 2nd cut, mixed together) (respectively, 271, 254, 235 g DM/kg, 10.9, 10.3, 9.1 MJ/kg DM, 129, 214, 185 g CP/kg DM, 513, 376, 444 g NDF/kg DM) . In half of the diets the forage part was made up of 70% grass silage and in the others it was made up of 70% red clover silage. The concentrate consisted of a mineral premix, and crimped barley was exchanged for rapeseed expeller (Farmarin Öpex, Suomen Rehu, Finland) to reach four increasing levels of crude protein (CP) in the concentrate rations. The control diet had no inclusion of rapeseed expeller. The rapeseed expeller process is allowed in organic milk production.

Table 1. Diet composition (g/kg DM), and CP concentration in the total diet

	Diets[1]							
	Grass Ctrl	Grass Low	Grass Medium	Grass High	Clover Ctrl	Clover Low	Clover Medium	Clover High
Grass silage	420	420	420	420	180	180	180	180
Clover silage	180	180	180	180	420	420	420	420
Crimped barley	300	230	160	90	300	230	160	90
Premix	100	100	100	100	100	100	100	100
Rapeseed expeller	0	70	140	210	0	70	140	210
Crude protein, g/kg DM	145	159	175	192	160	176	191	208

Diets[1]: GB=grass-based silage with only crimped barley, and no rapeseed expeller, GL = grass-based silage with low level of rapeseed expeller, GM = grass-based silage with medium level of rapeseed expeller, GH = grass-based silage with high level of rapeseed expeller, CB = clover-based silage with only crimped barley, and no rapeseed expeller, CL = clover-based silage with low level of rapeseed expeller, CM = clover-based silage with medium level of rapeseed expeller, CH = clover-based silage with high level of rapeseed expeller.

Measurements and Samplings

Feed intake was registered daily (Insentec B. V., Marknesse, Nederländerna). The cows were milked at 06.00 and 15.00 and milk yield registered accordingly. Test milking was performed during four consecutive milkings during the last week in each experimental period. The milk was analyzed for fat, protein, lactose, and urea. During three mornings the body weight of each cow was registered. Measurements of methane (CH4) and carbon dioxide (CO2) from the cows were done using a portable open-circuit head chamber system (GreenFeed system, C-Lock Inc., Rapid City, SD). The animals visit the system several times per day since they are given small amounts of concentrate at each visit. Details of the system and calculations are reported in Huhtanen et al. (2015). This method comparison was conducted with the cows in the present study.

Statistical analysis

The data were analyzed with the MIXED procedure of SAS (SAS Inc. 2002-2003, release 9.3; SAS Inst., Inc., Cary, NC). The statistical model included fixed effects of period, treatment and random effect of cow. For treatment comparison the following contrasts were used: comparison of grass silage and red clover silage, linear and quadratic effects of dietary CP concentration and interaction between forage type and dietary CP concentrations.

Domestic Protein Production Study

Design, Animals, and Experimental Feeds

In a cyclic change-over design (Davis and Hall, 1969) 24 lactating Swedish Red cows were fed 6 different diets over three periods containing 21 days each. The diets were fed as TMR in ad libitum and the ratio of forage and concentrate of 60:40. The forage consisted of a second cut grass silage (270 g DM/kg feed, 10.9 MJ/kg DM, 181 g CP/kg DM, 494 g NDF/kg DM). The concentrate ration consisted of crimped barley that was replaced with peas, untreated field beans or treated field beans to reach similar CP concentrations in all diet except the control diet (Table 2.).

Table 2. Ration ingredients (g/kg DM), and CP concentration in the total diet

	Diet[1]					
	C	RSE	PEA	UFB	TFB	TFB-MP
Grass silage	600	600	600	600	600	600
Barley	400	296	168	260	260	320
Pea	0	0	232	0	0	0
Rapeseed	0	104	0	0	0	0
Field bean not-heat treated	0	0	0	140	0	0
Field bean heat treated	0	0	0	0	140	80
Crude protein, g/kg DM	159	187	187	181	183	176

[1]C = control, RSE = rapeseed expeller, UFB = field bean not heat-treated, TFB = field bean heat-treated, TFB-MP = field bean heat-treated, same MP as UFB.

The heat-treatment procedure for the FB was done with a farm based roasting equipment (R-100E, Roastech, Blomfontein, South Africa). The machine was electrical heated using 380V AC. The machine was basically a drum with holes of 3 mm. The procedure was conducted at a temperature of 140 °C and a turning frequency of 50 Hz which gave a passage time at about 7.5-8 minutes. At the outlet of the drum a cooling container is mounted on the machine to allow air-cooling of the roasted product. The outlet was placed directly to bags so the heat treated FB could be collected and processed further.

Measurements and Samplings

Experimental procedures were similar as for the first study.

Statistical Analysis

The data were analyzed with the MIXED procedure of Statistical Analysis Systems (SAS for Windows, version 9.3, SAS Institute, Cary, NC). The statistical model included fixed effects of period, treatment and random effect of cow. For treatment comparison the following

contrasts were used: comparison of control and others, comparison of rapeseed expeller vs. others excluding the control, untreated field beans vs. peas, untreated field beans vs. treated field beans-MP, and untreated field beans vs. treated field beans.

Study of In Vitro Feed Protein Value

Experimental Design and Feed Samples

The in vitro experiment evaluated incubation of 34 different diet samples. The feed samples came from 8 different in vivo omasal flow studies. The diets were mixed from dietary ingredients in same proportions as were used in animal studies. Within and between 4 in vitro runs the feed samples were randomly distributed, along with 2 blank samples. The feed samples consisted of 0.500 g mixed silage and concentrate with CP concentrations ranging between from 13 to 20%. The silages were grass silage, red clover silage, and mixes of grass and red clover silages. Concentrate feeds were represented by different mixes of dry and crimpled barley, oats, molassed sugar beet pulp, rapeseed meal or expeller, field bean, dry or ensiled pea, blue lupine, soybean expeller, and mineral and vitamin supplements.

In Vitro Procedure

Rumen fluid was collected and filtered through 2 layers of cheese-cloth into pre-warmed thermos flasks flushed with CO_2. The collection was performed after the morning milking resulting in an at least 1 hour withholding of feed. In the laboratory pH was recorded for the fluid from each cow and 0.4 ml was sampled into 0.016 ml H_2SO_4 in Eppendorf tubes and kept frozen for later NH_3-N analysis. A mix of rumen fluid from all cows was further filtered through 4 layers of cheese-cloth during a constant flush of CO_2 adding up to 800-ml in a flask kept in 39ºC water-bath. The filtered rumen fluid was incubated with a carbohydrate mixture (1.6 g pectin dissolved in 100 ml of buffer (Menke & Steingass, 1988; 20:80 v/v) by constant stirring at 39ºC, 3.2 g maltose, 1.6 g starch, and 1.6 g xylose). The mixed fluid was stirred for a few minutes, 0.4 ml was sampled as described for rumen fluid, pH was measured, and then the stirrer was turned off and the fluid left in the water-bath for 30 min. After 30 min the top layer of feed particles was removed with a vacuum pump (15-20% of volume). Then the stirrer was turned on again and the fluid left to incubate with constant CO_2 stream for 2.5 h. Every hour during pre-incubation pH was measured and 0.4 ml of fluid was sampled as described for rumen fluid. After the pre-incubation the filtered rumen fluid was mixed with a buffered mineral solution (Menke & Steingass, 1988; 20:80 v/v) during constant stirring and flushing with CO_2. The buffer solution was modified to 38 g $NaHCO_3$, 1 g ($(NH_4)HCO_3$) with addition of distilled water up to1000-ml. The modification was done because we wanted to decrease the addition of ammonia to the system via the buffer. Buffered mineral solution was sampled as well as buffered rumen fluid in the same manner as described for rumen fluid. Previously, 0.500 g of substrate was weighed into 250-ml screw-cap bottles (Schott, Mainz, Germany). Buffered rumen fluid (60 ml) was added to each bottle placed in water baths (39ºC) with constant agitation for 30 h. The incubation was repeated in 4 consecutive runs.

Ammonia- N and Methane Measurements

Measurements of ammonia N (NH_3-N) were made at 0.5, 4, 8, 12, 24, and 30 hours after the first bottle was placed in the water bath. Sampling of liquid phase from the bottles was enabled due to the tubing that was connected to the bottles (Karlsson et al., 2009). A 0.4 ml sample from each bottle was taken with a syringe. Immediately after samling, the syring was filled with air and the liquid left in the tubing was pressed down back in the bottle and the incubation environment. Hence, no discarding of liquid needed and as little loss of liquid form the bottle due to sampling as possible was obtained. The liquid samples was transferred to test tubes filled with 0.016 ml of H2SO4 (96 %) for preservation, and kept on ice to stop further fermentation processes. The samples was kept frozen (-20ºC) until analysis. When time for analysis the samples were thawed to room temperature in lukewarm water. They were thawed in a sequence to keep them unfrozen for as short time as possible. When thawed the samples were centrifuged at 12500 RPM for 7 min. Supernatant (0.15 ml) was pipetted to vials containing MilliQ water (0.85 ml). Ammonia-N was analyzed with a continuous flow analyzer (AutoAnalyzer 3 HR, SEAL Analytical Ldt) following the instructions provided. Calculations of utilizable CP (uCP) at each time point were made according to Edmunds et al. (2012):

$$\text{uCP (g/kg DM)} = \frac{\text{NH3Nblank(mg)} + \text{Nsample(mg)} - \text{NH3Nsample(mg)}}{\text{weight (mg DM)}} \text{x } 6.25 \text{ x } 1000 \text{ (eqn. 1)},$$

where NH3Nblank is the average amount (mg) NH3N in the two blanks at the time of interest, Nsample is the amount (mg) of N in the sample at the start of the incubation and NH3Nsample is amount (mg) of NH3N in the incubation bottles at the time of interest for the treatment evaluated. Regression equation of natural logarithmic transformed uCP on time were developed for descending uCP concentrations (8, 24 and 30 h). uCP was then calculated for different (12, 16, 20 and 24 h) rumen retention times (RT) from intercept and slope of logarithmic equation:

$$\text{uCP (g/kg DM)} = \text{Exp (Intercept} + \text{Slope} \times \text{RT)} \quad \text{(eqn. 2)}$$

The total supply of uCP was calculated as:

$$\text{uCP supply (kg/d)} = \text{DM intake (kg/d)} \times \text{uCP (g/kg DM} \times 0.001 \text{ kg/g} \quad \text{(eqn. 3)}$$

Gas measurements was performed according to Ramin and Huhtanen (2012) using a fully automated gas in vitro system (Cone et al., 1996). Recording of gas production was done every 12 min and the gas production was corrected to normal air pressure (1013.5 h Pa). Gas (0.2 ml) was collected from the headspace of each bottle at the same timepoints as for NH_3-N measurements, using a gas tight syringe (Hamilton, Bonaduz, Switzerland). The sample was immediately analyzed for CH_4 concentration by gas chromatography (Varian Chromatography, USA).

Statistical Analysis

The data were analyzed with the MIXED procedure of SAS (SAS Inc. 2002-2003, release 9.3; SAS Inst., Inc., Cary, NC). The statistical model included fixed effects of diet, and random effect of bottle and run. Relationships between uCP and in vivo data (NAN flow, milk protein yield) were evaluated by mixed model regression analysis with random study effects. This approach removes random study effect and allows examining relationships within a study. Root means square errors (RMSE) and R^2 values were adjusted for random study effect.

RESULTS

Primary Silage vs. Regrowth Silage Production Study

The chemical composition of the experimetal feeds can be found in table 3. Increased dietary CP concentration increased dry matter intake (DMI) and milk yield even though the increase seemed to decline with a CP concentration corresponding to the medium-diets (Table 4.). Diets with higher CP level increased the milk protein yield and decreased the milk fat yield. Increased dietary CP level also increased the milk urea concentration and decreased the nitrogen efficiency.

Very few differences in production responses were found between high proportions of grass or red clover silages in the diets. Increased red clover proportion decreased milk protein concentration. The higher total dietary CP concentration with higher red clover proportion explains the increased milk urea concentration and decreased nitrogen efficiency for the same diets, compared to diets containing high grass silage proportion. There was no difference in methane production between diets with high grass or red clover proportion.

Table 3. Dietary chemical composition of the experimental feeds (g/kg DM unless stated otherwise)

	Grass silage	Clover silage, 1st cut	Clover silage, 2nd cut	Barley	Premix	Rapeseed expeller
DM, g/kg feed	271	254	235	598	839	842
CP	129	214	185	125	100	364
Neutral detergent fiber	513	376	444	168	228	340
iNDF[2]	91	106	155	65	92	140
Crude fat[3]	20	20	20	24	66	94
ME[4]	10.9	10.3	9.1	13.2	10.4	13.8

[1] Metabolizable protein calculated according to Spörndly (2003).
[2] Indigestible neutral detergent fiber.
[3] Crude fat for the silages are tabulated values (Spörndly, 2003), for concentrate ingredients crude fat was analyzed directly.

[4] Metabolizable energy concentrations of the silages calculated according to Lindgren (1979), calculations for the concentrate according to Spörndly (2003).

Table 4. Effects (n=12) of forage source and dietary CP concentration on dry matter and nutrient intake (kg/d unless stated otherwise)

	Diets[1]									Contrasts[2]				
	GB	GL	GM	GH	CB	CL	CM	CH	SEM	G v. C	Lin	Quad	F × Lin	F × Q
Intake														
DMI	17.4	18.6	19.5	19.5	18.0	18.7	19.7	19.2	0.57	0.69	< 0.01	0.10	0.38	0.95
CP	2.5	2.9	3.4	3.7	2.9	3.3	3.7	4.0	0.11	< 0.01	< 0.01	0.24	0.81	0.89
NDF[3]	6.5	7.1	7.7	7.9	6.2	6.7	7.2	7.3	0.23	< 0.01	< 0.01	0.18	0.36	0.92
pdNDF[4]	4.9	5.3	5.7	5.9	4.5	4.7	5.1	5.1	0.17	< 0.01	< 0.01	0.23	0.24	0.84
ME[5], MJ/d	199	213	224	226	200	207	220	214	6.4	0.14	< 0.01	0.12	0.31	0.92
Milk Yield														
Milk, kg/d	27.3	29.5	30.5	29.6	27.0	29.2	30.1	29.5	0.88	0.56	< 0.01	0.01	0.93	
ECM[6], kg/d	28.2	30.5	31.0	29.9	28.9	29.8	30.0	30.8	1.06	0.96	0.05	0.21	0.94	
Protein, g/d	918	1016	1037	1012	901	989	1019	993	31.1	0.19	< 0.01	< 0.01	1.00	
Fat, g/d	1152	1225	1248	1185	1216	1188	1189	1289	56.0	0.60	0.27	0.96	0.77	
Milk Concentration														
Protein, %	3.40	3.47	3.44	3.44	3.38	3.43	3.40	3.36	0.052	< 0.01	0.95	0.04	0.26	0.63
Fat, %	4.24	4.28	4.19	4.03	4.38	4.15	4.01	4.25	0.131	0.86	0.05	0.39	0.75	0.02
Urea-N, mg/dL	8.2	9.9	10.9	10.7	10.0	11.2	12.1	12.1	0.68	< 0.01	< 0.01	0.07	0.70	0.70
Urea, mmol/L	2.93	3.52	3.89	3.82	3.57	4.01	4.33	4.31	0.242	< 0.01	< 0.01	0.07	0.70	0.70
N-efficiency[7], g/kg	354	341	300	268	312	299	266	249	13.1	< 0.01	< 0.01	0.50	0.30	0.64
CH														
g/d	455	459	457	441	466	434	478	471	15.3	0.32	0.87	0.86	0.20	
g/kg DMI	25.9	25.0	23.7	22.3	26.0	23.7	24.5	25.3	0.78	0.24	0.03	0.23	0.05	
g/kg ECM	16.7	15.6	15.2	14.9	17.8	15.0	16.4	16.3	0.83	0.25	0.13	0.17	0.58	

[1] Diets: See footnote in table.

[2] Probability of the dietary treatment effects: G v. C = grass vs. clover; Lin = linear effect of dietary CP concentration; Quad = quadratic effect of dietary CP concentration; F × Lin = interaction between forage type and linear dietary CP concentrations; F × Q = interaction between forage type and quadratic dietary CP concentration was not significant ($P \leq 0.53$).

[3] Neutral detergent fiber

[4] potentially digestible NDF intake = NDF intake – indigestible NDF intake

[5] Metabolizable energy concentrations of the silages calculated according to Lindgren (1979), calculations for the concentrate according to Spörndly (2003).

[6] Energy corrected milk, calculated according to Sjaunja et al. (1990).

[7] Nitrogen efficiency = milk N/N intake.

Domestic Protein Production Study

The dietary chemical composition is found in Table 5. There were no differences in DMI between the diets (Table 6). Although, dietary inclusion of rapeseed expeller resulted in increased intake in CP and neural detergent fiber (NDF) compared to the other protein supplements. Rapeseed expeller also increased milk yield and milk protein concentration, but did not increase energy corrected milk compared to the other protein supplements. Heat-treated field beans had no effect on DMI, milk yield or milk protein concentration compared to untreated field beans when compared with similar dietary CP concentrations. Neither were there any production differences between treated and unreated field beans when they were compared with similar dietary MP concentration. Further, no differences were found between dietary inclusion of untreated field beans and peas concerning DMI and production results. The control diet had the lowest milk urea concentration and the highest nitrogen efficiency compared to all other diets, as well as similar production responses.

Table 5. Dietary chemical composition (g/kg DM unless otherwise noted).

	Diet[1]					
Item	C	RSE	PEA	UFB	TFB	TFB-MP
DM, g/kg of fresh matter	347	349	349	348	349	349
CP	159	187	187	181	183	176
Crude fat	26	33	24	25	25	25
NDF	400	415	365	395	390	393
iNDF	45	56	37	40	40	41
ME, MJ/kg of DM	11.8	11.8	12.1	11.9	12.0	11.9

[1]C = control, RSE = rapeseed expeller, UFB = untreated field bean, TFB = heat-treated field bean, TFB-MP = heat-treated field bean, with same MP as UFB.

Table 6. The effect of diet treatments on feed intake, milk yield and nutrient consumption of dairy cows.

	Ration[1]							Contrasts (P – value)[2]				
	C	RSE	PEA	UFB	TFB	TFB-MP	SEM[3]	C vs. O	R vs. O	UFB vs. PEA	UFB vs. TFB-MP	UFB vs. TFB
Intake												
DMI, kg/d	18.2	19.0	19.0	18.7	18.7	18.6	0.37	0.13	0.33	0.58	0.80	0.98
CP, kg/d	2.90	3.55	3.44	3.35	3.32	3.15	0.072	<0.01	<0.01	0.33	0.04	0.79
NDF, kg/d	7.11	7.66	6.94	7.23	7.23	7.26	0.154	0.31	<0.01	0.17	0.90	0.99
ME, MJ/d	219	224	231	223	223	222	4.50	0.18	0.98	0.17	0.87	0.97
Milk Yield												
Milk, kg/d	23.5	24.8	23.0	23.7	23.8	23.8	0.90	0.49	0.02	0.30	0.90	0.85
ECM, kg/d	24.6	26.6	24.9	25.8	25.8	25.3	0.91	0.18	0.17	0.40	0.97	0.62
Protein, g/d	873	913	833	863	873	887	28.2	0.95	0.02	0.24	0.35	0.71
Fat, g/d	993	1098	1043	1074	1075	1033	47.1	0.13	0.38	0.60	0.50	0.99
Milk Concentration												
Protein, %	3.76	3.73	3.66	3.69	3.69	3.75	0.053	0.07	0.049	0.29	0.44	0.07
Fat, %	4.33	4.48	4.54	4.61	4.55	4.41	0.163	0.70	0.17	0.72	0.71	0.30
Urea-N, mmol/L	3.01	3.79	3.94	3.90	4.42	3.57	0.154	<0.01	<0.01	0.16	0.80	0.03
N-efficiency, g/kg	294	240	245	278	254	255	8.60	<0.01	0.82	0.42	<0.01	0.09
CH_4												
g/d	390	383	397	389	403	406	9.6	0.53	0.09	0.45	0.12	0.20
g/DMI	21.4	20.4	21.0	20.8	21.6	22.0	0.57	0.71	0.11	0.78	0.10	0.22
g/kg ECM	16.0	15.2	16.7	15.9	16.1	16.2	0.75	0.99	0.13	0.30	0.71	0.80

[1]C = control, RSE = rapeseed expeller, UFB = untreated field bean, TFB = heat-treated field bean, TFB-MP = heat-treated field bean, with same MP as UFB. [2]C vs. O = C vs. other diets, R vs. O = RSE vs. other diets excluding C, [3]SEM = standard error of mean, [4]FE = feed efficiency (kg ECM) / (kg DMI), [5]MNE = milk nitrogen efficiency (milk protein yield × 6.38) / (protein intake × 6.25)

The concentration of uCP during the in vitro incubation followed what was expected (Figure 1). The concentration of uCP increased initially and sometime after 12 hours of incubation the concentration started to decrease. There was variation in uCP concentration between the diets which seem to be well explained by their CP concentration. For example at time point 12 the minimum uCP concentration was 164.8 g uCP/kg DM and the maximum was 223.3 g uCP/kg DM. Same diets also represented the minimum and maximum in dietary CP concentration, with respectively 133 and 197 g CP /kg DM. There was a good fit of a quadratic equation to the average of all diet uCP concentration least square means over time after incubation (Figure 2.).

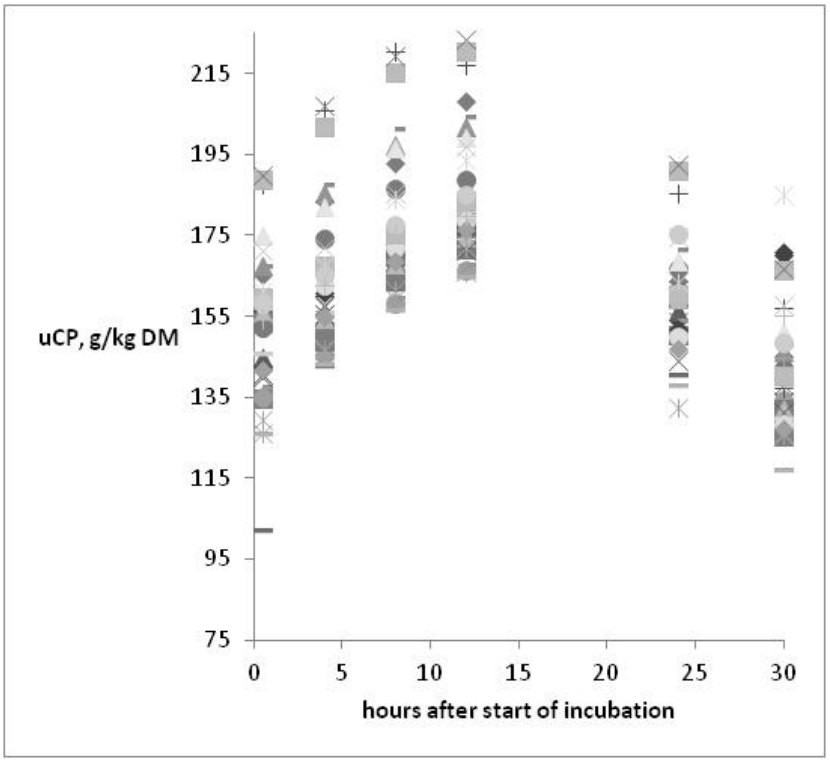

Figure 1. Least square means of utilizable crude protein from four in vitro runs testing 34 different diets at time point 0.5, 4, 8, 12, 24, and 30 hours after the start of the in vitro incubation.

Selected variables from the flow studies, uCP intakes and concentrations are presented in Table 7. Animal variables displayed large variation in intake, production and omasal CP flow. Mean uCP supply calculated using 20 or 24 h RT were close to observed in vivo data. Standard deviations were slightly smaller for uCP method compared to in vivo CP flow. This can at least partly reflect methodological differences between animal studies. The uCP supply was closely related to in vivo CP flow. There was a close relationship between uCP supply and omasal CP flow (Figure 3.). RMSE adjusted for random study effect ranged from 4.6 to 5.3 of observed mean CP flow being the smallest for uCP12 and the greatest for uCP24. The slope of fixed model (uCP16) was close to 1.0, but for mixed model regression the slope

(0.73) suggests that the uCP method overestimated the differences between the diets in omasal CP flow.

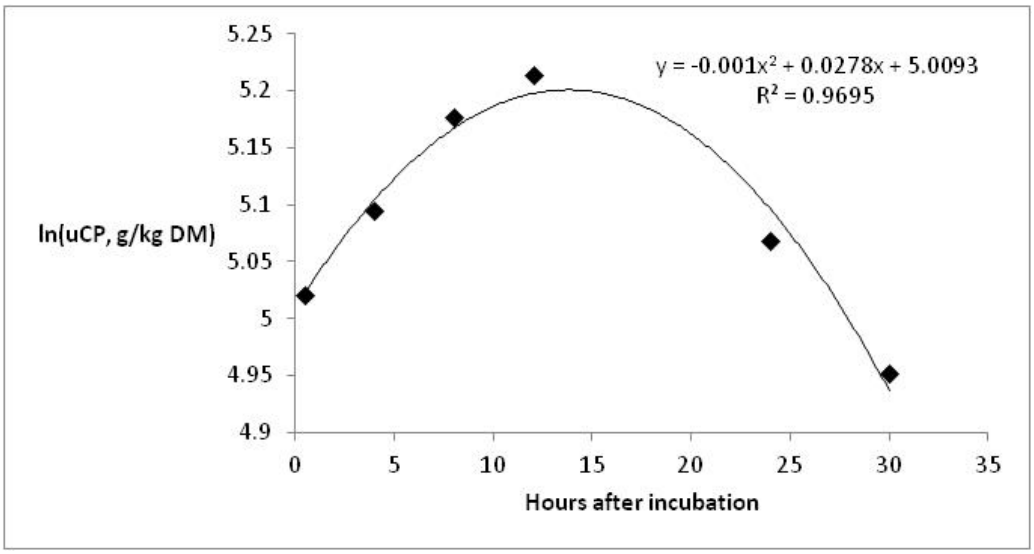

Figure 2. Fit of a quadratic equation to the average logarithmic utilizable crude protein concentration (g/kg DM) of all diet least square means by hours of incubation.

Table 7. Mean treatment data (n = 34) from omasal flow studies and estimated uCP supply and concentrations. The number in uCP refers to rumen retention time used in calculation.

	Mean	SD	Min	Max
Animal data				
DM intake, kg /d	19.6	2.00	15.8	23.6
Forage DM intake, kg/d	11.4	1.69	7.5	14.0
Milk yield, kg/d	27.7	4.73	19.9	35.8
Protein yield, g/d	898	116	702	1136
Omasal CP flow, kg/d	3.14	0.597	2.31	4.41
uCP supply, kg/d				
uCP12	3.69	0.511	2.66	4.86
uCP16	3.46	0.471	2.46	4.57
uCP20	3.24	0.437	2.28	4.29
uCP24	3.04	0.407	2.12	4.04
uCP, g/kg DM				
uCP12	188	15.7	168	227
uCP16	176	14.5	156	213
uCP20	165	13.6	145	200
uCP24	155	12.9	134	187

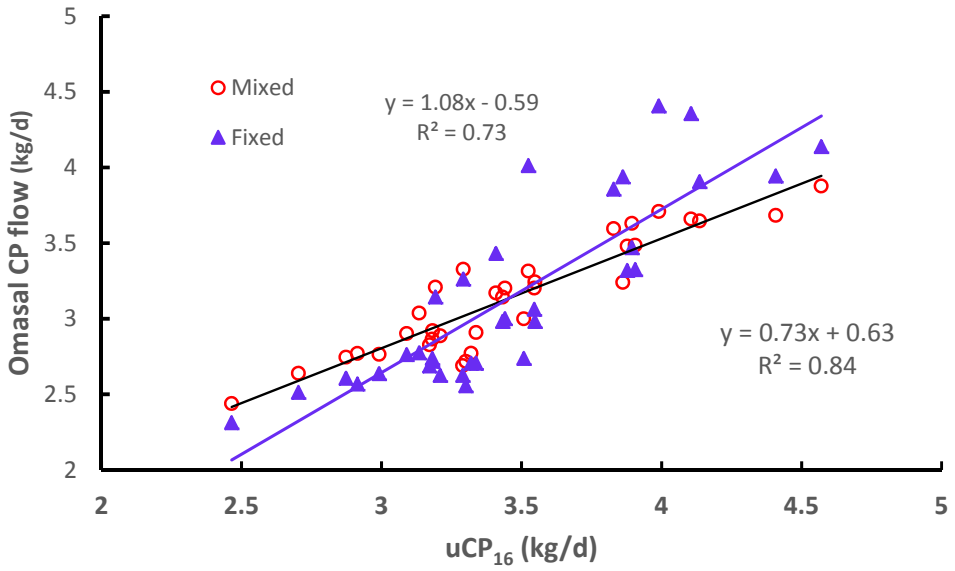

Figure 3. The linear relationship after 16 hour of in vitro incubation between the concentration of utilizable crude protein (kg/d) and the in vivo flow of crude protein (kg/d) at the omasum estimated by fixed and mixed regression models.

The relationship between estimated uCP concentration and omasal CP flow per kg DM intake is shown in Figure 4. The RMSE was the smallest for uCP_{12} (5.0% of observed mean) and the greatest (5.8%) for uCP_{24}. On the other hand, uCP_{20} and uCP_{24} were more accurate, i.e. the mean predicted value was closer to observed mean, compared to uCP_{12} and uCP_{16}.

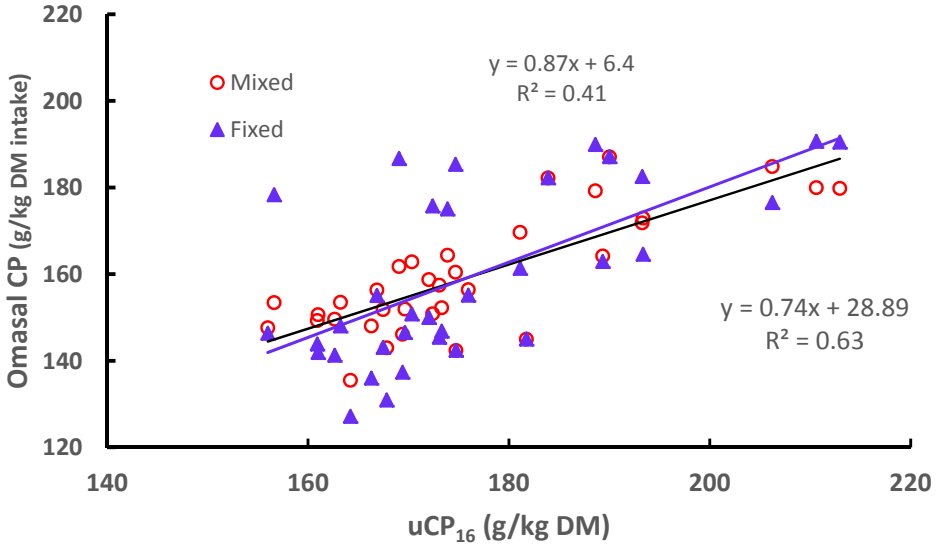

Figure 4. The relationship between dietary uCP_{16} concentration and omasal CP flow per kg DM intake when estimated by fixed and mixed regression models

The supply of uCP predicted milk protein yield reasonable well, especially when random variation between studies was considered by mixed model regression analysis (Figure 5). In fixed model analysis for example the stage of lactation can influence yield, but not necessarily uCP or omasal CP flow. The effect of RT in calculating uCP had no influenced on predictions of milk protein yield (RMSE 30.7 – 31.1 g/d). Interestingly, uCP predicted milk protein yield more precisely than omasal CP flow when estimated with mixed model regression (RMSE. Quadratic model slightly improved the fit of the model (RMSE = 27 g/d) compared to linear model indicating diminishing returns from increased protein supply. In bivariate model predicting milk protein yield from DM intake and uCP concentration the effect of uCP was statistically significant.

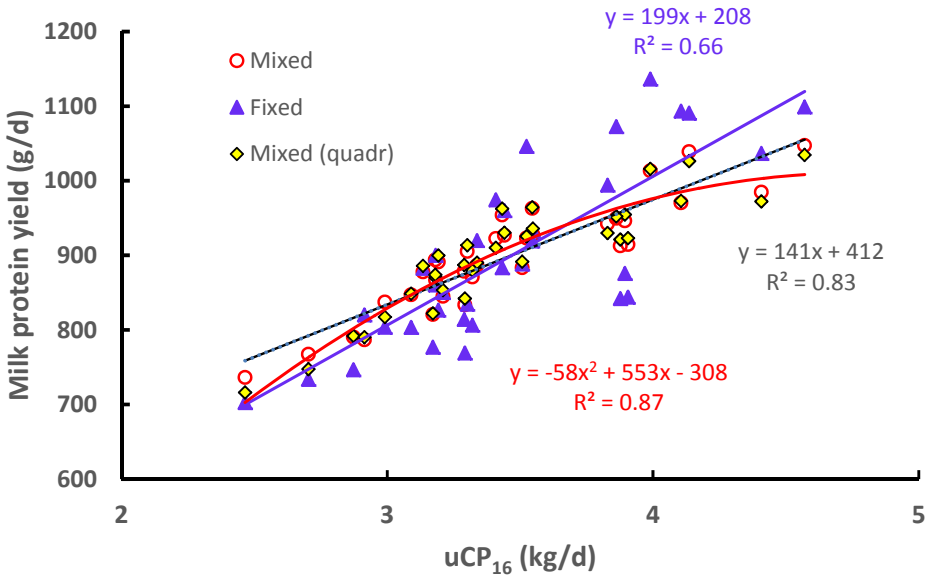

Figure 5. The linear relationship between the in vitro utilizable crude protein supply (kg/d) and milk protein yield (g/d) estimated by fixed and mixed regression models.

DISCUSSION

Primary growth vs. Regrowth Silage Production Study

Increasing the proportion of clover silage had no impact on the performance of cows. This can partly be due to lower digestibility of red clover silage as indicated by higher iNDF concentration. Milk production is often increased when the proportion of red clover in silage is increased. This is mainly due to greater intake potential of red clover silages. Feeding mixtures of grass and red clover silages have produced positive associative effects in intake (Huhtanen et al., 2007), i.e. cows fed mixtures grass and red clover eat more than the mean intake of grass and red clover silages when fed alone.

The efficiency of protein utilisation in the rumen is high with red clover silage based diets compared with grass silage based diets, mainly because of lower ruminal protein degradability and partly because of improved efficiency of microbial protein synthesis (Dewhurst et al., 2003; Vanhatalo et al., 2009). This can be due to polyphenol oxidase (PPO) in red clover that inhibits proteolysis both in the silo and in the rumen. However, the higher protein supply from the rumen has not been translated to increased milk protein yield. This can, at least partly, because energy intake can become a limiting factor. Vanhatalo et al. (2009) speculated based on lower concentrations of methionine blood plasma and omasal protein that the supply of methionine may limit milk protein production with diets based on red clover. However, omasal methionine flow was numerically greater with red clover diets compared with grass silage diets. Faecal CP output per kg DM intake was 16 g greater with red clover compared with primary growth grass silages in digestibility studies with sheep (Finnish dataset from digestibility studies). This suggests that part of the increased protein supply from red clover based diets is lost in faeces.

It could be expected that milk protein yield responses could be smaller with red clover compared to grass silages due to increased protein flow from the rumen. However, no interaction between forage type and level of protein supplementation was observed. This suggests possibilities to reduce the amounts of protein feeds by increasing the proportion of red clover in the diet are rather limited. On the other hand, our results indicated that very small increases in milk protein yield were obtained above the lowest level of supplementary feeding. Generally, milk protein yield responses to supplementary protein are rather small. In meta-analysis by Huhtanen et al. (2011) marginal response to incremental protein intake was 100 g/kg (soybean meal) and 130 g/kg (rapeseed feeds).

Methane production decreased or tended to decrease with increased protein level in the diet. This is consistent with earlier observations by Gidlund et al. (2015). The changes are partly related to increased intake that decreases methane per unit of intake, and partly to increased yield that dilutes total methane production per unit of product.

Domestic Protein Production Study

The effects of pea and field beans on milk production were disappointing; no improvements compared to control diet without supplementary protein were observed. This can partly reflect to low production level in this study. However, the higher production in cows fed rapeseed expeller compared to control diet without supplementary protein indicate that the cows were responsive to supplementary protein. The lack of response to pea is consistent with the results from omasal flow study (Ahvenjärvi et al., 2005). Despite increased protein intake omasal protein flow per kg DM intake did not increase compare to control diet without supplementary protein. This indicates high ruminal protein degradability and that a large proportion of incremental protein was lost from the rumen as ammonia. Heat treatment did not improve the productive value of field beans in the present study. It is possible that heat treatment was too mild. In a recent study at our department uCP of peas and field beans could be increased by stronger heat treatments (Vaga et al., 2016, submitted). However, mild / short

treatments didn't markedly increase uCP, whereas stronger / longer treatments were sometimes associated with increased acid detergent insoluble N that is considered to be indigestible in the small intestine. In their study increases in uCP were marginal when dietary CP concentration was increased from 13 to 18% by replacing barley with peas or field beans. More research is needed to find optimal level of heat treatment that decrease ruminal protein degradability, but does not destroy essential amino acids or decrease protein digestibility in the small intestine. Both peas and field bean are rather good sources of histidine that has demonstrated to be the first limiting amino acid in cows fed grass silage and grain based diets (Vanhatalo et al., 1999). However, to get full benefits of this ruminal protein degradability must be reduced by heat treatments. Peas, and especially field beans are very poor sources of methionine compared to rapeseed (0.9 and 0.6 vs. 1.8 g/kg CP; LUKE feed tables). This suggests that even if ruminal protein degradability could be reduced by heat treatments, very low methionine concentration can become a limiting factor. Milk protein is much higher in methionine than milk (2.6 g/kg CP) than legumes. Even if digestible uCP could be optimised by heat treatments, improved protein value must be demonstrated in feeding experiments. In meta-analysis based on large datasets milk protein response to untreated and heat-treated rapeseed feeds was similar (Huhtanen et al., 2011). In a recent study milk protein yield response to graded levels of heat-treated rapeseed meal (Expro) was poor (Gidlund et al., 2015) despite omasal flow study indicated low ruminal protein degradability (unpublished data from NJV)

Study of In Vitro Feed Protein Value

In vitro method determining uCP is a promising tool for determining protein value of the diet. Basically it estimates the protein supply from the rumen to small intestines, but it is not able to separate microbial protein and undegraded feed protein. The uCP method can overcome some problems related to the in situ method that is widely used to determine ruminal protein degradability. Microbial contamination of undegraded residues, losses of small particles and soluble NAN fractions and lower microbial activity within the bags compared with rumen digesta are methodological problems associated with the in situ nylon bag method. Problems related to these factors are less in the uCP method, since small particles and soluble NAN remain in the system, there is no need to separate microbial and feed protein and feeds are incubated freely dispersed in the flasks.

We used several incubation periods to find optimal time points. It appeared that early time points are not very useful; uCP concentrations increased to much higher levels than could be expected in vivo conditions. This can reflect rapid uptake of ammonia from rumen fluid to intracellular pools without incorporation to microbial cell protein. Three time points (12, 24 and 30 h) were used to develop regressions of natural logarithm of uCP on time. The mean correlation coefficient (0.97) was high suggesting that these time points were optimal for estimation of uCP using different retention time. It could be possible to use only one time point, but then uCP cannot be estimated for different passage rate / retention time.

Shorter RT resulted in best predictions of omasal NAN flow, but for predicting milk protein yield all RT resulted in similar predictions. Shorter RT overestimated uCP compared to in vivo data. This can be related to differences in the system; in vitro system is a batch culture without any outflow of microbes and/or nutrients, whereas rumen is a dynamic system with continuous outflow and inflow of urea through rumen wall and saliva, and intermittent inflow of nutrient (eating). Good relationships between uCP and omasal CP flow suggest that the method has potential to be used to evaluate protein value of the diet in laboratory conditions. Determining protein flow from the rumen either by duodenal or omasal sampling techniques is technically demanding and therefore part of the RMSE error reflects random variation in reference data. Even more promising was the good relationship between uCP supply and milk protein yield. Eventual goal of feed evaluation systems is to predict production responses. The relationship between uCP supply and milk was similar to that estimated between for omasal CP flow in a large (n=112) dataset from omasal sampling studies (Huhtanen et al., 2010)

CONCLUSIONS

1. Proportion of red clover in silage did not influence milk production or responses to supplementary protein.
2. Low level of supplementary protein increased markedly milk and milk protein yield, but only small (medium level) or even negative (high level) responses were observed at higher levels.
3. Methane production per unit of product can be decreased by optimal protein feeding, but beyond optimal protein levels N utilisation decrease.
4. No protein yield response was observed for peas or field beans, whereas rapeseed expeller increased yield. However, when rumen degradable N is limiting for rumen microbes legume protein feeds can be useful.
5. Provided that the requirements of rumen microbes are met (diet CP concentration 13-14%, milk urea 2.8 – 3.0 mmol/L) economic benefits of supplementary protein feeding in organic farms can be limited or even negative due to high prices of organically grown protein feeds.
6. In vitro method determining utilisable crude protein (uCP) can be a useful tool for screening the protein value of a large number of alternative diets. More research is need to evaluate and develop the method; for example to determine if the uCP values of dietary ingredients are additive.

REFERENCES

Ahvenjärvi, S., A. Vanhatalo, and S. Jaakkola. 2005. Herneen ruokinnallinen arvo marehtijalla [Nutritional value of field pea in dairy cow diets]. In: Herneen murskesäilönta ja kaytto märehtijoiden ruokinnassa. Loppuraportti. [Use of ensiled pea in dairy cow diets. Final report.] MTT Agrifood Research Finland.

Bauman, D.E., Mather, I.H., Wall, R.J., and Lock, A.L. 2006. J. Dairy Sci. 89:1235–1243.

Broderick, G.A., Huhtanen, P., Ahvenjärvi, S. Reynal, S.M., and Shingfield, K.J. 2010. J. Dairy Sci. 93: 3216–3230

Cone, J.W., van Gelder, A.H., Visscher, G.J.W., and Oudshoorn, L., 1996. Anim. Feed Sci. Technol. 61, 113–128.
Davis, A. W. and Hall, W. B. 1969. Cyclic change-over designs. Biometrika. 56, 283-293.
de Vries, M., and de Boer, I.J.M. 2010. Livest. Sci. 28: 1–11.
Dewhurst, R. J., Fisher, W. J., Tweed, J. K. S., and Wilkins, R. J. 2003. J. Dairy Sci. 86:2598-2611.
Edmunds, B., Südekum, K.-H., Spiekers, H., Schuster, M., and Schwarts, F. J. Anim. Feed. Sci. Tech. 175: 106-113.
Fagerberg B, Salomon E, and Jonsson S 1996. Swed. J. Agric. Res. 26:169-180.
Gidlund, H., Hetta, M., Krizsan, S. J., Lemosquet, S., and Huhtanen P. 2015. J. Dairy Sci. 98:1-14.
Hedqvist, H. and Udén, P., 2006. Anim. Feed Sci. Technol. 126: 1-21.
Huhtanen, P., Hetta, M., and Swensson, C. 2011. Can. J. Anim. Sci.91: 529-543
Huhtanen, P., Ahvenjärvi, S., Broderick, G.A., Reynal, S.M., Shingfield, K.J. 2010. J. Dairy Sci. 93:3203–3215.
Huhtanen, P., Rinne, M., Nousiainen, J. 2007. Animal, 1, 758-770.
Huhtanen, P., Cabezas-Garcia, E. H., Utsumi, S., and Zimmerman, S. 2015. J. Dairy Sci. 98:1-16.
Huhtanen, P. and Hristov, A.N. 2009. J. Dairy Sci. 92: 3222–3232.
Huhtanen, P., Rinne, M., and Nousiainen, J. 2008. Animal 2: 942–953.
Karlsson, L., Hetta, M., Udén, P., and Martinsson, K., 2009. Anim. Feed Sci. Technol. 153, 193-202.
KRAV, 2010. KRAVs marknadsrapport – 2010.
Menke, K.H. and Steingass, H., 1988. Anim. Research and Development, 28, 7-25.
Nousiainen, J., Rinne, M., and Huhtanen, P. 2009. J. Dairy Sci. 92: 5019–5030.
Ramin, M. and Huhtanen, P. 2012. Anim.FeedSci.Technol.174,190–200.
SCB, 2016. Statistikdatabasen, Försäljning av ekologiska livsmedel och alkoholhaltiga drycker in om handeln efter varugrupp. År 2004-2014.
SCB, 2013. HA24 Livsmedelsförsäljningsstatistik.
Vaga, M., Hetta, M, and Huhtanen P. 2016. Submitted to Anim. Feed Sci. Technol.
Vanhatalo, A., Kuoppala, K. Ahvenjärvi, S., and Rinne, M. 2009. J. Dairy Sci. 92: 5634-5644.
Vanhatalo, A., Huhtanen, P., Toivonen, V., and Varvikko, T. 1999. J. Dairy Sci, 82: 2674-2685.