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Ruminal Metabolism of Soluble Nitrogen in Dairy Cows

Doctoral Dissertation

Tomasz Stefański

Luke

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Abstract

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This work focuses on studying rapeseed meal (RM) protein degradation and utilization in dairy cows. Rapeseed meal was selected as a source of protein as it is widely used and cultivated in the European Union, and can be used to replace soybean meal in the rations of lactating dairy cows.

This thesis comprises three experiments. Two experiments were conducted using *in vitro* and *in vivo* techniques. The objectives of the work were to establish an *in vitro* method to study protein metabolism in the rumen (Publications I and II), to study the metabolism of ammonia N and soluble fractions of RM protein *in vivo* (III), and to study the efficiency of utilization of different N fractions for milk protein synthesis (IV).

The *in vitro* experiment documented in publication I involved a preliminary study to develop a mixture of carbohydrates to ensure high proteolytic activity and constant microbial N synthesis over the entire *in vitro* incubation period. In the main trial, the *in vitro* method was established to study the rate of ruminal degradation of RM protein based on observations of ^{14}N and ^{15}N isotope fluxes between ammonia N and non-ammonia N pools. In this study, feed protein was incubated with rumen fluid, mineral buffer and a carbohydrate mixture. The ammonia N pool was labelled with ^{15}N isotope, and the incubations were carried out for 10 h with 11 sampling times. The rate of RM degradation estimated with a six-pool model was 0.06/h with an effective protein degradation of 0.38. This approach of studying protein degradation *in vitro* seemed to be appropriate for determination of microbial N synthesis from ammonia N but it did not provide sufficient information on metabolic events involved in ruminal protein degradation and microbial protein synthesis from preformed amino acids.

A novel *in vitro* method developed to study the metabolism of soluble RM protein was presented in Publication II. In this experiment, unlabelled and ^{15}N labelled soluble fractions of RM were incubated for 10 h with 11 sampling times along with buffered rumen fluid and a carbohydrate mixture. A four-pool model involving pools of ^{14}N and ^{15}N isotopes of ammonia N, soluble non-ammonia N, and insoluble-N from unlabelled and ^{15}N labelled soluble RM incubations was used to estimate parameter values. The mean rate of soluble RM protein degradation was 0.126/h. There was no substantial difference in the rate of protein degradation and microbial N synthesis between the unlabelled and ^{15}N labelled soluble RM. In conclusion, combined data from incubations of unlabelled and ^{15}N labelled soluble RM provided sufficient information for estimation of parameter values in a complex dynamic model of soluble protein degradation. The results also indicated ruminal escape of soluble protein.

The ruminal *in vivo* metabolism of ^{15}N labelled ammonia N and a soluble N fraction of ^{15}N labelled RM protein introduced into the rumen were presented in Publication III.

Four lactating dairy cows equipped with rumen cannulae were used in this study. The cows consumed a total mixed ration (60% of silage and 40% of concentrates on DM basis) with 15.5% of crude protein on DM basis, with average rumen ammonia N concentration of 5.5 mg/100mL. The metabolism of ammonia N occurred at a very fast rate, with 99.4% of the original dose disappearing from the ammonia N pool in 4 h. The ammonia N was mainly incorporated into microbial N as 69% of the ^{15}N labelled ammonia N dose disappeared from the rumen as microbial N.

In the metabolism of soluble RM protein two steps were observed: 1) an almost instant uptake of more than half of the soluble non-ammonia N (SNAN) dose by the rumen bacteria 2) followed by slower degradation rate of the remaining fraction of the soluble RM protein. It was estimated that 8% of the soluble RM protein N escaped the rumen as feed N. SNAN had a higher initial uptake of the dose than ammonia N (AN) (56 vs. 16%). Also, the outflow as non-ammonia N from the rumen was higher for the SNAN than for AN treatment (89 vs. 69%). More N disappeared (outflow and absorption) from the rumen as ammonia N for the AN treatment than for SNAN treatment (31 vs. 11%). These observations suggested that SNAN was better utilized in the rumen than AN. Higher outflow of microbial N for the SNAN than for AN treatment (81 vs. 69%) indicated that preformed AA and small peptides stimulated microbial growth.

The efficiency of utilization of AN, soluble and insoluble fractions of RM protein N for milk protein synthesis were described in Publication IV. The average efficiency of N utilization for milk protein synthesis (milk N/N intake) in this study (32%) was in the higher end of the range reported in the literature (typically from 14% to 36% but in some cases up to 45%). The cumulative secretion of isotope ^{15}N in milk at 108 h post dose indicated that the three studied N fractions had different efficiency of N utilization for milk protein synthesis. The lowest efficiency of N utilization was estimated for AN (19%), followed by the soluble RM fraction (20%), and the highest efficiency of N estimated for the insoluble RM fraction (22%). These differences were smaller than could be expected based on the current protein evaluation systems.

Keywords: dairy cows, soluble protein metabolism, ammonia, isotope ^{15}N, rapeseed, efficiency of utilization of N for milk protein synthesis

Tiivistelmä

Tämän tutkimuksen tavoitteena oli selvittää rypsivalkuaisen aineenvaihduntaa ja hyväksikäyttöä lypsylehmien ruuansulatuksessa. Rypsivalkuainen valittiin tutkimuskohteeksi sen vuoksi, että se on Euroopan Unionin alueella laajasti viljelty ja käytetty rehuvalkuainen, jonka avulla voidaan korvata soijarouheen käyttöä lypsylehmien valkuaisrehuna.

Väitöskirja perustuu kolmeen kokeelliseen tutkimukseen, joista kahdessa käytettiin *in vitro* -menetelmiä ja yhdessä in vivo -menetelmiä. Kahden ensimmäisen tutkimuksen tavoitteena oli kehittää in vitro -menetelmä rehuvalkuaisen pötsiaineenvaihdunnan tutkimiseksi. Näiden tutkimusten tulokset on julkaistu väitöskirjan osajulkaisuissa I ja II. Kolmannen tutkimuksen tavoitteena oli selvittää ammoniumtypen sekä rypsin liukoisen valkuaistypen aineenvaihduntaa pötsissä. Lisäksi samassa tutkimuksessa selvitettiin ammoniumtypen ja rypsin valkuaistypen hyväksikäyttöä lypsylehmän maitovalkuaisen synteesissä. Kolmannen tutkimuksen tulokset on julkaistu väitöskirjan osajulkaisuissa III ja IV.

Väitöskirjan ensimmäisessä osajulkaisussa esitettiin in vitro -menetelmä, joka oli kehitetty rypsivalkuaisen pötsiaineenvaihdunnan tutkimiseen. Menetelmä perustuu typen ^{14}N- ja ^{15}N-isotooppien virtauksien havainnointiin ammonium- ja ei-ammoniumtyppipoolien välillä. Esitutkimuksessa kehitettiin ensin pötsimikrobien energia-aineenvaihdunnan tarpeita varten hiilihydraattiseos, jonka tarkoituksena oli varmistaa optimaalinen pötsimikrobien proteolyyttinen aktiivisuus ja tasainen mikrobivalkuaisen synteesi koko 10 h kestävän in vitro -kokeen ajan. In vitro -kokeessa rypsivalkuaista inkuboitiin 10 h ajan pötsinesteen, kivennäispuskuriliuoksen ja hiilihydraattien seoksessa, jonka ammoniumtyppipooli oli leimattu ^{15}N-isotooppia käyttäen. In vitro -kokeen aikana otettiin 11 näytettä, joiden perusteella saatuun havaintoaineistoon sovitettiin kuudesta poolista koostuva dynaaminen malli. Mallin avulla estimoitu rypsivalkuaisen hajotusnopeus pötsissä oli 0,06/h ja pötsihajoavuus 0,38. Tulosten perusteella in vitro -menetelmän arvioitiin soveltuvan mikrobisynteesin määrittämiseen, kun typpilähteenä on ammoniumtyppi, mutta menetelmän arvioitiin olevan riittämätön rehuvalkuaisen tai kokonaisten aminohappojen pötsiaineenvaihdunnan tutkimiseen.

Toisessa osajulkaisussa esitettiin uusi in vitro -menetelmä rypsivalkuaisen liukoisen fraktion pötsiaineenvaihdunnan tutkimiseksi. Tässä tutkimuksessa ^{15}N-leimattua ja leimaamatonta rypsin liukoista valkuaisfraktiota inkuboitiin pötsinesteen, puskuriliuoksen ja hiilihydraattien seoksessa 10 h ajan. Havaintoaineisto perustui inkubaation aikana kerättyihin 11 näytteeseen, joista määritettiin ^{14}N ja ^{15}N suhteelliset osuudet ammoniumtyppipoolissa, liukoisessa ei-ammoniumtyppipoolissa ja liukenemattomassa typpipoolissa. Havaintoaineistoon sovitettiin neljästä poolista koostuva dynaaminen malli, jonka avulla rypsin liukoisen valkuaisen hajotusnopeudeksi pötsissä estimoitiin 0,126/h. Leimaamattoman ja ^{15}N-leimatun rypsivalkuaisen välillä ei ollut merkittäviä eroja valkuaisen hajotusnopeudessa eikä mikrobisynteesin

nopeudessa. Tulosten perusteella arvioitiin, että ^{15}N-leimatun ja leimaamattoman rypsin liukoisen valkuaisen samanaikaisessa *in vitro* -inkubaatiossa saatiin riittävästi informaatiota useammasta tilamuuttujasta koostuvan dynaamisen mallin parametrien estimoimiseksi. Tulokset osoittivat, että lypsylehmän pötsistä virtaa hajoamatonta liukoista valkuaista alempaan ruuansulatuskanavaan.

Osajulkaisussa III esitettiin *in vivo* -tutkimuksen tulokset ^{15}N-leimatun ammoniumtypen ja rypsin liukoisen valkuaisfraktion aineenvaihdunnasta lypsylehmän pötsissä. Tutkimuksessa oli koe-eläiminä neljä pötsifistelöityä lypsylehmää, jotka söivät seosrehua, jonka kuiva-aineesta 60 % oli nurmisäilörehua ja 40 % väkirehuseosta. Dieetin raakavalkuaispitoisuus oli 15,5 % kuiva-aineessa ja lehmien pötsin ammonium-typpipitoisuus oli keskimäärin 5,5 mg/100 mL. Ammoniumtyppi metaboloitui pötsissä hyvin nopeasti siten, että lähes kaikki ammoniumtyppi (99,4 %) oli poistunut pötsinesteestä 4 h aikana. Suurin osa ^{15}N-leimatusta ammoniumtypestä syntetisoitui mikrobitypeksi ja poistui pötsistä mikrobivalkuaisena (69 %).

Liukoisen rypsivalkuaisen metaboliassa havaittiin kaksi vaihetta: 1) ensin yli puolet liukoisesta ei-ammoniumtypestä sitoutui lähes välittömästi pötsibakteereihin, ja 2) sen jälkeen jäljelle jäänyt solujen ulkoinen rypsin liukoinen valkuainen metaboloitui selvästi hitaammin. Tulokset osoittivat, että 8% rypsin liukoisesta valkuaisesta poistui pötsistä hajoamattomana rehuvalkuaisena. Ammoniumtyppeen verrattuna suurempi osa pötsiin annostellusta rypsin liukoisesta valkuaisesta sitoutui välittömästi mikrobivalkuaiseen (16 vs. 56 %). Lisäksi ammoniumtyppeen verrattuna suurempi osuus rypsin liukoisesta valkuaisesta virtasi ulos pötsistä ei-ammoniumtyppenä (69 vs. 89 %). Edelleen ammoniumtyppeen verrattuna pienempi osuus rypsin liukoisesta valkuaisesta poistui pötsistä ammoniumtyppenä ulosvirtauksen ja imeytymisen kautta (11 vs. 31 %). Näiden tulosten perusteella rypsin liukoisen valkuaisen hyväksikäyttö pötsissä oli tehokkaampaa kuin ammoniumtypen. Ammoniumtyppeen verrattuna suurempi osuus rypsin liukoisesta valkuaisesta virtasi pötsistä mikrobityppenä (69 vs. 81 %) osoittaen, että yksinkertaisiin typpiyhdisteisiin verrattuna aminohapot ja peptidit stimuloivat mikrobisynteesiä.

Neljännessä osajulkaisussa esitettiin ammoniumtypen sekä liukoisen ja liukenemattoman rypsivalkuaistypen hyväksikäyttö lypsylehmien maitovalkuaisen synteesissä. Tässä tutkimuksessa valkuaistypen hyväksikäyttö oli keskimäärin 32 %. Tutkimuskirjallisuudessa valkuaistypen hyväksikäyttö on vaihdellut tyypillisesti 14 ja 36 % välillä ja korkeimmillaan hyväksikäyttö on ollut 45%. Tutkimuksessa määritettiin ^{15}N-isotoopilla leimattujen typpifraktioiden kumulatiivinen eritys maidossa 108 h aikana. Tulokset osoittivat, että typpifraktioiden välillä oli eroja niiden hyväksikäytön tehokkuudessa. Ammoniumtypen hyväksikäyttöön verrattuna (19%) rypsin liukoisen valkuaisen hyväksikäyttö oli hieman suurempi (20%), mutta matalampi kuin rypsin liukenemattomalla fraktiolla (22 %). Erot typpifraktioiden välillä olivat kuitenkin pienempiä kuin nykyisten valkuaisen arviointijärjestelmien perusteella voitiin ennakoida.

Asiasanat: lypsylehmä, liukoinen valkuainen, aineenvaihdunta, ammoniakki, ^{15}N-isotooppi, typen hyväksikäyttö, maidontuotanto, maitovalkuainen

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Tomasz Stefański

List of orginal publications

This thesis is based on the following publications:

I. Ahvenjärvi, S., T. Stefański, and P. Huhtanen. 2009. In vitro method for determining the ruminal degradation rate of rapeseed meal protein using ^{15}N isotope labelled ammonia nitrogen. Animal Feed Science and Technology 153: 88–100.

II. Stefański, T., S. Ahvenjärvi, P. Huhtanen, and K. J. Shingfield. 2013. Metabolism of soluble rapeseed meal (Brassica rapa L.) protein during incubations with buffered bovine rumen content in vitro. Journal of Dairy Science 96: 440–450.

III. Stefański, T., S. Ahvenjärvi, A. Vanhatalo, and P. Huhtanen. 2020. Ruminal metabolism of ammonia-N and rapeseed meal soluble N fraction. Journal of Dairy Science 103: 7081–7093.

IV. Stefański, T., P. Huhtanen, A. Vanhatalo, and S. Ahvenjärvi. Utilization of nitrogen as ammonia, soluble or insoluble rapeseed meal for milk protein synthesis in lactating cows fed grass silage-based diets. Manuscript.

The publications are referred to in the text by their roman numerals.

Contribution

The contributions of all authors in the original publications of this thesis are presented in the following table.

	I	II	III	IV
Designing the experiments	SA, TS	SA, TS	PH, SA, AV	PH, SA, AV
Conducting the experiments and laboratory analysis	TS	TS	TS, SA	TS, SA
Data processing	TS	TS	TS	TS
Models development	SA, TS, PH	TS, SA, PH	PH, SA	
Manuscript	SA, TS, PH	TS, SA, PH, KS	TS, SA, PH, AV	TS, SA, PH, AV

AV = Aila Vanhatalo
KS = Kevin J. Shingfield
PH = Pekka Huhtanen
SA = Seppo Ahvenjärvi
TS = Tomasz Stefański

Abberviations

AA	Amino acids
AN	Ammonia nitrogen
ATP	Adenosine triphosphate
BN	Bacterial nitrogen
CNCPS	Cornell net carbohydrate and protein system
CP	Crude protein
DM	Dry matter
ECM	Energy corrected milk
EPD	Effective protein degradation
FSD	Reactional standard deviation
iRM	Insoluble rapeseed meal protein
L-sRM	Labelled soluble rapeseed meal protein
MN	Microbial nitrogen
MNE	Efficiency of utilization of N for milk protein synthesis
MP	Metabolizable protein
N	Nitrogen
NAN	Non-ammonia nitrogen
NDF	Neutral detergent fibre
NSC	Non-structural carbohydrates
OM	Organic matter
RM	Rapeseed meal
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
SNAN	Soluble non-ammonia nitrogen
SBM	Soybean meal
sRM	Soluble RM protein
TMR	Total mixed ration
U-sRM	Unlabelled soluble RM protein

Contents

1. Introduction

The capacity to digest human non-edible components (e.g. the fibrous feeds, by products) and convert them into meat and milk is one of the unique features of ruminant's digestive tract. The role of ruminants, especially dairy cows, will increase as the human population grows. Between 2010 and 2050 the demand for milk and meat is estimated to increase by 73 and 58% according to FAO (2011). For economic, environmental and political reasons there is an increasing pressure to decrease the use of imported protein sources, such as soybean meal (SBM) and increase use of domestic feed protein. One of the nitrogen rich plants commonly grown in the most of European Union countries is rapeseed (*Brassica napus subsp. Oleifera and Brassica rapa subsp. oleifera*). In recent years, it was suggested that rapeseed meal (RM) is a better source of protein for dairy cows than SBM (Huhtanen et al. 2011; Martineau et al., 2013; Broderick at al., 2015; Paula et al., 2018). Also, in previous studies RM has had better marginal milk protein yield responses than SBM (Huhtanen et al. 2011; Martineau et al., 2013; Broderick at al., 2015), and according to Paula et al. (2018), replacement of SBM with RM in isonitrogenous diets improve digestibility and decreases the urinary and milk urea N. This recent data indicates that replacing SBM with RM may reduce the environmental impact of lactating dairy cows in regards to N emissions. Moreover, by replacing SBM in the rations of lactating dairy cows RM decreases the proportion of human edible component in cows' diet as 20% of RM and 80% of SBM, respectively, is human edible (Wilkinson, 2011).

Another unique feature of ruminants is the ability to survive and have limited production only on non-protein N sources, such as urea and ammonium salts as demonstrated by Virtanen (1966). However, lactating dairy cows benefit from feed protein supplementation especially on diets with low crude protein (CP) levels (Rinne et al., 1999; Castillo et al., 2001; Olmos Colmenero and Broderick, 2006). Even so, the marginal efficiency is low especially on diets with high CP level (Castillo et al., 2001; Olmos Colmenero and Broderick, 2006; Huhtanen et al., 2011). A marginal N use efficiency of 136 and 98 (g/kg increase in CP intake) was reported for RM and SBM, respectively (Huhtanen et al., 2011). In general, increased protein supplementation on diets with high CP levels substantially decrease the efficiency of utilization of N for milk protein synthesis (MNE) (Broderick, 2003; Groff and Wu, 2005; Olmos Colmenero and Broderick, 2006; Rinne et al., 2015).

Microorganisms in the ruminants' foregut are able to break down fibrous material and feed protein to provide energy and protein components for the de novo synthesis of microbial protein and nutrients for the host animal (Van Soest, 1994, Broderick et al., 2010). Typically, microorganisms partly degrade feed protein to peptides, amino acids (AA) and finally to ammonia as described by Nolan (1993) (Figure 1). During rapid protein degradation, rumen microorganisms are unable to utilize all peptides, AA, and ammonia N produced during protein degradation. As a result, not all degraded protein N is used for the microbial protein synthesis.

Production of ammonia N during rapid protein degradation in excess of the rumen microorganisms' utilization ability leads to absorption of large proportions of the rumen ammonia N through the rumen wall to be excreted as urea in urine. An excessive amount of CP in a diet has several disadvantages with respect to environmental effects and animal requirements: increased N losses into the environment (NRC, 2001; Broderick, 2003), impaired reproductive performance (Butler, 1998; Shingfield et al., 1999), low efficiency of N utilization for milk protein production (Broderick, 2003; Huhtanen and Hristov, 2009; Calsamiglia et al., 2010), and economic losses associated with high cost of protein feeds and poor efficiency of N utilization. However, in previous studies it has also been discovered that part of the soluble protein escapes the ruminal degradation and supplies the host animal with peptides and AA (Chen et al., 1987; Hristov et al., 2001; Choi et al., 2002a; Reynal et al., 2007).

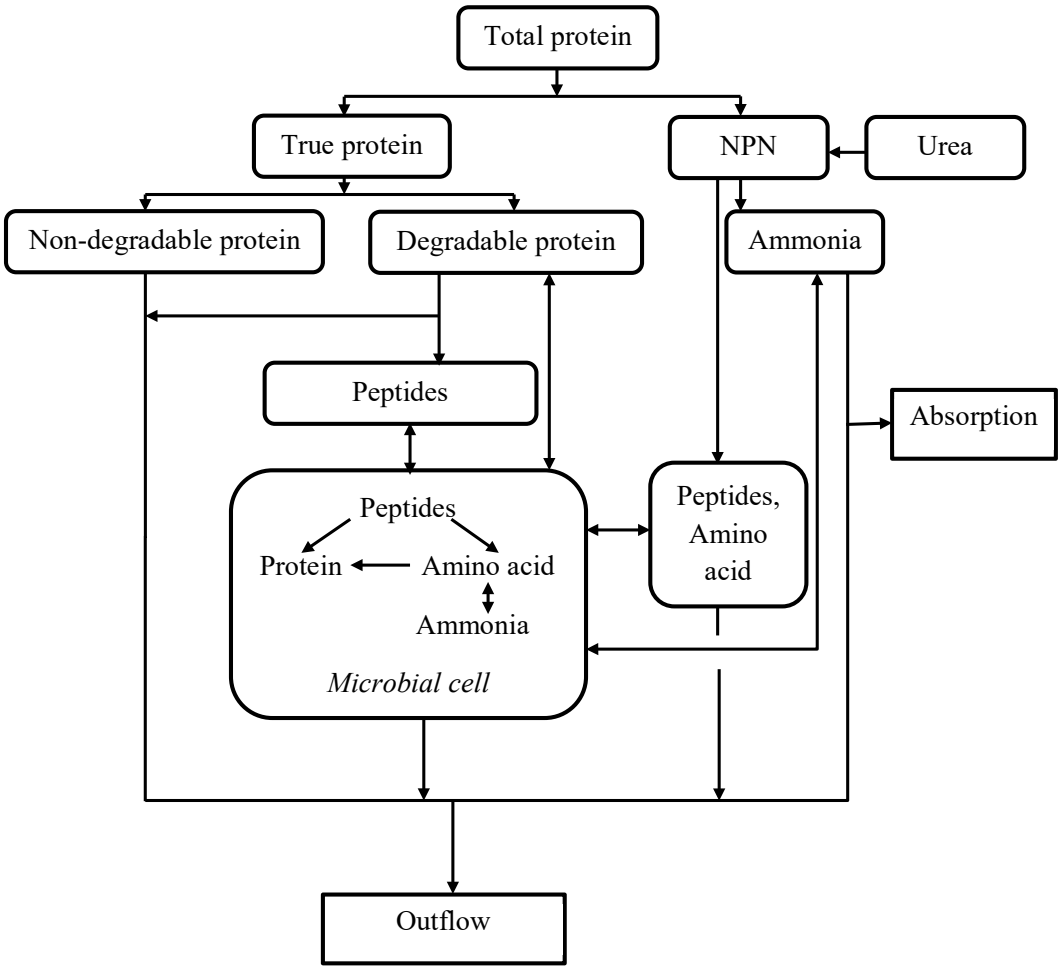

Figure 1. Schematic representation of N metabolism in the rumen. Adopted from J. V. Nolan (1993).

In the field of dairy cow nutrition, the focus has been increasingly on providing a ration that fulfils the nutritional requirements of the animal for optimal milk production, while taking into account the N emissions. The nutritional requirements of a dairy cow depend largely on the milk production level that is driven by the genetic merit and the stage of lactation. The ability of a dairy cow to increase milk yield in response to increasing high quality protein (e.g. RM, SBM) concentration in the ration is well established (Rinne et al., 1999; Huhtanen et al., 2011). Shingfield et al. (2001) did not report increases in milk yield with increase in diet CP concentration from grass silage. Furthermore, with a high CP level in the diet, the marginal responses to supplementary protein are poor (Huhtanen et al., 2011).

To maximize the economical profit, dairy farmers tend to provide diets with a high CP concentration to maximize the milk yield in spite of poor marginal responses to supplementary protein and low efficiency of N utilization on such diets. In lactating dairy cows, MNE varies considerably, with reported values ranging from 0.16 (Aarts et al., 2000) to 0.36 (Chase, 2004). Huhtanen and Hristov (2009) also reported high variation in MNE for lactating cows: from 0.14 to 0.45 in North American data and from 0.16 to 0.40 in North European data. According to Dijkstra et al. (2013) the theoretical maximum for MNE is 0.43.

However, increasing environmental awareness of consumers and farmers concerning the environmental and economical issues related to the overfeeding of N is likely to increase the farmer's interest in improving the N utilization efficiency of milk production. In addition to this, the changing legislation puts pressure on the farmers to reduce the N losses from the farm (Finland has agreed to reduce the NO_x emissions with 35% and NH_3 with 20% from the level of year 2005 by the year 2030, Ministry of the Environment, Finland, 2019).

One of the options for reducing the N pollution includes optimizing the dairy cow's diet to increase the MNE. To improve the N utilization efficiency of dairy cattle, a better understanding of the essential features of ruminal N metabolism is needed. Previous studies have indicated that soluble feed protein is not completely degraded in the rumen (Hedqvist and Udén, 2006; Ahvenjärvi et al., 2018) but the issue needs to be further elucidated. Furthermore, quantitative information on the microbial and undegradable feed protein available for absorption is essential to optimize the type and amount of protein required in ruminant diets.

Part of the general lack of improvement in the protein feeding systems for the lactating dairy cows in recent years may be attributed to the inaccurate information in tabulated values of feed components. There are several potential reasons for such inaccuracies: 1) the feed value may not be estimated correctly because of the inherent problems of the technique used to estimate it, 2) the feed value of the feed component may change over time as plant breeding introduces new varieties, feed manufacturing processes may be altered with consequences on by-products and feed ingredients used, and 3) the associative effects among feed ingredients.

Furthermore, the development in understanding of protein metabolism in the rumen should lead to better agreement between predicted and observed milk yield responses. Feed table values require periodical updates. In recent years, many new feed components, such as wood and biorefinery by-products (Halmemies-Beauchet-Filleau et al., 2018; Savonen et al., 2020; Kairenius et al., 2020) and new protein sources like microalgae (Lamminen, 2019) have been tested as dairy cow feed and may require feed value estimates.

Protein metabolism in the rumen may be estimated using *in vivo, in vitro* or *in sacco* methods. In theory, the *in vivo* technique is the most reliable method to study protein degradation because *in vitro* and *in sacco* methods fail to account for the complex dynamics of protein metabolism, absorption and outflow occurring in the rumen environment. *In vivo* methods have been widely studied and proposed by the scientists (e.g. Volden et al., 2002; Hedqvist and Udén, 2006; Ahvenjärvi et al., 2018) but compared to *in vitro* and *in sacco* methods, they are very expensive, and time consuming. For these reasons fast, cheap, and accurate alternatives for studying protein metabolism in the rumen are needed. However, the role of *in vivo* studies will remain to elucidate biological mechanisms, whereas the role of *in vitro* methods is to assess feed characteristics required by the static or dynamic feed evaluation models.

Because of the inherent problems of the *in sacco* technique such as low reproducibility, particle losses, microbial contamination, and the assumption that the soluble protein is completely degraded in rumen (Madsen and Hvelplund, 1994; Klopfenstein et al., 2001; Hristov et al., 2019), the technique is not used as widely as previously. Several studies have been published on the factors affecting the *in sacco* degradability (Broderick, 1994; Olaisen et al., 2003; Kamoun et al., 2014; De Jonge et al., 2013, 2015). Broderick and Cochran, (2000), and more recently Krizsan et al. (2015) published recommendations for *in sacco* degradability studies.

As discussed above, *in vitro* methods are needed to complement *in vivo* studies that are too costly for routine feed evaluation. Therefore, a number of *in vitro* methods to study the protein metabolism in ruminants have been introduced (e.g. Broderick, 1987; Raab et al., 1983; Hristov and Broderick, 1994) but none of them have been widely accepted and used for routine analysis. Usually, the *in vitro* systems are set up based on deliberate compromises between the *in vivo* environment and the need for a simple controlled system that can be studied in a laboratory. However, the *in vitro* methods should be validated by comparison with the *in vivo* results. Such evaluations have been presented very rarely (Yáñez-Ruiz et al., 2016), and if the results from these two methods have been compared, the differences between them have usually been large (Devant et al., 2001; Hatew et al., 2015). The *in vitro* methods are potentially good tools for screening and for comparative purposes (Hristov et al., 2019). The principle underlying *in vitro* studies is to assess intrinsic feed characteristics that determine the fate of protein in the rumen metabolism. One such feed characteristic assumed to be associated with rumen degradability is protein solubility. Crooker et al. (1978) introduced an *in vitro*

method based on protein N solubility, but it was discovered that protein solubility varies and depends on buffer composition, pH, time of extraction, and temperature (Crawford et al., 1978). In addition, the soluble proteins from different feedstuffs have different degradation rates (Hedqvist and Udén, 2006). Protein degradation is associated with ammonia N accumulation *in vitro*, whereas methods based on ammonia N release (Broderick, 1978) do not take into account microbial N uptake by the microorganisms. To overcome this problem, Broderick (1987) introduced a method based on inhibitors of ammonia and AA anabolism. However, later studies indicated that with longer incubation times the end product of degradation and the ''starvation of microorganisms'' seemed to affect the measured degradation rates (Broderick et al., 2004).

Instead of inhibiting microbial uptake of ammonia N it can be quantified. Various approaches to measure microbial N synthesis have been suggested. Rumen fermentation is associated with gas production that can be used to estimate the protein degradation rate (Raab et al., 1983; Karlsson et al., 2009). In this method, protein degradation is determined based on measurements of ammonia concentration and gas production via linear regression between gas production and ammonia N release. This method has gained popularity because it has high capacity and low cost (Bueno et al., 2005). The *in vitro* gas production method is especially sensitive to variation in the inoculum preparation and quality, ammonia concentration in the inoculum, and the microbial turn over.

Another method which enables measurement microbial N synthesis was introduced by Hristov and Broderick (1994). That method is based on using ^{15}N labelled ammonia to quantify microbial N synthesis. One of the advantages of this method is that it can be used in the *in vitro* but also in the *in vivo* method. In this method, the samples are analysed for ^{15}N enrichment of ammonia N, microbial N, and total solids N. The microbial protein synthesis is estimated based on the incorporation of isotope ^{15}N to microbial N, and the protein degradation rate is estimated based on the accumulation of ammonia N plus synthesis of microbial N. But if the microbial protein turnover is not accounted for the rate of degradation may be underestimated in this method. However, this method does not determine the direct incorporation of amino acids into microbial protein.

The shortcomings of the methods presented above have prevented them from being widely accepted. The most important inherent problems of the *in vitro* techniques are: 1) the accumulation of the end product of degradation and the starvation of microorganisms, in the inhibitors method, 2) the sensitivity to ammonia concentration in the inoculum and the microbial turn over in the gas production method, 3) the short incubation time with limited sampling time points and 4) the direct incorporation of amino acids into microbial protein is not taken into account.

The extent of ruminal metabolism of RM *in vivo* has been studied earlier (Ahvenjärvi et al., 2002; Choi et al., 2002b; Brito and Broderick, 2007). Because of the changes in the current understanding of soluble protein metabolism in the rumen, especially that the degradation rate of soluble protein is not infinite, and that part of the soluble protein

escapes rumen degradation, more accurate estimates of the rate and extent of soluble protein degradation are needed to optimize the protein feeding in ruminants.

2. Objectives and hypotheses

The main objective of the studies presented in this thesis was to study the ruminal metabolism of different N fractions *in vivo* and *in vitro*. Rapeseed meal was selected as a source of protein as it is widely used and cultivated in the EU. To study the ruminal metabolism of different N fractions *in vitro,* the method introduced by Hristov and Broderick (1994) was modified by using ^{15}N labelled feed protein, and then the degradation of the same protein was investigated *in vivo*.

The specific objectives of this thesis were:

- to develop an *in vitro* method to study RM protein degradation
- to study metabolism of RM protein fractions *in vivo*
- to study the efficiency of utilization of N from different N fractions for milk protein synthesis

The main hypotheses tested in this research were:

- An extended incubation period (10 h) with several time point measurements allows a detailed time course analysis of N transactions *in vitro.*
- *In vitro* incubations of ^{15}N labelled RM provide sufficient information for an accurate estimation of RM protein degradation.
- Simultaneous *in vitro* incubations of ^{15}N labelled and unlabelled soluble protein with rumen fluid provide sufficient information for an accurate estimation of soluble RM protein degradation.
- Non-protein-N is efficiently utilized by lactating dairy cows for microbial N synthesis on diets containing moderate amounts of crude protein.
- Utilization of soluble N fraction of RM in the rumen is better than that of ammonia N.
- Utilization of soluble and insoluble N fractions of RM for milk protein synthesis is better than that of ammonia N in lactating dairy cows.
- Soluble feed protein escapes the rumen representing a source of AA for animal.

3. Materials and methods

3.1. Animals and diets

The work documented in Publications I to IV were conducted as three separate experiments. Experiments 1 and 2 were conducted using *in vitro* methods and the results are presented in Publications I and II. The third experiment was conducted *in vivo* and the results from this study are presented in two Publications (III and IV). The experimental procedures used are described in detail in corresponding Publications, only a brief description of materials and method is presented here.

Three Finnish Ayrshire dairy cows equipped with rumen cannulas were used as rumen content donors for the *in vitro* experiments. The diets of donor cows were formulated based on typical feed ingredients in use by Finnish dairy farmers. The diets were based on grass silage and concentrates with the ratio of 60:40 (on DM basis), respectively. One donor cow in the second *in vitro* experiment was fed a mixture of grass silage and red clover (67:33 wt/wt on DM basis).

The *in vivo* experiment was performed with four Finnish Ayrshire dairy cows equipped with rumen cannulas in mid lactation. For the experimental cows in the *in vivo* study, diet was formulated based on the typical feed ingredients in use by Finnish dairy farmers, with 60:40 ratio of grass silage and concentrates, respectively. The cows produced 32.9 kg/d of energy corrected milk (ECM), consumed 22.4 kg/d of dry matter (DM) of total mixed ration (TMR) with 15.5% of CP level, with average ruminal ammonia N concentration of 5.5 mg/100ml. All experimental procedures were approved by the Animal Experiment Board in Finland (Hämeenlinna, Finland) in accordance with the guidelines established by the European Community Council Directives 86/609/EEC.

To study the metabolism of soluble fraction of RM *in vitro* (II) and *in vivo* (III), the same ^{15}N labelled RM was used. Labelled and unlabelled RM for all studies presented in this thesis were prepared from whole rapeseeds (Brassica rapa L. ssp. Oleifera DC, variety Valo; Boreal Plant Breeding Ltd., Jokioinen, Finland) grown in 6 experimental plots in Jokioinen, Finland. Three plots were assigned for the production of unlabelled rapeseeds and 3 used for the production of ^{15}N-labelled rapeseeds. Each plot was fertilized with 6.6 kg of N, 26.7 kg of P, and 30.8 kg of K/ha, respectively. Twelve days after sowing, each plot was fertilized with 1,132 g of ammonium sulphate or ammonium sulphate enriched with 10% of ^{15}N for the production of unlabelled and labelled rapeseed, respectively. The chemical composition (Table 1) of the RM investigated in all experiments was rather similar to commercial solvent extracted RM on the Finnish feed market (Table 1).

To prepare RM, whole rapeseeds were ground up, and oil was removed by repeated (n = 9) diethyl ether extraction. Extracted RM was allowed to stand overnight at room temperature, then dried at 60 °C for 24 h, and homogenized using a mortar and pestle. Detailed description of the process is presented in Publication II.

Table 1. Chemical composition (mean ± SD) and protein solubility of rapeseed meals (RM) used as treatments in the experiments

Publication	I and II	II, III and IV	
	Unlabelled	^{15}N Labelled	Commercial RM[1]
DM, g/kg	923	920	893 (±0.9)
In g/kg DM			
OM	916	917	923 (±6.0)
N	63.1	60.7	61.4 (±1.64)
Crude fat,	45	50	57 (±4.0)
NDF	271	266	290 (±4.5)
CNCPS[2], g/kg N			
A	250	218	-
B_1	208	205	-
B_2	440	483	-
B_3	40	29	-
C	62	64	-

[1] The data for the composition of commercial RM (n=14) was compiled from laboratory analysis carried out at Natural Resources Institute Finland (Luke).

[2] Characterization of protein based on the Cornell Net Carbohydrate and Protein System (Licitra et al., 1996); A, non-protein N; B1, buffer soluble true protein, B2, protein insoluble in buffer but soluble in neutral detergent; B3, protein insoluble in neutral detergent but soluble in acid detergent, and C, protein insoluble in acid detergent.

3.2. *In vitro* methods

The *in vitro* experiments were based on the principles outlined by Hristov and Broderick (1994) with some modifications: 1) the *in vitro* incubations were carried out for an extended period up to 10 h, 2) a new composition of carbohydrate mixture was developed to provide a constant supply of energy over the course of incubation (Figure 2). The incubation vessels were 120 ml spinner flasks, with a total volume of incubations mixture of 100 ml, and with the inclusion of substrate equal to 12.5 mg of N/100 mL. The incuba-

tions were repeated three times with one incubation vessel for each time point within each run. To prepare the inoculum, the rumen contents were collected at 2 h after the morning feeding. Rumen contents were pooled and squeezed through two layers of cheesecloth. To enrich the inoculum with particle associated microorganisms, retained rumen solids were washed four times through two layers of cheesecloth with a warm (39 ºC) buffer as recommended by Craig et al. (1984). The buffers used in the *in vitro* experiments were prepared according to Goering and Van Soest (1970). The rate of energy supply available to rumen microorganisms was determined based on 24-h gas production using an automatic gas measurement system (Huhtanen et al., 2008a).

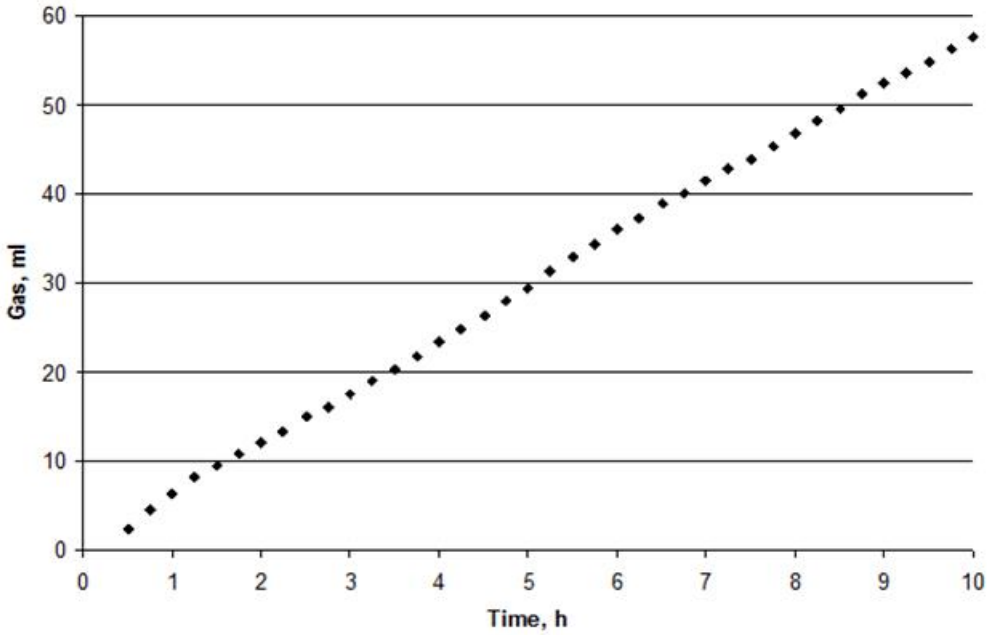

Figure 2. Cumulative gas production from carbohydrate mixture consisted of 60 mg of citrus fruit pectin, 80 mg of potato starch, and 360 mg of neutral detergent extracted grass silage used in the in vitro systems: Publication I and II.

3.2.1. Experiment 1

Incubation of unlabelled RM in the presence or ^{15}N labelled ammonium chloride with sampling times at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 h was carried out in Exp. 1. The incubations were terminated by adding sulphuric acid to a vessel, and the contents were freeze-dried and pool sizes of ^{15}N and ^{14}N were determined for ammonia N and non-ammonia N. To describe the kinetics of RM protein metabolism, a dynamic model (Figure 3) was fitted to the changes in the pool sizes of ^{15}N and ^{14}N in different N fractions using WinSAAm 3.0.7 software. Estimated parameter values were used to calculate the rate of degradation and the effective protein degradation.

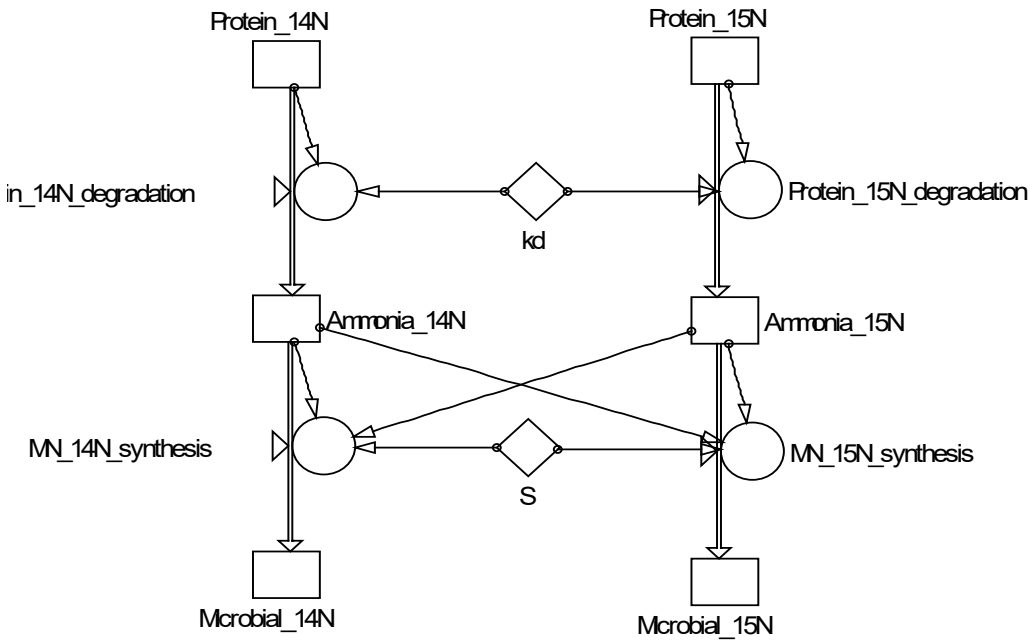

Figure 3. A model of protein N degradation to ammonia N and synthesis of ammonia N to microbial N, kd = a fractional rate (1/h) of protein N degradation to ammonia N, S = constant absolute rate (μg/h) of microbial N synthesis from ammonia N. Publication I.

3.2.2. Experiment 2

The Exp. 2 involved two simultaneous incubations with soluble RM protein (sRM): first one with unlabelled soluble RM protein (U-sRM) in combination with ^{15}N labelled ammonium chloride, and the second one with labelled soluble RM protein (L-sRM) in combination with unlabelled ammonium chloride. Samples were taken at 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 10 h. The incubations in Exp. 2 were terminated by placing the bottle in iced water, and the contents were separated by high speed centrifugation to solid and liquid fractions. The pool sizes of isotope ^{15}N and ^{14}N were determined for ammonia N, soluble non-ammonia N, and insoluble-N. To describe the kinetics of sRM protein metabolism, a dynamic model (Figure 4) was fitted to the changes in the pool sizes of ^{15}N and ^{14}N in these N fractions using WinSAAm 3.0.7 software. Estimated parameter values were used to calculate the rate of degradation and the effective protein degradation.

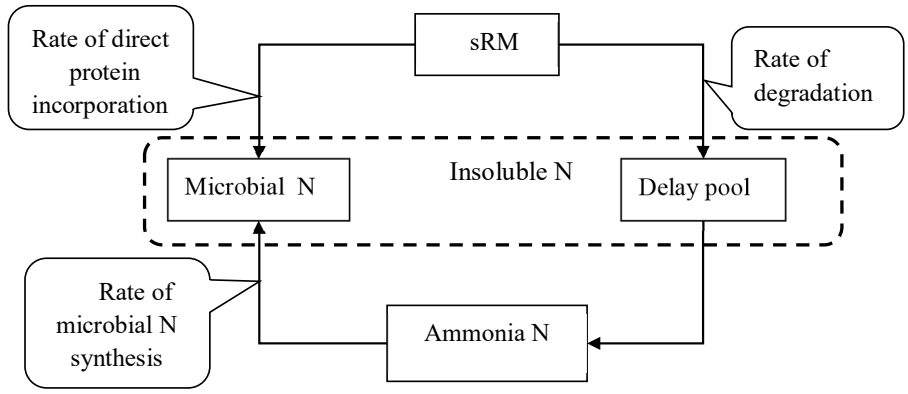

Figure 4. A model describing nitrogen transformations during degradation of soluble rapeseed meal (sRM) protein in vitro (Publication II)

3.3. *In vivo* experiment

W The in vivo experiment was designed to study the metabolism of three different N sources labelled with ^{15}N isotope: ammonia N, soluble and insoluble RM protein. The ruminal metabolisms of ammonia N and soluble RM protein are reported in Publication III. Ruminal metabolism of ^{15}N labelled insoluble RM-N was not included in this thesis. The efficiency of utilization of labelled N sources for milk protein synthesis is reported in Publication IV.

The study consisted of three periods. Each period lasted for 14 days with last 5 days dedicated for sampling. To facilitate rapid administration of labelled N and immediate sample processing, animals were split into two groups. The first group (two cows) received the treatment on day 9 of each period and the second group (two cows) the next day. Labelled N was administered as a pulse dose and mixed with the evacuated rumen contents. In total, three rumen evacuations were carried out to determine rumen pool sizes of soluble N, insoluble N and microbial N. Evacuations were conducted in each period on day 4, 9, and 12 in the first group of animals, and in the second group of animals one day later. Additionally, spot samples of rumen contents were collected before, immediately after and at 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 14, 17, 22, 27, 33, 39, 47, 55, 63, 72 and 82 h post administration of labelled N substrates for the analysis of ^{15}N and ^{14}N concentrations in N fractions. Liquid passage rate was determined based on LiCoEDTA marker dilution in rumen fluid. Rumen metabolism of labelled ammonia N was determined based on rumen pool sizes of ammonia-^{15}N and microbial-^{15}N. The metabolism of labelled soluble RM-N was determined based on rumen pool sizes of ammonia-^{15}N, microbial-^{15}N, and soluble RM-^{15}N.

To study the AN kinetics in vivo (III), a two-compartment model consisting of ammonia N and microbial N was developed (Figure 5A). The three-compartment model (Figure 5B) that consists of ammonia N, microbial N and SNAN was used to describe the SNAN kinetics in vivo (III) using WinSAAm 3.0.7 software. The estimated parameter val-

ues were then used to estimate (Powersim® 2.5 graphic modelling software) the proportions of N leaving the rumen via different pathways.

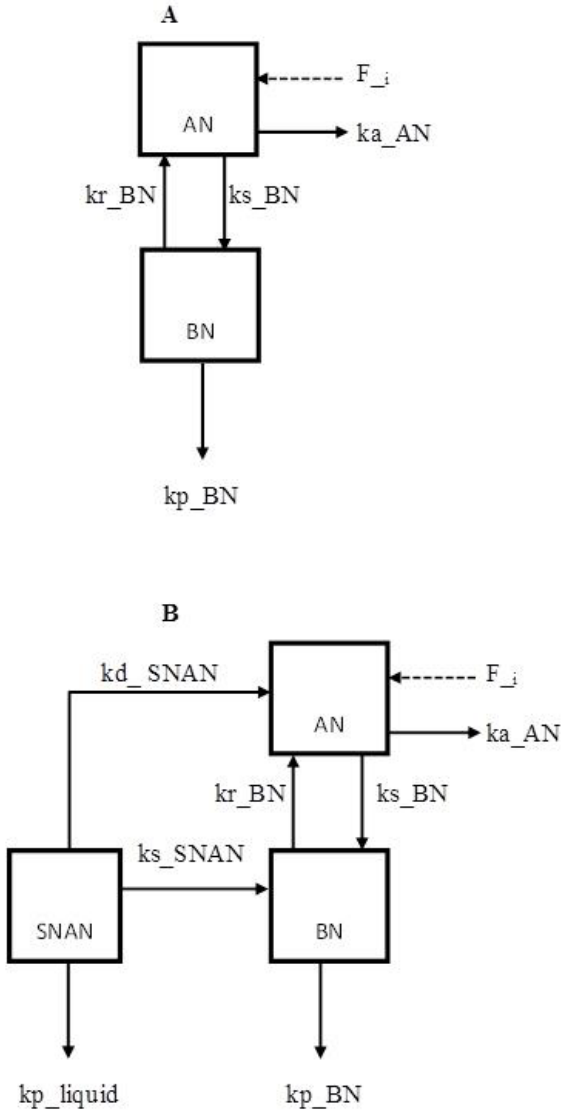

Figure 5. Schematic presentation of ammonia N (A) and soluble non ammonia N (B) models to describe ^{15}N metabolism in the rumen. AN, SNAN, and BN represent pool sizes (mg) of ammonia N, soluble non ammonia N and bacterial N, respectively; F_i = external input flux of ammonia N and urea N recycling (mg/d); ka_AN = fractional rate of ammonia N disappearance from the rumen (absorption + passage); ks_BN = fractional rate of ammonia N synthesis to bacterial N; ks_SNAN = fractional rate of direct uptake of soluble non ammonia N by bacteria; kd_SNAN = fractional rate of degradation of soluble non ammonia N to microbial N; kr_BN = fractional rate of bacterial N recycling to ammonia N; kp_liquid = fractional passage rate of liquid matter from the reticulo-rumen to the omasal canal; kp_BN = fractional passage rate of bacterial N from the reticulo-rumen to the omasal canal. Publication III.

The efficiency of N use for milk protein synthesis was investigated in the same in vivo experiment as described above. To study the diet MNE, N intake and milk N yield were determined (MNE; milk N/N intake). To investigate the MNE of different N fractions, the dose of ^{15}N isotope and the cumulative ^{15}N isotope secretion in milk over 108 h were determined, for AN, sRM-N, and insoluble rapeseed meal N (iRM-N) treatments. And the MNE for those fractions were calculated as: cumulative secretion of ^{15}N isotope in milk/dose of ^{15}N isotope, Publication IV.

4. Results and general discussion

4.1. Rapeseed chemical composition

Canola was developed in Canada in the 1970s from rapeseed plant (*Brassica napus, Brassica rapa or Brassica juncea*), and has very low levels of glucosinolates and erucic acid (Canola Council of Canada, 2019a) that have anti-nutritional properties. Now, modern varieties of rapeseed also have very low levels of glucosinolates and erucic acid and in Europe the modern varieties are called rapeseed. To avoid confusion for the reader not familiar with the subject, the term of rapeseed will be used, even if in original publication the term of canola was used.

In terms of OM, N, NDF and ether extract concentration the RM prepared for the studies were similar to the commercial products available on the Finnish feed market (Table 1, Ahvenjärvi et al., 1999; Luke, 2019). A more detailed analysis of protein composition according to CNCPS system (Sniffen et al., 1992) indicated that both RMs, the labelled and unlabelled, used in the current studies had higher proportions of soluble N fractions (A and B_1) and lower proportions of insoluble fractions (B_2, B_3, and C) than typically found in commercial products (Ahvenjärvi et al., 1999; Hedqvist and Udén, 2006).

The difference between the proportion of soluble and insoluble N fractions in the commercial RM and those used in the studies presented in this thesis (Publications II, III, and IV) may be caused by the rapeseed processing. During commercial rapeseed processing, steam and high temperatures are used to enhance oil removal (Canola Council of Canada, 2019b). Because only small quantities of labelled and unlabelled rapeseed were produced for those studies, rapeseeds were processed in our laboratory. No steam or heat was used during oil removal. Samadi et al. (2013) reported similar composition for untreated rapeseed seeds soluble N fraction (470 g/kg of CP) to the rapeseed meals reported in this thesis. The autoclaved rapeseed seeds (heated with moisture) had much lower proportions of soluble N fraction (140 g/kg of CP, Samadi et al., 2013).

4.2. Metabolism of rapeseed meal *in vitro*

The *in vitro* system introduced in Publication I was based on the principles outlined by Hristov and Broderick (1994). In this method, the samples are incubated for 6 h using two incubation times: 0 h at the beginning of the incubation and 6 h at the end of incubation. This method provides a limited number of time points, which does not allow a detailed time course analysis of ammonia N transactions *in vitro*. To increase the number of time points, the *in vitro* incubations were carried out for an extended period of time up to 10 h. To provide a constant energy supply for rumen microbes over the course of incubation, the composition of the carbohydrate mixture was modified.

To develop new composition of carbohydrate mixture that provides a constant supply of energy over the 10-h incubation period, small scale *in vitro* experiments using the

system described by Huhtanen et al. (2008a) were conducted. The rate of ATP yield for microbes was assumed to be directly related to the rate of fermentation gas production. Several commonly used energy sources were screened for their gas production profiles. In the next step, several carbohydrate mixtures were formulated and tested for their gas production profiles. Carbohydrate mixture that provided a constant rate of gas production over the 10h of incubation (Figure 2) was a mixture of 60 mg of citrus fruit pectin, 80 mg of potato starch, and 360 mg of neutral detergent extracted grass silage.

The *in vitro* metabolism of RM protein (I) was described using parameter values estimated by the model (Figure 3) based on temporal changes in ^{14}N and ^{15}N pool sizes of ammonia and NAN during the time of incubation. The rate of N degradation for RM estimated using the model was on average 0.06 /h, with the rate of microbial synthesis of 1.08 mg/h, and the EPD of 0.38 g/g (assuming an outflow rate of 0.09/h).

The ruminal degradability of RM N estimated in this study was lower than those estimated in a previous study for RM (0.50 g/g N) and rapeseed cake (0.41 g/g N) based on omasal canal flow measurements in dairy cows (Ahvenjärvi et al., 1999). In a later study, supplementation of grass silage-based diets with RM indicated that 0.72 of supplementary N was degraded in the rumen (Ahvenjärvi et al., 2002). Similar to that, Maxin et al. (2013) reported *in situ* rumen degradability of 0.48 g/g N (corrected for particle losses) for RM. Higher *in situ* rumen degradability of RM (0.64 g/g N) was reported by Heendeniya et al. (2012). Samadi et al. (2013) studying the effect of different heat treatments (autoclaving and dry heating) on rapeseed seed reported even higher *in situ* rumen degradability of rapeseed seed (0.75 g/kg N), but the *in situ* rumen degradability of autoclaved (heat and moisture) rapeseed seed was almost identical with the degradability estimated by Ahvenjärvi et al. (1999). However, based on higher proportion of soluble N fractions in the current RM, slightly higher ruminal degradability relative to commercial rapeseed products might have been expected.

To start from simple assumptions, the model used to describe the RM protein metabolisms in the rumen (I) was based on two hypotheses: 1) that all potentially degradable RM protein is degraded to ammonia N at a constant fractional rate and 2) that the rate of microbial N synthesis from ammonia N occurs at a constant absolute rate. The first hypothesis has an implicit assumption that potentially degradable protein constitutes of one entity in terms of degradation rate. This assumption can be questioned because soluble N fractions of protein are likely to have a higher rate of degradation than the insoluble fractions (Volden et al., 2002; Krishnamoorthy et al., 1983; Nocek et al., 1983). The second hypothesis is that the synthesis of microbial N from ammonia N is the major pathway between protein and microbial N pools must be questioned because there is evidence of direct incorporation of AA into microbial protein (Choi et al., 2002a; Reynal et al., 2007; II and III).

Despite the simplicity of the model it predicted accurately the synthesis of microbial N from the extracellular ammonia N. The model predicted well the pools of the ammonia ^{15}N and non-ammonia ^{15}N with small variation between the predicted and observed

trends in those pools. The parameter estimated for the rate of microbial N synthesis also had a small fractional standard deviation. However, any direct incorporation of AA into microbial N would result in higher proportion of ^{15}N synthesized into microbial N than the current model estimated and would result in underestimation of true degradation rate.

Using Powersim software, synthetic data was generated to assess the effect of direct incorporation of AA into microbial protein. A direct pathway from protein N to microbial N was added to the model presented in Figure 3. In this exercise it was estimated that 0.67 of the microbial N was synthesised from ammonia N and 0.33 from direct incorporation of the soluble protein. The proportion of microbial N synthesis from ammonia N varies in the literature, from 0.34 (Russell at al., 1983) to 0.82 (Hristov and Broderick, 1996). In Publication II, by means of using of ^{15}N ammonium N in one set of incubations and ^{15}N labelled sRM in another set of incubations, we estimated that 0.53 of microbial protein originated from ammonia N, while the remaining 0.47 was derived from direct incorporation of AA and small peptides. These results demonstrate that the simple model is likely to underestimate the rate of protein degradation if the direct utilization of AA and small peptides by microorganisms are not accounted for.

In Publication I, the microbial N synthesis was estimated based on the model (Figure 3) fitted to isotope transactions between microbial N and non-microbial N compartments. The microbial N synthesis can be quantified based on ^{15}N enrichment determined in microbial N and total NAN as suggested by Hristov and Broderick (1994). Using this approach in a preliminary study with ^{15}N labelled RM, bacteria samples harvested at 0 h of incubation were contaminated with feed protein as indicated by substantially higher ^{15}N enrichment of bacteria samples relative to the background levels. Attempts to modify the centrifugation method failed to alleviate this problem. Physical separation of the microbial sample from feed particle residues is always compromised by incomplete recovery of particle associated bacteria, which often have different enrichment levels relative to liquid associated bacteria (Ahvenjärvi et al., 2002). In this preliminary data, part of the 'contamination' was probably soluble protein adsorbed to the microorganism.

This first study (I) confirmed that the new carbohydrate mixture provides a constant energy supply for the rumen microorganism over a prolonged incubation time. As well as the multiply sampling during a prolonged incubation time allowed to conduct dynamic studies. The study presented in Publication I and in the preliminary trial indicated that when using an intact protein source, the data is difficult to interpret. For this reason, when planning the next studies, we decided to separate the protein source to soluble and insoluble protein fractions. It was hypothesized that: 1) the separated protein fraction will have less complex compositions, 2) the transaction between N pools during rumen metabolism will be less complex, 3) and the distinction between different N pools in the rumen will be more accurate.

4.3. Metabolism of solule fraction of rapeseed meal *in vitro* and *in vivo*

In Publication I, we reported the degradation of entire protein fraction without any fractionation according to solubility characteristics. Similarly, Hristov et al. (2001) reported the metabolism of ^{15}N labelled alfalfa silage and hay. This approach is potentially useful to study the metabolism of plant protein in ruminants but owing to the complex chemical composition of proteins and complicated transactions between different ruminal N pools, the data is difficult to interpret and sometimes the interpretation may be ambiguous. Also, there are substantial differences between soluble and insoluble N fractions in ruminal passage and degradation kinetics. Particulate matter has markedly lower passage rate than the soluble fraction (Gasa et al., 1991; Rinne at al., 2002; Hristov et al., 2003). Similarly, there are substantial differences between soluble and insoluble N fractions in degradation rate (Volden et al., 2002; Krishnamoorthy et al., 1983; Nocek et al., 1983).

Based on these profound differences between soluble and insoluble fractions and difficulties with interpretation of the data from entire protein, it seems justified to study the degradation kinetics of these fractions separately. It can be observed that the approach to studying the ruminal degradation of soluble and insoluble N fraction separately is gaining in popularity (Volden et al., 2002; Hedqvist and Udén, 2006; Ahvenjärvi et al., 2018). To study the metabolism of sRM we decided to start with the *in vitro* technique. Because none of the existing *in vitro* methods allows studying in detail different aspects (adsorption, direct incorporation of AA into microbial protein) of soluble protein metabolism in the rumen, a new *in vitro* method was developed.

New *in vitro* method. A new *in vitro* method was developed based on previous experiences using ^{15}N labelled ammonia to estimate microbial uptake of ammonia and protein degradability (Hristov and Broderick, 1994; Ahvenjärvi et al., 2009). This *in vitro* method introduced several novel aspects: length of incubations, method of introducing the ^{15}N isotope to the system, and the approach for data calculations. Usually, *in vitro* incubations are carried out between 4 h (Hedqvist and Udén, 2006) and 6 h (Hristov and Broderick, 1994: Hristov, 2002), but in this study (II) the incubations were carried out for 10 h. There is compelling evidence suggesting that rate of degradation of soluble protein is not infinite (III; Hedqvist and Udén, 2006; Ahvenjärvi et al., 2018) as assumed in the *in situ* methods. Therefore, a prolonged incubation may be beneficial in studying protein metabolism. In addition, extended incubation time allows to increase the number of time points, which in exchange allows for a more detailed time course analysis of protein N transactions *in vitro.*

In principle, there are two alternatives to introducing ^{15}N to the *in vitro* system. One method is based on using the ^{15}N labelled ammonium N and the second method using ^{15}N labelled protein sources. The novelty of our approach was that we used both meth-

ods of labelling simultaneously. At the same time two *in vitro* systems were studied. In one system unlabeled ammonia N was incubated in combination with ^{15}N labelled protein and in the second systems unlabelled protein was incubated in the presence of ^{15}N labelled ammonia. A crucial premise underlying this approach is that the protein source in both systems must be intrinsically identical. Use of this complementary approach allowed estimating both the extent of protein degradation and the microbial N synthesis, which was not possible if only one of the labelling methods was used. A model (Figure 4) with 4 pools was used to estimate the rate of sRM degradation based on combined data from simultaneous incubation of both systems (II).

Metabolism of soluble N fraction *in vitro*. In the *in vitro* study (II), the estimated rate of sRM protein degradation was 0.126 and the EPD was predicted at 0.45. In the same study (II), it was estimated that 0.53 of microbial protein originated from ammonia N, and the remaining 0.47 of microbial N was derived from direct incorporation of AA and small peptides.

In the literature, variable estimates for the *in vitro* degradation rates and extent of degradation for similar soluble N fractions have been estimated (Table 2). Hedqvist and Udén (2006) estimated the *in vitro* rate of sRM protein degradation at 0.19 and the EPD as 0.44. Bach et al. (2008) estimated that the EPD of sRM was 0.37 and for soluble soybean meal 0.30. In contrast, Hedqvist and Udén (2006) estimated the *in vitro* EPD of soybean meal as 0.73. In that study the sRM had 2.4 times slower degradation rate than the soluble N fractions of soybean meal. In contrast, Mahadevan et al. (1980) reported from the *in vitro* study that sRM had 1.4 times faster degradation rate than the soluble N fraction from soybean meal. The reports on comparisons of the *in vitro* degradation rate of soluble and insoluble fractions are also inconsistent. For example, Nocek et al. (1983) reported that insoluble soybean meal N degraded at an almost three times slower rate than the soluble soybean meal N. In contrast, Mahadevan et al. (1980) reported that the soluble and insoluble soybean meal N degraded at the same rate. In that study the sRM was degraded twice as fast as the iRM.

Table 2. Degradation rate and effective protein degradation (EPD) of soluble protein fraction of different feeds.

Soluble fraction	$K_d(h^{-1})$[1]	EPD[2]	Method	Reference
Casein	0.49	0.75	*In vitro*	Peltekova and Broderick, 1996
Casein	1.00	0.85	*In vitro*	Hedqvist and Udén, 2006
Wheat DDGS[4]	0.62	0.79	*In vitro*	Hedqvist and Udén, 2006
Soybean meal	0.46	0.73	*In vitro*	Hedqvist and Udén, 2006
Soybean meal	_	0.30	*In vitro*	Bach et al., 2008
Pea seed	0.39	0.71	*In vitro*	Hedqvist and Udén, 2006
Lupine seed	0.34	0.56[5]	*In vitro*	Hedqvist and Udén, 2006
Rapeseed cake	0.19	0.54	*In vitro*	Hedqvist and Udén, 2006
Rapeseed meal	0.19	0.44[5]	*In vitro*	Hedqvist and Udén, 2006
Rapeseed meal	_	0.37	*In vitro*	Bach et al., 2008
Rapeseed meal	0.13	0.45	*In vitro*	Stefański et al., 2013 (II)
Rapeseed meal	0.73	0.82	*In vivo*	Stefański et al., 2020 (III)
Linseed cake	0.18	0.46[5]	*In vitro*	Hedqvist and Udén, 2006
Fish meal	_	0.99	*In vitro*	Bach et al., 2008
Ryegrass	0.49	0.75	*In vitro*	Hedqvist and Udén, 2006

Birdsfoot trefoil Norcen	0.46	0.74	*In vitro*	Hedqvist and Udén, 2006
White clover	0.46	0.74	*In vitro*	Hedqvist and Udén, 2006
Red clover	0.33	0.67	*In vitro*	Hedqvist and Udén, 2006
Mixture, grass[6]	2.02	0.92	*In vivo*	Volden et al., 2002
Timothy grass silage [8]	1.227	0.87[7]	*In vivo*	Ahvenjärvi et al., 2018
Timothy grass silage [8]	0.129	0.45	*In vitro*	Vaga and Huhtanen, 2018
Timothy grass silage [9]	0.111	0.41	*In vitro*	Vaga and Huhtanen, 2018
Alfalfa silage	0.285	0.64	*In vitro*	Peltekova and Broderick, 1996
Timothy grass, dry	0.112	0.41	*In vitro*	Vaga and Huhtanen, 2018
Alfalfa hay	0.273	0.63	*In vitro*	Peltekova and Broderick, 1996

[1] Fractional degradation rate

[2] Effective protein degradation rate, calculated as $K_d/(K_d + K_p)$, where K_p = rate of passage. K_p = 0.16 unless specified otherwise.

[3] Ruminal escape of soluble protein (1-EPD)

[4] Dried distillers' grain with solubles from wheat

[5] EPD calculated as $\exp(-K_pL) \times k_d / (K_d + K_p)$, were L = discrete lag time (h)

[6] Mixture of timothy and meadow fescue, K_p = 0.166, complex model

[7] K_p = 0.178

[8] Preserved with formic aside base additive

[9] Ensiled without additiv e

As discussed above, measurements from different *in vitro* studies show that the degradation rates of soluble proteins are very variable. This suggests that either there are true differences in the rate and extent of degradation among the same feed or the differences between the *in vitro* methods result in large differences in estimated values. Usually, differences in the rate and extent of degradation between feeds within a study tend to indicate true differences in these parameters. However, the differences between studies and *in vitro* methods cannot be reliably attributed to either source of variation. The differences among feeds can be attributed to the composition of the soluble protein (proportions of AA, peptides and soluble protein). As an example, grass silage SNAN is mainly comprised of AA and peptides (Ahvenjärvi et al., 1999; Hedqvist and Udén, 2006), whereas rapeseed SNAN mainly consists of soluble true protein (Ahvenjärvi et al., 1999; Volden et al., 2002; Hedqvist and Udén, 2006). According to Wallace et al. (1997) the degradation rates vary between peptides. In addition physical structure of the soluble protein may affect the degradation rate (Broderick et al., 1988; Peng et al., 2014; Prates et al., 2018). Samadi et al. (2013) studied the effect of heat-induced changes to protein molecular structure (amide I, amide II, ratio of amide I to II, α-helix, β-sheet, ratio of α-helix to β-sheet) on yellow and brown canola seed utilization and availability. In that study the *in situ* protein degradabilities were positively correlated with heat-induced protein molecular structure changes of α-helix to β-sheet ratios, and the intestinal digestibility of protein was negatively correlated with the protein α-helix to β-sheet ratios. Similar to that, studies on the effects of heat-induced changes in protein molecular structure on protein degradability of pea (Yu et al., 2015), flaxseed (Doiron at al., 2009; Khan et al., 2015), camelina (Pang at al., 2014), and barley (Prates et al., 2018) authors concluded that the changes in rumen protein degradability and intestinal digestibility were associated with changes in protein molecular structures. To validate the data from the in vitro method and to study the metabolism of soluble N fraction in more detail, an in vivo experiment was conducted, using the same ^{15}N labelled RM.

Metabolism of the soluble N fractions *in vivo*. In the *in vivo* study (III), the rate of degradation of sRM was estimated to be 0.73 and the EPD was 0.82. We also estimated that 0.51 of microbial protein originated from ammonia N, and correspondingly 0.49 from direct incorporation of amino acids and small peptides in the AN treatment.

In the study of Hedqvist and Udén (2006), authors attempted to estimate the rate and the EPD for soluble N fraction of rapeseed cake and peas *in vivo*. After a pulse dose of soluble N fractions to the rumen, the soluble N fraction concentrations were measured. However, only 0.33 and 0.26 of the initial (dose + rumen soluble N) N concentrations were recovered 30 min after the dose of soluble N fraction of rapeseed cake and peas, respectively. Furthermore, 1 h after the dose the concentrations of soluble N fractions returned to the base line. Because of that, the authors were not able to estimate the degradation rates and the EPD for those N fractions. In a recent *in vivo* study, similar to that of Publication III, Ahvenjärvi et al. (2018) reported very high rate of degradation

(1.23 per h) for soluble N fractions of grass silage. In addition the EPD estimated from the reported value was very high (0.87) but the value was similar to the EPD of sRM (0.82) reported in our *in vivo* study (III).

Microbial protein. Using a dynamic model, we estimated that 0.69 and 0.81 of N outflow from the rumen as bacterial N in that study (III). It is well established that bacteria that ferment structural carbohydrates use ammonia N as a sole N source for protein synthesis, whereas bacteria that ferment non-structural carbohydrate (NSC) use a wider range of N substrates (ammonia N, AA, and small peptides) for protein synthesis. In the CNCPS model, presence of AA improves the microbial growth efficiency of NSC fermenting bacteria up to 18.7% (Russell et al., 1992). The stimulative effects of free AA and peptide on microbial protein synthesis have been confirmed in previous studies (Atasoglu et al., 2001; Walker et al., 2005). As discussed above, the proportion of microbial protein synthesis from ammonia N and direct incorporation of soluble dietary N fractions is not constant, because the proportion of the soluble N fractions and ammonia N differs according to the diets and is known to vary over the time (Wallace and McKain, 1990; Williams and Cockburn, 1991; Atasoglu et al., 1999). Hristov et al. (2009) studying the effects of lauric acid and coconut oil on ruminal fermentation reported that proportion of microbial N originating from ammonia N ranged from 0.42 to 0.50. Foley et al. (2006) estimated the proportion of microbial N originating from ammonia N ranged from 0.56 to 0.63 when feeding dairy cows with different grain types. In earlier reports the proportion ranged from 0.34 (Russell et al., 1983) to 0.82 (Hristov and Broderick, 1996). Ahvenjärvi et al. (2018) reported from the *in vivo* study with pulse dose of ammonia N to the rumen that 0.53 of microbial N derived from ammonia N. In that study, the proportion of microbial protein that originated from ammonia N with the soluble grass silage N treatment increased to 0.62. Similarly, in our *in vivo* study (III) the proportion increased from 0.51 to 0.85 with the AN and SNAN treatments, respectively. This data indicates that the presence of free AA and peptide have stimulative effects on microbial protein synthesis.

Comparison of *in vitro* and *in vivo* SNAN metabolism. When comparing the rate of degradation and the EPD of sRM *in vitro* (II) and *in vivo* (III) it can be observed that both of them were higher (5.8 and 1.9 times higher, respectively) in the *in vivo* study. Part of the explanation may be that the observed proportion of sRM adsorbed to microbes was 2.8 times higher in the *in vivo* (III) than in the *in vitro* study (II). In addition the *in vitro* EPD for sRM (Table 2) reported by Hedqvist and Udén (2006) and Bach et al. (2008) was lower than the *in vivo* estimate (III). The same trend can be observed when comparing the *in vivo* and *in vitro* soluble protein degradation from timothy grass silage (Ahvenjärvi et al., 2018; Vaga and Huhtanen, 2018). This data suggests that *in vitro* EPD values are underestimated for these feeds. Meta-analysis of Hristov et al. (2012) on variability of the *in vitro* systems indicated that the estimates from *in vitro* systems for different parameters (e.g. volatile fatty acid concentration, digestibility of organic matter) are usually lower than those observed *in vivo*. This meta-analysis also indicated that the variabil-

ity of the *in vitro* estimates is higher than in the *in vivo* data. The rather low estimates of *in vitro* EPD leads to high estimates of the proportion of SNAN escaping the rumen compared to the *in vivo* data. This suggests that if the data was used for diet optimization the production responses to inclusion of these protein feeds might be lower than anticipated by EPD. If a cow consumes 2.2 kg RM (III), assuming that 0.25 of RM is soluble N, and using data from Table 2 (EPD 0.37 and 0.82 for *in vitro* and *in vivo,* respectively) it can be estimated that using the EPD from *in vitro* system overestimates the milk production from RM supplementation by 1.5 kg/d.

The inherent problems of in vitro methods. Many factors may explain or contribute to the low estimates of protein degradation rates derived from the *in vitro* systems. One factor affecting the *in vitro* estimates is inoculum (Mould et al., 2005). Low microbial activity usually observed in the *in vitro* systems may explain the lower estimates from those systems. This may be attributed to lower proportion of particle associated bacteria in strained rumen fluid than in the rumen contents. Furthermore, the microbial community in the *in vitro* system changes over time (Hristov et al., 2012; Danielsson et al., 2014) and also differs from that of the *in vivo* donor animals as it becomes less diverse over the course of in vitro incubation (Mansfield et al., 1995; Martínez et al., 2010; Mateos et al., 2015). Another factor affecting the inoculum quality is the diet of donor animals (Broderick et al., 2004). If the tested protein is not included in the donor animal's diet the bacteria in the inoculum are not accustomed to metabolizing that protein. In addition the time of collecting the inoculum has big influence on its quality. Collecting the inoculum before morning feeding decreases the effect of the diet of donor animal and increases the microbial activity of rumen inoculum compared to 2 or 4 h after feeding (Broderick et al., 2004). It could be speculated that in the inoculum collected after morning feeding bacteria have received a morning meal in the rumen and have adsorbed protein and are not 'hungry' for a new protein meal for a while. On the contrary, pre incubation of inoculum to deplete N reserves and increase the microbial activity probably leads to 'N starvation of microbes'. In addition, adsorption plays an important role in protein metabolism and possibly also in outflow of soluble protein from the rumen as will be discussed further in chaprer 4.4 and 4.5. There are probably also some unidentified factors in the *in vitro* conditions that do not fully correspond to those *in vivo*.

The *in vitro* system presented in Publication II was also probably affected by the factors described above. Despite the hypothetical advantages compared to previous *in vitro* systems, the current *in vitro* approach also suffered from the limitations discussed above. Accurate measurements are of little benefit unless the *in vitro* conditions fail to simulate the *in vivo* environment and provide realistic estimates of protein degradation rate and EPD.

4.4. Adsorption of soluble N to microbes

Adsorption. According to the previous findings, the event of adsorption of soluble N fraction to rumen bacteria plays a very important part in the extracellular ruminal protein metabolism. The extracellular processes involve protein solubilisation, adsorption onto bacterial cell surfaces, hydrolysis, and transportation into microbial cells (Russell et al., 1991). The solubilisation is a process where protein is exposed to saliva during mastication, and that is intensified during transition into the rumen. This step is followed by adsorption of soluble N to the surface of rumen bacteria (Nugent and Mangan, 1981; Wallace, 1985), and hydrolysis of soluble protein to peptides and AA and transportation of these intermediates inside the bacterial cells (Russell et al., 1991; Nolan, 1993). The excess of ammonia N and AA released within the cell are excreted to extracellular space (Stevenson, 1978; Nolan, 1993; Firkins et al., 2015). This feature of proteolytic bacteria indicates a constant turnover between bacterial and extracellular pools. In recent years, increasing numbers of Publications have reported the phenomenon of adsorption, and it can also be observed that the extent of adsorption of soluble protein N to rumen microbes has varied between the reports from 0.010 to 0.039 of bacterial N pool size (Wallace, 1985; Hedqvist and Udén, 2006; Publication III).

Adsorption of SNAN. The phenomenon of adsorption of SNAN was observed in all the experiments reported in this thesis. In the preliminary studies using ^{15}N labelled RM (I) part of the contamination of bacterial samples by the RM protein at 0 h of incubation with rumen fluid was probably explained by the soluble protein adsorbed to the microbial mass. In Publication II, the adsorption was observed in both incubations. It was estimated that 0.20 of ^{15}N labelled soluble RM N was adsorbed to bacterial mass. In Publication III, 0.56 (0.039 of bacterial N) of ^{15}N labelled soluble RM N dose was immediately adsorbed to bacteria.

Studies of Nugent and Mangan (1981) on ^{14}C-labelled fraction I leaf protein from lucerne and later study of Wallace (1985) on ^{14}C-labelled casein indicated rapid adsorption of soluble protein onto microbial cells. Furthermore, Wallace (1985), based on the *in vitro* studies with rumen inoculum, estimated that the capacity for protein adsorption did not exceed 10 µg/mg of bacterial protein with the implication that microbial N recovered by centrifugation may contain up to 0.01 of soluble dietary protein. In the *in vivo* work with dairy cows, Hedqvist and Udén (2006) reported that 0.33 and 0.26 of rapeseed cake and pea soluble proteins were recovered 30 min after dosing to rumen content, respectively. Assuming 200 g of ruminal bacterial N pool size and using the data reported by Hedqvist and Udén (2006), the estimated extent of adsorption corresponded to 0.036 and 0.039 of bacterial N pool size for rapeseed cake and pea soluble proteins, respectively. The authors speculated that the results may have been overestimated by quick escape of a fraction of the dose administered into the rumen as indicated by lower observed Co concentration than that expected at 30 min after dosing. However,

the extent of adsorption in that study was similar to that reported in Publication III for the soluble RM protein (0.039). In Publication (III), the calculations were based on direct measurements of isotope ^{15}N in bacteria. In a recent study with dairy cows on ruminal metabolism of ^{15}N labelled grass silage soluble N fraction, Ahvenjärvi et al. (2018) reported that 0.20 of the doses of soluble fraction of grass silage ^{15}N were adsorbed to microbial N within 30 min after the dose. Assuming 200 g of rumen pool size of microbial mass N it corresponded to 0.013 of microbial N. The extent of adsorption reported in that publication was in close agreement with the maximum extent of adsorption proposed by Wallace (1985), but lower than the value reported in Publication III or by Hedqvist and Udén (2006). Ahvenjärvi et al. (2018) also reported an unexpected decrease (by 0.16) in bacterial ^{15}N enrichment 3 h after feeding. This observation could be explained by adsorption of unlabelled soluble protein to bacteria thus decreasing their ^{15}N enrichment. Similar to findings of Ahvenjärvi et al. (2018), we also observed (III) lower ^{15}N bacteria enrichment 1 h after feeding compared to the mean of previous and subsequent samples, but the extent of the decrease was lower (from 0.02 to 0.10).

Absorption of ammonia N. The phenomenon of instant uptake of N by rumen bacteria appears also with ammonia N. In Publication I, 0.17 of ^{15}N labelled ammonia N was detected immediately in the NAN (probably absorbed into bacterial cells). Similarly, analysing the model performance with the ^{14}N pools of NAN and ammonia N in that publication, it could be observed a constant under prediction of ^{15}NAN and over prediction of ammonia ^{15}N at the beginning of incubation with opposite effects towards the end of the incubation. These observations suggest rapid incorporation of the ammonia ^{15}N in the beginning of the incubation. In Publication II, 0.08 of ammonia ^{15}N was detected immediately in bacterial mass. In the *in vivo* experiment (Publication III), 0.16 of the ammonia N dose was immediately detected in microbial N, accounting for 0.005 of total microbial N pool.

Ahvenjärvi et al. (2018) reported that 0.04 of the ammonia ^{15}N dose was detected in microbial N within 30 min after dose and it corresponded to 0.0016 of microbial N. The extent of absorption of ammonia N reported in that publication was in close agreement with the maximum extent of instant absorption proposed by Wallace (1985), but lower than the value reported in Publication III. Rumen bacteria seems to efficiently absorb ammonia N to maintain sufficient concentration of intracellular ammonia N even if extracellular ammonia N concentrations are low. Russell and Strobel (1987) suggested that rumen bacteria may actively transport ammonia N from extracellular pool to maintain sufficient intracellular concentration. In a recent study of Ahvenjärvi and Huhtanen (2018) efficient absorption of ammonia N from extracellular space was reported when diet CP concentration was low (127 g/kg of DM). Due to efficient absorption of ammonia N from extracellular space rumen ammonia N concentrations did not respond to increasing levels of urea N supplement (up to 49 g/d) and only the highest level of urea N supplement into the rumen (66 g/d) increased ammonia N concentration in extracellular space. These findings suggest that bacterial uptake of AN is dependent on diet CP con-

centration. On diets with limited N supply, the microbes use AN from extracellular space very efficiently but when the N supply increases the efficiency of utilization decreases. In addition Marini and Van Amburgh (2003) reported efficient capture of ammonia N from extracellular space by rumen bacteria on a diet with very low CP concentration (91 g/kg DM). The extent of rapid absorption of ammonia N by rumen microorganism may be affected mainly by diet CP concentration in the *in vivo* and composition of buffer and inoculum in the *in vitro* systems.

Adsorption *in vitro* and *in vivo.* The phenomenon of protein adsorption to microbial cells complicates the distinction between microbial and feed N pools, or between rumen degradable and undegradable protein. The process of adsorption of soluble protein onto microbial cells may play an important role in the escape of soluble protein out of the rumen if protein is adsorbed into cells but not metabolized before entering the omasal canal.

The results obtained from both *in vitro* and *in vivo* methods confirm the phenomenon of protein adsorption and allow estimating the extent of adsorption of soluble N by microorganisms. However, the quantities of adsorbed soluble N estimated from the *in vitro* system may be difficult to extend to *in vivo* conditions. In the *in vitro* system the inoculum is diluted rumen fluid depleted in N, probably leading to 'N starvation of microbes. For instance, in Publication II, assuming that all insoluble N was microbial mass and 0.20 of ^{15}N labelled soluble RM N was adsorbed to bacteria the extent of adsorption was 0.36 of bacterial N. In a recent work, Udén (2013) using macro *in vitro* method (buffer and 5 to 7.5 kg of rumen contents as inoculum and fermentation medium), reported that only 0.56 of casein was recovered at first sampling (3 min). In these studies, reported by Udén (2013), with increasing casein dose, the proportion of recovered dose decreased. However, the absolute adsorption (loss from the medium) of casein was relatively constant at 358 mg/kg (SD = 47) of rumen contents (Udén, 2013). Assuming 100 kg of rumen content and 200 g of rumen pool size of microbial mass N, the absolute adsorption corresponded to 0.18 of microbial N. This estimate is much lower than that from study II (0.36) probably because the macro *in vitro* method used large amount of rumen contents as inoculum, operated under rumen-like condition, and the N starvations of microbes was not occurring. For these reasons it could be expected that the estimates from the macro *in vitro* method would be similar to the *in vivo* conditions, but they were more than 4.5 times higher than the estimates from *in vivo* (0.036 – 0.039). One of the reasons for higher estimates of protein adsorption presented by Udén (2013) compared to our estimates (III) may be the unique structure of casein. Both micelles and gel forms have highly active surface forming properties (Horne, 2002). Using ^{15}N labelled casein in similar study could yield more information on the fate of instant casein disappearance from rumen fluid. This data suggests that also the protein structure may influence the extent of adsorption of soluble N by microorganisms. In addition the ideal AA profile may play a role in the increased adsorption of casein protein. If the composition of feed N affects the extent of soluble protein adsorption, it would explain some of the

variation in the extent of adsorption of soluble protein by microorganisms from different feeds in similar conditions. As an example, in the *in vivo* studies with grass silage-based diet with 155 g/kg of CP, Ahvenjärvi et al. (2018) reported adsorption of soluble grass silage protein up to 0.013 of rumen microbial protein mass, but in Publication III sRM was adsorbed up to 0.039 of rumen microbial protein mass. Grass silage SNAN in composed mainly from AA and peptides (non-protein N), whereas sRM is mainly true protein. If the protein structure and composition of feed N influence the extent of adsorption it complicates even more the process of prediction of protein adsorption. More research is needed to confirm this speculation.

4.5. Outflow of soluble rumen undegradable protein

SNAN outflow in the *in vivo* studies. Microbial protein and rumen undegradable protein (RUP) are the two sources of absorbed amino acids in ruminants and basis of the modern protein evaluation systems (e.g. Luke 2019). Together they are called metabolizable protein. The metabolizable protein systems have been developed in recent decades to improve the protein feeding in ruminants by accounting for the contribution of these sources to the AA supply. Most of the feeding systems estimate the supply of microbial N and RUP based on the estimates of protein degradation *in situ*. This commonly used method proposed by Ørskov and McDonald (1979) assumes that all protein, peptides and AA capable of escaping the bag have an infinite degradation rate with no escape of SNAN out of the rumen. However, our measurements (II and III) and several earlier studies (Chen et al., 1987; Choi et al., 2002a, 2002b; Reynal et al., 2007; Ahvenjärvi et al., 2018) have shown that such an assumption is not realistic but a portion of SNAN escapes the rumen undegraded.

In the *in vivo* study (III), it was estimated that 0.08 of the SNAN dose escaped the rumen as SNAN. Using fluid kinetics method, Chen et al. (1987) estimated that 0.061 of N intake escaped the rumen as SNAN. Based on measurements of digesta flow entering the omasal canal Choi et al. (2002a and 2002b) reported that for 9 grass silage based diets the escape of SNAN as a proportion of N intake was 0.067. Similarly, in cows fed grass silage-based diets between 0.05 to 0.08 of the dietary SNAN escaped the rumen depending on the harvest date and the level of concentrates (Choi et al., 2003). Reynal et al. (2007) reported that depending on the dietary CP source, on average 0.10 of total AA flow from the rumen was dietary SNAN. Also Hristov and Broderick (1996) reported that 0.11 of N intake escaped the rumen as SNAN on alfalfa silage-based diet. In the study similar to that presented in Publication III (ruminal pulse dose of ^{15}N labelled soluble N fraction from grass silage), Ahvenjärvi et al. (2018) reported that 0.13 of the grass silage SNAN dose escaped the rumen as SNAN.

Choi et al. (2002a) observed a positive intercept for the relationship between SNAN intake and SNAN flow entering the omasal canal suggesting that a substantial proportion of SNAN flow entering the omasal canal originated from microbial N. In addition studies

using ^{15}N as a microbial N marker demonstrated that part of the SNAN escaping the rumen was of microbial origin (Choi et al., 2003; Reynal et al., 2007). Choi et al. (2002a; 2003) following ruminal administration of ^{15}N ammonia N, reported that microbial N contribution to omasal canal SNAN flow ranged from 0.61 to 0.71.

SNAN outflow in the *in vitro* studies. Using the *in vitro* method in Publication II we estimated that 0.57 of SNAN fraction from RM escaped ruminal degradation. In that report the estimated ruminal escape of SNAN fraction from RM was calculated according Hedqvist and Udén, 2006 (i.e. 1-EPD). There is extensive *in vitro* literature demonstrating that part of SNAN may escape the rumen degradation and contribute to the supply of AA available for the host animal (Table 2). Hedqvist and Udén (2006) estimated that ruminal escape of SNAN ranged from 0.15 to 0.56 in concentrate feeds. In that report the estimated ruminal escape of SNAN fraction from RM was 0.56. In addition Bach et al. (2008) reported that 0.63 of SNAN from RM escaped the rumen.

The extent of SNAN escaping the rumen is mainly dependent on the rate of SNAN degradation and the passage rate of liquid fraction out of the rumen. As was discussed in chapter 4.3, the degradation rates of soluble proteins are very variable. In addition the phenomenon of adsorption may play an important role in the extent of SNAN escape as it was discussed in the previous chapter (4.4). If the level of CP in the diet influences protein uptake and degradation by the rumen microbes it means that the extent of SNAN degradation and that of protein in general, is not a constant but a variable dependent on external factors.

Overall, the extent of degradation in the rumen and escape of SNAN from the rumen indicate that this fraction may be more important in fulfilling the AA requirements of ruminants than predicted by the current feeding systems. If the feeding system does not account for the SNAN escaping the rumen it may not estimate correctly the MP. According to our data (III), a system that ignores SNAN escape underestimates the MP of solvent extracted RM by 2%. This example highlights the need to account for the contribution of rumen undegradable SNAN to the estimates of feed MP.

SNAN outflow in protein evaluation systems. In recent years some protein evaluation systems have attempted to overcome this shortcoming by assuming a constant degradation rate for the SNAN fraction. In the NorFor system (Volden, 2011), an assumption was made that the SNAN fraction is degraded at a constant rate of 2.0/h. Van Duinkerken et al. (2011) assumed a corresponding value of 1.5/h. The upgraded version of Cornell Net Carbohydrate and Protein System (CNCPS) uses rates of 2.0/h for soluble non-protein N fraction (PA1) and 0.10 to 0.40 for soluble true protein N fraction (PA2) as described by Van Amburgh et al. (2015). These attempts are likely to increase the accuracy of MP estimates, but may not account for all SNAN escaping the rumen. In our *in vivo* study (III) the first-order disappearance rate of labelled RM SNAN (PA1 + PA2 in the upgraded CNCPS system) during the first 1.5 h after dosing was on average 0.52 (SD=0.070) per h. This rate includes degradation of SNAN to microbial N, uptake to microbial pool and passage out of the rumen. These observations suggest that the degra-

dation rate of RM SNAN is rather slow compared to the constants assumed in previous updates to MP systems.

Potentially, a fraction of soluble protein entering the rumen may adsorb to microbial cell surfaces but escape the rumen associated with microbial cells prior to further metabolism in the rumen or synthesis into microbial protein. A clear definition of microbial protein synthesis is necessary. If it is accepted that microbial protein synthesis is the "Process of protein synthesis from AA and ammonia N" then adsorption is not protein synthesis. The definition of microbial protein synthesis has direct consequences on the definition of rumen degradability. As a biological phenomenon SNAN adsorption to bacterial cells may change the intestinal digestibility and the AA profile of microbial protein entering the small intestine, since the intestinal digestibility and AA profile of SNAN and microbial protein are different (Table 3). However, the quantitative significance of this phenomenon still remains to be explored.

Since the profile of AA composition of milk differs from that of soluble RM and bacterial protein (Table 3), the profile of AA composition of these proteins is important when considering the utilization for milk protein synthesis. Histidine has been reported to be the first limiting AA in cows fed grass silage-based diets (Vanhatalo et al., 1999; Kim et al., 1999: Korhonen et al., 2002a; Huhtanen et al., 2002). In the North American diets based on maize and lucerne, Met supplementation has consistently improved milk protein yields (Zanton et al., 2014). Increase in DMI, milk and milk protein yields were reported in low CP diet supplemented with His, Met and Lys (Lee et al., 2012; Giallongo et al., 2016: Nursoy et al., 2018). The concentration of His in soluble RM protein is greater (proportionally 1.37) than in the microbial protein (Table 3). Consequently, the outflow of soluble RM protein is not only increasing the supply of AA to the host animal, but also because of its higher His concentration compared to bacterial protein, it may improve the profile of AA absorbed from the intestine and stimulate the milk protein synthesis.

Using the *in vivo* study presented in this thesis (III) as an example where cows consumed 2.2 kg/d of RM, it can be estimated that the SNAN escaping the rumen could support production of 0.3 l milk per day. This estimate was based on assumptions that SNAN concentration in RM protein is 250 g/kg and 0.08 of SNAN escapes ruminal degradation (III). It could be speculated that also part of the SNAN adsorbed to microbial cells escapes the rumen degradation, and further contributes to AA supply to the small intestine. That SNAN adsorbed to microbial N may be of higher value to the animal than microbial N. SNAN consists of 100% AA and they are fully available for the host animal, whereas microbial N consists of 80% AA and 20% of nucleic acids (NRC, 1985, 2001; Sok et al., 2017).

Table 3. Amino acid composition (g of AA/100 g CP) of soluble RM fraction (sRM), rumen microbial and cow milk protein.

Amino acid	sRM[1]	Microbial protein[2]	Milk protein[3]
Essential AA			
Arg	5.7	4.8 (±0.10)	3.7
His	2.7	1.9 (±0.19)	2.9
Ile	3.6	5.7 (±0.12)	6.2
Leu	6.4	7.8 (±0.23)	10.3
Lys	5.4	7.6 (±0.60)	8.8
Met	2.0	2.5 (±0.26)	3.0
Phe	3.5	5.2 (±0.21)	5.2
Thr	3.8	5.6 (±0.16)	4.6
Val	4.7	6.0 (±0.24)	6.9
Nonessential AA			
Ala	4.3	7.2 (±0.53)	3.5
Asp	7.1	11.9 (±0.15)	3.7
Cys	2.5	1.4 (±0.20)	0.8
Glu	20.1	12.7 (±0.32)	9.8
Gly	4.7	5.6 (±0.24)	1.9
Pro	6.3	3.5 (±0.28)	10.5
Ser	3.8	4.5 (±0.04)	6.7
Tyr	2.3	5.1 (±0.5)	5.9
Essential AA[4]	37.8	47.2	51.6
Nonessential AA[5]	51.0	51.9	42.8
BCAA[6]	14.8	19.5	23.4
Total AA[7]	88.8	99.0	94.3

[1]Soluble fraction of RM from Publication III, g of AA/100 g CP.

[2]Average with SD from data presented by: Le Hénaff, 1991; Clark et al., 1992; Korhonen et al., 2002b; Sok et al., 2017, g of AA/100g of true protein.

[3]Lapierre et al., 2012, g of AA/100g of true protein.

[4]Essential AA (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val).

[5]Nonessential AA (Ala, Asp, Cys, Glu, Gly, Pro, Ser, and Tyr).

[6]BCAA = branched-chain AA (Val, Ile, and Leu).

[7]Total AA = EAA + NEAA.

There is some evidence that the rumen protein degradation may be a saturable process or at least with increasing levels of protein dose the degradation rate decreases and the soluble protein outflow increases. Volden et al. (2002) reported that with increasing doses of soluble fraction of grass silage N the *in vivo* degradation rates decreased and the outflow increased. In addition Udén (2013) in a more recent report from macro *in vitro* study indicated that the highest dose of casein had the lowest degradation rate, which in turn suggested a higher protein outflow. The same author also reported that the adsorption per kg of rumen contents did not increase with increasing casein doses, but after reaching the maximum was constant. These findings may indicate that if protein supplement was provided as sufficiently large meals it could increase outflow of protein from the rumen. However, this phenomenon still remains to be explored.

4.6. Utilization of different N fractions for milk protein synthesis

Efficiency of utilization of N for milk protein synthesis. In recent years, the dairy industry has faced increasing pressures to lower the environmental impact of milk production and to optimize the use of ruminant's unique digestive system. Diets containing excessive amounts of CP increase feed cost, release extra N to the environment (NRC, 2001), and lower the efficiency of N for milk protein production (Broderick, 2009; Danes et al., 2012). The MNE is a very good indicator of the environmental impact of milk production with regards to N pollution. It is a simple ratio (milk N yield/N intake) that indicates how a large part of N intake is converted into milk protein and how much is excreted in urine and faeces. Increasing live weight and pregnancy reduce the N excretion in urine and faeces and these factors must be taken into account when comparing studies conducted in different stages of lactation. In lactating cows MNE varies considerably, with reported values ranging from 0.16 (Aarts et al., 2000) to 0.36 (Chase, 2004) and up to 0.45 (Huhtanen and Hristov, 2009). Concentration of CP in the diets is considered to be the most important factor influencing the MNE in lactating cows (Huhtanen et al., 2008c; Huhtanen and Hristov, 2009). With increasing concentrations of CP in the diet the MNE decreases (Castillo et al., 2001; Olmos Colmenero and Broderick, 2006; Huhtanen and Hristov, 2009). Dijkstra et al. (2013) speculated on the theoretical biological maximum for MNE in lactating cows (0.43). This estimate is based on the conversion ratio of proteins in ruminants on standard diets. In theory, higher MNE could be achieved in diets with high levels of good quality, human edible proteins (Dijkstra et al., 2013). In addition in diets with low CP or when the diets are limited in terms of some AA, supplementary AA can improve MNE (Bequette et al., 1998; Haque et al., 2012).

When formulating the diet for lactating dairy cows the profitability is usually the most important factor affecting the choice of feed ingredients and level of CP in the diet. Little consideration is given to the MNE because the maximum milk yield is seldom if ever achieved under the same conditions as maximum MNE. On the contrary, increasing

milk yield by increasing CP in the diet are usually associated with decreases in MNE as it was discussed above.

Diet MNE. Publication IV focused on the MNE from the whole diet and from different N fractions for milk protein synthesis. In that study, the average MNE for the whole diet was 0.32, which is in the upper end of the range of MNE reported in the literature. Because of small (0.5 kg/d) live weight gain of the cows the diet MNE was probably slightly underestimated. If all MP used for growth would be directed to milk protein synthesis the MNE would increase by 0.02 units up to 0.34. This estimate was based on requirements of 233 g of metabolizable protein per 1 kg body gain in lactating cows (Luke, 2019). In that study (IV), the CP concentration (155 g/kg DM) of the diet was close to the optimal in regard to the balance between the estimated supply of RDP and microbial N requirements. Broderick et al. (2010) reported that the degradation of CP in the rumen and flow of microbial N into the omasal canal per day was similar when cows were fed diets with 147 g/kg DM. This estimate was based on 32 independent studies. Using this study (IV) as example to calculate the N excretion from milk production, it can be estimated that 0.66 of N intake were excreted as urine and faeces (1 – MNE (0.32) - weight gain (0.02)). It can be further calculated that for each kg of ECM cows excreted in urine and faeces 11 g of N. In total, 368 g N/d was excreted in urine and faeces (Publication IV). If, hypothetically, the MNE increased by 0.07 in the study presented in Publication IV, without increasing CP level, it would decrease the loss of N in urine and faeces for each kg of ECM to 8 g, and the total loss to 320 g N/d. It can be estimated that for each kg of increase in ECM the loss of N in urine and faeces decrease by approximately 7 g. In the study of Krizsan et al. (2017) increasing CP level (by 48 g/kg) decreased the MNE by 0.07 (Table 4). In this study, for each kg of ECM, cows excreted in urine and faeces 10 and 13.5 g of N. In total 210 and 351 g N/d was excreted for the lowest and the highest level of CP level in the diet, respectively. The ECM yield in that study increased by 5 kg/d with increasing the CP level in the diet from 132 to 180 g/kg DM. It can be calculated that for each kg of increase in ECM the loss of N in urine and faeces increased by 28 g (Krizsan et al., 2017). In this study, increases in CP level increased milk yield but it was associated with very high N losses. In some cases when the CP level of diet is very high, decreases in protein intake have not affected the milk yield. McCormick et al. (1999) reported that decreasing CP levels from 231 to 177 g/kg DM did not affect milk yield. It can be estimated that in the study of McCormick et al. (1999) the MNE increased from 0.22 to 0.26, and the N losses decreased by 160 g N/d. To improve the MNE of a diet, without changing the CP level, the N conversion efficiency needs to be improved. This can be achieved by e.g. supplementation of the diet with individual AA or using different feed components to improve the AA profile of absorbed AA compared to AA profile of MP. Wang et al. (2010) reported that supplementing a lactating dairy cow diet (CP, 165 g/kg DM) slightly limited in MP with Lys and Met increased milk yield by 3.8 kg/d. In that study the MNE increased from 0.26 to 0.30. It can be estimated that in this study (Wang et al., 2010) the N excreted in urine and faeces decreased from 15.4 to 12.5 g N/kg of

milk, and the total N loss decreased by 27 g/d. Also in the study of Lee et al. (2012) the MNE was improved by lowering the diet CP level (from 157 to 136 g/kg) and supplementing the diet with individual AA (Lys, Met, and His). In that study (Lee et al., 2012) the MNE increased from 0.29 to 0.34, and the total N losses decreased by 38 g/d. The discussion presented above indicates that it is possible to improve the MNE of lactating dairy cows without reducing milk yield, and as to decrease N pollution from dairy sector without compromising the economy of milk production.

Feed components MNE. To study the efficiency of utilization of different N fractions or individual feed ingredients, the plant material needs to be labelled so as to follow the fate of that component. By calculating the cumulative secretion (Figure 6) of ^{15}N in milk after the pulse dose of ^{15}N labelled N fractions to the rumen we estimated that 0.19, 0.20, and 0.22 of N administered as ammonia N, soluble and insoluble RM was utilized for milk protein synthesis (^{15}N MNE), respectively (IV).

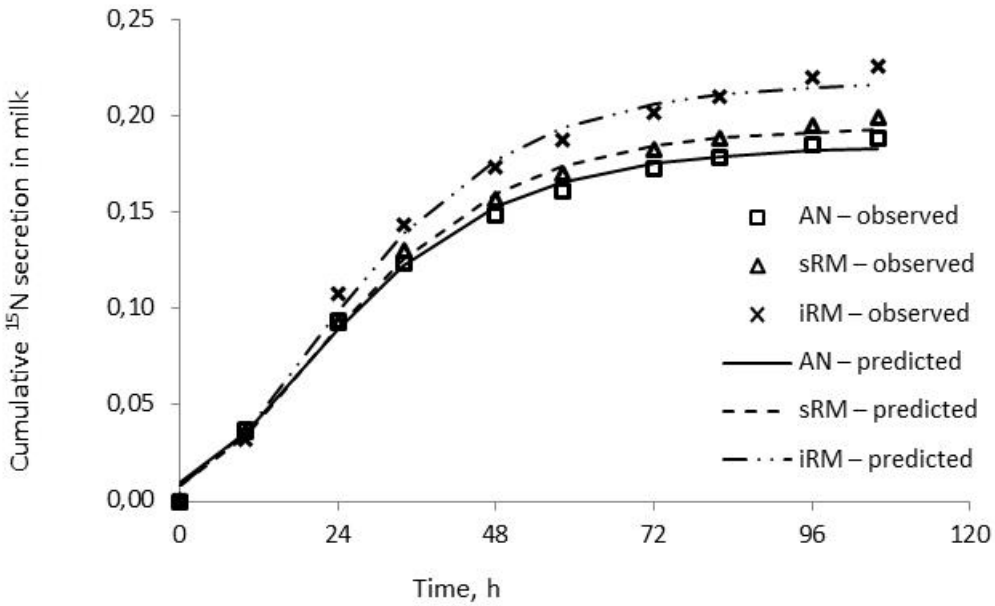

Figure 6. Mean cumulative secretion of ^{15}N in milk as a proportion of total ^{15}N administered by pulse dosing of ^{15}N labelled ammonium-N (AN), or soluble (sRM) and insoluble (iRM) rapeseed protein in the rumen. Symbols are measured values and lines are predicted values with Gompertz non-linear mixed model.

In this study, the cumulative secretion was followed up to 108 h post dosing. Figure 7 presents the rate of secretions of ^{15}N atom-% excess in milk (IV). In the study presented in Publication IV the cows were milked twice daily at 10 and 14 h intervals. The high-

est rate of ^{15}N secretion in milk was observed at 24 h after dosing for all N fractions. Data from the last observations indicated that the plateau in cumulative secretions of ^{15}N in milk was not reached at 108 h. Small increases in the cumulative values indicated that the MNE for the N fractions may have been slightly underestimated especially for the insoluble N fraction. This conclusion was based on the observation that at the end of collection period, the ^{15}N atom-% in excess of background levels in milk was 50% higher than in the soluble N fraction. In addition Barros et al. (2017) reported that 80 h after stopping feeding cows with ^{15}N enriched feed ingredients the ^{15}N enrichment in milk was still decreasing. To have more accurate measurements of cumulative secretion of ^{15}N in milk, the collection time should have been prolonged. Hristov and Ropp (2003) used 155 h collection period and Hristov et al. (2004) used 120 h collection period for calculations total cumulative secretions of ^{15}N in milk.

In the study of Hristov et al. (2004) and Agle et al. (2010), the highest rates of ^{15}N secretion in milk from a pulse dose of ^{15}N labelled ammonia N were observed at 15 h after the dose. In these studies, the milking interval was 5 h. These results suggest that to accurately study the rate of N transfer from different N fractions or feed ingredients in milk N, more frequent milking intervals than 10 and 14 h may be required.

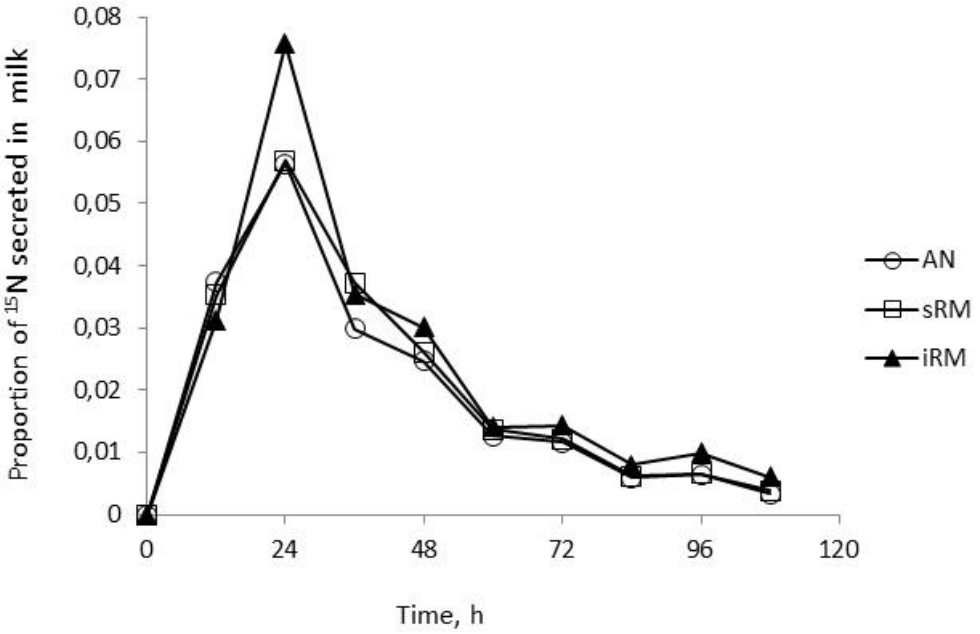

Figure 7. Mean secretion of ^{15}N in milk as a proportion of the total amount of ^{15}N administered in the rumen in the form of ^{15}N labelled ammonium-N (AN), or soluble (sRM) and insoluble (iRM) RM.

The MNE for ^{15}N labelled ammonia N (Table 4) estimated in IV was consistent with previous reports from dairy cows on moderate level of CP (Hristov et al., 2004, 2009;

Agle et al., 2010; Ahvenjärvi and Huhtanen, 2018). The data on MNE and ^{15}N MNE (Table 4) clearly indicate more efficient utilisation of rumen ammonia N for milk protein on the diet with low CP (Agle et al., 2010; Krizsan et al., 2017). Diets with low CP and RDP usually have better utilization of ruminal ammonia N for microbial protein synthesis (Krizsan et al., 2017).

Table 4. Efficiency of utilization of N (MNE), and ^{15}N labelled ammonia N for milk protein synthesis (^{15}N MNE).

Diet CP level, g/kg	MNE	^{15}N MNE	Reference
154	0.23	0.21	Agle et al., 2010
134	0.28	0.23	Agle et al., 2010
129	0.28	0.27	Agle et al., 2010
127 – 145	0.36 – 0.33	0.18[1]	Ahvenjärvi and Huhtanen 2018
195	0.18	0.13[2]	Hristov and Ropp, 2003
191	0.23	0.14[3]	Hristov and Ropp, 2003
183	0.16	0.16	Hristov et al., 2004
158	0.18	0.17	Hristov et al., 2004
221	0.15 – 0.13	0.10 – 0.08[4]	Hristov et al., 2005
159	0.22 – 0.23	0.16 – 0.18[5]	Hristov et al., 2009
180	0.27	0.12	Krizsan et al., 2017
164	0.29	0.12	Krizsan et al., 2017
148	0.30	0.13	Krizsan et al., 2017
132	0.34	0.16	Krizsan et al., 2017
155	0.32	0.19	Stefański et al., (IV)

[1]Marginal efficiency of urea N utilization

[2]Diet with non-structural carbohydrate

[3]Diet with ruminally fermented fibre

[4]Effect of different carbohydrate on ruminal ammonia N utilization

[5]Effect of different fatty acids on ruminal ammonia N utilization

There is limited amount of information on the utilization efficiency of N from individual feed components for the milk protein synthesis. As indicated by the data from Publication IV there are differences in the MNE between different N fractions due to differences in ruminal degradation and N utilization in the rumen. Recent reports of Barros et al. (2017) focusing mainly on relative partitioning of N of individual feeds within a diet into milk, urinary and faecal N in lactating cows, showed that N from different feed ingredients was not partitioned in the same manner for these exit pools. The ratio of urine/milk ^{15}N excretion was higher for alfalfa silage followed by corn silage and the lowest for concentrate ingredients. Silages also had a higher ratio of feces/milk ^{15}N than concentrates (Barros et al., 2017). These results can be explained by differences in rumi-

nal N metabolism of these feeds, the changes in CP level of the diet will probably change the relative partitioning of N from these feeds to exit pools, because the ruminal N metabolism of these feeds will change. The reported differences between the MNE of ammonia N and the different N fractions of RM (IV) were smaller that could be expected based on the ruminal metabolism of these N fractions reported in the literature. This can be explained by a very high efficiency of utilisation of ammonia N for microbial protein synthesis (0.61) reported in this study (III). As discussed above, with low ruminal ammonia N concentrations the efficiency of ruminal utilisation of ammonia N is high and as a consequence the MNE is likely to be relatively high.

There is some evidence suggesting that there may be also other factors increasing the variation in the MNE for separate feed components. Gannes et al. (1998) using changes of enrichments of stable isotopes between diets and body tissues reported that nutrients from different feed ingredients do not contribute equally to specific tissues.

Comparison of the MNE of diet and ^{15}N MNE. The ^{15}N MNE accounts only for milk protein synthesis from the AA absorbed from the gastrointestinal tract and the milk protein derived from body tissue proteins are ignored. In contrast, the diet MNE is calculated based on total N intake and N secretions in milk. Wilson et al. (1988), using the C_3 and C_4 isotope in plant material, estimated that 0.14 to 0.19 of casein carbon was derived from body tissue protein in lactating cows. According to these authors, the estimates were probably underestimated, owing to the rapid turnover of some newly deposited body tissues originating from labelled feed.

The differences between the diet MNE and ^{15}N MNE could be attributed to deposition of labelled AA in body tissue protein with slow turnover (slower than 108 h milk collection period, IV). The increases in the background levels of ^{15}N in milk in periods 2 and 3 compared to period 1 indicate the existence of slow turnover body tissue N pool. Also these observations indicate that parts of the ^{15}N labelled AA released from slow turnover body tissue were used for milk protein synthesis (IV).

Comparison of the diet MNE and ^{15}N MNE indicated that from 0.60 to 0.70 of milk protein was synthesized from AA absorbed from the gastrointestinal tract either directly or via rapid turnover body pools and that 0.30 to 0.40 of milk protein originated from slow turnover body tissues (IV). Using the rate of body tissue protein-N synthesis (2.35 g/d per body weight$^{0.75}$) estimated by Oldham (1984), it can be estimated that 290 g/d of body tissue protein-N was synthesized and catabolized daily in these cows (IV). In addition to that we estimated that approximately 0.18 to 0.24 of tissue protein turnover was directed to milk protein synthesis.

5. Concluding remarks

The current approach to investigate intact protein degradation *in vitro* presented in Publication I based on measurements of ^{14}N and ^{15}N isotope fluxes between microbial N and non-microbial N pools did not provide sufficient information to obtain realistic estimates of the rate of RM degradability in the rumen. A more comprehensive assessment of the metabolic pathways involved in ruminal degradation RM is needed. This approach seemed appropriate for determining microbial N synthesis from ammonia N, but measurements of the direct incorporation of amino acids into microbial N is required to adequately characterize the metabolic events involved in ruminal degradation of RM.

The *in vitro* method presented in this thesis (I, II) with prolonged incubation time up to 10 h, and multiple time point measurements allows detailed time course analysis of N transactions *in vitro*, and provides sufficient amount of information to develop dynamic models of N metabolism. In addition the novel approach to introduce the isotope ^{15}N to the *in vitro* system reported in Publication II, provided sufficient information to allow for an accurate estimation of key parameters in a complex dynamic model of soluble RM protein degradation. The results suggest that the new *in vitro* method with the novel approach to introduce the isotope ^{15}N to the system could be applied to the evaluation of ruminal degradation characteristics of other feed soluble proteins.

The results presented in this thesis from experiments I to III confirm that the non-protein N was efficiently utilized for microbial N synthesis. This data also revealed that a considerable proportion of the ammonia N dose was instantly absorbed into bacterial N. The process of instant uptake occurred at such a high rate that it was complete within minutes (by 0.1 h). When rumen ammonia N concentrations are low rumen bacteria absorb extracellular ammonia N efficiently in order to maintain sufficient intracellular microbial N concentrations. This phenomenon was observed in all experiments presented in this thesis.

Utilization of soluble N fraction from RM in the rumen was better than that of ammonia N as indicated by greater predicted non-microbial N flow from the rumen and smaller ammonia N absorption. Adsorption to microbial cells and uptake to intracellular pools rather than degradation to ammonia N explained rapid disappearance of labelled soluble RM N from rumen fluid.

The data from studies II and III revealed that a large proportion of soluble rapeseed protein N was instantly adsorbed to the microbial cells. These observations suggest that the distinction between feed and microbial or undegradable and degradable protein is not unequivocal as microbes may convey feed protein into the intestines without fur-

ther metabolism that could be considered true ruminal degradation. If the adsorbed soluble protein is not degraded to AA and then synthesized to microbial protein, the AA profile of those proteins will remain the same as in the original feed. The AA profile of those soluble proteins may or may not be as good as microbial protein AA profile, but it will be completely available for post-ruminal absorption.

The data presented in Publication IV were in agreement with the generally accepted view that ammonia N has a lower efficiency of N utilization for milk protein production compared with soluble and insoluble N fractions of RM. However, the differences were much smaller than would be expected, due to high efficiency of utilization of ammonia N for microbial N synthesis.

The data presented in Publication IV indicated that using ^{15}N labelled feed ingredients it is possible to study the immediate use of feed N to milk protein synthesis. Comparisons between the whole diet MNE and that of ^{15}N labelled protein sources can be used to estimate body tissue contribution to milk protein synthesis.

In the *in vitro* and *in vivo* experiments, the results indicated a substantial escape of dietary soluble protein N (0.08) from the ruminal degradation. The results were consistent with the previous observations reported in the literature and highlight ruminal escape of soluble protein as an important source of amino acids in ruminants.

The current work indicated that the results obtained *in vitro* are rather different from those obtained from *in vivo* studies. It indicates also that it is difficult to establish an *in vitro* system that produces data comparable to the *in vivo* data before we fully understand the metabolic events occurring *in vivo*. Also new *in vitro* methods should be validated against *in vivo* studies before implementation in practise. And finally, because the protein degradation may be affected by the protein composition and the protein physical structure, to truly validate the *in vitro* method the same material should be studied *in vivo*.

The data presented in this thesis and in the literature suggest that the concept of distinction between RDP and RUP is an inadequate representation of feed characteristics required to predict ruminal metabolism of individual feed ingredients in dynamic rumen environment. Dynamic models that represent the essential steps in ruminal N metabolism combined with accurate estimates of key parameter values are necessary.

6. Future research

Future research is needed on the metabolism of soluble feed protein in the rumen, and the proportion of soluble feed protein that escapes rumen degradation. Because the degradation rate of soluble feed protein is much slower than it was assumed earlier and part of it escapes ruminal degradation and may be an important source of amino acids in ruminants, more research is needed to estimate the soluble feed protein degradation rates and escape from the rumen. Also the effects of diet CP level on the soluble feed protein degradation rates should be studied.

More systematic research is needed on the quantitative role of protein adsorption to microbes on ruminal N metabolism. More emphasis should be dedicated on the protein adsorption onto microbial cells, and the effects of protein meal frequency and size (g/d) on ruminal N metabolism and on post-ruminal AA supply.

Detailed studies on the effects of concentration gradients of ruminal ammonia N and bacterial intracellular ammonia N on the ruminal ammonia N absorption into microbial cells are required.

Studies focusing on the effect of CP levels in the diets on the variation in the proteolytic activity in the rumen could contribute to improvements in understanding of the N metabolism in the rumen and, as a consequence, the feed utilisation efficiency in the ruminants.

Research on factors affecting the changes in microbial population over time and the proteolytic enzyme activity in the in vitro system compared to the in vivo conditions could contribute to improvements in the in vitro techniques for estimation of feed values for ruminant animals.

More systematic research is needed to develop a widely accepted, fast, cheap, and accurate method for feed value estimations. None of the techniques currently used for feed value estimations are widely accepted because of the inherent problems associated with those techniques.

Future research is needed on the metabolism of insoluble feeds protein in the rumen although it was not included in this thesis.

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